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# NEUROD polymorphism Ala45Thr is associated with Type 1 diabetes mellitus in Czech children

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## Abstract

Association of the NEUROD Ala45Thr polymorphism with Type 1 diabetes mellitus (DM) has been found in some but not all populations. We performed a study on the association of two NEUROD exon 2 polymorphisms, the Ala45Thr and the Pro197His, with childhood-onset Type 1 DM in the Czech population. We compared 285 children with Type 1 DM diagnosed under the age of 15 years with 289 non-diabetic control children. The genotypes were determined using novel real-time allele-specific PCR assays in the TaqMan format, and data were analysed using logistic regression. The numbers of subjects with codon 45 genotypes Ala/Ala, Ala/Thr, Thr/Thr were 95, 145, 45 among cases and 117, 130, 42 among controls. Thr45 phenotypic positivity was associated with a significant risk of Type 1 DM (OR = 2.01, CI 95% 1.25–3.24) in a multivariate logistic regression model involving also the insulin gene –23HphI genotype and the presence of Type 1 DM-associated HLA-DQB1\*0302-DQA1\*03 (DQ8) and DQB1\*0201-DQA1\*05 (DQ2) molecules. No association was observed for the Pro197His mutation which was carried by 5.3% cases and 5.9% controls. Our results confirm that the NEUROD Ala45Thr polymorphism is associated with childhood-onset Type 1 DM.

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**Keywords:** NEUROD; Genetic association; Type 1 DM; Real-time PCR

## 1. Introduction

Type 1 diabetes mellitus (DM) is a consequence of autoimmune destruction of insulin-producing

pancreatic  $\beta$ -cells. The genetic background of the disease is complex, with multiple genes involved in the pathogenesis [1]. The major part of the disease risk or protection is conferred by the HLA class II genes, with significant contributions of other loci. One of the loci linked to Type 1 diabetes is IDDM7 mapped to 2q31–33 [2–5]. It contains a plausible candidate gene, NEUROD, mapped to 2q32 [6]. NEUROD is a differentiation factor for

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neurogenesis [7], and an activator of insulin gene transcription in pancreatic  $\beta$ -cells [8]. Based on data from knock-out mice, NeuroD is also essential for  $\beta$ -cell morphogenesis and differentiation [9], and it may even have a role in  $\beta$ -cell apoptosis [10].

Apart from rare mutations causing autosomal dominant forms of Type 2 diabetes [11], the NEUROD gene has three sequence variants: the common Ala45Thr [10], and the less prevalent Pro197His and Ser259Ser [12]. Association of the Ala45Thr polymorphism with Type 1 DM has been demonstrated in Japanese adult-onset patients [10], in Japanese children [13], and in Danish patients [12]. However, the diabetes-associated allele in the Japanese datasets is the Thr45, while in the Danes it is the Ala45. There are also several populations with no association between Type 1 DM and NEUROD Ala45Thr: white Americans [14], French [15] and one study in Japanese patients [16].

The aims of the present study were (1) to perform a case-control association study of the NEUROD Ala45Thr and Pro197His polymorphisms in childhood-onset Type 1 DM, (2) to investigate whether the NEUROD-associated susceptibility interacts with insulin gene polymorphism or with HLA risk factors, (3) to demonstrate that real-time PCR with sequence-specific primers is a rapid and accurate alternative to conventional techniques of typing for single nucleotide polymorphisms (SNP).

## 2. Material and methods

### 2.1. Patients and controls

The association was investigated in a case-control study. Phenotypic and genotypic frequencies of the studied polymorphisms were compared between Type 1 diabetic patients and non-diabetic control subjects of a similar age. All patients and control subjects held Czech citizenship and declared themselves as Caucasians. No other criterion of ethnicity was applied. The study was approved by the local Ethics Committee.

We examined 285 children (144 boys, 141 girls) who developed Type 1 DM under the age of 15 years, and were followed on at the Second Department of Paediatrics of Motol University Hospital, Prague. Diagnosis was based on the WHO criteria, abrupt onset, and proneness to ketosis. The age at DM onset was  $7.5 \pm 3.9$  years (mean  $\pm$  S.D.); the distribution by 5-year age-at-diagnosis bands 0–4, 5–9 and 10–14 years was 89, 106, and 90 cases, respectively.

