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Genetics

Mutation spectrum of the rhodopsin gene among patients with autosomal dominant retinitis pigmentosa

(guanine nucleotide-binding protein receptor/photoreceptor/retinal degeneration)

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ABSTRACT **We searched for point mutations in every exon of the rhodopsin gene in 150 patients from separate families with autosomal dominant retinitis pigmentosa. Including the 4 mutations we reported previously, we found a total of 17 different mutations that correlate with the disease. Each of these mutations is a single-base substitution corresponding to a single amino acid substitution. Based on current models for the structure of rhodopsin, 3 of the 17 mutant amino acids are normally located on the cytoplasmic side of the protein, 6 in transmembrane domains, and 8 on the intradiscal side. Forty-three of the 150 patients (29%) carry 1 of these mutations, and no patient has more than 1 mutation. In every family with a mutation so far analyzed, the mutation cosegregates with the disease. We found one instance of a mutation in an affected patient that was absent in both unaffected parents (i.e., a new germ-line mutation), indicating that some "isolate" cases of retinitis pigmentosa carry a mutation of the rhodopsin gene.**

Retinitis pigmentosa is the name given to a group of hereditary degenerative diseases of the retina characterized in their early stages by nyctalopia and constricted visual fields and in their later stages by the abnormal accumulation of pigmentation in the mid-peripheral retina. Affected patients have abnormal electroretinograms (ERGs) (1, 2). The disease usually leads to blindness during middle age. It currently affects between 50,000 and 100,000 people in the United States (3, 4). There is considerable genetic heterogeneity, with the disease being transmitted as an autosomal dominant trait in some pedigrees, as an autosomal recessive trait in others, and as a chromosome X-linked trait in still others.

Some patients with autosomal dominant retinitis pigmentosa carry a mutant rhodopsin gene. We have previously reported four separate mutations in the rhodopsin gene, designated Pro-23 → His, Thr-58 → Arg, Pro-347 → Leu, and Pro-347 → Ser (5–8). In addition, Inglehearn *et al.* have reported a fifth mutation, deletion of Ile-255, that also correlates with the disease (9). These previously described mutations were found by directly sequencing the exons of the gene in leukocyte DNA. This is a highly sensitive technique, but because it is relatively laborious, only a few dozen unrelated patients had been comprehensively analyzed. Here we report the results from screening a large number of patients, using a slightly less sensitive but faster technique, called "SSCP" for single-strand conformation polymorphism (10). In our experience, this technique detects approximately 90% of mutations due to single-base substitutions. The results provide a more precise measure of the proportion of patients who carry mutations of this gene and reveal that autosomal dominant retinitis pigmentosa can be caused by any of a number of mutations of the rhodopsin gene.

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METHODS

Ascertainment of Patients. We studied 150 patients from separate families with autosomal dominant retinitis pigmentosa from the United States and Canada. Patients had reduced or absent rod ERG responses to flashes of light and other signs of retinitis pigmentosa. We determined the genetic type of disease through a family history and, in some cases, by the ophthalmological examination and ERG testing of relatives. We collected between 10 and 50 ml of venous blood from each patient, using EDTA as an anticoagulant. Leukocyte nuclei were prepared from the blood samples and stored at −70°C for up to 6 yr before DNA was purified from them.

SSCP. We synthesized pairs of oligonucleotide primers, with each pair of primers designed to amplify the translated sequence within one or two exons of the rhodopsin gene by the polymerase chain reaction (see Table 1 for the sequences of the oligomers). For each analysis we added ≈100 ng (1–2 μl) of leukocyte DNA to 49 μl of 20 mM Tris (pH 8.4 or 8.6) containing 0.75–1.5 mM $MgCl_2$, 50 mM KCl, 0.02 mM dATP, 0.02 mM dTTP, 0.02 mM dGTP, 0.002 mM dCTP, 1 μCi (7 nM) of [α-^{32}P]dCTP, 0.1 μg of bovine serum albumin per ml, and 0.5 unit of *Taq* DNA polymerase. The pH, Mg concentration, and the annealing temperatures were tailored for each pair of oligomers to give optimal amplification as follows: for exon 5 and both halves of exon 1, pH 8.4, 1.0 mM $MgCl_2$, and 50°C; for exon 2, pH 8.6, 1.5 mM $MgCl_2$, and 50°C; for exon 3, pH 8.4, 0.75 mM $MgCl_2$, and 52°C; and for exon 4, pH 8.4, 1.0 mM $MgCl_2$, and 52°C. Each target sequence was amplified by 30 cycles of the polymerase chain reaction performed in a programmable temperature cycler (Ericomp, San Diego). To reduce the size of the assayed fragments to <200 bases and thereby increase the sensitivity of the SSCP technique, the amplified DNA in most cases was subsequently cleaved by adding 10–20 units of a restriction endonuclease and incubating at the appropriate temperature overnight. Table 1 lists the restriction endonuclease used for each set of primers and the resulting sizes of the assayed DNA fragments.

