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Research

Associations of XRCC4, eNOS, and PER3 VNTR variants with Childhood Acute Lymphoblastic Leukemia in Turkish Patients

Türk Hastalarında XRCC4, eNOS ve PER3 VNTR Varyantlarının Çocukluk Çağı Akut Lenfoblastik Lösemi ile İlişkisi

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ABSTRACT

Objective: The genetic factors responsible for the etiopathogenesis of childhood acute leukemia have been extensively investigated. High-resolution expression analysis of the whole genome, and results of gene studies including whole genome sequencing, copy number changes of DNA, loss of heterozygosity and epigenetic changes revealed the classification of acute lymphoblastic leukemia (ALL). A variable number of tandem repeats (VNTRs) can regulate many biological processes, including gene transcription, protein function, morphological development, and cancer formation. They may also play a role in many disorders in humans such as labile repeat expansions. In this paper, our aim was to compare the genotype and allele frequencies in VNTR variants of XRCC4, eNOS, and PER3 between pediatric ALL patients and healthy controls.

Methods: Seventy-four high-risk pediatric ALL patients (82.4% B-ALL, 17.6% T-ALL) who were consecutively admitted to the Pediatric Hematology Units of İstanbul Medical Faculty and Yeni Yuzyıl Medical Faculty and 100 healthy volunteers were included in this case-control study. VNTRs of three genes were analyzed using the polymerase chain reaction method.

Results: The frequency of the eNOS VNTR 4a/4a genotype was found to be higher in the pediatric patients with ALL compared to the healthy controls (p=0.044) and the risk factor for childhood ALL was found to be 8.382 (95% confidence interval =0.985-71.262). The frequency of eNOS 4/a allele was found to be higher in the childhood ALL group compared to the controls (p=0.013). The frequencies of the 5R/5R genotype and 5R allele of the PER3 VNTR were found to be significantly lower in the childhood ALL patients (p=0.039 and p=0.015, respectively).

Conclusion: Our results show that functional variants of the *eNOS* and *PER3* genes may have an important relationship with the etiopathogenesis of childhood ALL. Further studies including larger groups and different ethnic populations are needed to determine the effect of VNTR variants on the risk of developing childhood ALL.

Keywords: VNTR, eNOS, XRCC4, PER3, childhood ALL

ÖZ

Amaç: Çocukluk çağı akut lösemi etiyopatogenezinden sorumlu olan genetik faktörler kapsamlı bir şekilde araştırılmıştır. Tüm genomun yüksek çözünürlüklü ekspresyon analizi, ve tüm genom dizilimi, DNA'nın kopya sayısı değişiklikleri, heterozigotluk kaybı, epigenetik değişiklikler gibi gen çalışmalarının sonuçları, tüm akut lenfoblastik lösemi (ALL) hastalarının sınıflandırılabilmesini sağladı. Değişken sayıda tandem tekrarları (VNTR) gen transkripsiyonu, protein fonksiyonu, morfolojik gelişim, kanser davranışı ve fizyoloji gibi birçok biyolojik süreci modüle edebilirler. İnsanlarda, kararsız tekrar açılımlarını da içeren birçok bozukluktan da sorumlu olabilirler. Bu çalışmada çocukluk çağı ALL'li hastalarda XRCC4, eNOS ve PER3'ün VNTR varyantlarındaki genotip ve alel frekanslarını sağlıklı kontrollerle karşılaştırmayı amaçladık.

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Gereç ve Yöntem: İstanbul Tıp Fakültesi Pediatrik Hematoloji Ünitelerine ve Yeni Yüzyıl Tıp Fakültesi'ne ardışık olarak başvuran toplam 74 yüksek riskli çocukluk çağı ALL hastası (%82,4 B-ALL, %17,6 T-ALL) ve 100 sağlıklı gönüllü bu olgu-kontrol çalışmasına dahil edildi. Polimeraz zincir reaksiyonu yöntemi kullanılarak üç gen VNTR bölgesi analiz edildi.

