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Evolutionary biology

Molecular evolution of the hyaluronan synthase 2 gene in mammals: implications for adaptations to the subterranean niche and cancer resistance

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The naked mole-rat (NMR) *Heterocephalus glaber* is a unique and fascinating mammal exhibiting many unusual adaptations to a subterranean lifestyle. The recent discovery of their resistance to cancer and exceptional longevity has opened up new and important avenues of research. Part of this resistance to cancer has been attributed to the fact that NMRs produce a modified form of hyaluronan—a key constituent of the extracellular matrix—that is thought to confer increased elasticity of the skin as an adaptation for living in narrow tunnels. This so-called high molecular mass hyaluronan (HMM-HA) stems from two apparently unique substitutions in the hyaluronan synthase 2 enzyme (HAS2). To test whether other subterranean mammals with similar selection pressures also show molecular adaptation in their *HAS2* gene, we sequenced the *HAS2* gene for 11 subterranean mammals and closely related species, and combined these with data from 57 other mammals. Comparative screening revealed that one of the two putatively important HAS2 substitutions in the NMR predicted to have a significant effect on hyaluronan synthase function was uniquely shared by all African mole-rats. Interestingly, we also identified multiple other amino acid substitutions in key domains of the HAS2 molecule, although the biological consequences of these for hyaluronan synthesis remain to be determined. Despite these results, we found evidence of strong purifying selection acting on the *HAS2* gene across all mammals, and the NMR remains unique in its particular HAS2 sequence. Our results indicate that more work is needed to determine whether the apparent cancer resistance seen in NMR is shared by other members of the African mole-rat clade.

1. Introduction

The naked mole-rat (NMR) *Heterocephaus glaber* is emerging as an important 'non-model' organism for the study of longevity and healthy ageing. A range of physiological and molecular/biochemical adaptations underpin the lack of senescence observed in this small Hystricomorph rodent that can live for 32 years—10 times longer than a mouse and more than five times longer than predicted for its body size [1].

There has been considerable interest in the ability of NMRs to resist cancer [1,2], and recently, a mechanism involving the production of a high molecular mass hyaluronan (HMM-HA) has been proposed [3]. Hyaluronan (HA) is a glycosaminoglycan and its presence in a variety of tissues and the extracellular matrix has been linked to many cellular processes, such as cell division, motility and morphogenesis [4], and also implicated in the development of some

cancers [5]. HMM-HA is up to six times the mass of the largest human HA. The novel anti-cancer mechanism identified in the NMR has been termed early contact inhibition (ECI). This is a process whereby cell growth occurring when cells come into contact with each other, or with the extracellular matrix, is arrested at much lower densities than in the mouse. Contact inhibition is lost in cancer cells, and the loss of ECI makes cells more susceptible to malignant transformation [6]. ECI is controlled by the interaction of HA with the CD44–NF2 pathway, which mediates contact inhibition [3]. In addition, NMRs also produce a novel tumour suppressor protein (an isoform of INK4) in response to HMM-HA stimulation. Induction of *INK4* is associated with contact inhibition and leads to cell-cycle arrest, contributing to tumour resistance [7]. In the NMR, it has been postulated that selection for its characteristic loose, elastic skin is an adaptation to living underground in tight tunnels and confined spaces, and that the elasticity of the skin is facilitated by HMM-HA [3]. Thus, the cancer resistance imparted by HMM-HA may be a secondary and fortuitous consequence of the primary function of HMM-HA under selection.

Hyaluronan synthase 2 (HAS2) is one of three characterized membrane-embedded HA synthases responsible for the synthesis of HA from intracellular precursors, and deposition into the extracellular matrix [8]. Tian *et al.* [3] showed that HMM-HA is produced in NMRs by a uniquely modified version of the *HAS2* gene, and accumulates owing to extremely low hyaluronidase activity. Specifically, two serine substitutions at highly conserved sites in the cytoplasmic domains of exons 2 and 4 appear to confer upon the protein molecule the ability to produce HMM-HA: when NMR HAS2 was overexpressed in a human HEK293 cell culture, secretion of HMM-HA was observed [3].

Given the link between NMR cancer resistance, HMM-HA and specific mutations in HAS2, together with the advantages of an elastic skin in the subterranean niche, we predict that production of HMM-HA may not be unique to NMRs, and that discovery of other HAS2 mutations may be of potential interest to cancer research. This study, therefore, aims to test for possible parallel signatures of adaptive evolution in the *HAS2* gene in other mammals with shared selection pressures; in particular, other members of the African mole-rat family (Bathyergidae), as well as divergent subterranean insectivorous mammals within the superorders Afrotheria and Laurasiatheria.