Five microliters of the labeled, digested DNA was diluted with 40 μl of 10 mM EDTA/0.1% SDS. Five μl of this was diluted with 5 μl of gel loading buffer (80% formamide/89 mM Tris/2 mM EDTA/89 mM boric acid, pH 8.0, with bromphenol blue and xylene cyanol added as indicator dyes) and was denatured by incubating at 95°C for 3 min. The DNA sample was cooled on ice for at least 2 min. Aliquots of 2.5–3 μl were loaded onto each of two nondenaturing 6% polyacrylamide gels, one with and one without 10% glycerol. After electrophoresis at room temperature or at 4°C, the gels were dried and exposed to x-ray film without an intensifying screen.

Abbreviations: ERG, electroretinogram; SSCP, single-strand conformation polymorphism.

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Table 1. Oligomers used for polymerase chain reaction amplification of each exon in the human rhodopsin gene

Exon	Primer pairs (sense/antisense)	Size of amplified fragment, bp	Restriction endonuclease	Size of digested fragments, bp
1				
5′ end	5′-AGCTCAGGCCTTCGCAGCAT-3′ 5′-AGAGGCGTGCGCAGCTTCTT-3′	279	*Bst*NI	155, 124
3′ end	5′-CTCTACGTCACCGTCCAGCA-3′ 5′-GAGGGCTTTGGATAACATTG-3′	320	*Bst*NI	180, 140
2	5′-GAGTGCACCCTCCTTAGGCA-3′ 5′-TCCTGACTGGAGGACCCTAC-3′	290	*Rsa* I	187, 103
3	5′-CTGTTCCCAAGTCCCTCACA-3′ 5′-CTGGACCCTCAGAGCCGTGA-3′	260	*Msp* I	132, 106
4	5′-TCTGGACCCGGGTCCCGTGT-3′ 5′-CCTGGGAGTAGCTTGTCCTT-3′	342	*Bst*NI	173, 166, 3
5	5′-ACTCAAGCCTCTTGCCTTCC-3′ 5′-GCCACAGAGTCCTAGGCAGG-3′	155	No digestion	155

bp, Base pairs.

Follow-Up Studies Performed for Variant Bands Seen by SSCP. Whenever we observed a variant band by the SSCP technique, we searched for the responsible DNA sequence variation by sequencing the corresponding leukocyte DNA sample after amplification by the polymerase chain reaction using methods previously described (11). To determine if the sequence variation cosegregated with the disease in a family, relatives of the patient were contacted and asked to donate blood. The leukocyte DNA samples from the relatives who volunteered were assayed for the variant sequence seen in the propositus either by the SSCP technique or by digestion with an endonuclease if the normal or variant sequence involved a digestion site (6). Whenever possible and especially in cases with uncertain history, appropriate relatives were examined ophthalmoscopically and by electroretinography to establish whether or not they were affected. Finally, leukocyte DNA samples from 100 control individuals without retinitis pigmentosa or a family history of the disease were also screened by SSCP analysis so that DNA polymorphisms unrelated to retinitis pigmentosa could be suitably classified.

