RSPO4 Is the Major Gene in Autosomal-Recessive Anonychia and Mutations Cluster in the Furin-Like Cysteine-Rich Domains of the Wnt Signaling Ligand R-spondin 4

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Congenital anonychia is a rare autosomal-recessive disorder characterized by the absence of finger- and toenails. Recently, we and others identified the secreted Wnt signaling ligand R-spondin 4 (*RSPO4*) as the first gene known to be responsible for inherited anonychia. R-spondins are secreted proteins that activate the Wnt/β-catenin signaling pathway. This puts anonychia on the growing list of congenital malformation syndromes caused by Wnt signaling pathway defects. Here, we expand the *RSPO4* mutational spectrum by identification of the previously unknown mutations c.190C>T (p.Arg64Cys) in exon 2 and c.301C>T (p.Gln101X) in exon 3, thereby corroborating *R-spondin 4* as the major protein in autosomal-recessive anonychia. Almost all *RSPO4* mutations detected so far affect the highly conserved exons 2 and 3. Thus, we postulate that *RSPO4* mutations preferentially cluster in the furin-like cysteine-rich domains of R-spondin 4, which is in line with experimental data proposing that for β-catenin stabilization, a shortened protein comprising just these two regions is sufficient.

INTRODUCTION

Congenital anonychia (OMIM 206800) is defined as the absence of finger- and toenails (Baran *et al.*, 2001). Usually, anonychia and its milder phenotypic variant hyponychia occur as a feature of genetic syndromes and may be associated with significant skeletal and limb anomalies. Well-known examples are Coffin–Siris syndrome (OMIM 135900), several ectodermal dysplasias (OMIM 129490, 300291, and 224900), nail–patella syndrome (OMIM 161200), and brachydactyly type B (OMIM 113000). Most of these disorders are transmitted in an autosomal-dominant mode. In contrast, isolated, non-syndromic anonychia without additional features is a rare entity that usually follows autosomal-recessive inheritance with variable expression even within a given family. The encountered nail phenotypes range from no nail field to a nail field of reduced size with an absent or rudimentary nail (Littman and Levin, 1964).

Recently, we and others identified homozygous and compound heterozygous mutations in the secreted Wnt signaling component R-spondin 4 (*RSPO4*) (OMIM 610573) as the cause of autosomal-recessive anonychia congenita (Bergmann *et al.*, 2006; Blaydon *et al.*, 2006). *RSPO4* spans approximately 44 kb of genomic DNA and comprises five exons that encode a secreted protein of 234 amino acids (Kim *et al.*, 2006). The R-spondin protein family was only recently described and comprises four independent gene products in mammals, which are widely expressed and predicted to share a common organization with substantial structural homologies (Kazanskaya *et al.*, 2004; Blaydon *et al.*, 2007). Noteworthy, each of the five exons constitutes a predicted structural domain. Whereas the N-terminal signal peptide sequences encoded by exon 1 only share a relatively low degree of conservation, the two adjacent cysteine-rich furin-like domains encoded by exons 2 and 3 and the single thrombospondin motif that is encoded by exon 4 display significant similarity among protein family members. Finally, the C-terminal basic region encoded by exon 5 is of varying length and scores as a nuclear localization signal.

R-spondins have been shown to activate the Wnt/β-catenin signaling pathway, which is evolutionarily conserved and pivotal for embryonic development by the regulation of

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Abbreviations: LEF, lymphoid enhancer factor; LRP, low-density lipoprotein-related receptor; RSPO, R-spondin; TCF, T-cell factor

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cell morphology, proliferation, and motility (Logan and Nusse, 2004; Mulholland *et al.*, 2005; Kim *et al.*, 2006; Nam *et al.*, 2006). In accordance, inappropriate activation of Wnt/β-catenin signaling has been demonstrated in several human cancers (Katoh, 2005). As recently shown, all four human R-spondin family members as well as a splice variant of *RSPO4* can induce proliferation of intestinal epithelium (Kim *et al.*, 2006). However, current knowledge about the precise role of R-spondin 4 in Wnt signaling is limited. From recent data on its related protein family member Rspo3, it can be hypothesized that the functions of R-spondins essentially overlap with those of the canonical Wnt ligands by binding to the Frizzled family receptors and the low-density lipoprotein-related receptor (LRP) receptors 5 or 6, ultimately leading to gene activation by β-catenin and the T-cell factor (TCF)-lymphoid enhancer factor (LEF) transcription factor complex (Nam *et al.*, 2006).