Bulgular: Çocukluk çağı ALL'li olgular sağlıklı kontrollere göre daha yüksek eNOS VNTR 4a/4a genotipine sahipti (p=0,044) ve çocukluk çağı ALL'si için (Olasılık oranı: 8.382 %95 güven aralığı =0.985-71.262) risk faktörü olarak gösterildi. eNOS 4a alelinin sıklığı, çocukluk çağı ALL grubunda kontrollere göre daha yüksekti (p=0,013). Çocukluk çağı ALL hastalarında sırasıyla PER3 VNTR'nin 5R/5R genotipi ve 5R alelinin frekansları önemli ölçüde daha düşük saptandı (p=0,039, p=0,015).

Sonuç: Sonuçlarımız, *eNOS* ve *PER3* genlerinin fonksiyonel varyantlarının çocukluk çağı ALL etiyopatogenezi ile önemli bir ilişkisi olabileceğini düşündürmektedir. VNTR varyantlarının çocukluk çağı ALL gelişme riski üzerindeki etkisini belirlemek için daha büyük gruplar ve farklı etnik kökenler ile daha ileri çalışmalara ihtiyaç vardır.

Anahtar Kelimeler: VNTR, eNOS, XRCC4, PER3, çocukluk çağı ALL

INTRODUCTION

The genetic factors responsible for the etiopathogenesis of childhood acute leukemia have been extensively studied. Results of gene studies, high-resolution whole genome expression analysis, copy number changes of DNA, loss heterozygosity epigenetic changes and whole genome sequencing enabled the recognition of new genetic changes so that all acute lymphoblastic leukemia (ALL) patients could be classified.

Nitric oxide (NO) is synthesized from L-arginine by the nitric oxide synthase (NOS) enzyme and is a dual molecule that can have a tumor protective or stimulating effect depending on its local concentration. There are three main isoforms of the NOS enzyme: neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). Over the past decade, clinical trials have shown that NOS2 expression is associated with many cancers. Overexpression of NOS2 is present in >50% of patients with glioma, melanoma, breast, prostate, pancreatic, liver, cervical, ovarian, nasopharyngeal, lung, stomach, colon, and esophageal cancers (1). These studies drew attention to the increased angiogenic and metastatic potential of NOS2 (2). High NO flux causes genotoxicity and protein modification. It has been shown that high NO levels may lead to deamination, leading to a transition from C to T in DNA (3,4). Further research has shown that high NO levels can inhibit specific DNA repair systems, particularly thiol-dependent ones such as alkyl transferase and zinc finger proteins. Oxidation of carcinogenic nitrosamines via cytochrome P450 (CYP450) generate DNA alkylating metabolites that cause DNA damage (2).

DNA repair defects may induce further cancer progression by causing genetic instability in the genome (5). Three major DNA-based excision repair genes act interactively in DNA repair processes; X-ray repair cross-complement 1 and 4 (XRCC1 and XRCC4) and xeroderma pigmentosum complement group D (6). Polymorphisms of these genes can alter gene transcription rate, the stability of mRNA, or protein functions. It is thought that variations in these genes may cause cancer development by affecting an individual's capacity to repair damaged DNA (7).

Mechanisms related to the circadian clock are extremely important in terms of cell cycle, DNA damage and tumor suppression (8). At the molecular level, circadian clocks consist of the products of "clock genes" regulated in the transcription-translation regulatory system. Some clock genes encode transcriptional activators, while others encode proteins that can inhibit their expression. The circadian clock is associated with the clock genes of the circadian rhythm and consists of two transcription factors: CLOCK (circadian locomotor output loops caput) is a histone acetyl transferase that is activated when heterodimerized with 1 (brain and muscle hydrocarbon receptor nuclear receptor-antigen 1). It provides the transcription of Period (*PER 1, 2,* and *3*), and Cryptochrome (*Cry 1* and *Cry 2*) genes (9-14).

Per proteins contain two consecutive PAS domains and can interact with one another and with other proteins through these regions. PER3 is under clock control but is not required for rhythm production. However, PER1 and PER2 are the central components of the clock (8,13,14).

Disruption of the circadian clock is instrumental in the development of different human cancers. Disruption of the circadian rhythm causes modifications that change cell proliferation and lead to oncogenesis and cancer (15,16).

We hypothesized that genotype and allele frequencies in the variants of XRCC4 intron 3 variable number of tandem repeat (VNTR), eNOS intron 4b/a VNTR and PER3 exon 18 (54 bp repeats VNTR) are linked to childhood ALL.

