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Adaptive evolution of *MRGX2*, a human sensory neuron specific gene involved in nociception☆

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Abstract

MRGX2, a G-protein-coupled receptor, is specifically expressed in the sensory neurons of the human peripheral nervous system and involved in nociception. Here, we studied DNA polymorphism patterns and evolution of the *MRGX2* gene in world-wide human populations and the representative nonhuman primate species. Our results demonstrated that *MRGX2* had undergone adaptive changes in the path of human evolution, which were likely caused by Darwinian positive selection. The patterns of DNA sequence polymorphisms in human populations showed an excess of derived substitutions, which against the expectation of neutral evolution, implying that the adaptive evolution of *MRGX2* in humans was a relatively recent event. The reconstructed secondary structure of the human *MRGX2* revealed that three of the four human-specific amino acid substitutions were located in the extra-cellular domains. Such critical substitutions may alter the interactions between *MRGX2* protein and its ligand, thus, potentially led to adaptive changes of the pain-perception-related nervous system during human evolution.

Keywords: GPCRs; Human-specific substitution; Darwinian positive selection; Drug target

1. Introduction

With the completion of the human genome sequencing project, hundreds of orphan G-protein-coupled receptors (GPCRs) have been reported (Civelli et al., 2001; Vassilatis et al., 2003). The GPCRs belong to one of the largest gene super-families which have been exploited extensively for drug targets (Civelli et al., 2001; Grazzini et al., 2003). *MRG* (mas-related gene) is a recently identified GPCRs family. It has 32 murine and 4–7 human genes (*hMRGX1-hMRGX7*) (Dong et al., 2001; Lembo et al., 2002; Grazzini et al., 2003; Choi and Lahn, 2003). The *MRG* gene family is expressed only in a specific subset of nociceptive sensory neurons that are known to detect painful stimuli (Dong et al., 2001; Robas et al., 2003). Recently, a series of ligands have been identified to be the *MRG* receptors. For example, *MRGA1* and *MRGA4* could be optimally activated by RFamide neuropeptides (Han et al., 2002). However, the detailed

Abbreviations: *MRG*, mas-related gene; GPCR, G-protein-coupled receptor.

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physiological functions of the *MRG* receptors and the underlying mechanisms of signal transduction are yet to be understood.

MRGX2 is a member of the *MRG* gene family. Human *MRGX2* gene is located on chromosome 11p15. It is a two-exon gene encoding a protein of 330 amino acids (the first exon is untranslated). Immuno-histochemical and quantitative PCR data showed that *MRGX2* had a limited expression profile in the sensory neurons, which is similar to other members of the *MRG* gene family (Robas et al., 2003). However, as the only identified high potency receptor for cortistatin, the pharmacological profile of *MRGX2* is distinct from the other characterized members of the *MRG* family. It was reported that *MRGX2* might play a crucial role within the pathway of nociception, therefore, suggesting a potential gene target for drug design (Robas et al., 2003).

Humans diverged from African apes about 5–7 million years ago. Genetically, humans and chimpanzees share nearly 99% DNA sequence similarity (Fujiyama et al., 2002; Hellmann et al., 2003; Shi et al., 2003), which seems to contradict the marked biological and phenotypic divergence between them, e.g., the highly developed cognitive abilities in humans. One of the major challenges in the rapidly progressing genome comparative studies between humans and chimpanzees is to identify and determine those sequence differences that contribute to the species-specific phenotypic traits (Enard et al., 2002; Evans et al., 2004a,b; Wang and Su, 2004; Stedman et al., 2004).

Genes undergone strong Darwinian positive selection in the human lineage are the keys of understanding the unique biological functions of humans. At molecular level, signature of positive selection can be detected by comparative sequence analysis between humans and nonhuman primates. In our effort in identifying positively selected genes in the human genome, we found that *MRGX2* showed an elevated amino acid substitution rate between human and chimpanzee, implying possible adaptive evolution of *MRGX2* in the human lineage. It was reported that all the human *MRGX* genes are closely related with each other resulted from recent gene duplications that postdated primate-rodent divergence (Choi and Lahn, 2003; Robas et al., 2003). The *MRG* gene family has undergone strong Darwinian positive selection in mammalian species, therefore, an implication of adaptive evolution of the pain-perception-related nervous system (Choi and Lahn, 2003). As potentially a newly evolved member of the *MRG* gene family, the human *MRGX2* might play a role in the evolution of human nervous system.