In this study, we sought to confirm the major role of R-spondin 4 in autosomal-recessive anonychia by further

delineating the *RSPO4* mutational spectrum in individuals afflicted with congenital non-syndromic anonychia.

RESULTS AND DISCUSSION

In this study, we analyzed two unrelated families of Turkish and Kazakh origin, respectively (Figures 1 and 2). Haplotype analysis was compatible with linkage to *RSPO4* on chromosome 20p13 in both of these families. Owing to parental consanguinity in the Turkish pedigree, homozygous haplotypes could *a priori* be expected for the disease-causing locus in this family. In contrast, parental consanguinity was considered unlikely in the Kazakh family. This assumption was in line with the result of heterozygous haplotypes obtained for the closely flanking marker D20S117 in both affected individuals (Figure 1). Thus, identification of the novel homozygous nonsense mutation c.301C>T (p.Gln101X) in exon 3 of the *RSPO4* gene in these individuals was rather surprising. If one assumes that the parents are indeed non-consanguineous, it is tempting to speculate that
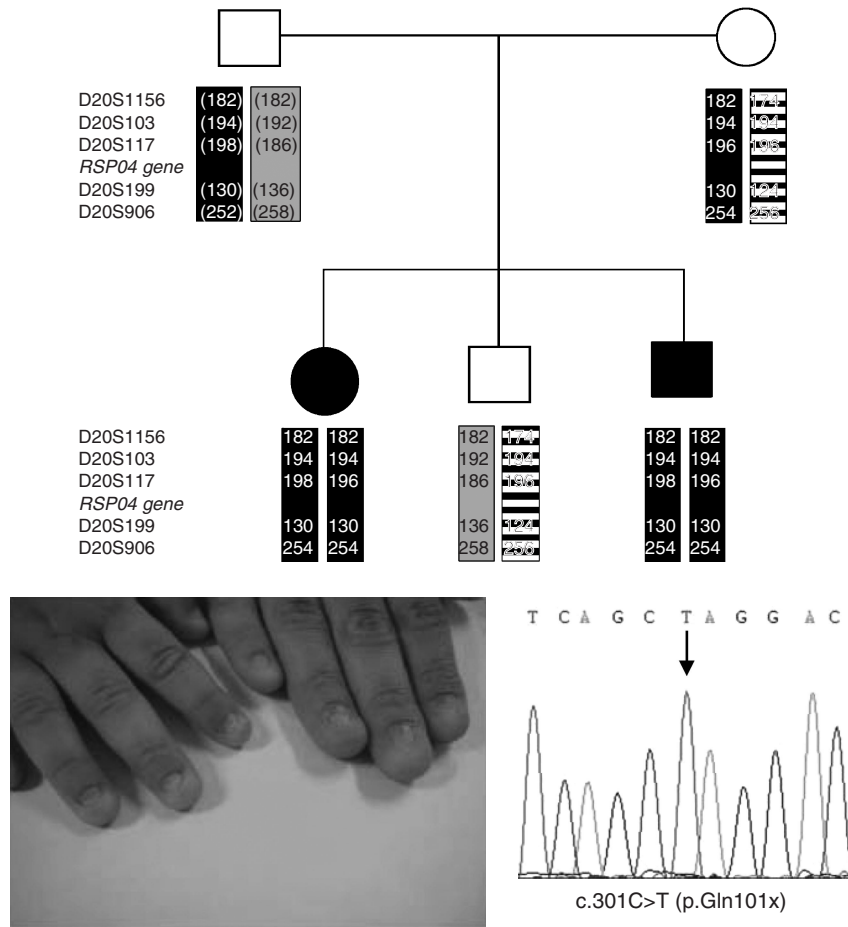