RESULTS

We found 21 variant bands by SSCP analysis. DNA sequence analysis showed that all were due to single-base substitutions. Of these, one was an *Rsa* I restriction fragment length polymorphism (RFLP) in intron 3 that we previously reported (6). Another was a *Sac* II RFLP in the 5′ untranslated region secondary to an adenosine or guanosine found 26 bases upstream of the initiation codon. Allele frequencies were A = 0.71 and G = 0.29, based on 117 control individuals of mixed North American descent. The remaining 19 were due to changes in the DNA sequence within the open reading frame of the rhodopsin gene (see Table 2). There were 15 nucleotide transitions (7 instances of C → T, 5 of G → A, 2 of T → C, and 1 A → G) and 4 transversions (C → A, C → G, G → T, and T → G). The most frequent dinucleotide sequence affected was CpC on either the sense or antisense strand (see Table 2). Of the C → T and G → A transitions, 7 occurred at the dinucleotide CpG. Two of the C → T transitions, found in codons 120 and 173, resulted in no modification of the encoded amino acid sequence. The one within codon 120 was found in the affected members of a family who also had the Val-345 → Met mutation, and the other was found only in a "control" individual who did not have retinitis pigmentosa. We deemed these to be rare variants, and we did not study them further.

Each of the remaining 17 sequence variations altered a single amino acid. None of these was found among a group of 100 control individuals without retinitis pigmentosa. Among these 17 mutations were 4 that we reported previously (5–8). Forty-three of the 150 patients (29%) carried 1 of these 17 mutations, and no patient had more than 1 mutation. Only 3 of the mutations were found more than once among our set of 150 unrelated patients: Pro-23 → His, 19 cases; Gly-51 → Val, 2 cases; and Pro-347 → Leu, 8 cases. In every family with a mutation so far analyzed, the mutation coseg-

Table 2. Mutations found in the coding region of the rhodopsin gene

Exon	Mutation: Codon	Mutation: Sequence	Amino acid change	No. of families	CpG	CpC
1	17	ACG→ATG	Thr*→ Met°	1	Yes	No
1	23	CCC→CAC	Pro°→ His⁺	19	No	Yes
1	23	CCC→CTC	Pro°→ Leu°	1	No	Yes
1	51	GGC→GTC	Gly*→ Val°	2	No	Yes
1	58	ACG→AGG	Thr*→Arg⁺	1	Yes	No
1	89	GGT→GAT	Gly*→Asp⁻	1	No	Yes
2	125	CTG→CGG	Leu°→Arg⁺	1	No	No
2	167	TGC→CGC	Cys*→Arg⁺	1	No	No
2	171	CCA→CTA	Pro°→ Leu°	1	No	Yes
3	181	GAG→AAG	Glu⁻→Lys⁺	1	Yes	No
3	186	TCG→CCG	Ser*→ Pro°	1	No	No
3	188	GGA→AGA	Gly*→Arg⁺	1	No	Yes
3	190	GAC→AAC	Asp⁻→Asn*	1	Yes	No
3	190	GAC→GGC	Asp⁻→ Gly*	1	No	No
5	345	GTG→ATG	Val°→ Met°	1	No	Yes
5	347	CCG→TCG	Pro°→ Ser*	1	No	Yes
5	347	CCG→CTG	Pro°→ Leu°	8	Yes	Yes
		Rare variants unrelated to retinitis pigmentosa				
1	120	GGC→GGT	Gly*→ Gly*	1	Yes	No
2	173	GCC→GCT	Ala°→ Ala°	0	Yes	Yes

The superscript symbol after each three-letter amino acid abbreviation indicates its type of R group: *, polar; o, nonpolar; −, negatively charged; +, positively charged.

regated with the disease. Fig. 1 shows the structure of each pedigree that we analyzed, excluding pedigrees with 1 of the 4 mutations that we reported earlier (5–8). Fig. 2 shows an example of a typical SSCP analysis of a pedigree, in this case a family with Pro-171 → Leu. The relatives of the propositi who carried the Cys-167 → Arg and Ser-186 → Pro mutations refused to participate in this study, so that no cosegregation analysis could be performed.

In one pedigree we observed the genesis of a mutation. Fig. 3 illustrates family no. 5864, in which individual II-2 carried a C → T transition in codon 347 (Pro-347 → Leu) and passed this defect to one of her children, who also has retinitis pigmentosa. This mutation was absent in both of the parents of II-2. Analysis with 20 RFLPs revealed no indication of incorrectly designated paternity (confidence level >100,000:1; data not shown). We concluded that in this family the Pro-347 → Leu defect arose as a new germ-line mutation in II-2.

