Evolution of Bitter Taste Receptors in Humans and Apes

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Bitter taste perception is crucial for the survival of organisms because it enables them to avoid the ingestion of potentially harmful substances. Bitter taste receptors are encoded by a gene family that in humans has been shown to contain 25 putatively functional genes and 8 pseudogenes and in mouse 33 putatively functional genes and 3 pseudogenes. Lineage-specific expansions of bitter taste receptors have taken place in both mouse and human, but very little is known about the evolution of these receptors in primates. We report the analysis of the almost complete repertoires of bitter taste receptor genes in human, great apes, and two Old World monkeys. As a group, these genes seem to be under little selective constraint compared with olfactory receptors and other genes in the studied species. However, in contrast to the olfactory receptor gene repertoire, where humans have a higher proportion of pseudogenes than apes, there is no evidence that the rate of loss of bitter taste receptor genes varies among humans and apes.

Introduction

Mammals are able to perceive five basic tastes: sweet, salty, sour, bitter, and umami (Kinnamon and Cummings 1992; Lindemann 1996). These perceptions are mediated by taste receptor cells clustered in taste buds on the surface of the tongue and palate. Electrophysiological studies in rats have shown that salt and sour tastants activate ligand-gated cation channels (Heck, Mierson, and DeSimone 1984; Kinnamon, Dionne, and Beam 1988). In contrast, sweet, bitter, and umami tastes are mediated by receptors belonging to the G protein–coupled receptor hyperfamily, characterized by seven transmembrane domains (Striem et al. 1989; Wong, Gannon, and Margolskee 1996).

Mammalian bitter taste receptors (T2Rs) are encoded by a gene family that consists of a few dozen members (Conte et al. 2002; Shi et al. 2003) which share 20% to 70% sequence identity between mouse and human at the amino-acid level. Twenty-five and 33 putative bitter taste receptor genes were found in the human and mouse genomes, respectively (Conte et al. 2002; Shi et al. 2003), and 4 of the mouse genes have been directly shown to be activated by bitter tastants (Chandrashekar et al. 2000; Bufe et al. 2002).

The coding regions of T2Rs are ~900 base pairs (bp) long and are not interrupted by introns. In humans, they are organized in two main clusters; one on chromosome 12p13 and one on chromosome 7q31 (Adler et al. 2000). In addition, a single gene (T2R1) is found on human chromosome 5p15 (Reed et al. 1999).

The ability to taste bitter compounds is thought to be critical to survival as it helps to avoid the ingestion of potentially poisonous and harmful substances. One might therefore hypothesize that these genes evolve under strong, perhaps species-specific, evolutionary constraint. A comparison of the human and mouse bitter taste receptor repertoires revealed several species-specific gene duplications (Conte et al. 2003; Shi et al. 2003), and it has been shown in mammals that the rate of nonsynonymous substitution (dN) is higher than the rate of synonymous substitutions (dS) in the T2R extracellular domains (Conte et al. 2003; Shi et al. 2003) that are putatively involved in ligand binding (Adler et al. 2000; Gilbertson, Damak, and Margolskee 2000).

Primates are a group of mammals that exhibit exceptional ecological and dietary diversity (Fleagle 1999). We have previously shown that Old World monkeys, apes, and humans differ remarkably in their fractions of intact olfactory receptor (OR) genes (Gilad et al. 2003). To better understand how taste perception may vary among humans and apes, we sequenced the T2R gene repertoires of a human and all extant ape species (chimpanzee, bonobo, gorilla, orangutan) as well as a rhesus macaque and a baboon.

Materials and Methods

DNA Samples

DNA samples from one human, two chimpanzees (*Pan troglodytes troglodytes*), and one bonobo (*Pan paniscus*) were prepared from cell lines obtained from lymphocytes transformed with Epstein-Barr virus. DNA samples from two gorillas (*Gorilla gorilla*), two orangutans (*Pongo pygmaeus*), one rhesus (*Macaca mulatta*), and one baboon (*Papio hamadryas*) were isolated from liver.