Figure 1. **Pedigree and *RSPO4* linkage data of the Kazakh family studied.** The recombination rates of the informative flanking markers (distally, D20S117 paternally and D20S1156 maternally and D20S199 proximally for both parental haplotypes) in this family are approximately 3 cM for the paternal and approximately 5.5 cM for the maternal haplotypes. The father of this family was not available for analysis. Therefore, paternal haplotypes in this kindred were deduced from the haplotypes of his children. Bottom left: the right hand of the affected 2-year-old son (left) and the right hand of the affected 15-year-old daughter of this family. The skin in the region of the absent nails was normal, and the nail bed, nail matrix, and fold were present in all fingers and toes (data not shown). Bottom right: electropherogram depicting the novel homozygous nonsense mutation c.301C>T (p.Gln101X) in exon 3 of the *RSPO4* gene.
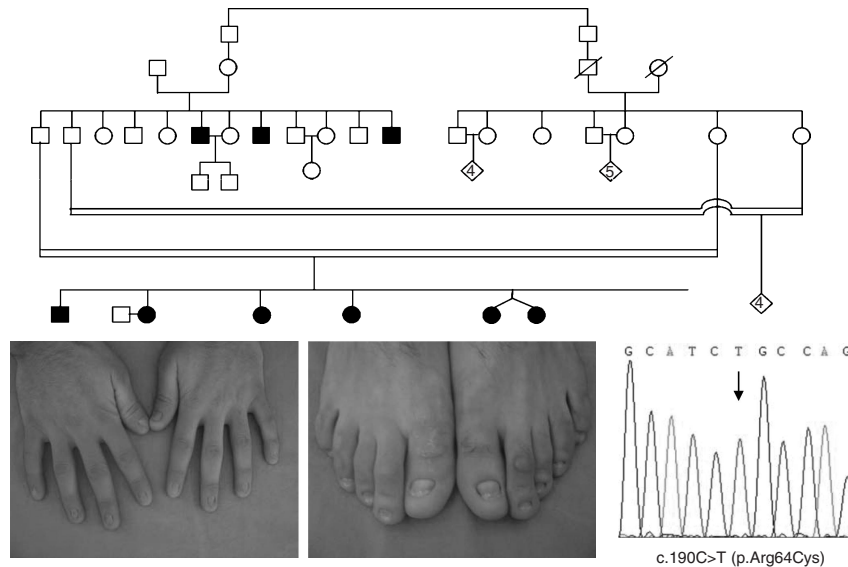
Figure 2. **Pedigree of the Turkish consanguineous family with a total of nine affected individuals.** Bottom left: hands and feet of affected individual V-1 showing isolated total congenital anonychia with absence of finger- and toenails. Bottom right: electropherogram depicting the novel, non-conservative homozygous missense mutation c.190C>T that leads to an amino-acid exchange from arginine to cysteine at position 64 (p.Arg64Cys) in exon 2 of the *RSPO4* gene.

the paternal and maternal mutation in this kindred arose independently. However, it might be more likely that the parents are distantly related (possibly without knowing it) and that a paternal marker mutation for D20S117 with expansion from 196 base pairs (bp) to 198 bp occurred (Buschiazzo and Gemmell, 2006). The latter hypothesis is further corroborated by the finding of homozygous haplotypes in both patients for all other typed *RSPO4* microsatellite markers and homozygosity for the known non-coding single-nucleotide polymorphism c.1-304C>G (rs6077512, http://genome.ucsc.edu) in the proximal 5′ untranslated region of the *RSPO4* gene.

Direct sequencing of the coding region and adjacent 5′ untranslated region of the *RSPO4* gene in the propositus of the Turkish family also revealed the aforementioned known homozygous SNP c.1-304C>G. Furthermore, we could identify the novel, non-conservative homozygous missense mutation c.190C>T that leads to an amino-acid exchange from arginine to cysteine at position 64 (p.Arg64Cys) in exon 2 of the *RSPO4* gene and was shown to segregate with the phenotype in this family (Figure 2). The affected R-spondin 4 arginine residue was found to be strongly conserved in several species throughout evolution (Figure 3). Moreover, this mutation was not detected in 200 ethnically matched Turkish control chromosomes tested by restriction enzyme analysis, thereby further corroborating its pathogenic character.

