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Cytokine Gene Polymorphisms in Italian Preterm Infants: Association Between Interleukin-10 –1082 G/A Polymorphism and Respiratory Distress Syndrome

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ABSTRACT: In this study, we determined the genotype frequencies of polymorphisms of cytokine genes and investigated their association with the risk of respiratory distress syndrome (RDS) in preterm infants. Genetic polymorphisms in the cytokines interleukin (IL)-10, IL-8, and tumor necrosis factor (TNF) α, were studied in 342 white Italian newborns (112 without RDS, 66 prematurely born with RDS, and 164 infants born at term who were included as healthy controls). The polymorphisms were analyzed by polymerase chain reaction (PCR) restriction fragment length polymorphism (RFLP). The IL-10 mRNA levels were analyzed according to genotype by quantitative real-time PCR (QRT-PCR) in Epstein-Barr virus–transformed lymphoblastoid cell lines (EBV-LCLs) of 42 full-term healthy infants. Logistic regression analysis demonstrated the risk of RDS to be significantly lower in preterm infants with an IL-10 –1082 GG/GA genotype than in those with an AA genotype [odds ratio (OR) = 0.48, 95% confidence interval (CI): 0.24–0.95, $p = 0.03$]. QRT-PCR analyses showed that the IL-10 mRNA levels were significantly higher in 27 IL-10 –1082 GG/GA carriers compared with 15 IL-10 –1082 AA carriers ($p = 0.03$). We conclude that the IL-10 –1082 GG/GA polymorphism may have a role in RDS development in premature infants. (***Pediatr Res* 61: 313–317, 2007**)

Neonatal RDS is mainly caused by structural immaturity of the lung and a deficiency in pulmonary surfactant (the complex lipoprotein mixture that is produced by type II alveolar epithelial cells). The surfactant itself is made up of 90% phospholipids and 10% surfactant proteins (SPs), the latter of which include SP-A, SP-B, SP-C, and SP-D (1). Furthermore, genetic factors appear to have pivotal roles in RDS, as has been suggested by genetic studies of twins and by epidemiologic data.

The most promising candidates for this surfactant deficiency that have been identified to date lie in the genes coding for the lung-specific protein components of the pulmonary surfactant, and especially those for SP-A and SP-B. Indeed, polymorphisms in these genes have been associated with susceptibility to RDS (2–4), although, overall, the etiology of RDS is considered to be multifactorial and possibly multigenic.

Apart from a structural immaturity of the lung and surfactant deficiency and dysfunction, the current evidence shows unequivocally that lung inflammation is involved in RDS (5,6). Cytokines may be the regulators of surfactant metabolism in the preterm infant (7), with SP-A and SP-B being involved in the maintenance of an infection-free and inflammation-free lung (8). Interstitial inflammation, as revealed by immunohistochemistry in 40 infants who died in the first week of life due to acute RDS, has been shown to be maximal at 72 h of age, although it had been present within hours of birth (9). Moreover, it has been shown that in preterm infants with RDS, the activation of circulating polymorphonuclear leukocytes has a role in the pathogenesis of this syndrome (10–12). In contrast, neither the mRNA nor the protein for the counterregulatory cytokine IL-10 were detectable in similar samples obtained during the first month of life compared with those obtained from infants born at term (13,14). Therefore, it appears that proinflammatory and anti-inflammatory cytokines are involved in the development of RDS.

The promoter regions of a number of key cytokine genes contain polymorphisms that can directly influence cytokine production (15). Based on these observations, we hypothesized that abnormal cytokine production arising from specific polymorphisms may be involved in the development of RDS. Therefore, we undertook a case-control association study in preterm newborns to determine whether polymorphisms associated with the differential expression of IL-10, IL-8, and TNF-α are associated with susceptibility to RDS.