2. Material and methods

We generated new *HAS2* sequence data from 11 subterranean mammal species representing five divergent families (table 1;

Table 1. Sample list, taxonomy and accession numbers/EMSEMBL Transcript ID for the *HAS2* sequences included in our analysis.

scientific name	common name	order; family	accession number
Homo sapiens	human	Primates; Hominidae	U54804.1
Pan troglodytes	chimpanzee	Primates; Hominidae	XM528222.4
Pan paniscus	bonobo	Primates; Hominidae	XM3820492.1
Pongo abelii	orangutan	Primates; Hominidae	XM3777303.1
Nomascus leucogenys	northern white-cheeked gibbon	Primates; Cercopithecidae	XM3256164.2
Macaca fascicularis	crab-eating macaque	Primates; Cercopithecidae	XM5564005.1
Macaca mulatta	rhesus macaque	Primates; Cercopithecidae	XM1098841.2
Chlorocebus sabaeus	green monkey	Primates; Cercopithecidae	XM8001453.1
Saimiri boliviensis boliviensis	squirrel monkey	Primates; Cebidae	XM3933098.1
Callithrix jacchus	common marmoset	Primates; Callitrichidae	XM2759268.2
Otolemur garnettii	Garnett's greater galago	Primates; Galagidae	XM_3782374.1
Tarsius syrichta	Philippine tarsier	Primates; Tarsiidae	XM8056607.1
Tupaia chinensis	Chinese tree shrew	Scandentia; Tupaiidae	XM_6157522.1
Lipotes vexillifer	Yangtze River dolphin	Cetacea; Lipotidae	XM_7445586.1
Physeter catodon	sperm whale	Cetacea; Physeteridae	XM_7106932.1
Odobenus rosmarus divergens	walrus	Carnivora; Odobenidae	XM_4416640.1
Leptonychotes weddellii	Weddell seal	Carnivora; Phocidae	XM_6739332.1
Canis lupus familiaris	dog	Carnivora; Canidae	XM_539153.4
Panthera tigris altaica	tiger	Carnivora; Felidae	XM_7075592.1
Felis catus	cat	Carnivora; Felidae	XM_4000089.2
Ailuropoda melanoleuca	panda	Carnivora; Ursidae	XM_2927908.1
Mustela putoriua furo	ferret	Carnivora; Mustelidae	XM_4804975.1
Myotis davidii	David's mouse-eared bat	Chiroptera; Vespertilionidae	XM_6769087.1
Myotis lucifugus	little brown bat	Chiroptera; Vespertilionidae	XM_6085251.1

(*Continued.*)

Table 1. (*Continued.*)