The control group consisted of 289 non-diabetic children aged  $8.5 \pm 3.9$  years. These subjects were consecutively recruited from patients who underwent minor surgical interventions between January and October 1999 at the Department of Paediatric Surgery (Motol University Hospital, Prague). Control subjects with any history of autoimmune disorders, connective tissue disorders, glucose intolerance, endocrine diseases, or with increased erythrocyte sedimentation rate were excluded.

DNA was extracted from freshly drawn or frozen EDTA-anticoagulated blood using the salting-out method [17], and stored at  $-20$  °C until use.

### 2.2. NEUROD Ala45Thr and Pro197His genotyping using real-time PCR with sequence-specific primers

The two polymorphisms of the NEUROD gene were typed for using polymerase chain reaction with sequence-specific primers (PCR-SSP) in real-time PCR TaqMan format. Real-time PCR detects the PCR product simultaneously with its synthesis in the reaction [18], using a specific fluorescent probe annealing to a sequence within the region flanked by the primers. The fluorescent probe is decomposed as the Taq polymerase proceeds with synthesis of a new strand. Decomposition of the TaqMan probe results in an increase in fluorescent signal so that the quantity of the specific amplicon can be assessed at any given moment of the PCR program. Apart from its immediate utility for gene quantification, this format may also be used for rapid allelic discrimination using the principal property of PCR-SSP: perfectly matched primers yield PCR product more likely and in earlier PCR cycles than primers with a 3'-terminal mismatch.

To discriminate between alleles of a SNP, two PCR reactions per sample are utilised, one for each allelic alternative. Similarly to the classic PCR-SSP format, each allele-specific reaction contains two primer pairs: one pair specifically detects the particular allele of the SNP, the other pair serves as an internal control of amplification. While in classical PCR-SSP, the two products (allele-specific and control) are distinguished by their different lengths using gel electrophoresis, the real-time PCR detects the two amplification products using differently labelled specific probes.

### 2.2.1. The Ala45Thr polymorphism

The primer sequences, the probe sequence, and their concentrations in each reaction are presented in Table 1. For specific detection of the codon 45 SNP, two tests were used for each sample: one specific for the A allele (encoding Thr45), the other for the G allele (encoding Ala45). Similarly, for the codon 197 polymorphism, one primer detected the C, and the other the A allele. The primers detecting the SNP were destabilised at the third position from the 3'-end to increase specificity (Table 1, underlined). An internal control reaction amplifying a conserved region of the albumin gene was present in each tube to disclose failure of PCR. To allow simultaneous detection of the allele-specific and the control PCR products in

one tube, the NEUROD amplicon was detected by a FAM labelled probe, while the albumin amplicon was detected using a VIC labelled probe.

The PCR was run in a 15 µl volume, with 1 × PCR buffer (Platinum, Gibco BRL), 2 mM MgCl<sub>2</sub>, 100 µM each dNTP, 1 µM 6-carboxy-X-rhodamin (Molecular Probes), 75–100 nM of sequence-specific (NEUROD) primers, 40 nM control (albumin) primers, 200 nM NEUROD FAM-labelled probe, and 150 nM albumin VIC-labelled probe, 0.3 U Platinum Taq DNA polymerase (Gibco, BRL), and 1 µl DNA. All reactions were performed on the ABI 7700 (TaqMan) machine (Applied Biosystems) with the SEQUENCE DETECTOR 1.7 software. After 5 min initial denaturation at 94 °C, 40 cycles were run, each of 15 s at 94 °C, 1 min at 62 °C, 30 s at 72 °C. The signal was collected in two dye layers (FAM for the NEUROD specific probe, VIC for the albumin-specific probe) using the Spectral Compensation option of the software, and normalised to the passive standard (6-carboxy-X-rhodamin).

For each reaction, cycles were determined in which the NEUROD signal and the control signal exceeded a given threshold over the noise (threshold cycles). An earlier threshold cycle means more effective amplification of the sequence. The threshold cycle was read separately for the FAM signal (NEUROD probe), and the VIC signal (albumin).

