First evidence for functional vomeronasal 2 receptor genes in primates

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Two classes of vomeronasal receptor genes, V1R and V2R, occur in vertebrates. Whereas, V1R loci are found in a wide variety of mammals, including primates, intact V2R genes have thus far only been described in rodents and marsupials. In primates, the V2R repertoire has been considered degenerate. Here, we identify for the first time two intact V2R loci in a strepsirrhine primate, the grey mouse lemur (Microcebus murinus), and demonstrate their expression in the vomeronasal organ. Putatively functional orthologues are present in two other strepsirrhines, whereas, both loci are pseudogenes in a range of anthropo-poid species. The functional significance of the loci is unknown, but positive selection on one of them is consistent with an adaptive role in pheromone detection. Finally, conservation of V2R loci in strepsirrhines is notable, given their high diversity and role in MUP and MHC detection in rodents.

1. Introduction

The vomeronasal organ (VNO) in mammals is an olfactory organ specialized for the perception of pheromones and predator cues (kairomones) [1,2]. Whereas, rodents possess large VNOs and huge repertoires of both classes of vomeronasal receptors (V1Rs and V2Rs), the size and functionality of the VNO vary immensely in primates. It is well developed in strepsirrhines (lemurs and lorisoids), smaller but still functional in tarsiers and most New World monkeys, and completely non-functional in Old World monkeys, apes and humans [3,4]. Correspondingly, the gene repertoire of V1Rs is huge in strepsirrhines (78 and 214 estimated intact genes in galago and mouse lemur, respectively; [5]), but only contains a few putative functional genes in anthropoid primates (e.g. five in humans although not expressed in the VNO) [5,6]. In contrast, to date, functional V2Rs have only been identified in rodents and marsupials, and the V2R repertoire in primates was reported to be degenerate [7]. However, the multiple exonic structure of V2Rs in comparison with single exon V1Rs has been considered a hindrance to their identification in databases, and previous studies have only reported catarrhine primate genomes (macaque, chimpanzee and human) [7]. Here, for the first time, we search for V2R loci in available primate genomes with a primary focus on strepsirrhine primates to determine whether V2R function occurred only later in primate evolution. We also use expression of putative V2Rs in the grey mouse lemur to confirm probable functionality, and investigate patterns of natural selection on putative functional loci.

2. Material and methods

(a) Data collection

We used BLAST (Basic Local Alignment Search Tool) to search for V2R loci in the three available strepsirrhine genomes (Otolemur garnettii, Genbank AAQR 0000
Table 1. Sequences for two V2R genes with number of available base pairs from available genome sequences (percentage of total length in round brackets); mouse sequences are VmV2R1 and VmV2R2, respectively; Microcebus sequence information was obtained by Sanger sequencing; second line shows obtained full-length exons (incomplete exons in square brackets); superscript ‘ps’ denotes exon 2 sequence pseudogenized.

<table>
<thead>
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<th>gene</th>
<th>Mus</th>
<th>Microcebus</th>
<th>Daubentonia</th>
<th>Otollemur</th>
<th>Tarsius</th>
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<td>2739 bp (100)</td>
<td>2319 bp (85)</td>
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<tr>
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<td>2418 bp (100)</td>
<td>1927 bp (82)</td>
<td>2888 bp (98)</td>
<td>1145 bp (52)</td>
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<tr>
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<td>123456</td>
<td>123456</td>
<td>123456</td>
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</tbody>
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0000.3; Daubentonia madagascariensis, AGTM 0000 0000.1; Microcebus murinus, ABDC 0000 0000.1. All exons (usually six) of the 122 intact mouse V2R genes derived from the UCSC Genome Browser [8] were used in BLAST searches. For potentially functional V2R genes (open reading frame, no stop codon), we used exon 6 to search for orthologues in the 12 further primate species with available genome data (Tarsius syrichta, Callithrix jacchus, Saimiri boliviensis, Macaca fascicularis, Macaca mulatta, Papio anubis, Nomascus leucogenys, Pongo abelii, Gorilla gorilla, Pan paniscus, Pan troglodytes and Homo sapiens). Exon 6 encodes the transmembrane region, which is highly conserved compared with the N-terminal of the receptor [9], and has been the main focus in other studies [10]. Moreover, exon 5 was identified whenever exon 6 was available, either because it was found on the same shotgun read or in the neighbouring read (Genbank accession numbers in electronic supplementary material, S3). Sequences were aligned and analysed in MEGA v. 5 [11]. Phylogenies were reconstructed using neighbour joining trees with maximum composite likelihood.

(b) Confirmation of transcription

We extracted total RNA from the VNO of a male grey mouse lemur (M. murinus) to confirm the expression of V2R genes in a strepsirrhine primate. The animal was euthanized for veterinary reasons; the VNO was removed immediately after death and stored in RNA later (Qiagen). RNA was extracted with the RNeasy Micro Kit (Qiagen) and transcribed into cDNA using a Quantitect Reverse Transcription Kit (Qiagen) and N6 primer, according to manufacturer’s instructions. RT-PCR and sequencing of TRPC2, which encodes a cation channel protein expressed almost exclusively in the VNO, were used to confirm successful cDNA synthesis from VNO tissue.

Several pairs of internal primers on different exons and two external primers (close to the 5'- and 3'-end of exon 1 and exon 6, respectively), were designed to amplify the complete V2Rs (total length of more than 2400 bp) in several ampiclons with MyTaq DNA polymerase (Bioline) using sequence information from the three strepsirrhine data (for PCR details, see electronic supplementary material, S3). PCR products were sequenced on both the strands using BigDye Terminator v. 3.1 (Applied Biosystems) under standard conditions and run on an Applied Biosystems 3500 capillary sequencing machine. Consensus sequences of single genes were built with SeqMan v. 5.05 (DNASTAR Inc., Madison, WI, USA). Sequences were aligned and analysed using MEGA v. 5.