scientific name	common name	order; family	accession number
Myotis brandtii	Brandt's bat	Chiroptera; Vespertilionidae	XM_5885867.1
Eptesicus fuscus	big brown bat	Chiroptera; Vespertilionidae	XM_8143356.1
Pteropus alecto	black flying fox	Chiroptera; Pteropodidae	XM6916584.1
Elephantulus edwardii	Cape elephant shrew	Macroscelidea; Macroscelididae	XM_6879317.1
Orycteropus afer afer	aardvark	Tubulidentata; Orycteropodidae	XM_7943422.1
Trichechus manatus latirostris	Florida manatee	Sirenia; Trichechidae	XM_4372979.1
Procavia capensis	rock hyrax	Hyracoidea; Procaviidae	ENSPCAG00000005792
Loxodonta africana	African elephant	Proboscidea; Elephantidae	XM_003408169
Echinops telfairi	lesser hedgehog tenrec	Afrosoricida; Tenrecidae	XM_004697409
Amblysomus hottentotus[a]	golden mole	Afrosoricida; Chrysochloridae	KR057419
Oryctolagus cuniculus	European rabbit	Lagomorpha; Leporidae	AB055978.1
Ochotona princeps	American pika	Lagomorpha; Ochotonidae	XM_6982381.1
Spermophilus tridecemlineatus	thirteen-lined ground squirrel	Rodentia; Sciuridae	XM_5316195.1
Mus musculus	mouse	Rodentia; Muridae	U52524.2
Rattus norvegicus	rat	Rodentia; Muridae	AF008201.1
Peromyscus maniculatus bairdii	prairie deer mouse	Rodentia; Cricetidae	XM_6982381.1
Cricetulus griseus	Chinese hamster	Rodentia; Cricetidae	XM_7638417.1
Mesocricetus auratus	golden hamster	Rodentia; Cricetidae	XM_5082729.1
Tachyorychtes splendens[a]	East African root rat	Rodentia; Spalacidae	KR057420
Nannospalax galili	blind mole-rat	Rodentia; Spalacidae	XM_008837986.1
Fukomys zechi[a]	Ghana mole-rat	Rodentia; Bathyergidae	KR057425
Fukomys damarensis[a]	Damaraland mole-rat	Rodentia; Bathyergidae	KR057427
Georychus capensis[a]	Cape dune mole-rat	Rodentia; Bathyergidae	KR057424
Cryptomys hottentotus[a]	common mole-rat	Rodentia; Bathyergidae	KR057422
Bathyergus janetta[a]	Namaqua dune mole-rat	Rodentia; Bathyergidae	KR057423
Bathyergus suillus[a]	dune mole-rat	Rodentia; Bathyergidae	KR057421
Heliophobius kapiti[a]	silvery mole-rat	Rodentia; Bathyergidae	KR057426
Heterocephalus glaber	naked mole-rat	Rodentia; Bathyergidae	XM_004883123
Thryonomys swinderianus[a]	cane rat	Rodentia; Thryonomyidae	KR057428
Hystrix africaeaustralis[a]	Cape porcupine	Rodentia; Hystricidae	KR057429
Ctenomys perrensi[a]	tuco-tuco	Rodentia; Ctenomyidae	KR057430
Cavia porcellus	guinea pig	Rodentia; Caviidae	XM_003463665
Chinchilla lanigera	chinchilla	Rodentia; Chinchillidae	XM5410908.1
Sorex araneus	common shrew	Eulipotyphla; Soricidae	XM4607644.1
Talpa europaea[a]	European mole	Eulipotyphla; Talpidae	KR057431
Condylura cristata	star-nosed mole	Eulipotyphla; Talpidae	XM_4679612.1
Erinaceus europaeus	European hedgehog	Eulipotyphla; Erinaceidae	XM7520808.1
Vicugna pacos	alpaca	Artiodactyla; Camelidae	XM_6211418.1
Bos taurus	cow	Artiodactyla; Bovidae	XM_174079.2
Capra hircus	goat	Artiodactyla; Bovidae	XM_5688873.1
Sus scrofa	pig	Artiodactyla; Suidae	XM_214053.1
Equus caballus	horse	Perissodactyla; Equidae	XM_1081801.1
Ceratotherium simum simum	white rhinoceros	Perissodactyla; Rhinocerotidae	XM_4431107.1
Dasypus novemcinctus	nine-banded armadillo	Cingulata; Dasypodidae	XM_4480750.1
Monodelphis domestica	opossum	Didelphimorphia; Didelphidae	XM_1370252.2
Ornithorhynchus anatinus	duck-billed platypus	Monotremata; Ornithorhynchidae	XM_1505190.2

[a]Species sequenced for this study.