In this study, we sequenced the *MRGX2* gene in worldwide human populations and the representative nonhuman primate species. Our results demonstrated a strong Darwinian positive selection in humans, which may contribute to the adaptive changes of sensory system during human evolution.

2. Material and methods

2.1. DNA samples

We sequenced 30 human individuals from the major continental populations, including Africans (10 individuals), Europeans (10 individuals), and East Asians (10 Chinese individuals). In addition, a total of seven nonhuman primate species were sequenced, which reflect a 25 million-year history of primate evolution (Goodman et al., 1998). The nonhuman primate panel includes three great ape species (two chimpanzees—*Pan troglodytes*, two gorillas—*Gorilla gorilla* and one orangutan—*Pongo pygmaeus*), one lesser ape species (one white-browed gibbon—*Hylobates hoolock*), three Old World monkey species (two rhesus monkeys—*Macaca mulatta*, one black leaf monkey—*Trachypithecus francoisi*, and one Yunnan golden monkey—*Rhinopithecus bieti*). The divergence times between humans and the nonhuman primate species are 5–6 million years (myr) for chimpanzee, 6–7 myr for Gorilla, 14 myr for orangutan, 18–20 myr for lesser apes and 23–25 myr for Old World monkeys (Goodman et al., 1998). The human sample IDs are Africans: AB01, AB02, AB04, AB06–08, AB11, AB13–15; Europeans: E150, E153, E161–164, PG1305, PG1319, PG1628, PG1634; Chinese: KHC32–41. All DNA samples were obtained from the collections in the Kunming Cell Bank of CAS, the Kunming Blood Center, and Stanford University.

2.2. PCR and sequencing

The whole coding region (993 bp) of *MRGX2* was sequenced for all the human and nonhuman primate samples. In addition, a 533-bp fragment of intron immediately upstream of the second exon was sequenced for human and chimpanzee samples. Primers were designed by comparing the published human, chimpanzee and mouse sequences in Ensembl (Ensembl genome browser at http://www.ensemble.org and http://pre.ensembl.org/Pan_troglodytes/). The primer sequences are:

*MRG*_F 5′-ACAGTGAGTGGGGGTGTTTGG-3′
*MRG*_seq1 5′-TGTTTTGTGGCAAGGAGACC-3′
*MRG*_seq2 5′-CTGTGATGACCTGTGCCTACCTTG-3′
*MRG*_seq3 5′-CAAAGAACTTCCCTTCCAAGATGC-3′
*MRG*_R 5′-TGTGGTGGAAGCCTGTGAGTAAG-3′
Intron_R 5′-CGGCAGCGATACCAGATG-3′
Intron_F 5′-TGAGGGAGGACAACAGAAGTG-3′
Intron_seq 5′-TGAGGACACAGAAATGGATAA-3′

Sequencing was performed in both directions by forward and reverse primers using the BigDye terminator sequencing kit on an ABI 3100 automated sequencer (ABI, Inc.).

2.3. Data analysis

DNA sequences were firstly aligned using software package DNASTAR (DNASTAR, Inc.) and then checked manually. The

program MEGA 2.0 was used for the phylogenetic analysis (Kumar et al., 2001). The ancestral sequences were inferred by using Zhang's method in software ANC-GENE (Zhang and Nei, 1997). PHASE 2.0 program was used to infer the haplotypes in humans whose sequences have more than two heterozygous sites (Stephens et al., 2001). The haplotypes of nonhuman primates were obtained by randomly separating the bialleles at the heterozygous sites into two chromosome copies. This will not affect it serving as outgroup haplotype because it is usually homozygous at the sites where DNA polymorphisms are observed in the human populations. The synonymous and non-synonymous substitution rate (K_s and K_a) were calculated based on Pamilo–Bianchi–Li's method, in which the transitional/transversional substitution bias was taken into account (Pamilo and Bianchi, 1993; Li, 1993). The Z-test was used to detect the deviation of the K_a/K_s ratios from neutrality (Kumar et al., 2001). The MacDonald–Kreitman test and the Fay and Wu's H test were used to detect selection (McDonald and Kreitman, 1991; Fay and Wu, 2000). These tests were developed to detect whether the patterns of sequence variations in a population are consistent with the expectation of neutral evolution.