Table 1  
Sequences and concentrations of the primers and probes used in the NEUROD typing

Reaction	Specific primers	Control primers, specific and control probe present in each reaction
Ala 45	N-Ala45 forward: AG AAG GAG GAC GAC CTC G <u>C</u> A G; N-common reverse*: TCT CAA TTT AAA ACG CTC CAG; each 75 nM	NEUROD probe: FAM-ATG AAC GCA GAG GAG GAC TCA CTG AGG AAC-TAMRA, 200 nM
Thr 45	N-Thr45 forward: AG AAG GAG GAC GAC CTC G <u>C</u> A A; N-common reverse*: TCT CAA TTT AAA ACG CTC CAG; each 100 nM	Control (albumin) primers: forward TGA AAC ATA CGT TCC CAA AGA GTT T; reverse CTC TCC TTC TCA GAA AGT GTG CAT AT; 40 nM each
Pro 197	N-197Pro reverse: G CAT GTC CTG GTT CTG C <u>T</u> T AG; N-common forward: AAG AAG GAG GAC GAC CTC GAA; each 100 nM	
His 197	N-197Pro reverse: G CAT GTC CTG GTT CTG C <u>T</u> T AT; N-common forward: AAG AAG GAG GAC GAC CTC GAA; each 100 nM	Control (albumin) probe: VIC-TGC TGA AAC ATT CAC CTT CCA TGC AGA T-TAMRA; 150 nM

Deliberate mismatches increasing specificity are underlined. Primer 'N-common reverse' (marked with asterisk\*) was originally designed by Awata et al. [16].

The allelic-specific reaction was deemed positive if the NEUROD signal emerged earlier than the control (albumin) signal, or not later than two cycles behind the control signal. The allelic-specific reaction was deemed negative if the NEUROD specific signal did not appear, or if it appeared ten or more cycles behind the albumin signal. A reaction was inconclusive if amplification of neither albumin nor NEUROD was seen in a particular well, or if the NEUROD signal lagged behind albumin by three to nine cycles. It should be noted, however, that false reactivity occurred most often more than 15 cycles after the specific signal, so that it was easy to distinguish between alleles by comparing the pair of the allele-specific threshold cycles. An example of results of two samples is shown in Fig. 1.

#### 2.2.2. *The Pro197His reaction*

The Pro197His reaction was carried out using the same PCR setup as for the Ala45Thr with primers listed in Table 1. The His197 reaction was run in every sample to find this infrequent allele, while the Pro197 reaction was run only to confirm heterozygosity in subjects carrying the His197 allele.

#### 2.2.3. *Specificity of the novel method*

Specificity of the novel method was verified using several previously typed samples (courtesy of L. Hansen, Steno Diabetes Center, Denmark), and also 200 samples simultaneously typed for the Ala45Thr polymorphism using PCR with subsequent digestion of the product with restriction enzyme (PCR-restriction fragment length polymorphism, RFLP) according to Awata et al. [16]. The results were concordant in 198/200 samples, the two discordant samples being considered as an error in reading the RFLP electrophoresis pattern.

#### 2.3. *Genotyping of the insulin gene and the HLA-DQ loci*

To adjust for potentially modifying or interacting factors, genotypes of the HLA-DQB1, -DQA1 and the insulin gene were determined in patients and control subjects. The HLA-DQB1 and -DQA1 genotypes were typed for using PCR with se-

quence-specific primers [19]. The genotype of the insulin gene was determined using –23HphI restriction fragment length polymorphism according to Undlien et al. [20], because this polymorphism is known to be in tight linkage disequilibrium in European populations with the etiological VNTR within the insulin gene promoter.

#### 2.4. *Statistical analysis*

The risk of Type 1 DM was first calculated using odds ratios with 95% confidence intervals from the phenotypic, genotypic and allelic frequencies of the NEUROD variants. Analysis was performed for the whole dataset, and then stratified according to sex, age at diabetes onset, and genetic risk factors other than NEUROD. Then the NEUROD Ala45 and Thr45 positivity was entered into a multivariate logistic regression model together with presence of the HLA-DQ risk molecules DQB1\*0302-DQA1\*03 (DQ8), DQB1\*0201-DQA1\*05 (DQ2) and positivity of the protective allele of the insulin gene –23HphI, with Type 1 diabetes as the dependent variable. Two-way interaction terms between the NEUROD and the other genetic factors were tested. The calculations were performed using the STATVIEW 5.0 software (SAS Institute Inc).