DISCUSSION

This study was undertaken to explore the range of mutations that were capable of producing autosomal dominant retinitis pigmentosa. We surveyed 150 patients for mutations within the rhodopsin gene. Our results show that any of a large number of mutations of the rhodopsin gene can cause autosomal dominant retinitis pigmentosa. Together with the abnormality reported by Inglehearn *et al.* (9)—namely, deletion of Ile-255—18 different mutations have been reported to correlate with the disease.

This number of mutations allows a preliminary compilation of the mutation spectrum of the gene, at both the DNA and the protein levels. All but 1 of the 18 known mutations are single-base substitutions, the only exception being the three-base deletion identified by Inglehearn *et al.* (9). As at most human loci where single-base substitutions have been assessed, transitions greatly outnumber transversions (12), in this case by 13 to 4. This bias for transitions occurs despite the fact that there are only four possible transitions compared to eight possible transversions. One interesting feature of these mutations is that they frequently occur within the sequence CpC. Ten of the 19 base changes, including mutations and rare variants, affect this dinucleotide sequence. Adjacent pyrimidines such as CpC are known to be hot spots for transitions induced by ultraviolet light (13, 14). However, since ultraviolet light has little access to the human germ line, it is unlikely to be the cause of the numerous transitions observed in the rhodopsin gene. A partial explanation for the excess of transitions is the apparent predisposition of a methylated cytidine in the dinucleotide CpG to mutate to a T (15). For example, 4 of the 17 mutations and 2 of the rare variants that we observed are C → T or G → A transitions

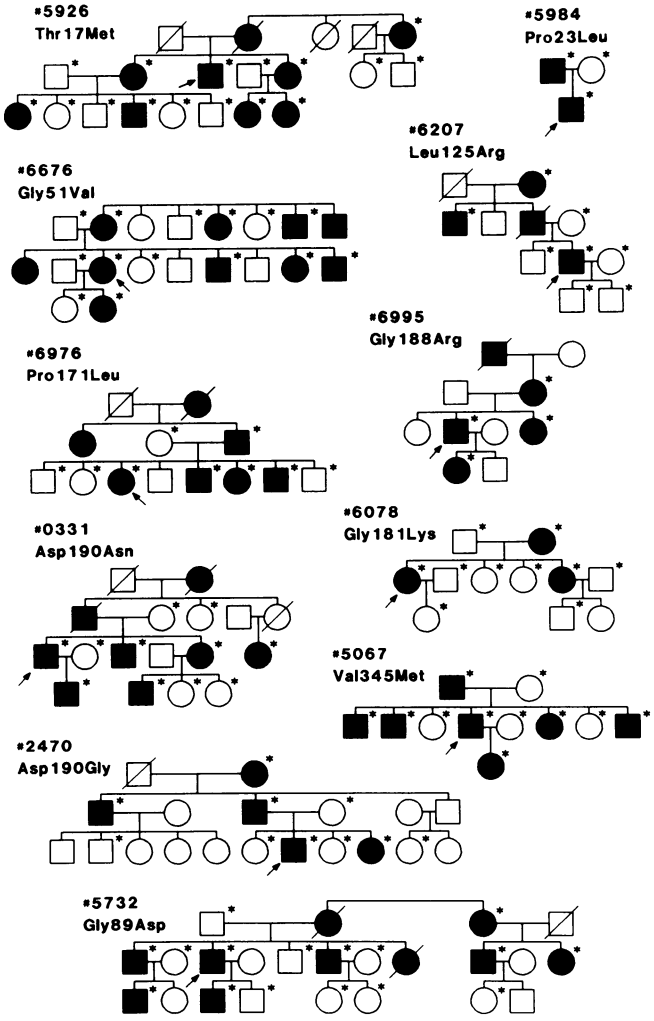

FIG. 1. Pedigrees with autosomal dominant retinitis pigmentosa found to have a missense mutation in the rhodopsin gene. Asterisks denote individuals whose leucocyte DNA was analyzed. Arrows point to propositi. Families with mutations that were described in previous publications are not presented here—i.e., Pro-23 → His, Thr-58 → Arg, Pro-347 → Leu, and Pro-347 → Ser (5, 6). The relatives of the patients with Cys-167 → Arg and Ser-186 → Pro changes refused to donate blood; those pedigrees are also not presented here.