The protein structure analysis was conducted by utilizing the tools from the ExPASY Molecular Biology Server hosted by North Carolina Supercomputing Center (http://us.expasy.org/). The software RbDe (http://icbtools.med.cornell.edu/viseur/viseur.html) was used to reconstruct the 2D structure of *MRGX2*.

3. Results and discussion

3.1. Sequence substitution patterns in diverse primate lineages

We identified 117 nucleotide variant sites in the 993-bp coding region of *MRGX2* based on the alignment of sequences of 30 humans and seven nonhuman primate species including Old World monkeys, lesser apes and great apes. A total of 69 amino acid variant sites (20.9% of amino acid sequence, 69/330) were observed (refer to supplementary material for protein sequence alignment). The non-synonymous substitution (i.e., amino acid changing) rate (K_a) of *MRGX2* was 1.7% between humans and chimpanzees, which is two times higher than the reported average divergence (0.51%) across the genome (Shi et al., 2003), indicating rapid amino acid substitutions in *MRGX2* after human and chimpanzee diverged from each other about 5–7 million years ago (Goodman et al., 1998). Based on the well-accepted phylogenetic relationship of primate species (Goodman et al., 1998), we calculated the K_a/K_s ratios (K_s, synonymous substitution rate) of different evolutionary lineages using Pamilo–Bianchi–Li's method (Pamilo and Bianchi, 1993; Li, 1993) (Fig. 1). The one-tailed Z-tests showed that most of the primate lineages followed either neutral evolution ($K_a/K_s=1$) or purifying selection (functional constraint, $K_a/K_s<1$) (Fig. 1). In contrast, the K_a/K_s ratio in the human lineage reflected a strong positive selection, e.g., all 4 substitutions were non-synonymous ($K_a/K_s=\infty$, $p=0.022$, one-tailed Z-test). Hence, our data indicated that the substitution pattern in humans was likely driven by Darwinian positive selection leading to the adaptive changes of *MRGX2* during recent human evolution. Note that the chimpanzee and gorilla lineages also showed relatively large K_a/K_s ratios (Fig. 1), however, the statistical tests were not significant for the deviation from neutral expectations ($p>0.05$, one-tailed Z-test), which were likely caused by relaxation of purifying selection in these two species though positive selection cannot be ruled out.

Previous study showed that the Z-test is too liberal when the numbers of substitutions are small (Zhang et al., 1997). When the Fisher's Exact Test was performed, the human

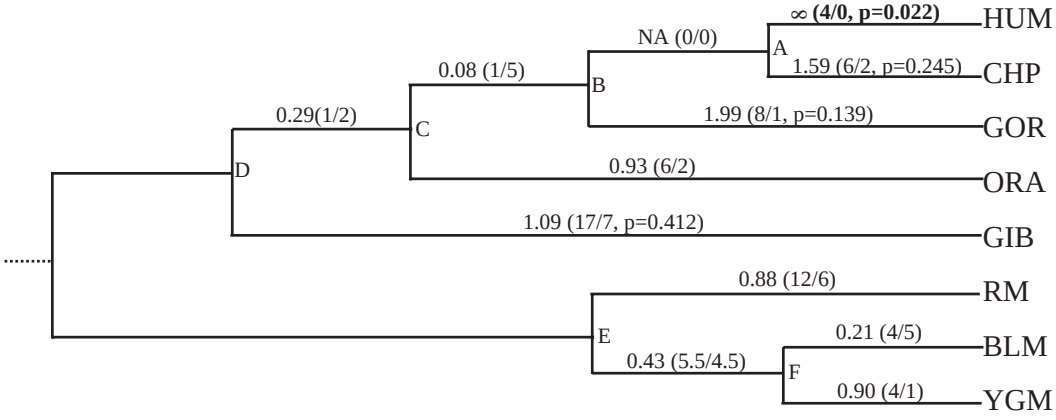


Fig. 1. The K_a/K_s ratios of different evolutionary lineages in primates. The K_a/K_s calculation was based on only the coding sequences using Pamilo–Bianchi–Li's method (Pamilo and Bianchi, 1993; Li, 1993). The one-tailed Z-test was used to detect the deviation of the K_a/K_s ratios from neutral expectation ($K_a/K_s=1$), and the P-values were shown in parentheses. The numbers in parentheses refer to the number of non-synonymous/synonymous substitutions observed in each lineage. The labels (A–F) refer to the internal nodes. The consensus sequence of humans was used in the analysis. For nonhuman primates, if polymorphic sites exist, the ancestral alleles were used. HUM—human, CHP—chimpanzee, GOR—gorilla, ORA—orangutan, GIB—white-cheeked gibbon, RM—rhesus monkey, BLM—black leaf monkey, YGM—Yunnan golden monkey.