### 3. Results

The genotypic, phenotypic and allelic frequencies of the NEUROD Ala45Thr polymorphism in cases and control subjects are shown in Table 2. None of the crude differences were statistically significant. Stratification of the dataset according to sex, age at diabetes onset, and presence of the HLA risk molecules DQ8 and DQ2 revealed no significant association of the NEUROD polymorphisms. There was no influence of the NEUROD Ala45Thr polymorphism on age at Type 1 DM onset, either.

The NEUROD association was then tested using multivariate logistic regression. Best fit was found for a model which included NEUROD Thr45 and Ala45 phenotypic positivity together with genetic factors associated with Type 1 DM in

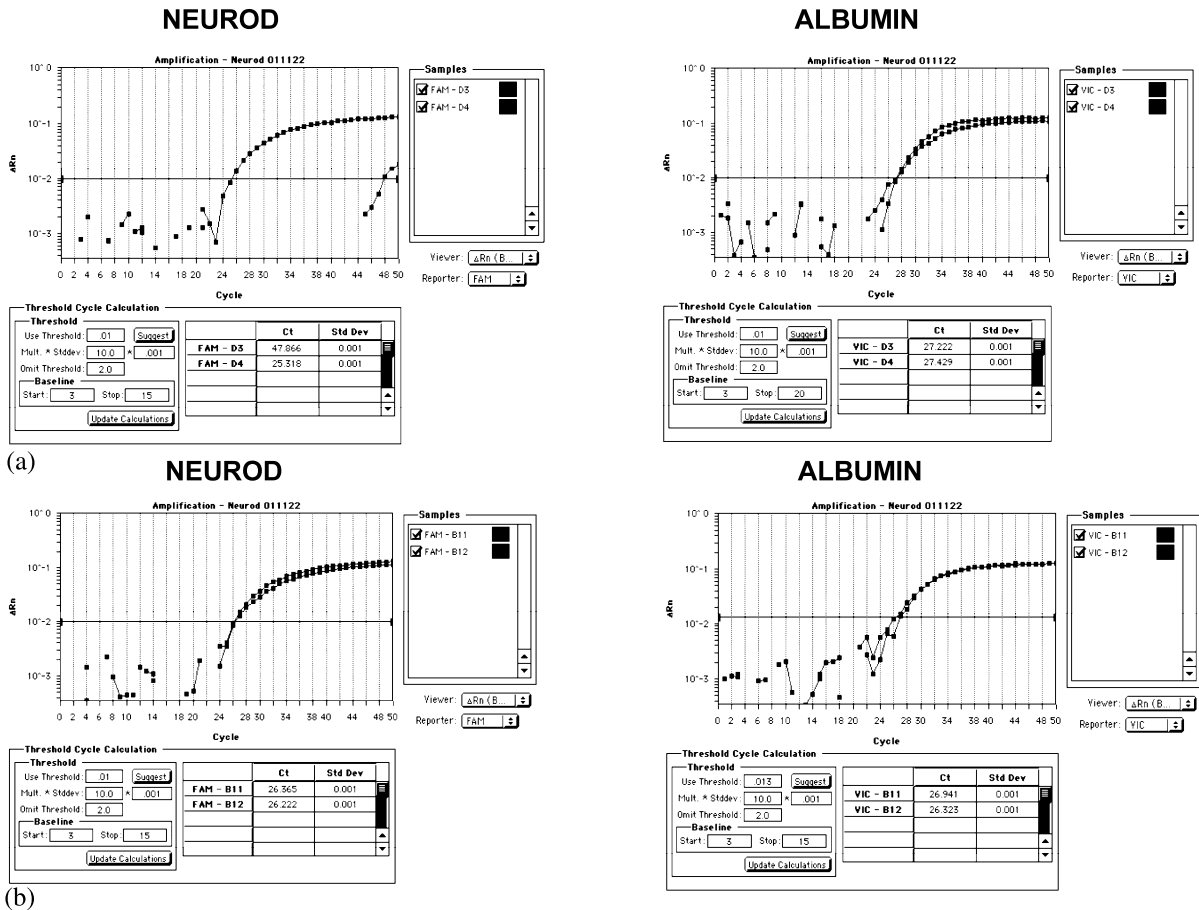


Fig. 1. An example of the real-time amplification curves. The left charts show the signals of the FAM-labelled NEUROD-specific probe in the Ala-, and Thr-specific reactions. The right charts show the VIC-labelled albumin control signal. Vertical axis, signal of the probe on a logarithmic scale; horizontal axis, PCR cycle. (A) NEUROD Thr45 homozygote. Left, the Thr45-specific signal emerges at cycle 25, while the Ala45-specific signal shows a weak cross-reactivity 22 cycles later. The PCR run was extended to 50 cycles to demonstrate this late cross-reactivity. Right, the control threshold cycle is equal in both reactions. (B) NEUROD Ala45/Thr45 heterozygote. The Ala45 and the Thr45-specific signals emerge equally at cycle 26 (left), the control threshold cycle is also equal in both reactions (right).

the Czech population: the HLA-DQB1\*0302-DQA1\*03 positivity, the DQB1\*0201-DQA1\*05 positivity [21], and presence of the protective allele of the insulin gene polymorphism (data on association of these factors with Type 1 DM are shown in Table 3). In this model, Thr45 positivity is associated with risk of Type 1 diabetes, OR = 2.01 (CI 95% 1.25–3.24), while Ala45 positivity is not associated with the disease, OR = 0.96 (CI 95% 0.51–1.82), Table 4. The NEUROD Thr45-asso-

ciated risk is detectable only when the HLA-DQB1\*0302-DQA1\*03, and DQB1\*0201-DQA1\*05 are included in the model. None of the two-way interaction terms was significant; therefore, interactions were not included in the final model.