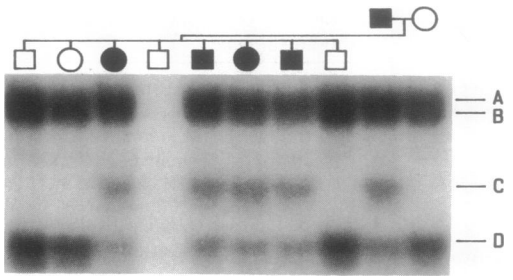

FIG. 2. SSCP analysis of family no. 6976 (Pro-171 → Leu). Sequence analysis revealed that the variant band (labeled "C") seen in the affected individuals in this family is due to a C → T substitution in codon 171. No DNA was available from the middle, unaffected brother.

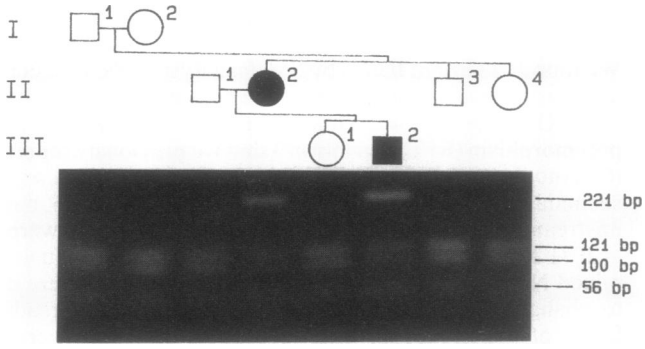

FIG. 3. New germ-line mutation (Pro-347 → Leu) in pedigree no. 5864. Sequence analysis previously showed that subject II-2 carries the mutation Pro-347 → Leu. The normal rhodopsin sequence has a recognition sequence for the restriction endonuclease *Msp* I encompassing codon 347 (6). To test her relatives for this mutation, we digested with *Msp* I an amplified DNA fragment containing the coding sequence in exon 5. The undigested band of 221 bp indicates that the mutation is present. II-2 has the mutation, as does her son III-2, but neither of her parents does.

within the dinucleotide sequence CpG, yet the CpG sequence accounts for only 5% of the open reading frame.

The propensity of the CpG sequence to mutate may explain the absence of a founder effect among unrelated patients carrying the Pro-347 → Leu mutation, which is due to a C → T transition in a CpG sequence. We previously reported no linkage disequilibrium at the CpA-repeat polymorphism in the first intron of the rhodopsin gene among this group of eight patients (6). Here we document also that in one of these cases, family no. 5864, the mutation arose *de novo* (see Fig. 3). Combining this result with the CpA-repeat data, it appears that the Pro-347 → Leu defect in the eight families we studied arose independently in at least three different founders. This is in contrast to our previously reported analysis of patients with Pro-23 → His (due to a C → A transversion). The patients with the Pro-23 → His mutation all share the same uncommon allele at the CpA-repeat polymorphism in intron 1, strongly supporting the notion that this mutation arose only once in a common ancestor of all patients encountered so far (6). Similar evidence links the two families with Gly-51 → Val to a common founder (data not shown). Hence, except for Pro-347 → Leu, each of the 17 mutations reported here probably originated in a single ancestor. The only evident "hot spot" for new mutations is the middle nucleotide, cytidine, in codon 347. Furthermore, the documentation of a new germ-line mutation in a case of autosomal dominant retinitis pigmentosa considerably strengthens the argument that the missense mutation is the cause of the disease, rather than a separate mutation at some tightly linked locus.

By considering the amino acids affected by these mutations, one can obtain some insight into possible mechanisms responsible for the ensuing retinal degeneration. Based on current models for the structure of opsin, 3 of the 18 mutations involve amino acids on the cytoplasmic side of the protein (amino acids 345 and 347), 7 in transmembrane domains, and 8 on the intradiscal side (see Fig. 4). Many of the mutations replace amino acids that should be important for maintaining the normal tertiary structure of rhodopsin. For example, rhodopsin mutations Glu-181 → Lys, Ser-186 → Pro, Gly-188 → Arg, Asp-190 → Asn, and Asp-190 → Gly are all near Cys-187, the residue in the second intradiscal loop that forms a disulfide bond with Cys-110 in the first intradiscal loop. This disulfide bond is thought to be essential for the proper conformation of the protein, and a missense mutation altering the cysteine residue at the comparable location in green cone opsin is also pathogenic (16). It is conceivable that changes in amino acids surrounding Cys-187 prevent the formation of this disulfide bond, thereby disturbing the protein's structure.

