Sciencexpress Report

A Melanocortin 1 Receptor Allele Suggests Varying Pigmentation Among Neanderthals

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The melanocortin 1 receptor (MC1R) regulates pigmentation in humans and other vertebrates. Variants of MC1R with reduced function are associated with pale skin color and red hair in humans primarily of European origin. We amplified and sequenced a fragment of the MC1R gene (*mc1r*) from two Neanderthal remains. Both specimens have a mutation not found in ~3700 modern humans. Functional analyses show that this variant reduces MC1R activity to a level that alters hair and/or skin pigmentation in humans. The impaired activity of this variant suggests that Neanderthals varied in pigmentation levels, potentially to the scale observed in modern humans. Our data suggest that inactive MC1R variants evolved independently in both modern humans and Neanderthals.

One gene responsible for skin and hair color variation in humans is *mc1r*, which encodes a seven transmembrane G protein coupled receptor (GPCR) (Fig. 1) (*1*). Red hair and pale skin result from both complete and partial loss-of-function alleles in the human MC1R (huMC1R) by altering the balance between eumelanin and pheomelanin synthesis (*2*). Thus, we hypothesize that the retrieval of *mc1r* sequences from extinct species can potentially provide information on their phenotypic traits.

Neanderthals are an extinct hominid group that lived in Eurasia ~400,000 - 28,000 years ago (*3*). Recently, metagenomic approaches recovered about one megabase of the Neanderthal genome (*4, 5*), implying that amplification of nuclear DNA by the polymerase chain reaction (PCR) may be feasible in well preserved Neanderthal remains. We studied two Neanderthal fossils, Monti Lessini (Italy) (*6*) and El Sidrón 1252 (Spain) (*7*). Both samples have a low degree of amino acid racemization (<0.10) and high amino acid content (>20,000 ppm) suggesting good DNA preservation (*8*). Amplifications of the hypervariable region 1 of the mitochondrial (mt) DNA showed that endogenous Neanderthal DNA was preserved in these individuals (*6, 9*).

We assumed that retrieval of Neanderthal nuclear DNA sequences from these samples was possible if the amplicon length was short and large numbers of clones were generated. Due to the low divergence between Neanderthals and modern humans, it is impossible to distinguish contamination if the sequences are identical or polymorphisms are shared between the species (*10*). Therefore, we focused on identifying Neanderthal specific substitutions. We successfully amplified a 128 base pair (bp) fragment of *mc1r* (*11*), from the Monti Lessini sample. Most clones were identical to the modern human sequence, most likely representing contamination of the Neanderthal bone with modern human DNA. However, one of the 25 clones had an A to G substitution at nucleotide position 919, resulting in an Arg to Gly change at amino acid position 307, not previously observed in modern humans (*12, 13*). We hypothesized that this was not due to PCR error as most errors are C to T (or G to A) changes, due to cytosine deaminations in the template DNA (*14, 15*).

As the number of amplifiable ancient DNA molecules increases exponentially with decreasing amplification length (*16*), we designed primers to amplify a shorter DNA fragment and performed four different amplifications for El Sidrón 1252 and one for Monti Lessini. We sequenced at least ten clones per reaction. The Arg307Gly substitution was present in all amplification products in frequencies ranging from 7 to 25% (fig. S1). As the human-like sequences probably at least

partially represent contamination with modern human DNA, we cannot decide whether the two individuals are homozygous for Arg307Gly or heterozygous. Thus, we concentrated on authentication of the Arg307Gly variant. The likelihood of an incorrect nucleotide, due to post-mortem damage or PCR errors, decreases as the number of independent amplifications increases (*14*). Therefore we amplified a different Monti Lessini extract in two additional laboratories with different primer sets. The Arg307Gly substitution was found in all three laboratories for the Monti Lessini sample, but only in Barcelona for the El Sidrón sample. Altogether, we observed this substitution in 9 of 12 amplifications with frequencies ranging from 4 to 36% (table S1). Moreover, there is no known damage in ancient DNA resulting in A to G substitutions (*15*). Even if our results were due to a previously unknown kind of template damage that occurs at the same frequency as cytosine deamination (2%) (*14*), the probability of obtaining the same result in nine out of twelve independent amplifications is ~ 10^{-13} (*11*). These results suggest that the Arg307Gly substitution is a reproducible, albeit minority, sequence not attributable to damage.

The Arg307Gly substitution has not been described in more than 2800 modern humans that have been fully sequenced for *mc1r* (*12, 13*). We genotyped this polymorphism in the *CEPH Human Diversity Panel* (*17*) (fig. S2), as well as in all persons involved in the excavation and genetic analysis to determine if this variant exists in extant humans (*11*). No individual had the Gly307 allele. If the Gly307 allele occurs in modern humans, it must be at very low frequency, and it is unlikely that such a rare variant would appear as contamination in three separate laboratories. We additionally investigated if non-human contamination could explain this result. BLASTN was used to compare the longest sequence with the Arg307Gly variant and showed that it was most like human, with 98% sequence similarity, followed by primate sequences with progressively decreasing identity (table S4). None of the sequences in GenBank matched perfectly to the Arg307Gly variant and non-primate mammalian sequences differ considerably, excluding other common sources of contamination as possible origin of the sequence. Therefore, we concluded that the Arg307Gly substitution represents an endogenous Neanderthal sequence.