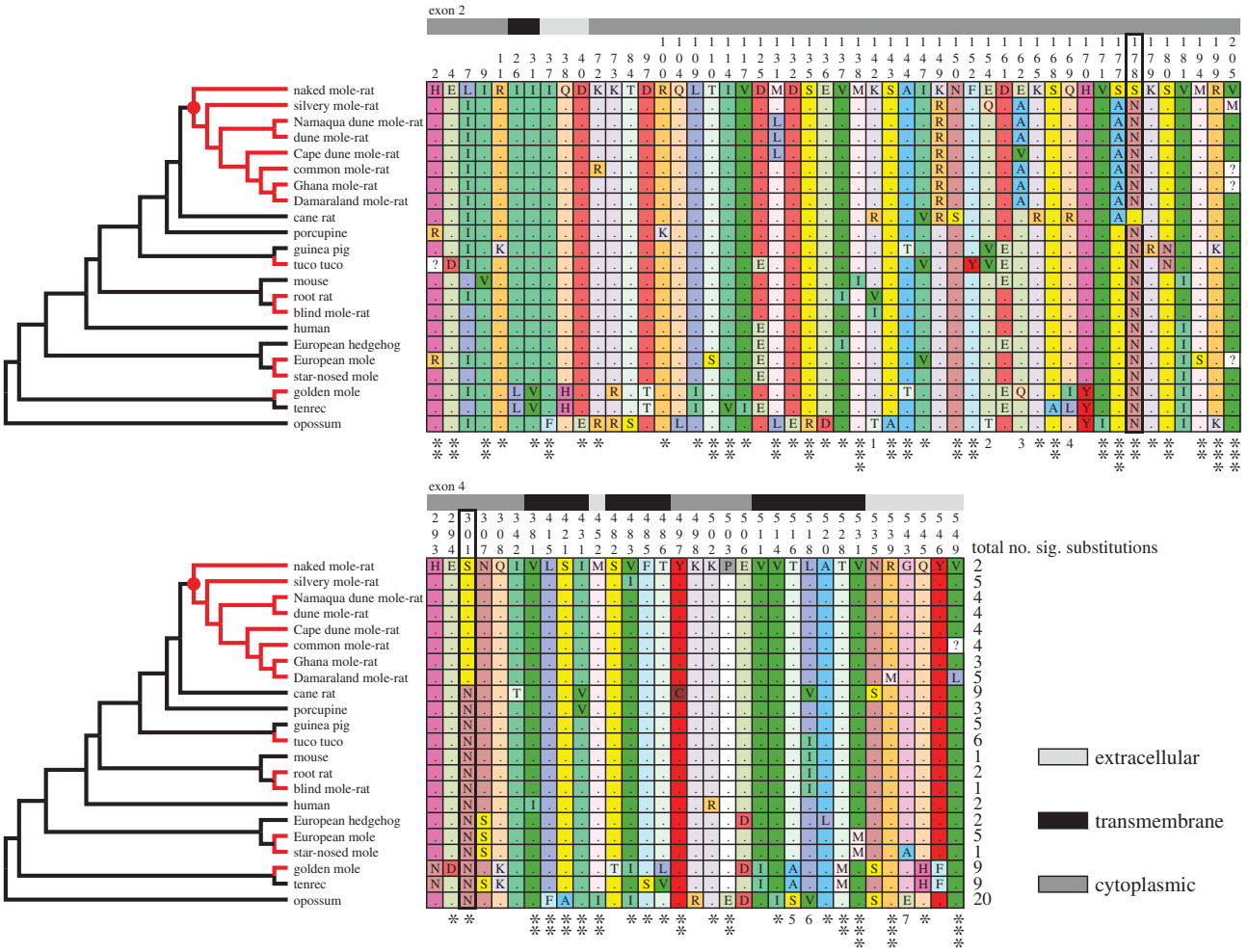

Figure 1. Phylogenetic relationships and corresponding HAS2 sequences of five clades containing subterranean mammals (red branches), including non-subterranean ingroup comparisons, the human and a marsupial (opossum) outgroup (black branches). The Bathyergidae are the monophyletic clade denoted by the red circle. Adjacent panels show the respective variable amino acids for exons 2 and 4 (site numbers indicated above columns). Shaded bars indicate the relative locations of sites in the molecule (extracellular, transmembrane or cytoplasmic). The key amino acid residues at sites 178 and 301—that facilitate production of HMM-HA in NMRs—are indicated by the bold border. Asterisks below sites denote significance of substitutions estimated by MAPP analysis: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; ns, not significant; 1: R = ns, V = **, I = *, T = **; 2: T = **, V = *, Q = ns; 3: V = ***, A = **, Q = ns; 4: R = **, I = ns, L = ns; 5:A = *, S = ns; 6: V = *, I = ns; 7: E = *, A = ns. Numbers at the end of the respective sequence alignments denote the number of substitutions per taxon that are predicted to have a significant impact on protein function.

electronic supplementary material, Methods). These were combined with new sequences from two close outgroups of the Bathyergidae (cane rat and Cape porcupine), and a further 57 representative mammalian *HAS2* sequences from all species available at the time of the study on GenBank (table 1). Sequences were aligned manually for analysis using Mesquite [9] and genetic distances calculated using MEGA v. 6 [10]. To test for signatures of selection acting along 519 codons of *HAS2* (exons 2 and 4) across all 70 mammal species included in our study, we implemented site, branch-site and clade models with the codeml package in PAML v. 4.4 [11], using a mammal species tree topology based on published studies [12–14]. Amino acid polymorphisms were analysed using MAPP [15], which implements a predictive statistical framework to score the physico-chemical impact of substitutions in multiple alignments of orthologues. Novel sequences have been deposited in GenBank.