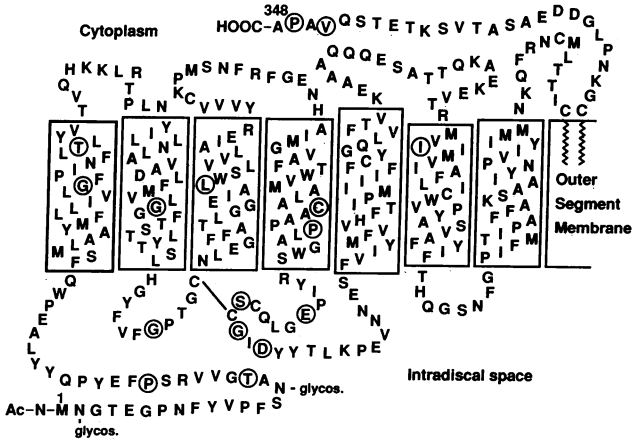

FIG. 4. Schematic model of human opsin. Circled amino acids are altered by mutations in the rhodopsin gene in patients with autosomal dominant retinitis pigmentosa described here and elsewhere (5, 6, 9).

The seven mutant amino acids in transmembrane domains also might alter the structure of the protein, since four of these mutations change nonpolar, hydrophobic residues to charged residues that should be unstable in the lipid bilayer. One of the remaining three mutant residues in transmembrane domains replaces a proline residue, Pro-171→Leu. Proline is known to be important in producing bends in a peptide chain, so it is possible that the tertiary structure of the mutant opsin is deranged. Inglehearn *et al.* (9) also argue that the deletion mutation of Ile-245 they observed is likely to disturb the structure of the transmembrane domain normally containing that residue.

Why should abnormalities in the tertiary structure of rhodopsin lead to photoreceptor degeneration? Rhodopsin is one of the major proteins continually synthesized by the rod inner segment. A singular feature of photoreceptors is that they have no need to degrade the large amount of opsin that they make, since the outer segment discs are ingested by the neighboring retinal pigment epithelial cells. Perhaps mutant rhodopsin molecules that cannot assume the proper three-dimensional structure are unable to be transported to the outer segment and thence to the pigment epithelium, but instead are retained by the inner segment, which has no efficient mechanism for catabolizing them. Ultimately, this large protein load causes the death of the photoreceptors. In support of this hypothetical scenario is the mutation Thr-17 → Met, described here, that removes a glycosylation site near the amino terminus of the protein. Glycosylation of asparagine requires the amino acid sequence Asn-Xaa-(Ser or Thr), where Xaa is any amino acid except proline. Therefore, replacement of Thr-17 would prevent glycosylation normally occurring at Asn-15. Previous studies have found that unglycosylated rhodopsin molecules are not incorporated into rod outer segments. Instead, they inappropriately accumulate in the extracellular space between the inner and outer segments (17–19).

The mutations Val-345 → Met, Pro-347 → Leu, and Pro-347 → Ser do not appear to fit this hypothetical pathogenic mechanism, for it is difficult to understand how these two amino acids near the carboxyl terminus of the molecule could interfere with the tertiary structure of the protein and prevent transport to the outer segment. Perhaps a different mechanism explains the toxicity of these mutant opsins. On the other hand, Hargrave and O'Brien have postulated that these two residues may be necessary for proper intracellular transport of the protein, based on an analogy with the required sequence at the carboxyl terminus of proteins that localize to the endoplasmic reticulum (20).

A better understanding of the mechanism(s) by which mutant opsins cause photoreceptor death will probably await further studies of these opsins. In the meantime, knowledge of the spectrum of mutations that can cause retinitis pigmentosa should be helpful for the genetic diagnosis of patients with the disease and for their genetic counseling.

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