PATIENTS AND METHODS

Blood sample collection and study population. From 1999 to 2004, blood samples were obtained for genotype analysis from 342 white newborn infants from Southern Italy, after informed parental consent. The study protocol was approved by the local institutional review board. There were 178 newborn infants (112 without RDS, 66 with RDS) born prematurely (<36 wk of gestation) (Table 1) and 164 born at term without a family history of neonatal

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Abbreviations: EBV-LCLs, Epstein-Barr virus–transformed lymphoblastoid cell lines; **NF-κB,** nuclear factor κB; **QRT-PCR,** quantitative real-time PCR; **SNPs,** single nucleotide polymorphisms; **SP,** surfactant protein

Table 1. *Characteristics of the infants with RDS and the premature controls*

	RDS	No RDS
Total no. of cases	66	112
Females, no.	39	67
Males, no.	27	45
Gestational age, wk*	30.4 ± 0.29; 31	32.3 ± 0.22; 33
Birth weight, g†	1437 ± 61.08, 1437	1846 ± 62.90, 1700

*Mean ± SEM; median (RDS: 24–35 wk; no RDS: 24–35 wk).
†Mean ± SEM; median (RDS: 580–2575 g; no RDS, 600–3590 g).

RDS (healthy controls). None of the infants included in the analysis was transfused.

The diagnosis of RDS was made based on the reported clinical (grunting, retraction, flaring, need for supplementary O_2 for >48 h or the need for exogenous surfactant therapy), radiographic (diffuse reticulogranular pattern and air bronchograms), and/or pathologic (diffuse atelectasis and hyaline membranes) criteria. None of the newborn infants had had treatment with prophylactic surfactant. Also, antenatal steroid therapy was not administered to any, and none developed bronchopulmonary dysplasia (BPD).

DNA extraction. Genomic DNA was isolated from ethylenediamine tetraacetic acid anticoagulated whole blood specimens using the Wizard Genomic DNA Purification Kit (Promega Corporation, Madison, WI).

PCR. PCR amplifications of all the promoters for the IL-10, IL-8, and TNF-α genes were performed with the primer sequences given in Table 2. The PCR mixtures contained 100 ng genomic DNA, 0.1 mmol/L each of the deoxynucleotide triphosphates, 12.5 pmol each of the primers, and 1 U of *Taq* polymerase (Applied Biosystems, Branchburg, NJ) in a final volume of 25 μL. PCR was performed using a 9700 Gene Amp PCR System Thermal Cycler (Applied Biosystems), according to the following thermocycler conditions: 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, T_A (annealing temperature) for each single nucleotide polymorphism (SNP) for 30 s (Table 2), and 72°C for 30 s, with a final extension at 72°C for 7 min.

Genotyping. The IL-10 –1082 G/A (rs1800896), IL-10 –592 A/C (rs1800872), IL-8 –251 A/T (rs4073), and TNF-α –308 G/A (rs1800629) SNPs were genotyped by RFLP. The RFLP assays were performed in 15-μL reaction volumes containing the PCR products and the specific restriction enzyme for each of the SNPs. In the presence of the IL-10 –1082 G allele, *Ear I* (New England Biolabs, Ipswich, MA) cut its 155-bp PCR product into two bands of 128 and 27 bp. In the presence of the IL-10 –592 A allele, *Rsa*I (New England Biolabs) cut its 154-bp PCR product into two bands of 79 and 75 bp. Similarly, for the IL-8 –251 A allele, *Mfe I* (New England Biolabs) cut its 223-bp PCR product into two bands of 113 and 110 bp, and for the TNF-α –308 G allele, *Nco*I (New England Biolabs) cut the 224-bp PCR product into two bands of 208 and 16 bp. These digestion products were visualized in 3.5% agarose gels that were stained with ethidium bromide. This genotyping by RFLP was also confirmed by automated DNA sequencing.

Generation of cell lines. EBV-LCLs were established by culturing peripheral blood mononuclear cells in the presence of 100 μg/mL cyclosporine (Sigma Chemical Co. Aldrich, Milan, Italy) with the EBV supernatant harvested from the B95-8 cell line (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). The EBV-LCLs were maintained in RPMI 1640 medium supplemented with 20% fetal bovine serum, 2 mmol/L glutamine, 20 U/mL penicillin, and 0.2 mg/mL streptomycin (CELBIO, Pero, Milan, Italy).

RNA isolation and reverse transcription. Total cellular RNA was extracted from the cultured cells by the guanidinium isothiocyanate method, using the TRIzol reagent (16). All the reverse transcriptase reactions were performed using SuperScript II RNase H-Reverse Transcriptase (Invitrogen, San Diego, CA), with oligo-dT priming, according to the manufacturer's instructions.