The phenotypic positivity of the T (His) allele of the Pro197His polymorphism did not differ significantly between individuals with Type 1 DM (15/285, 5.3%) and control subjects (17/289, 5.9%).

Table 2  
NEUROD Ala45Thr genotypic, phenotypic and allelic frequencies in children with Type 1 DM, and in healthy control subjects

	Type 1 DM ( <i>n</i> = 285)	Controls ( <i>n</i> = 289)	OR	CI 95%
<i>Genotypic frequencies</i>				
Ala/Ala	95 (33%)	117 (40%)	0.74	0.52–1.03
Ala/Thr	145 (51%)	130 (45%)	1.27	0.91–1.76
Thr/Thr	45 (16%)	42 (15%)	1.10	0.70–1.74
<i>Phenotypic frequencies</i>				
Ala positive	240 (84%)	247 (85%)	0.91	0.57–1.43
Thr positive	190 (67%)	172 (60%)	1.36	0.97–1.91
	<i>n</i> = 570	<i>n</i> = 578		
<i>Allelic frequencies</i>				
Ala allele	335 (59%)	364 (63%)	0.84	0.66–1.06
Thr allele	235 (41%)	214 (37%)	1.19	0.94–1.51

#### 4. Discussion

Childhood-onset Type 1 DM is associated with the NEUROD Ala45Thr but not with the Pro197-His polymorphism in the Czech population. This positive association confirms reports of an association of this allele in Japanese adult-onset [10] and Japanese childhood-onset Type 1 diabetic patients [13]. However, in a study in Danish Caucasians [12], positive association with Type 1 DM was found for the Ala45, not the Thr45 allele. Data from a functional study presented in the same paper show that the known allelic variants of NEUROD have similar transcriptional activity on the human insulin promoter. As has recently been discussed in greater detail by Mochizuki et al. [13], these results stress that the Ala45Thr probably does not play an etiological role within IDDM7, being more likely to be in linkage disequilibrium with another yet unidentified polymorphism. NEUROD is not the only candidate within the IDDM7 locus. The locus includes also the inter-

leukin-1 gene cluster, HOXD8, GAD1, and GALNT3, but sufficient evidence of linkage or association for any of the genes is still missing [22]. GALNT3 would be a particularly promising candidate. This gene encodes *N*-acetyl-galactosaminyltransferase-T3, which might influence autoimmunity by glycosylating autoantigens. No linkage of GALNT3 with Type 1 DM was, however, found in a study of 241 Danish IDDM multiplex families [23].