Data Mining

The T2R sequences from human were retrieved from the National Center for Biotechnology Information (NCBI) using the Entrez-protein database. Each protein was used as a query in two *tblastn* searches to look for potentially new bitter taste receptor sequences: one against the NCBI "*nr*" database with organisms restricted to human, and another search using the Celera human genome assembly. All overlapping sequences were merged. The nucleotide sequence was then obtained using the protein coordinates. The Entrez-nucleotide database was used to find all the T2Rs known from the mouse. These sequences were then BLASTed against the rat genome to find the orthologous sequences.

Polymerase Chain Reaction (PCR) Amplification

The 35 genes that were considered in this study have a single coding exon of approximately 900 bp. Specific PCR primers were designed for each gene, in the regions flanking the coding sequence. A list of all primers is available online as Supplementary Material. With four different primer combinations, the amplification of T2R45 failed to give the

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expected product in human. Amplifications were done for each species separately to avoid DNA contamination. PCR amplification (50 μl) contained a standard buffer (10 mM Tris-HCl, 5 mM $MgCl_2$), the four deoxynucleotide triphosphates (0.25 mM each), primers (0.5 pM each), 1 unit of *Taq* polymerase, and genomic DNA (70 ng). Polymerase chain reaction conditions were as follows: 35 cycles of denaturation at 94°C for 30 s, annealing for 45 s at 57° and 59°C depending on the primers, and extension at 72°C for 2 min. The first step of denaturation was 45 s. A final extension step was carried out at 72°C for 3 min. When an insertion/deletion was found following analysis, the PCR products were cloned with the TOPO TA-cloning kit (Invitrogen, Paisley, Scotland). Once the bacteria had grown on agar plates, 10 white colonies were picked and a new amplification was performed with 40 cycles beginning with a denaturing temperature of 94° for 1 min, followed by primer annealing at 57°C for 1 min, and an elongation temperature of 72°C for 2 min.

DNA Sequencing

The PCR primers were also used for sequencing. Additional sequencing primers were designed to anneal every 400 bp for complete coverage in both orientations. Following DNA amplification, PCR products were purified with the Millipore (Genomics, Bedford, Mass.) system, and the quantity of purified DNA was estimated by electrophoresis in a 1% agarose gel and by measurement with a spectrophotometer. Approximately 10 ng of the purified sample (1–4 μl) was used as a sequencing template. These templates were diluted in water to a volume of 7 μl, and 1 μl primer (5 pM) and 2μl dye-terminator were added. Sequencing was done by using the BigDye Terminator Cycle Sequencing kit and the ABI Prism 3700 (Applied Biosystems, Darmstadt, Germany).

Sequence Analysis

Chromatograms were imported to Sequencher 4.0 (Gene Codes Corporation) for assembly into contigs. Coding region disruptions were identified separately for each species. All sequences are available in GenBank under accession numbers AY724810–AY725023.

We concatenated all the sequences from the genes and pseudogenes amplified in all species and estimated the number of synonymous substitutions on each lineage using the PAML software package (Yang 1997). We then calculated the gene silencing rate for each lineage by dividing the number of gene silencing events per lineage by the number of synonymous substitutions and compared each value to the mean pseudogeneization rate (total number of pseudogeneization events/total number of synonymous substitutions) with a Fisher exact test.

Using PAML, we inferred ancestral sequences and estimated dN/dS ratios on each lineage for concatenated sequence of all the genes found to be intact in all species. We used the lineage specific model and a likelihood ratio test to test whether a model allowing different rates for the different lineages (model 1) is significantly more likely than a null model (model 0). For the analysis of individual genes, dN/dS ratios were estimated over the whole tree by the modified Nei and Gojobori method (Nei and Gojobori 1986) using PAML. To define the different protein domains of the T2R receptors, we aligned the amino-acid sequences of all the genes intact in all species to find conserved motifs. We also created a Kyte-Doolittle plot (Kyte and Doolittle 1982) for each protein to identify the hydrophobic transmembrane domains. We superimposed these hydrophobicity profiles and found out that some genes had the third extracellular loop shifted compared to other. We thus built two alignments and defined the different domains for the two groups of genes separately (fig. 2 in the Supplementary Material online). The dN/dS ratios were then estimated for the extracellular, transmembrane, and intracellular domains of each protein using PAML. For each further step, we first tested the distributions of dN/dS for normality with a Kolmogorov-Smirnow test. Because normality could not be rejected for any of the distributions ($P > 0.05$), we continued by using a one-sample *t*-test to ask whether the means are significantly different from one, and an independent *t*-test to compare pairwise means.