QRT-PCR analysis. QRT-PCR was performed using the SYBER green method, with an Applied Biosystem Model 7900HT Sequence Detection System. The primers were designed with the Primer Express 2.0 program (Applied Biosystems), and the sequences were as follows: IL-10 (forward) 5′-ACCTGCCTAACATGCTTCGAG-3′, (reverse) 5′-TGTCCAGCTGATCCTTCATTTG-3′, and β-actin (forward) 5′-CGTGCTGCTGACCGAGG-3,' (reverse) 5′-GAAGGTCTCAAACATGATCTGGGT-3′. All the PCRs were performed in duplicate. The relative gene expressions were calculated using the $2^{(-\Delta Ct)}$ method, in which Ct indicates the cycle threshold, the fractional cycle number at which the fluorescent signal reaches the detection threshold. β-Actin was used as the internal control (17). The ΔCt was calculated using the differences in the mean Ct between the IL-10 gene and the internal controls.

Statistical analysis. A comparison of the genotypic and allelic frequencies between the groups was performed using the χ^2 test with Yates correction for the specific case of 2×2 tables. Multivariate logistic regression analysis was performed to investigate whether heterozygosity or homozygosity for particular alleles could explain the risk of RDS. ORs of the genotypic variables and confounders for RDS (gestational age at birth, gender) included in the analysis were estimated using a logistic regression model, where the presence or absence of RDS was the dependent variable. For QRT-PCR, the data were presented as median and mean ± standard error of the mean (SEM). The significance of the relative gene expression differences was determined using the Mann-Whitney *U* test. The Shapiro-Wilk test was used for testing normality. Statistical significance was established at $p = 0.05$.

RESULTS

Allele and genotype frequencies. DNA samples from 64 prematurely born infants with RDS and 112 premature control infants were available for the genotype analysis of the selected polymorphisms. Their clinical data are given in Table 1.

Table 3 summarizes our findings on the allelic and genotypic distributions of these cytokine gene SNPs in all the samples collected. The Hardy-Weinberg equilibrium was checked in all three of the groups (RDS, no RDS, and healthy controls) by χ^2 tests for each polymorphism. All the markers were in Hardy-Weinberg equilibrium.

The allele and genotype frequencies of the IL-10 –592 A/C, IL-8 –251 A/T, and TNF-α –308 G/A polymorphisms were not significantly different between the two preterm infant groups (Table 3). The genotypic and allelic distributions of the polymorphism at position –1082 of IL-10 were instead significantly different in the preterm infants with RDS compared with the preterm infants without RDS ($p = 0.03$ and $p = 0.02$, respectively) (Table 3). To better define the independent contributions of the heterozygosity and homozygosity for the G allele to genetic susceptibility to RDS, we performed multivariate logistic regression analysis. When the genotypic

Table 2. *Characteristics of the cytokine gene polymorphisms, primer sequences and PCR conditions*

Gene	Promoter position of the polymorphism	Allele	Phenotype	Primer sequences 5′-3′	T_A(°C)
IL-10	–1082	G	High	F: CCAGGTAGAGCAACACTCCT	55
		A	Low	R: CTCTTACCTATCCCTACTTCCGC	
IL-10	–592	C	High	F: GTGGAAACATGTGCCTGAGA	58
		A	Low	R: ATGAGGGGGTGGGCTAAATA	
IL-8	–251	A	High	F: TATGCCATTAAAAGAAAATCATCCAT	60
		T	Low	R: TCAAATACGGAGTATGACGAAAGTTT	
TNF-α	–308	A	High	F: AATAGGTTTTGAGGGCCATG	55
		G	Low	R: TCATCTGGAGGAAGCGGTAG	

T_A: Annealing temperature for each individual SNP; F: forward; R: reverse.