METHODS

Study Population

Seventy-four high-risk pediatric ALL patients, who were consecutively admitted to the Pediatric Hematology Units of İstanbul Medical Faculty and Yeni Yüzyıl Medical Faculty, were included in this study. One hundred healthy volunteers were included as a control group.

Ethics statement: This study was approved by the Clinical Research Ethics Committee of İstanbul University, İstanbul Faculty of Medicine (no: 242064, date: 23.11.2020). The authors assert that all procedures contributing to this work comply with the ethical standards of Istanbul University and the Helsinki Decleration of 1975, as revised in 2008. Informed consent form was not obtained because the study was retrospective.

DNA Extraction and Genotyping

The peripheral blood samples of the patients were obtained at the time of diagnosis procedures before treatment was applied. Genomic DNA was extracted from whole blood using the Plus Blood Genomic DNA Purification test kit (GeneMark, USA).

The VNTRs of three genes, including *XRCC4*, *eNOS*, and *PER3*, were analyzed using the polymerase chain reaction method. Gene polymorphisms were detected with the polymerase chain reaction method (17-19) (Table 1).

Statistical Analysis

The data were analyzed using the SPSS software version 21. Descriptive statistics included the mean and standard deviation for the continuous variables. Nominal variables were summarized as frequency and percentage. Odds ratio (OR) and corresponding 95% confidence interval (CI) were used to determine the strength of the association. Consequently, we presented the ORs and 95% CIs for associating MBL genotypes with the clinical parameters. The association of the alleles and homozygosity was compared with the chi-square test (χ^2) or Fisher's Exact test, and Bonferroni correction was used. The two groups were in accordance with the Hardy-Weinberg equilibrium ($p>0.05$). A value of $p<0.05$ was accepted to be statistically significant.

RESULTS

A total of 74 childhood ALL patients and 100 controls were included in this study. Demographic and clinical characteristics of the patients are shown in Table 2. The statistical analysis showed no significant relationship for alleles and frequencies of XRCC4 genotype between the patients and controls ($p>0.05$) (Table 3).

The frequency of the eNOS VNTR 4a/4a genotype was found to be higher in the pediatric patients with ALL compared to the healthy controls ($p=0.044$) and the risk factor for childhood ALL was found to be 8.382 (95% CI=0.985-71.262). The frequency of eNOS 4a allele was found to be higher in the childhood ALL group compared to the controls ($p=0.013$) (Table 3). The frequencies of the 5R/5R genotype and 5R allele of the PER3 VNTR were found to be significantly lower in the childhood ALL patients ($p=0.039$ and $p=0.015$, respectively) (Table 3). Forty six percent of the male patients and 15.8% of the female patients carried the 4R/5R genotype of PER3 VNTR. The difference between the two groups was statistically significant ($p=0.026$, OR=4.543, 95% CI=1.174-17.579) (Table 4). No statistically significant correlation was found between the *XRCC4*, *eNOS*, *PER* genes, and disease relapse ($p>0.05$) (Table 5). The frequency of the eNOS 4a/4a genotype was found to be higher in the childhood T-ALL group (30.8%) compared with the childhood B-ALL group (3.3%) ($p=0.010$) (Table 6).

DISCUSSION

The pathophysiology of ALL is a very complex relationship with various factors (genetic, immune, environmental and drugs) at different levels. NO plays a crucial role in regulating cancer progression. Several studies have shown that the NO and NOS systems play important roles in carcinogenesis. Some studies are attempting to uncover the potential to modulate NO levels to increase the efficacy of currently available treatments against lymphoma, leukemia, and myeloma. It is thought that NO modulation could aid hematological cancer management, either by directly targeting tumor cells or by activating the immune system to eliminate cancer cells. *eNOS* gene polymorphisms significantly influence serum NO concentrations (20). Polymorphisms T786C and G894T affect eNOS regulation and have been associated with various diseases. Sickle

Table 1. XRCC4 (intron 3), eNOS (intron 4) and PER3 VNTR primer sequences, and amplification conditions