Results

T2R Gene Repertoires

We performed a database search with known bitter taste receptor genes in order to identify the entire T2R gene repertoire in humans. We found 25 intact full-length bitter taste receptor–like genes and 10 pseudogenes. Of these, two pseudogenes (Ps9 and Ps10) were not previously identified (Bufe et al. 2002; Conte et al. 2002; Shi et al. 2003).

With the exception of T2R1 on chromosome 5, all human bitter taste receptor genes are clustered on chromosomes 7 and 12. An analysis of the phylogenetic relationships among bitter taste receptor genes confirms that genes on the same chromosome are more closely related to each other than genes across chromosomes (Conte et al. 2003; Shi et al. 2003; figure 1 in Supplementary Material online). Moreover, the sequence similarity among the T2R genes on chromosome 12 is higher than among the T2R genes on chromosome 7 (61% versus 47%, respectively, χ^2, $P \ll 10^{-3}$). This observation supports the suggestion that these genes evolve by tandem gene duplications and that more recent duplication events occurred among the T2R genes on chromosome 12 than on chromosome 7 (Conte et al. 2003; Shi et al. 2003).

We designed gene-specific primers for the coding regions of all 35 human bitter taste receptor genes, and amplified them from one human individual, from all major ape species, as well as from rhesus and baboon (table 1). Of the 90% successfully amplified genes, one gene in chimpanzee and another gene in gorilla carried polymorphisms that result in coding region disruptions in one of the two alleles amplified in an individual. In further analyses, these genes were considered as intact.

The fraction of T2R pseudogenes is 29% for human and ranges from 15% to 28% for the apes and the Old World monkeys (table 1). The proportion of pseudogenes

Table 1
Taste Receptor Genes Analyzed and the Fractions of Pseudogenes in Each Species

Locus	Human	Bonobo	Chimpanzee	Gorilla	Orang	Rhesus	Baboon	Chromosomal location	dN/dS
T2R1	–	–	–	–	–	ND	ND	5	0.6
T2R3	–	–	–	–	–	–	–	7	0.59
T2R4	–	–	–	–	–	–	–	7	0.88
T2R5	–	–	–	–	–	–	–	7	0.91
T2R7	–	–	–	–	–	–	–	12	0.5
T2R8	–	–	–	–	–	–	–	12	0.89
T2R9	–	Stop513	–	Stop267	–	–	–	12	0.86
T2R10	–	–	–	–	–	ND	–	12	0.81
T2R13	–	–	–	–	–	–	–	12	1.58
T2R14	–	–	–	–	–	–	–	12	1.11
T2R16	–	–	–	–	–	–	–	7	0.81
T2R38	–	–	–	–	–	–	–	7	0.43
T2R39	–	–	–	–	Stop81	–	–	7	0.76
T2R40	–	–	–	–	–	–	–	7	0.75
T2R41	–	–	–	–	–	–	–	7	0.85
T2R43	–	ND	–	ND	ND	–	–	1	0.95
T2R44	–	–	–	–	–	Stop255	–	1	1.23
T2R46	–	–	–	–	ND	–	–	1	0.96
T2R47	–	–	–	–	–	Stop363	ND	1	1.22
T2R48	–	Stop765	ND	–	–	–	Stop114	12	1.48
T2R49	–	–	–	–	–	–	–	12	1.48
T2R50	–	–	–	–	–	–	–	12	0.87
T2R55	–	–	–	–	–	–	–	12	1.11
T2R56	–	–	–	–	–	–	–	7	0.9
Ps1	Stop627	–	–	–	–	–	–	7	0.89
Ps2	Stop759	ND	–	ND	–	ND	ND	12	
Ps3	Stop120	Stop120	Stop120	Stop120	Stop120	Stop120	Stop120	7	
Ps4	Stop465	Stop465	Stop465	Stop465	–	ND	ND	12	
Ps5	Stop60	Stop60	Stop60	Stop60	Stop60	Stop60	Stop60	12	
Ps6	Stop216	Stop216	Stop216	Stop216	Stop216	ND	ND	12	
Ps7	Stop87	Stop87	Stop87	Stop87	Stop87	ND	ND	12	
Ps8	Stop771	Stop771	Stop771	Stop771	–	ND	ND	12	
Ps9	Stop465	–	–	–	–	–	Stop849	7	0.73
Ps10	Stop48	Stop48	Stop48	Stop48	Stop48	ND	ND	12	
Fraction of pseudogenes	29%	28%	21%	25%	21%	15%	15%		