Table 3. *Absolute allele and genotype frequencies in white newborns in southern Italy*

Geneticpolymorphisms	Prematurely born: No RDS (n = 112), No. (%)	Prematurely born: RDS (n = 64), No. (%)	Full term Controls* (n = 164), No. (%)	p†
IL-10–1082 G/A				
Genotypes				
AA	38 (33.9)	35 (54.7)	73 (44.5)	0.03
GA	58 (51.8)	23 (35.9)	66 (40.2)	
GG	16 (14.3)	6 (9.4)	25 (15.2)	
Alleles				
A	134 (59.8)	93 (72.7)	212 (64.6)	0.02
G	90 (40.2)	35 (27.3)	116 (35.4)	
IL-10 –592 A/C				
Genotypes				
AA	9 (8.0)	5 (7.8)	13 (7.9)	0.99
AC	53 (47.3)	30 (46.9)	74 (45.1)	
CC	50 (44.6)	29 (45.3)	77 (47.0)	
Alleles				
A	71 (31.7)	40 (31.3)	100 (30.5)	0.97
C	153 (68.3)	88 (68.7)	228 (69.5)	
TNF-α –308 G/A				
Genotypes				
AA	2 (1.8)	2 (3.1)	0 (0.0)	0.71
GA	16 (14.3)	7 (10.9)	27 (16.5)	
GG	94 (83.9)	55 (85.9)	137 (83.5)	
Alleles				
A	20 (8.9)	11 (8.6)	27 (8.2)	0.93
G	204 (91.1)	117 (91.4)	301 (91.8)	
IL-8 –251 A/T				
Genotypes				
AA	17 (15.2)	11 (17.2)	36 (22.0)	0.23
TA	53 (47.3)	37 (57.8)	74 (45.1)	
TT	42 (37.5)	16 (25.0)	54 (32.9)	
Alleles				
A	87 (38.8)	59 (46.1)	146 (44.5)	0.22
T	137 (61.2)	69 (53.9)	182 (55.5)	

* The genotype and allele frequencies of healthy controls were not significantly different compared with the non-RDS patients.

† Significance of difference between non-RDS and RDS groups.

variables (classified into two categories: AA and GG or GA) were included in the model, after the correction for gestational age (number of weeks) and gender, they were seen to be independently associated with RDS. The data showed that the risk of RDS was significantly lower in preterm infants with the GG and GA genotypes than with those with the AA genotype (OR = 0.48; 95% CI: 0.24–0.95; p = 0.03). This correction for gestational age and gender in the multivariate analysis excluded the possibility that the association of the IL-10 –1082 GG/GA genotype with a lower risk of RDS was influenced by different gestational age and sex distributions among the RDS and non-RDS groups. None of these polymorphisms (IL-10 –1082 G/A, IL-10 –592 A/C, IL-8 –251 A/T, TNF-α –308 G/A) differed significantly in allele or genotype distribution between the control premature (n = 112) and term (n = 164) infants.

Differential IL-10 gene expression associated with the –1082 G/A polymorphism. To determine whether any variant of IL-10 –1082 G/A could affect IL-10 expression, we established EBV-LCLs from the peripheral blood mononuclear cells of 42 full-term healthy infant controls: 15 with the AA genotype, and 27 with the GG/GA genotype (seven with the GG and 20 with the GA genotype). The QRT-PCR analyses were then applied, which showed that IL-10 mRNA levels were significantly higher in the IL-10 –1082 GG/GA carriers (n = 27; median, 1.43; mean, 1.81 ± 0.19) than in the IL-10 –1082 AA carriers (n = 15; median, 1.01; mean, 1.13 ± 0.19) (p = 0.03) (Fig. 1). The $2^{(-\Delta Ct)}$ values of the GG/GA group were not normally distributed, as seen by the Shapiro-Wilk test ($p < 0.05$ for GG/GA group; p = 0.41 for AA group); we therefore used a nonparametric test (Mann-Whitney U) to compare the mRNA levels between these two groups.