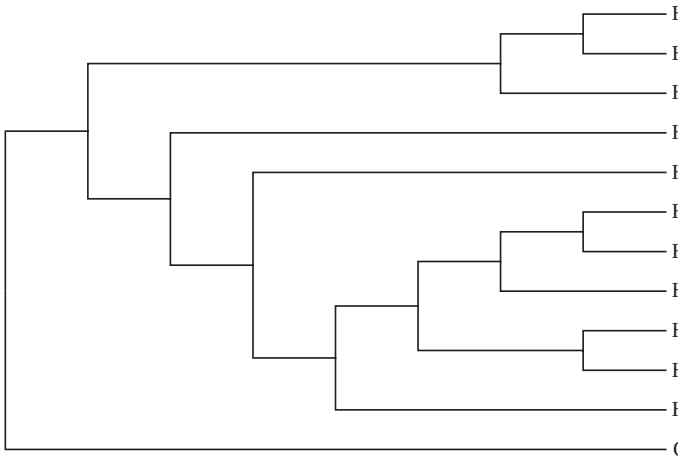
Table 1
The sequence polymorphisms of *MRGX2* in human populations

Polymorphic site	Allele frequencies			
	Africans ($n=20$)	Asians ($n=20$)	Europeans ($n=20$)	Total ($n=60$)
C/**A** (intron)	0.150	0.200	0.250	0.200
G/**A** (intron)	0.150	0.200	0.250	0.200
T/**C** (intron)	0.850	0.750	0.750	0.783
G/**A** (intron)	0.750	0.750	0.700	0.733
C/**T** (intron)	0.850	0.750	0.750	0.783
G/**C** (intron)	0.850	0.750	0.700	0.767
C/**A** (intron)	0.850	0.750	0.750	0.783
C/**T** (intron)	0.150	0.250	0.250	0.217
G/**A** (intron)	0.850	0.750	0.750	0.783
A/**C**^{46th} (Asn/His)	0.100	0.350	0	0.150
A/**G**^{185th} (Asn/Ser)	0.200	0.350	0.300	0.283
T/**G**^{195th} (synonymous)	0.050	0.300	0	0.267
T/**C**^{232nd} (Phe/Leu)	0.050	0	0	0.083

The characters in bold represent the derived alleles. The allele frequencies are that of the derived alleles.

lineage did not show deviation from neutrality ($P>0.05$, Fisher's Two-tailed Exact Test). Consequently, the observed rapid evolution in the human lineage could also be explained by relaxation of functional constraint. To further test if positive selection occurred in the human lineage, we performed the MacDonald–Kreitman's neutrality test which was developed to compare the mutation patterns (number of non-synonymous mutations vs. synonymous mutations) between and within species and to detect whether positive selection explains the genetic divergence between species (McDonald and Kreitman, 1991). There are only four polymorphic sites in the coding region of *MRGX2* in the human populations (1 synonymous and 3 non-synonymous). To increase the number of polymorphic sites for the test, we included 533-bp intron sequence which is immediate upstream of exon 2 and treated as synonymous sites. In this non-coding fragment, nine DNA polymorphic sites were identified in human populations, one polymorphic site in chimpanzees, and two substitutions between humans and chimpanzees. When the non-coding region substitutions were treated as synonymous, the non-synonymous/synonymous substitution ratio between-species (human vs. chimpanzee) (11/4) is significantly larger than the non-synonymous/synonymous polymorphisms within species (4/11) ($p=0.027$, two-tailed Fisher's Exact Test), a clear indication of positive selection in the human lineage. Table 1 shows the allele frequencies of the 13 polymorphic sites in the human populations. There are six polymorphic sites in the intronic region showing high frequencies (>70%) of the derived alleles in all continental populations. We performed Fay and Wu's *H* test (Fay and Wu, 2000). The results indicated an excess of common derived mutations in human populations ($H=-2.921$, $p=0.007$), which is significantly deviated from the expectation of neutral evolution and again is consistent with the proposed positive selection in humans. Next, we inferred the haplotypes in human populations using *PHASE* (Stephens et al., 2001) (Fig. 2). A total of eleven haplotypes were obtained with Hap10 being the dominant haplotype (65% on average). The phylogenetic relationship among the eleven haplotypes was constructed using parsimonious method (the chimp sequence was used as outgroup) (Nei and Kumar, 2000). Surprisingly, Hap10 was shown to be a recently evolved lineage carrying six derived mutations, a strong signal of genetic hitchhiking which is consistent with the results of Fay and Wu's test, and suggesting possible recent selection of *MRGX2* in current human populations.