RSPO4 mutations identified so far comprise the entire spectrum of small mutations with deletions and insertions leading to a frameshift, nonsense, splice, and missense mutations. Intriguingly, no genotype–phenotype correlation can be drawn for the type or position of the causative mutation. Of note, the majority of *RSPO4* mutations detected so far affect the highly conserved furin-like cysteine-rich

domains encoded by exons 2 and 3 (Figure 3; Bergmann *et al.*, 2006; Blaydon *et al.*, 2006). This is in accordance with experimental data suggesting that for *β*-catenin stabilization, a shortened variant of R-spondin 4 comprising just these two furin-like regions is sufficient (Kazanskaya *et al.*, 2004; Kim *et al.*, 2006). *RSPO4* mutation analysis in further families will answer the intriguing question if mutations in other domains than the highly conserved furin-like regions result in a similar clinical phenotype or may allow for a residual protein function conceivably giving rise to a milder expression pattern with, for example, hyponychia or selective absence of single nails. However, currently it is rather speculative to predict the specific effects of the detected *RSPO4* mutations on the stability of mRNA and the mutant protein. Notwithstanding, two different types of mutations can generally be differentiated: (i) those resulting in amino-acid substitutions due to missense mutations, and (ii) those leading to premature termination codons. Non-conservative amino-acid substitutions that affect evolutionarily highly conserved functional domains (such as the furin-like cysteine-rich domains in R-spondin 4) are likely to strongly perturb proper function of the encoded protein (Krawczak and Cooper, 1996). Premature termination codons may preferably result in nonsense-mediated mRNA decay, following detection of faulty open reading frames and subsequent elimination of ''imperfect messages'' by a conserved mammalian surveillance mechanism (Hentze and Kulozik, 1999).

We performed extensive *in silico* analyses to further endorse the pathogenicity of the six *RSPO4* missense mutations identified to date, in detail p.Arg64Cys, p.Gln65Arg, p.Cys73Tyr, p.Cys95Phe, p.Cys107Arg, and p.Cys118Tyr (Bergmann *et al.*, 2006; Blaydon *et al.*, 2006; and this study). First, striking evidence for the pathogenicity of these aforementioned changes is proposed
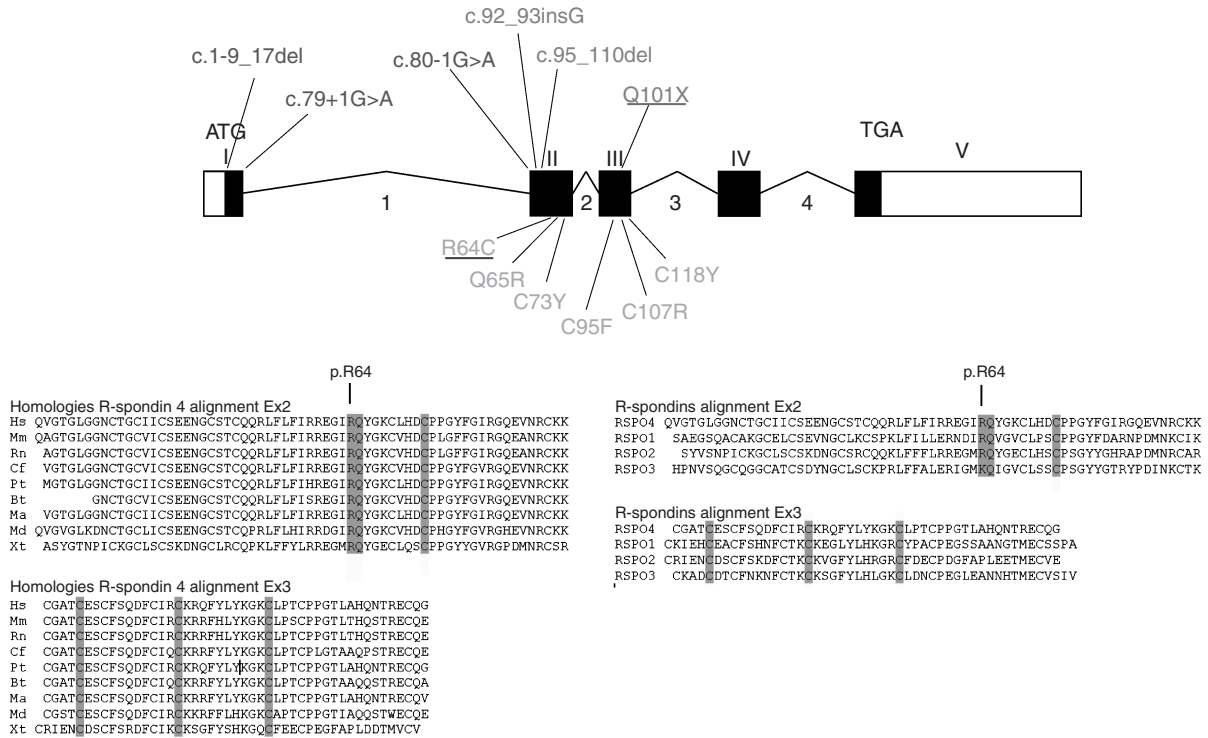
Figure 3. **Simplified scheme (not drawn to scale) of the *RSPO4* gene that spans approximately 44 kb of genomic DNA and comprises five exons that encode a secreted protein of 234 amino acids.** (Exons are depicted as bars: coding sequence = blackened bar, non-coding exonic sequence = white bar; introns are displayed by lines). *RSPO4* mutations detected in this study (*underlined*) and those described in the literature are shown (blue = splice mutations, red = truncating mutations, green = missense mutations). Bottom left and right: *in silico* characterization of known *RSPO4* missense mutations (the affected arginine residue (p.R64) in the Turkish family is indicated). Multiple protein sequence alignment, generated with ClustalW, of the furin-like cysteine-rich domains encoded by exons 2 and 3. Residues affected by *RSPO4* missense mutations are shown on a gray background. Sequence comparison shows that the identified *RSPO4* missense mutations affect amino acids that are highly conserved through evolution (bottom left) and among members of the R-spondin protein family (bottom right). Abbreviations and protein annotations used: Hs, *Homo sapiens* NP_001025042; Mm, *Mus musculus* BC116367.1; Rn, *Rattus norvegicus* ENSRNOP00000052312; Cf, *Canis familiaris* ENSCAFP00000010193; Pt, Pan troglodytes ENSPTRP00000022516; Bt, Bos taurus ENSBTAP00000039099; Ma, Macaca mulatta ENSMMUP00000037654; Md, *Monodelphis domestica* ENSMODP00000024370; Xt, *Xenopus tropicalis* ENSXETP00000007765. R-spondin 4 NP_001025042, R 1 NP_001033722, R 2 NP_848660, and R 3 NP_116173.