VNTR	Primer sequence	Annealing
XRCC4 (intron 3)	5′-TCCTGTTACCATTTCAGT GTTAT-3′ 5′-CACCTGTGTTCAATTCCAGCT T-3′	55 °C and 32 cycles
eNOS (intron 4)	5′-AGGCCCTATGGTAGTGCCTTT-3′ 5′-TCTCTTAGTGCTGTGGTCAC-3′	57 °C and 35 cycles
PER3	5 -TGTCTTTTCATGTGCCCTTACTT-3 5 -TGTCTGGCATTGGAGTTTGA-3	60 °C and 35 cycles

VNTR: Variable number of tandem repeat

cell disease, a clinically diverse chronic hemolytic anemia, involves impaired nitric oxide bioavailability (21). This study found the frequency of the eNOS 4a allele to be higher in the childhood ALL patient group. Simultaneously, the frequency of the eNOS VNTR 4a/4a genotype was found to be higher as a risk factor in the pediatric ALL group compared to the control group. The frequency of the eNOS 4a/4a genotype was higher in the childhood T-ALL group compared to the childhood B-ALL group. XRCC4 encodes a DNA repair protein that preserves genome stability by repairing a double strand breaks using the error-prone method. XRCC4 is generally expressed as a protein (334 amino acids) involved in DNA ligase IV and the enzyme DNA-dependent protein kinase in repairing DNA double strand breaks. Defects in the protein-coding gene cause disruption of the DNA repair process and accumulation of DNA damage in the cell that can cause cancer development (22,23).

Table 2. Demographic details of the patients. Values are either mean ± SD or n (%)

Parameters	Mean ± SD or n (%)
Total patients (n=74)	
Age, years	8.07±5.09
Female/male	24 (27.9%)/50 (72.1%)
Type ALL (B-ALL/T-ALL)	61 (82.4%)/13 (17.6%)
WBC count	50,000 (20,000-978,000)
Follow-up period	7.4±4.86 (1-15) years

WBC: White blood cell, SD: Standard deviation, ALL: Acute lymphoblastic leukemia

This study is the first to report of XRCC4 gene polymorphism in cALL in our population. We did not find any significant difference between pediatric ALL patients and healthy control groups in terms of the distribution of genotypes and alleles in XRCC4 VNTR.

Wu et al. (24) found differences in the frequency of XRCC4 G-1394T and intron 3 genotype between childhood

Table 3. The distribution of genotypes and alleles of XRCC4, eNOS, and PER3 variants in patients with childhood ALL and controls

		Patients	Controls	OR	95% CI	p
XRCC4	**Genotypes**	n=74	n=100			
	DD	17 (23.0%)	28 (28.0%)	0.766	0.382-1.538	0.488[&]
	ID	37 (50.0%)	43 (43.0%)	1.326	0.724-2.425	0.441*
	II	20 (27.0%)	29 (29.0%)	0.906	0.463-1.773	0.865*
	Allele					
	D	71 (48.0%)	99 (49.5%)	0.940	0.614-1.439	0.828[&]
	I	77 (52.0%)	101 (50.5%)	-	-	-
eNOS	**Genotypes**	n=74	n=96			
	4a/4a	6 (8.1%)	1 (%1.1)	8.382	0.985-71.267	0.044*
	4a/4b	18 (24.2%)	18 (%18.7)	1.393	0.665-2.914	0.449*
	4b/4b	50 (67.7%)	77 (%80.2)	0.514	0.255-1.035	0.075[&]
	Allele					
	4a	30 (20.3%)	20 (10.4%)	2.186	1.185-4.034	0.013[&]
	4b	118 (79.7%)	172 (89.6%)	-	-	-
PER3	**Genotypes**	n=69	n=97			
	4R/4R	40 (58.0%)	41 (42.3%)	1.884	1.008-3.521	0.058[&]
	4R/5R	26 (37.7%)	42 (43.3%)	0.791	0.421-1.489	0.523*
	5R/5R	3 (4.3%)	14 (14.4%)	0.269	0.074-0.977	0.039*
	Allele					
	4R	106 (76.8%)	124 (64.0%)	1.870	1.143-3.059	0.015[&]
	5R	32 (23.2%)	70 (36.0%)	-	-	-

*OR (95% CI) was adjusted for age and sex, [&]Fisher's Exact test. CI: Confidence interval, OR: Odds ratio, ALL: Acute lymphoblastic leukemia
Data written in bold was found to be statistically significant (p<0.05).

leukemia and control groups. They noted that deletions of the G allele of G-1394T and intron 3 were clear risk factors for susceptibility to childhood leukemia. They suggested that the G allele of XRCC4 G-1394T and deletion of intron 3 might be responsible for pediatric leukemia and might be useful in the early detection of cALL (24).