NOTE.—For the pseudogenes, the last base of the first stop codons in the reading frames are given. The nomenclature is the one from Shi et al. (2003). Ps: pseudogenes. ND: genes for which no PCR products were obtained.

is not significantly different across species (χ^2, $P = 0.78$), even if the two segregating pseudogenes mentioned above are considered as pseudogenes (χ^2, $P = 0.76$). The same conclusion holds when the non-amplified genes are considered all as intact or as pseudogenes (χ^2, $P = 0.88$ and $P = 0.47$, respectively).

We assigned mutational events that inactivate putatively intact genes to lineages using parsimony and estimated the gene silencing rates of each lineage by dividing the number of pseudogenization events by the number of synonymous substitutions on each lineage across all genes that are intact in all species (fig. 1). If only inactivating events that can be unambiguously assigned to a lineage are considered, the sum of the two lineages that lead to the common ancestors of the apes and humans, and the baboon and rhesus macaques, respectively, stand out as having experienced no detected pseudogenization events but, rather, a large number of synonymous substitutions ($P = 0.004$). However, when genes that we failed to amplify are considered pseudogenes, this difference is no longer significant ($P = 0.18$), and the gene silencing rates on the separate lineages are not different relative to the mean of the entire phylogeny (Fisher's exact tests not significant).

Mode of Evolution of T2R Genes

Among the successfully amplified genes, 15 have an intact coding region in all seven species (table 1). We concatenated these 15 genes in each species and estimated the dN/dS ratio on each lineage using two models of protein evolution. Under the null model, there is one dN/dS ratio for all lineages, whereas under the alternative model, each lineage is allowed a separate dN/dS ratio. This model should tell us if there are differences in the selective pressures acting on the different lineages. Using a likelihood ratio test, we failed to reject the null model of equal dN/dS ratios in all lineages (χ^2 test, $P = 0.69$), suggesting that T2R genes as a group did not evolve under marked species-specific selective pressures.

For the 26 genes where intact coding regions were found in five or more species, we estimated an "overall" dN/dS ratio for each gene, excluding lineages where a gene is carrying inactivating substitutions (table 1). The overall dN/dS ratios for the entire tree for each gene vary between 0.43 and 1.58 (table 1), and the mean dN/dS for all genes is 0.93. This is significantly higher than the average dN/dS ratio of 0.21 for primates (Yang and

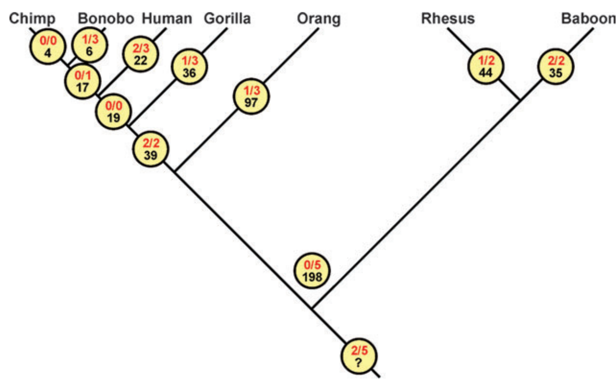

FIG. 1.—T2R pseudogene accumulation and number of synonymous substitutions per lineage in primates. The number of gene silencing events is given in red. The first number gives unambiguously assigned events and the second number the events inferred if genes not amplified are considered pseudogenes. The number of synonymous substitutions estimated to have occurred in genes intact in all species is given in black.

Nielsen 1998; Zhang 2000) and is not significantly different from one (one-sample *t*-test, $P = 0.22$).