by the evolutionarily highly conserved character of affected amino acids, as demonstrated by multiple protein sequence alignment that was generated with the program ClustalW (http://ebi.ac.uk/clustalw/) using translation from genome assemblies and expressed sequences (Figure 3, bottom left). This program was also used to study the conservation of these mutated *RSPO4* amino acids among the four known human R-spondin proteins (Figure 3, bottom right). PolyPhen is a bioinformatics tool that predicts a possible impact of an amino-acid substitution on the structure and function of a human protein based on straightforward physical and comparative considerations (http://genetics.bwh.harvard.edu/pph/ or http://coot.embl.de/PolyPhen/). The results of PolyPhen analysis strongly suggested that all tested missense mutations would probably result in a dysfunctional protein with position specific independent count score differences > 2, respectively. Utilization of the ConSeq prediction software (http://conseq.bioinfo.tau.ac.il) corroborated these findings. The conservation score (1 = variable, 9 = conserved) of affected R-spondin 4 residues was 8 for the substitutions

p.Arg64Cys and p.Gln65Arg, and 9 for the mutations p.Cys73Tyr, p.Cys95Phe, p.Cys107Arg, and p.Cys118Tyr, respectively. Structurally, highly conserved residues 64, 65, and 73 were evaluated as exposed, whereas the highly conserved residues 95, 107, and 118 were predicted to be buried. At last, we checked the SIFT version 2 bioinformatics software (http://blocks.fhcrc.org/sift/SIFT_seq_submit2.html) that also predicted convincing data with strong SeqRep fractions and intolerable amino-acid changes with regard to RSPO4 protein structure and function.

Conclusively, we have demonstrated that *RSPO4* is the major gene in autosomal-recessive anonychia and that mutations mainly affect the highly conserved exons 2 and 3 encoding the furin-like cysteine-rich domains of R-spondin 4.