Cancer research in human and animal models has shown that endogenous factors contributing to the development

Table 4. Distribution of genotypes and alleles of the PER3 variant in male and female patients with childhood ALL

PER3	Male patients n=50 (%)	Female patients n=19 (%)	OR	95% CI	p
Genotypes					
4R/4R	25 (50.0)	15 (79.0)	0.266	0.077-0.916	**0.033&**
4R/5R	23 (46.0)	3 (15.8)	4.543	1.174-17.579	0.026*
5R/5R	2 (4.0)	1 (5.3)	0.750	0.063-8.791	1.000*
Allele					
4R	73 (73.0)	33 (86.8)	0.409	0.144-3.059	1.158&
5R	27 (27.0)	5 (13.2)			

*OR (95% CI) was adjusted for age and sex, &Fisher's Exact test. CI: Confidence interval, OR: Odds ratio, ALL: Acute lymphoblastic leukemia
Data written in bold was found to be statistically significant (p<0.05).

Table 5. The distribution of genotypes and alleles of XRCC4, eNOS, PER3 variants in childhood ALL patients with and without relaps

		With relaps	Without relaps	OR	95% CI	p
XRCC4	**Genotypes**	n=11	n=63			
	DD	2 (18.2%)	15 (23.8%)	0.941	0.229-6.237	0.682&
	ID	5 (45.5%)	32 (50.8%)	0.721	0.493-1.045	0.743*
	II	4 (36.4%)	16 (25.4%)	0.763	0.217-0.834	0.449*
	Allele					
	D	9 (40.9%)	62 (49.2%)	0.738	0.247-0.874	0.472&
	I	13 (59.1%)	64 (50.8%)	-	-	-
eNOS	**Genotypes**	n=11	n=63			
	4a/4a	1 (9.1%)	5 (7.9%)	1.160	0.123-11.006	0.999*
	4a/4b	2 (18.2%)	16 (25.4%)	0.652	0.127-3.346	0.954*
	4b/4b	8 (72.7%)	42 (66.7%)	1.133	0.320-5.554	0.895&
	Allele					
	4a	4 (18.2%)	26 (20.6%)	0.854	0.266-2.774	0.791&
	4b	18 (81.8%)	100 (79.4%)	-	-	-
PER3	**Genotypes**	n=11	n=58			
	4R/4R	7 (63.6%)	33 (56.9%)	1.326	0.349-5.034	0.750&
	4R/5R	4 (36.4%)	22 (37.9%)	0.935	0.245-3.365	1.000*
	5R/5R	0 (0.0%)	3 (5.2%)	0.689	0.033-14.287	1.000*
	Allele					
	4R	18 (81.8%)	88 (75.9%)	1.432	0.447-4.587	0.783&
	5R	4 (18.2%)	28 (24.1%)			

*OR (95% CI) was adjusted for age and sex, &Fisher's Exact test. CI: Confidence interval, OR: Odds ratio, ALL: Acute lymphoblastic leukemia
Data written in bold was found to be statistically significant (p<0.05).

Table 6. The distribution of genotypes and alleles of XRCC4, eNOS, PER3 variant in patients with childhood B-ALL and childhood T-ALL