The mean dN/dS ratio for the T2R gene cluster on chromosome 7 is significantly lower than for the cluster on chromosome 12 (0.77 and 1.07, respectively, $P = 0.004$). Moreover, the mean dN/dS ratio for the genes on chromosome 12 is not significantly different from one (one-sample *t*-test, $P = 0.19$), whereas the ones on chromosome 7 are (one-sample *t*-test, $P < 10^{-3}$).

We defined the different domains of the T2R proteins using conserved motifs in multiple alignments of the amino acid sequences and hydrophobicity plots (fig. 2 in the Supplementary Material online) and estimated dN/dS ratios for the parts of the genes encoding protein domains across the entire tree, using the same approach as above for individual T2R genes. The mean dN/dS for the domains across genes are 0.82, 0.74, and 1.44 for the intracellular (IC), transmembrane (TM), and extracellular (EC) domains, respectively.

Discussion

We studied the repertoires of bitter taste receptor genes in human as well as in six non-human primates. The proportions of pseudogenes do not differ among the species studied, although this conclusion is less secure for the Old World monkeys because several genes failed to be amplified in these species (table 1). Thus, the proportions of T2R pseudogenes, and probably the total number of genes, do not differ radically among apes and humans.

The mean dN/dS ratio for the T2R genes in the primates studied is 0.93 and thus was surprisingly high given an average dN/dS ratio for genes in primates of 0.21 (Yang and Nielsen 1998; Zhang 2000). This could mean that the bitter taste receptor genes underlie little or no constraints in primates. However, when 26 orthologous pairs of bitter taste receptor genes are compared between rat and mouse, the average dN/dS ratio is 0.56 and thus is substantially higher than the dN/dS ratio of 0.13, which was found for 48 random genes in these rodents (Yang and Nielsen 1998; Zhang 2000). The fact that the rate of amino acid replacements in primates is higher than in rodents may occur because the effective population sizes in apes are smaller than in rodents and purifying selection is thus less effective (Zhang 2000; Weinreich 2001). Thus, it would seem that the evolution of bitter taste receptor genes is characterized by a high rate of amino acid substitution in mammals.

An interesting question is whether the high dN/dS ratios typical of T2R receptor gene evolution reflect a low level of selective constraint on bitter taste receptors in general, or if they reflect positive selection acting on parts of the proteins. The facts that the average dN/dS ratio of T2R transmembrane domains is lower than one, while the dN/dS ratio for the extracellular domains that is putatively involved in ligand binding is higher than one (albeit not significantly so), suggest that not only a low level of functional constraint but also positive selection may play a role in the evolution of T2R genes. Also of relevance is the finding that the mean dN/dS ratio for T2R genes, as well as the proportion of pseudogenes, is significantly higher on chromosome 12 than on chromosome 7 in all ape species. Because recent duplications of T2R genes are more common on chromosome 12 than on chromosome 7 (fig. 1 in the Supplementary Materials online), this suggests that bitter taste receptors underlie functional constraints that differ and depend on whether closely related receptors exist in the organism.

It is interesting to compare our observations to the evolution of olfactory receptor genes (OR) in humans and apes. It has been shown that when a sample of 50 OR genes was studied—i.e., a number similar to what we studied here—humans were found to accumulate OR coding region disruptions roughly threefold to fourfold faster than apes and Old World monkeys (Gilad et al. 2003). However, an alternative explanation is that purifying selection is less efficient in humans than in other primates due to the smaller human effective population size (Chen and Li 2001; Kaessmann et al. 2001). If the latter explanation is correct, we would also expect a more rapid accumulation of pseudogenes in humans in gene families other than OR genes. In contrast, we find no evidence that the bitter taste receptor gene repertoire has experienced relaxed constraint in humans relative to the apes. This supports the notion that the higher proportion of OR pseudogenes in humans is caused by reduced olfactory needs. Although substitutions outside the coding regions, as well as differences in threshold sensitivity (Simmen and Hladik 1998; Hladik, Pasquet, and Simmen 2002) or gene expression, may influence the function of bitter taste receptors, our results suggest that while human olfactory capability is reduced compared to apes as a result of an increase in the fraction of pseudogenes, there has been no significant reduction in the repertoire of bitter taste receptors in humans.

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