3. Results and discussion

Overall measures of variation among 70 taxa revealed p-distances (nucleotide) ranging from 0.08 (chimp versus bonobo) to 19.53% (Philippine tarsier versus Opossum) and from zero (e.g. human versus chimp) to 15.26% (Cape elephant shrew versus opossum) for amino acid substitutions (see electronic supplementary material, table S2 for distances and table S3 for a complete amino acid alignment for all taxa). Site, branch-site and clade models implemented in PAML did not find evidence for positive selection; instead, all models suggest that *HAS2* is under purifying (negative) selection with ω values of less than 1 across all mammals ($p < 0.001$; see electronic supplementary material, Results). Despite this, we identified multiple amino acid substitutions in key domains of the HAS2 molecule, including those previously described for the NMR (see figure 1; electronic supplementary material, table S3), although we observed no obvious substitutions shared across subterranean mammals. Of particular interest are the residues at sites 178 and 301 that facilitate production of HMM-HA in NMRs. Our results show that the serine substitution at site 178 in NMRs is only present in one other mammal, the cane rat, a close outgroup to the Bathyergidae, and thus perhaps arose convergently. Interestingly, however, the neighbouring site 177 has a serine residue substituted with an alanine in the cane rat and all bathyergids

except the NMR. Replacement of a serine (which is readily phosphorylated and often important in the active site of enzymes) with an alanine is likely to have functional significance ($p < 0.001$ in MAPP analysis). The serine substitution at site 301 of the NMR is present in all bathyergid genera, but no other mammals, and is a shared derived character (synapomorphy) for the group (electronic supplementary material, figures S1–S3). There are also a number of other unique substitutions in particular species of the Bathyergidae/cane rat (e.g. sites 149 and 162; figure 1). Analysis of these polymorphisms using MAPP revealed multiple significant mutations (figure 1). Among the bathyergids, the Damaraland and silvery mole-rats ranked highest with five significant substitutions, followed by four in common, Cape dune, dune and Namaqua dune mole-rats, and three in the Ghana mole-rat. The NMR had just two—the aforementioned substitutions at sites 178 and 301 (figure 1; electronic supplementary material, figure S4 and table S3). The blind mole-rat HAS2 sequence is unremarkable and similar to the mouse and root rat, despite the fact that this species has also been reported to produce HMM-HA [3]. Thus, the mechanism of HMM-HA production in blind mole-rats may differ from that of NMRs, and cancer resistance also reported in this species appears to be mediated by a different mechanism [16,17]. It is noteworthy that these substitutions were relatively low within the context of the entire mammal dataset examined, where a maximum value of 21 substitutions predicted to have a significant effect was observed in the Cape elephant shrew (electronic supplementary material, table S3 and Supplementary results).

These results raise interesting questions regarding the functional significance of the observed changes in the HAS2 amino acid sequence, and whether there is any correlation with longevity and cancer resistance (where known). Within the Bathyergidae, so far no other taxa are known to live as long as NMRs. Species such as the dune mole-rats (genus *Bathyergus*) and *Georychus* generally have short lifespans in the order of 4–6 years ([18], N. C. Bennett 2014, unpublished data), although two *Georychus* are known to have lived for 10 and 11 years, respectively, in captivity [19]. The Silvery mole-rat (*Heliophobius*) and some *Fukomys* (e.g. Damaraland and Zambian mole-rats) may commonly live more than 7 and 10 years, respectively [19,20], and sometimes up to 15+ years [20]. The absence of cancer has only been noted in NMRs, but there is a paucity of studies on other species in this context. Thus, the role of HAS2 and HA in other species remains unclear, but it is likely that multiple factors contribute to longevity. Nevertheless, our results and analysis provide the basis for further studies to establish the functional significance of HAS2 variants, using *in vitro* methods to characterize the different versions of HA produced, and the role they may play in cancer resistance.

Ethical statement. All procedures involving live animals and sample collection described in this manuscript were conducted in accordance with appropriate national and provincial guidelines, permits and regulations.

Data accessibility. New DNA sequence data have been deposited in GenBank accession numbers: KR057419–KR057431 (table 1).

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Authors' contributions. C.G.F. conceived of, designed and coordinated the study, carried out the molecular laboratory work and sequence alignments, participated in data analysis and drafted the manuscript; K.T.J.D. designed primers, participated in data analysis and bioinformatics; S.J.R. participated in data analysis; N.C.B. provided samples and funding. All authors contributed to critical assessment of the results, manuscript revisions and gave final approval for publication.

Conflict of interest. We have no competing interests.

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