		B-ALL	T-ALL	OR	95% CI	p
XRCC4	**Genotypes**	n=61	n=13			
	DD	13 (21.3%)	4 (30.8%)	0.609	0.161-2.299	0.479[&]
	ID	32 (52.5%)	5 (38.4%)	1.766	0.518-6.013	0.542*
	II	16 (26.2%)	4 (30.8%)	0.800	0.216-2.962	0.739*
	Allele					
	D	58 (47.5%)	13 (50.0%)	0.906	0.388-2.114	0.832[&]
	I	64 (52.5%)	13 (50.0%)	-	-	-
eNOS	**Genotypes**	n=61	n=13			
	4a/4a	2 (3.3%)	4 (30.8%)	0.086	0.013-0.535	0.010*
	4a/4b	18 (29.5%)	1 (7.7%)	4.636	0.558-38.468	0.166*
	4b/4b	41 (67.2%)	8 (61.5%)	0.911	0.249-3.332	1.000[&]
	Allele					
	4a	22 (18.0%)	9 (34.6%)	0.415	0.163-1.154	0.068[&]
	4b	100 (82.0%)	17 (65.4%)	-	-	-
PER3	**Genotypes**	n=57	n=12			
	4R/4R	34 (59.6%)	6 (50.0%)	1.478	0.423-5.157	0.541[&]
	4R/5R	20 (35.1%)	6 (50.0%)	0.540	0.157-1.898	0.347*
	5R/5R	3 (5.3%)	0 (0%)	1.606	0.077-33.130	0.984*
	Allele					
	4R	88 (77.2%)	18 (75.0%)	1.128	0.408-3.138	0.794[&]
	5R	26 (22.8%)	6 (25.0%)	-	-	-

*OR (95% CI) was adjusted for age and sex, [&]Fisher's Exact test. CI: Confidence interval, OR: Odds ratio, ALL: Acute lymphoblastic leukemia
Data written in bold was found to be statistically significant (p<0.05).

of disruption of circadian rhythms contribute to the development of cancer in mammals (25). Previous studies, it has been reported that circadian expression is altered in chronic myeloid leukemia (CML). In two different studies, expression changes of clock genes were shown in acute leukemia, BMAL1 expression was shown to be down-regulated by methylation in patients with AML and ALL (26) and PER2 expression was shown to be down-regulated in patients with AML (27).

The CRY1, CRY2, PER1, PER2, PER3, brain and muscle aryl hydrocarbon receptor nuclear translocator (BMAL1) genes have previously been shown to be associated with CML (28).

In this study, the frequencies of the 5R/5R genotype and 5R allele of the PER3 VNTR were foundto significantly lower in the pediatric ALL patients.

We determined that 46% of the male patients and 15.8% of the female patients carried the 4R/5R genotype of PER3 VNTR. The differences between the two groups were statistically significant. The fact that PER3 is the most down-regulated gene and recovery of PER3 correlates with better clinical outcomes in patients with acute leukemia, raises the possibility that deregulation of multiple molecular pathways may play a role in the development of acute leukemia, and at least one of them is tissue-specific inactivation of the *PER3* gene (29). Yang et al. (30) analyzed the expression of nine core circadian clock genes in patients with acute leukemia, and their data showed that different genes were dysregulated in AML and ALL. In both diseases, PER3 was the most down-regulated gene and improved PER3 expression was associated with better clinical outcomes (30).

A limitation of this study was the small number of patients. We anticipate that it may be more meaningful to work with a higher number of patients.

CONCLUSION

This study is one of the first studies investigating the relationship between *XRCC4, eNOS,* and *PER3* gene variants and cALL in our country. Our findings show that the *eNOS* and *PER3* genes may have a significant association with the etiopathogenesis of childhood ALL in Turkish subjects. Downregulation of circadian clock genes, particularly PER3, may promote proliferation of blastic cells, resulting in deregulation of the cell cycle.

ETHICS

Ethics Committee Approval: This study was supported by the Clinical Research Ethics Committee of İstanbul University, İstanbul Faculty of Medicine (no: 242064, date: 23.11.2020). This study was approved by the ethical review boards of the İstanbul University and conducted in accordance with the standards of the Declaration of Helsinki.

Informed Consent: Informed consent form was not obtained because the study was retrospective.

Authorship Contributions

Surgical and Medical Practices: R.O., M.G., A.A., Concept: R.O., S.P., Y.O., H.Ş.Ç., Design: R.O., S.P., Y.O., H.Ş.Ç., Data Collection or Processing: R.O., M.G., S.P., Y.O., Z.K., F.A., Analysis or Interpretation: R.O., S.P., Y.O., H.Ş.Ç., Z.K., Literature Search: R.O., M.G., S.P., Y.O., H.Ş.Ç., A.A., Z.K., F.A., Writing: R.O., S.P., H.Ş.Ç., F.A.

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