The Arg307Gly mutation is positioned at the cytoplasmatic surface of the MC1R within the so-called helix 8 or 4th intracellular loop (*18, 19*) (Fig. 1A). Mutations of conserved basic residues within this amphipathic helix alter receptor function in numerous GPCR (*19, 20*). Although Arg307 is replaced by Lys in fox, cow and sheep and by Met in mouse (*21*), the position has been shown to be intolerant to most mutations (*22*) (fig. S5). To investigate whether the Arg307Gly substitution affects the function of human MC1R, both wild-type and the Arg307Gly variant were expressed in COS-7 cells, and basal and agonist-induced intracellular cAMP levels were determined. Human MC1R responded to the natural agonist α-MSH with a ~4-fold increase in intracellular cAMP levels (Fig. 1B and table S2). In contrast, cells expressing the Arg307Gly variant had intracellular cAMP levels reduced to 40% of the wild-type levels and 50% less than wild-type when activated by an agonist. Whereas basal cAMP levels of the wild-type strongly correlated with the amount of transfected plasmid DNA, this was not the case for the basal activity of Gly307 (fig. S3). Analysis of the stimulation curves (Fig. 1B) showed that the EC50 values did not differ between the human and the Arg307Gly variants. To exclude the possibility that the observed differences resulted from overexpression in COS-7 cells, we established stable cell lines with a single expression cassette of either MC1R variant at a predefined locus (*11*). These stably transfected CHOK1 cells also showed reduced agonist-induced cAMP levels for the Arg307Gly variant (table S2).

In order to determine if these reduced basal and agonist-induced cAMP levels observed in Arg307Gly were caused by either lower cell surface expression levels (fig. S4) or reduced G-protein coupling properties, we determined MC1R protein expression levels with ELISA and performed binding assays on intact cells (*11*). Total expression of the full length receptor protein did not differ between the two variants (table S3). In contrast, radioligand binding and ligand-binding-independent measurements revealed a significantly reduced cell surface expression of the Gly307 variant (table S3). However, we observed no difference in the ability of both receptor variants to bind α-MSH (table S3). Altogether, our data support that the Arg307Gly allele has a partial loss of function caused by a reduced cell surface expression of receptor protein (*23, 24*) and an altered G protein coupling efficacy.

Alleles conferring partial loss of function of MC1R have been associated with pale skin color and red hair in humans (*1, 12*). We tested a functional cAMP assay on extant partially functional huMC1R alleles associated with pale skin color and red hair. We confirmed partial activity of these variants, indistinguishable from that of Arg307Gly (table S2). Pale skin color and lighter hair are more likely when MC1R alleles are in a homozygote or compound heterozygote stage. Although we cannot decide currently whether the analyzed individuals were homozygous for Arg307Gly or heterozygous, we can obtain a minimum frequency for this variant in Neanderthals. If we assume that both individuals were heterozygous we obtain a minimum allele frequency for Arg307Gly of 50% for the two individuals investigated. From this figure, the minimum frequency of the mutation in all Neanderthals that is compatible with observing two mutant alleles when four alleles are sampled at random is 0.1 for P >

0.05. This translates into at least 1% homozygous Neanderthal individuals that may have had reduced pigmentation levels possibly even similar to the pale skin color and/or red hair observed in modern humans. These results once more raise the question whether reduced pigmentation may have been advantageous in Europe for example via UV-light mediated vitamin D synthesis, or whether it just reflexes a loss of constraint for the *mc1r* gene in regions of reduced solar irradiation (*25*). Our data do not support the hypothesis that phenotypic similarities between these two human groups is explained by gene flow (*26*) and support convergent evolution of reduced function MC1R alleles, as suggested between modern European and Asian populations (*27, 28*).

References and Notes

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29..We thank C. Green for many suggestions and critical reading of the manuscript, S. Ptak for help with the statistical analysis, and the Ministry of Education and Science of Spain and the Generalitat de Catalunya (C.L.F. and J.B.), the Max Planck Society (D.H., N.R., M.S., and M.H.), the Deutsche Forschungsgemeinschaft, Bundesministerium für Bildung und Forschung, the IZKF Leipzig (H.R., C.S., and T.S.) and the Studienstiftung des Deutschen Volkes (C.S.) for financial support. The excavation at El Sidron is supported by the Autonomous Government of Asturias. The Neanderthal *mc1r* sequence has been deposited at GenBank, accession number 1024875.

Supporting Online Material

www.sciencemag.org/cgi/content/full/1147417/DC1
Materials and Methods
Figs. S1 to S5
Tables S1 to S4
References

5 July 2007; accepted 12 October 2007
Published online 25 October 2007; 10.1126/science.1147417
Include this information when citing this paper.

Fig. 1. Structure and activity of MC1R. **(A)** Depicted are the seven transmembrane helices of the MC1R. The Arg307Gly mutation, found in two Neanderthals, is positioned within the so called helix 8 or 4th intracellular loop. This amphipathic helix contains many conserved basic residues and is part of the intracellular receptor carboxy terminus. (**B)** Partial loss of function of the Arg307Gly variant. For functional characterization, COS-7 cells were transiently transfected with constructs coding for the wild-type (huMC1R) and the Arg307Gly variant. As a control, cells transfected with a plasmid encoding the green fluorescent protein (GFP) were used. Transfected cells were tested for agonist-induced cAMP accumulation (*11*). Shown are the mean ± SEM of three independent representative assays, each performed in duplicate.

A

extracellular
plasma
membrane
1
2
3
4
5
6
7
8
Arg307Gly

B

cAMP (amol/cell)
200
150
100
50
0
-16
-14
-12
-10
-8
-6
-4
α-MSH (log mol/L)
wild-type
Arg307Gly
GFP