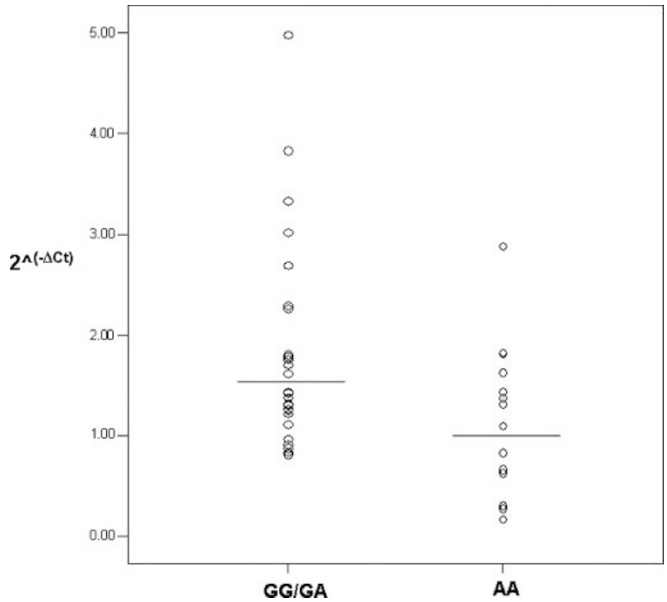

Figure 1. Differential IL-10 gene expression associated with the –1082 G/A polymorphism in EBV-LCLs in peripheral blood mononuclear cells of 42 full-term healthy infant controls: 15 with the AA genotype and 27 with the GG/GA genotype (20 with GA, seven with GG). QRT-PCR analysis of IL-10 gene expression showed significant increases in the GG/GA subjects (median, 1.43; mean 1.81 ± 0.19), with respect to the AA subjects (median, 1.01; mean, 1.13 ± 0.19) (p = 0.03). The y axis of $2^{(-\Delta Ct)}$ represents the relative gene expression of IL-10. The ΔCt values were calculated using the differences in the mean Ct values between the IL-10 gene and the internal control of β-actin.

DISCUSSION

Here, we investigated the cytokine IL-10 –1082 G/A, IL-10 –592 A/C, IL-8 –251 A/T, and TNF-α –308 G/A gene promoter polymorphisms and determined their relationships to the occurrence of RDS in a population of preterm newborns in southern Italy. The data show that the IL-10 –592, IL-8 –251 and TNF-α –308 genotypes did not differ significantly between the RDS and non-RDS groups. In contrast, significant differences were seen for the frequencies of the IL-10 –1082 genotypes and alleles. Furthermore, our results show that the IL-10 –1082 GG/GA genotype carriers were significantly more frequent in the non-RDS group than in the RDS group after correction for gestational age and sex. The comparison between the control preterm and term neonates showed that there were no differences in the frequencies of the same

polymorphisms, indicating that prematurity is unlikely to be a confounder in our analyses.

IL-10 is an important immunoregulatory cytokine that is mainly produced by monocytes, macrophages, T cells, and B cells. It controls the inflammatory processes by suppressing the expression of proinflammatory cytokines, chemokines, adhesion molecules, and antigen-presenting and costimulatory molecules in monocytes/macrophages, neutrophils, and T cells (18). Several studies of complex diseases have indicated a major role for IL-10 in chronic inflammatory disorders that are characterized by the predominance of cytokines such as IL-1, IL-6, IL-8, IL-12, and TNF. These included psoriasis, Crohn's disease, multiple sclerosis, and rheumatoid arthritis (18,19).

The precise mechanisms involved in the regulation of IL-10 production remain to be determined, although inherited factors appear to have important roles. A difference in IL-10 secretion in association with a SNP in the –1082 position of the gene promoter has been demonstrated. In particular, an association of the –1082 G allele with a high IL-10–producing ability has been shown, through assessments of both mRNA and protein (20–22). Here, we see that EBV-LCLs from full-term healthy infant controls carrying the IL-10 –1082 G allele (the IL-10 –1082 GG/GA genotypes) have increased IL-10 mRNA levels compared with those with an IL-10 –1082 AA genotype. The IL-10 –1082 G/A SNP is located within an Ets binding site. The –1082A allele confers a higher binding affinity to the transcription factor PU.1, which inhibits gene expression and leads to decreased IL-10 expression in individuals carrying this allele (23); thus, it is plausible that the IL-10 –1082 GG and GA genotypes also have high expression rates in the premature lung.