Note that there are two polymorphic sites showing between-population allele frequency differences (Table 1), including the A to C (Asn to His) and T to G (synonymous) variations in the coding region where the frequencies in East Asian populations are significantly higher than those in Europeans ($p<0.05$, Fisher's One-tailed Exact Test). The



	Africans	Asians	Europeans	Total
HAP01	1	3	0	4
HAP02	2	1	5	8
HAP03	0	1	0	1
HAP04	0	0	1	1
HAP06	1	0	0	1
HAP05	1	0	0	1
HAP07	0	2	0	2
HAP08	0	1	0	1
HAP09	0	0	1	1
HAP10	14	12	13	39
HAP11	1	0	0	1

Fig. 2. The human haplotype relationship. The phylogenetic tree was constructed using the maximum parsimony method in Mega 2.0 (Nei and Kumar, 2000).

between-population divergence at these two loci is either due to population substructures or regional selection, which calls for further population analysis.

3.2. Functional implications of sequence substitutions

There are four fixed amino acid substitutions in humans which are conserved in the entire nonhuman primate species studied, an indication of functional importance of these human-specific changes (Fig. 3). It is well known that the GPCR has a characteristic structure of seven transmembrane domains. Fig. 3 shows the reconstructed 2D structure of the human *MRGX2* by the secondary structure prediction program *HMMTOP* Version 2.0 (Tusnády and Simon, 1998; Tusnády and Simon, 2001). Seven α-helix regions corresponding to the seven transmembrane domains were clearly revealed (Fig. 3a). Using the *RbDe* program, a snake-like diagram was generated based on the 2D prediction result (Campagne and Weinstein, 1999; Konvicka et al., 2000; Skrabanek et al., 2003) (Fig. 3b). It was shown that three of the four human-specific substitutions were located in the extra-cellular domains and the other one in the C-terminus of the third transmembrane domain. This observation is consistent with the previous report showing

(a)

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seq  MDPTTPAWGT ESTTVNGNDQ ALLLLCGKET LIPVFLILFI ALVGLVGNGF VLWLLGFRMR RNAFSVYVLS LAGADFLFLC 80
pred OOOOOOOOOO OOOOOoooos ooooooooos HHHHHHHHHH HHHHHHHHHH HHHHHiiiii iiiHHHHHHH HHHHHHHHHH

seq  FQIINCLVYL SNFFCSISIN FPSFFTTVMT CAYLAGLSML STVSTERCLS VLWPIWYRCR RPRHLSAVVC VLLWALSLLL 160
pred HHHHHHHHoo ooooooooos ooooHHHHHH HHHHHHHHHH HHHHiiiiii iiiiiiiiii iiiiHHHHHH HHHHHHHHHH

seq  SILEGKFCGF LFSDGDSGWC QTFDFITAAW LIFLFMVLCG SSLALLVRIL CGSRGLPLTR LYLTILLTVL VFLLCGLPFG 240
pred HHHooooooo ooooooooos oooooHHHHH HHHHHHHHHH HHHHHiiiii iiiiiiiiii iiiiiHHHHH HHHHHHHHHH

seq  IQWFLILWIW KDSDVLFCHI HPVSVVLSSL NSSANPIIYF FVGSFRKQWR LQQPILKLAL QRALQDIAEV DHSEGCFRQG 320
pred HHHHHHHHHH ooooooooos ooHHHHHHHH HHHHHHHHHH HHHHHiiiii iiiiiiiiii IIIIIIIIII IIIIIIIIII