(c) Mode of selection on V2R genes

We used codon-based site-specific substitution models (codeml in PAML v. 4.4) [12] to estimate $d_{NS}/d_{S}$ ratios (non-synonymous versus synonymous substitution rates) and to test for positive selection in V2R genes in strepsirrhines. The likelihood of model M1a that does not allow positive selection was compared with that of model M2a that allows positive selection ($d_{NS}/d_{S} > 1$) using a likelihood ratio test. We also used branch models to estimate different $d_{NS}/d_{S}$ ratios in separate primate branches (for details on all PAML analyses, see studies of Hohenbrinck et al. [13]).

3. Results and discussion

(a) V2Rs in strepsirrhines and tarsiers

BLAST searches revealed two potentially functional V2R (greater than 80% sequence, intact open reading frame) in the three strepsirrhine genomes, which we name VN2R1 and VN2R2 (table 1, more details in electronic supplementary material, S2). VN2R1 was also found to be potentially intact in the Philippine tarsier, T. syrichta, whereas, VN2R2 was a pseudogene in this species with a frameshift and stop codon in exon 2. Data were missing from some exons, notably exon 4 of VN2R2 which is very short (22 bp).

The expression of both genes in the VNO of the mouse lemur was confirmed by RT-PCR, and full-length cDNAs with intact reading frames were obtained by sequencing (Genbank accession nos KC208006 and KC208007). Thus, at least two intact V2R genes were conserved in strepsirrhine lineages, and these are most likely functional in the VNO. This may be an underestimate of the total number of intact V2Rs (for example, gaps in the genome of the grey mouse lemur mean that 105 intact V1Rs were found out of an estimated 214; [9]), but the total is still very small compared with the large V2R repertoire in mice (greater than 120 loci), showing much lower importance of V2Rs in strepsirrhines. It is also small when compared with the V1R repertoire in strepsirrhines, which have become the dominant class of vomeronasal receptors.

Phylogenetic reconstructions show that VN2R1 groups with mouse V2R family C, whereas, VN2R2 belongs to V2R family D (figure 1). No strepsirrhine members of mouse families A or B was found, which is interesting since family A is by far the most diverse in mice containing over 80 per cent of all V2Rs. In mice, one V2R gene of families A, B or D is generally coexpressed with one V2R gene of family C in non-random combinations [14], suggesting the possibility that the two strepsirrhine V2Rs are themselves coexpressed.

Selection analyses revealed that a proportion of codons of VN2R2 significantly evolved under positive selection in strepsirrhines (model M2a $d_{NS}/d_{S} = 22.82$, $p_3 = 1.4\%$; likelihood ratio statistics, LRS = 6.9, $df = 2$, $p = 0.031$), whereas, VN2R1 did not show signs of positive selection (LRS = 0.0, $p = 1.0$; detailed results can be seen in electronic supplementary material S4).
Degeneration of VN2R1 and VN2R2 in anthropoid primates

We found homologues of exons 5 and 6 of VN2R1 in 11 anthropoid primate species spanning New World monkeys, Old World monkeys, apes and humans, but all of these were pseudogenes, with at least one exon containing reading frame shifting deletions and/or non-sense mutations. The phylogenetic reconstruction of these sequences recapitulates the primate species phylogeny (figure 2; [15]).

The estimated $d_{\text{NS}}/d_{\text{S}}$ ratio in branch tests was close to 1 in the anthropoid branch ($d_{\text{NS}}/d_{\text{S}} = 0.94$) as expected for pseudogene evolution, while $d_{\text{NS}}/d_{\text{S}}$ was lower in the mouse (0.28), the strepsirrhine branch (0.53) and the tarsier (0.35), indicating purifying selection as the main mode of selection that is conserving the function of these $V2R$ genes. For VN2R2, we found further orthologous sequences only in orangutans, both chimpanzee species and humans (all pseudogenes; results not shown). Because only one of the two $V2R$ genes was intact in tarsiers, it appears that pseudogenization of $V2R$ genes began before the split of tarsiiformes and anthropoids and continued in the anthropoid stem lineage. Interestingly, both genes are pseudogenized in New World monkeys, although, this clade still possesses a functional VNO in most genera (including *Callithrix* and *Saimiri*; [3,4]). We suggest dissimilar importance of $V1R$s and $V2R$s through primate evolution with $V2R$s having started to pseudogenize earlier than $V1R$s.

Functional significance of $V2R$s in strepsirrhines

The conservation of two $V2R$s, including positive selection in one of them, strongly suggests important functions in strepsirrhines. In rodents, $V2R$s bind mostly non-volatile molecules and have numerous functions. Intriguingly, these include detection of several of the most important classes of pheromones in rodents, including MUPs (major urinary proteins; [2,16]), ESP1 (exocrine gland-secretory peptide 1; [17,18]) and MHC (major histocompatibility complex) class I peptides [19]. In addition to intraspecific communication, mice can also detect MUPs from predators using $V2R$s [2,20]. It is known that mouse lemurs react to olfactory cues from predators [21,22] and females exercise post-copulatory mate choice based on MHC constitution [23]. Given their conservation over millions of years of evolution and the importance of pheromonal communication in strepsirrhines [24], we can speculate that the intact $V2R$s in strepsirrhines likely have an...
important role in pheromone detection. In conclusion, detection of V2Rs in vertebrate genomes appears to be more straightforward than commonly assumed. Expression data from VNO tissue are important corroborative evidence for V2R (or V1R) function.

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References