The association we found for the Thr45 allele is rather weak and is significant only after considering other genetic risk factors as covariates. We analysed our data using logistic regression in a model which included the NEUROD Ala45 and Thr45 phenotypic positivity, as well as HLA-DQB1\*0302-DQA1\*03 (DQ8) and DQB1\*0201-DQA1\*05 (DQ2) phenotypic positivity, and positivity of the insulin gene protective allele. Absence of significance for any of the two-way interaction terms in the regression analysis suggests that the NEUROD variants act independently on the HLA

Table 3  
Phenotypic frequencies of the HLA-DQB1\*0302-DQA1\*03 (DQ8), DQB1\*0201-DQA1\*05 (DQ2) molecules, and of the insulin gene –23HphI ‘–’ allele (lacking the restriction site) which were previously found associated with Type 1 DM in the Czechs

Phenotypic frequencies	Type 1 DM ( <i>n</i> = 285)	Controls ( <i>n</i> = 289)	OR	CI 95%
HLA-DQB1*0302-DQA1*03	184 (65%)	49 (17%)	8.92	6.03–13.2
HLA-DQB1*0201-DQA1*05	149 (52%)	58 (20%)	4.36	3.01–6.32
Protective allele –23HphI ‘–’ of the insulin gene	63 (22%)	137 (47%)	0.31	0.22–0.45

Table 4

Risk of Type 1 DM associated with the NEUROD Ala45 and Thr45 phenotypic positivity analysed using multivariate logistic regression in a model which includes genetic factors previously associated with Type 1 DM

Genetic factor	OR	CI 95%	P
Thr45 phenotypic positivity	2.01	1.25–3.24	0.0040
Ala45 phenotypic positivity	0.96	0.51–1.82	0.90
HLA DQA1*03-DQB1*0302 positivity	16.4	9.95–27.0	< 10 <sup>-4</sup>
HLA DQA1*05-DQB1*0201 positivity	9.19	5.57–15.2	< 10 <sup>-4</sup>
Protective allele –23HphI ‘-’ of the insulin gene	0.27	0.17–0.43	< 10 <sup>-4</sup>

and insulin genotypes, unlike in the recent study in Japanese children [13]. However, drawing conclusions on potential interaction or epistasis based on two studies in children of different ethnic origin and genetic background would be premature. More NEUROD association studies which will also take account of the other genetic risk factors are, therefore, needed.

The underlying reason for adjusting, stratification or matching for the HLA-conferred risk is that non-HLA factors may be overshadowed by the strong effect of the HLA risk genotypes. Mochizuki et al. [13] handled the HLA-conferred risk by dividing the patients into groups according to HLA-DRB1\*0901 positivity which confers the risk of Type 1 DM in Japanese. Another frequently used strategy in studies of non-HLA associations is matching controls according to the HLA-conferred risk [24]. Usefulness of this has, however, been disputed [25]. Matching for the risk conferred by the HLA and the insulin gene was used in a study by Owerbach et al. [14] who examined putative genes on 2q31–35, finding no association of the NEUROD Ala45Thr with Type 1 DM in American Caucasians.

We used a novel real-time PCR-SSP assay for determining the NEUROD genotypes. Generally, allelic discrimination in the real-time TaqMan format can be accomplished using two different approaches. One approach uses allele-specific probes (e.g. [26]) which requires one probe for each allele of the SNP. We used the other approach—adapting a PCR-SSP assay to the real-time format by adding a probe annealing to a conserved sequence of the PCR product. In the latter approach, specificity is maintained by relatively inexpensive primers, not by the expensive

TaqMan probes, so that the assay can be conveniently used in multiallelic systems like HLA [27]. Our assays used one TaqMan probe for both the NEUROD Ala45Thr and the Pro197His typings, since the 3′-ends of the allele-specific primers are oriented towards each other. This would also enable determining the phase of the Ala45Thr-Pro197His SNPs if needed.

In conclusion, our study shows a significant association of the NEUROD Thr45 variant with Type 1 DM. This association is present only when the positivity for the HLA-DQ Type 1 DM-associated molecules is included among covariates in the regression model. The Ala45Thr variant is unlikely to be the etiological mutation since the alleles associated with Type 1 DM differ between populations.

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