seq  TPEMSRSSLV 330
pred IIIIIIIIII
```

(b)

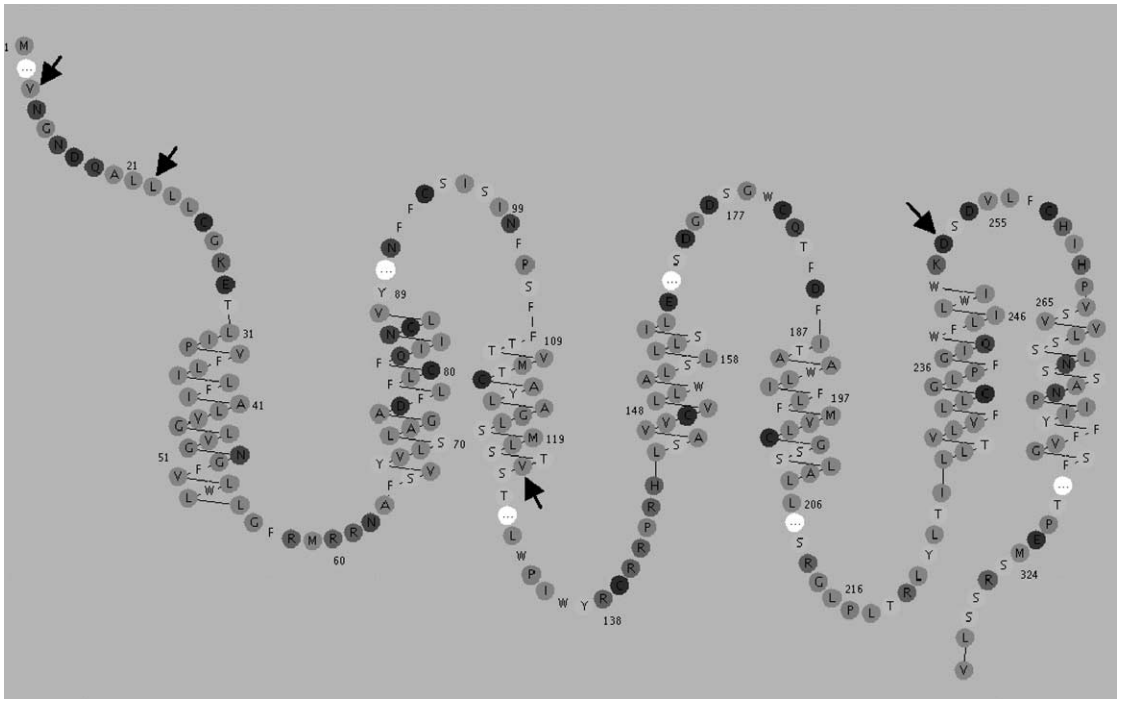

Fig. 3. The reconstructed secondary structure of human *MRGX2*. (a) The predicted secondary structure by HMMTOP Version 2.0 (Tusnády and Simon, 1998, 2001). The seven putative transmembrane domains were highlighted. The symbols, "I", "i", "o", "O", and "H" refer to inside loop (I), inside tail (i), membrane helix (H), outside tail (o) and outside loop (O). Tails were suggested to interact with the inside or outside parts of the membrane, while loops do not. Two tails between helices can form a short loop, but longer loops are formed by tail–loop–tail sequences. (b) The snake-like diagram reconstructed by the *RbDe* program (Campagne and Weinstein, 1999; Konvicka et al., 2000; Skrabanek et al., 2003). The four human-specific mutations were indicated by arrows. The numbers indicate the positions of the amino acid sequence. The white balls represent the omitted amino acids.

significant excess of amino acid changes in the extracellular domains of genes in the *MRG* family, an indication of adaptive evolution (Choi and Lahn, 2003). As the extracellular domains of GPCRs are usually involved in ligand/receptor recognition, the three human-specific substitutions could potentially modify the interaction between *MRGX2* protein and its ligand. The two human-specific substitutions in the arm region of *MRGX2* (the first 30 amino acids at the N terminus) are particularly intriguing because this extracellular domain usually serves as the recognition site for ligand of many GPCRs, e.g., the thrombin receptor (Flower, 1999). Therefore, we speculated that the adaptive sequence changes of human *MRGX2* may contribute to the functional modification of human sensory system, especially the sensory pathway of detecting pain stimuli, which could be a refined self-protection mechanism developed during human evolution. Consequently, the genetic uniqueness of the human *MRGX2* calls for comparative neurological studies on the pain sensory system between humans and nonhuman primates.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.gene.2005.03.001.

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