MATERIALS AND METHODS
Patients
Detailed clinical data of the Kazakh family described in this study have been published previously (Rigopoulos *et al.*, 2006), whereas

the clinical characteristics of the consanguineous Turkish pedigree were recently presented at the 15th Congress of the European Academy of Dermatology and Venereology (EADV) in Rhodes, Greece (Koc *et al.*, 2006). All molecular data are described for the first time in this study.

Briefly, all affected individuals had isolated total congenital anonychia (Figures 1 and 2). In both families, the parents were unaffected. Further, family and medical histories were unremarkable, respectively. Patients were of normal height and proportionate stature and not different in any aspect except for anonychia from their healthy siblings. X-rays of the upper and lower limbs did not show any abnormality. No additional malformations were found on clinical examination, neither of ectodermal structures nor of sensory or inner organs. All subjects were of normal intelligence. We obtained blood samples from all patients and their parents and extracted genomic DNA using standard procedures. Signed and informed consent was obtained from patients and family members using protocols approved by the Institutional Ethics Review Board at the University of Aachen and collaborating institutions. Experiments were carried out with institutional approval and in adherence to the Declaration of Helsinki Principles. As the father of the Kazakh family was not available for analysis, paternal haplotypes in this kindred were deduced from the haplotypes of his children.

Haplotype analysis

In both multiplex families, we initially performed linkage analysis with microsatellite markers located in the *RSPO4* region on chromosome 20p13 (see Figure 1 for exemplification of the Kazakh family). Primers for PCR amplification were as published in the Genome Database (http://www.gdb.org) and are available on request. Microsatellite markers were analyzed on an ABI PRISM 3130 genetic analyzer (Applied Biosystems, Darmstadt, Germany) with a universal fluoresceinated primer ComF (5′-TACGCATCC CAGTTTGAGACG-3′) as follows (Warner *et al.*, 1996). Each microsatellite marker was amplified in a three primer PCR reaction in which the forward primer of the two specific primers was tailed with the sequence corresponding to the universal fluorescent primer ComF (specFcom). A 10:1 molar ratio of primers ComF to specFcom was used to ensure that primer specFcom is exhausted during the early amplification cycles.

Mutation analysis

We performed direct sequencing of the entire coding region including exon–intron boundaries and the proximal adjacent 5′ untranslated region (1 kb upstream of the ATG initiation codon) of the *RSPO4* gene (GenBank: NM_001029871; mutation numbering +1 corresponds to the A of the ATG translation initiation codon). Genomic DNA from affected individuals was amplified by PCR with oligonucleotide primers complementary to flanking intronic sequences. Primers were designed using the Primer3 program (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi) (primer sequences and PCR conditions are available on request). PCR products were purified with the DNA Clean & Concentrator™-5 kit (Zymo Research, Orange, CA) and sequenced employing ABI BigDye chemistry (Applied Biosystems). The same primers as for PCR were used as sequencing primers. Samples were run and analyzed on an ABI PRISM 3130 genetic analyzer (Applied Biosystems). Once a mutation had been identified, segregation of

the altered allele was tested by direct sequencing of parental probes. The missense mutation detected in this study was not present in 200 ethnically matched Turkish control chromosomes tested by Bts*CI* restriction enzyme analysis (New England Biolabs, Beverly, MA).

Web resources

The human genome databases are available at http://genome.ucsc. edu/ and http://www.ncbi.nlm.nih.gov/. Primer 3 is available at http://www.broad.mit.edu/cgi-bin/primer/primer3_www.cgi/. The Online Mendelian Inheritance in Man (OMIM) database is available at http://www.ncbi.nlm.nih.gov/omim. The GenBank database is available at http://www.ncbi.nlm.nih.gov/Genbank/. ClustalW alignment is available at http://www.ebi.ac.uk/clustalw/. Databases for *in silico* analysis of amino-acid substitutions are available at http:// genetics.bwh.harvard.edu/pph/ or http://coot.embl.de/PolyPhen/ (PolyPhen prediction tool), http://conseq.bioinfo.tau.ac.il (ConSeq software), and http://blocks.fhcrc.org/sift/SIFT_seq_submit2.html (SIFT version 2).

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