To date, there have been a few studies that have documented the differences in IL-10 detection and quantities in premature infants with RDS, in terms of whether they develop BPD (13,24,25). However, there have been no previous studies that have looked for an association between genetic susceptibility to neonatal lung disease and IL-10 gene polymorphisms. Only in a study by Yanamandra *et al.* (26) was it possible to show that the IL-10 –1082 A allele (for lower IL-10 production) had a minor effect on the combined outcome of death or BPD. Therefore, it appears that low IL-10 production and the consequent increase in the proinflammatory cytokines are associated with a favorable outcome for BPD. This hypothesis is in contrast with previous studies that have demonstrated that multiple proinflammatory and chemotactic factors are present in the air spaces of ventilated preterm infants and that these factors are found in higher concentrations in the air spaces of infants who subsequently develop BPD. Factors such as macrophage inflammatory protein-1 and IL-8 persist in the air spaces, and counterregulatory cytokines, such as IL-10, may be decreased, resulting in unregulated and persistent inflammation (27,28). We show that heterozygosity or homozygosity for the IL-10 –1082 G allele (for higher IL-10 production) is more frequent among infants without RDS, suggesting a protective role in this pathology. Although contrary to the data of Yanamandra *et al.* (26), our data are consistent with previous findings that have seen no decrease in or absence of IL-10 expression at the levels of mRNA and protein in cells obtained through bronchoalveolar lavage from lungs of premature neonates with hyaline membrane disease (13,14). Moreover, the development of recombinant IL-10 as a potential anti-inflammatory agent in the treatment of hyaline membrane disease has been suggested (29). The IL-10 –1082 GG genotype has been reported to be a protecting factor against other pulmonary diseases, such as acute RDS and active tuberculosis (30,31). However, further studies are needed to confirm that our observations do indeed apply to other patient populations. Other SNPs in the promoter region of the IL-10 gene may also be associated with increased or decreased IL-10 production. For instance, recent studies have indicated that the T–3575A, G–2849A, and C–2763A SNPs are associated with susceptibility to systemic lupus erythematosus (32), and leprosy and disease severity in leprosy (33). It is therefore important to investigate the roles of these and other SNPs and haplotypes in RDS in future studies.

Surfactant deficiency in preterm infants leads to RDS, although some of the available data also suggest participation of local inflammation and its interaction with the surfactant system. The SPs SP-A and SP-D are important in the innate host defense system against pathogenic microorganisms and in the modulation of acute inflammation (8). It has been demonstrated that when IL-1α is given intra-amniotically to immature rabbit or sheep, it increases SP-A and SP-B mRNA levels and decreases the severity of RDS after premature birth (34,35). Additionally, another study revealed that IL-1α has a biphasic influence on the expression of the SPs, thus increasing SP-A and SP-B mRNA in early premature lung and decreasing the expression SP-B and SP-C in the transitional and mature lung (36). Watterberg *et al.* (37) noted that in early premature infants with intrauterine infection, the incidence of RDS is lower than that expected based on their very early birth. Hallak and Bottoms (38) showed that premature infants who were more mature than in the series presented by Watterberg *et al.* (37) had an increased incidence of RDS in intrauterine infection. Moreover, circulating polymorphonuclear leukocytes are activated in preterm infants with RDS, and this appears to have a role in pathogenesis, with leukocyte activation present only 2 h after birth (10–12). We hypothesize that due to a specific genotype of IL-10 –1082 G/A polymorphism in our population study, the low proinflammatory cytokine levels correlate with a lower risk of RDS. This hypothesis is supported by a number of studies demonstrating that IL-10 inhibits nuclear factor-κB (NF-κB) activity (39,40). Many of the proinflammatory cytokines and costimulatory proteins that have been demonstrated to be suppressed by IL-10 are known to be regulated by the NF-κB transcription factor. Thus, it has been seen that patients with RDS had an increased expression of NF-κB in their alveolar macrophages on d 2 and 4, compared with a control group, and that the IL-1β and IL-8 levels were closely correlated to NF-κB expression (41). Therefore, we believe that the IL-10 –1082 GG/GA genotype is potentially associated with a decreased NF-κB activity that results in the down-expression of proinflammatory cytokines. Further studies need to be performed to address this hypothesis.

In conclusion, this study suggests that the IL-10 –1082 GG/GA genotype is associated with a decreased risk of RDS. This association is biologically acceptable because the G allele has been associated with increased IL-10 production and decreased production of proinflammatory cytokines, which have been associated with increased severity of RDS in preterm infants. Our results may contribute toward the development of new therapies.

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