Mutations in mitochondrial aldehyde dehydrogenase (ALDH2) change cofactor affinity and segregate with voluntary alcohol consumption in rats

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Genetic factors influence alcohol consumption and alcoholism. A number of groups have bred alcohol drinker and non drinker rat strains, but genetic determinants remain unknown. The University of Chile rat lines UChA (low drinkers) and UChB (high drinkers) display differences in the relative K_m for NAD^+ of mitochondrial aldehyde dehydrogenase (ALDH2) but no V_{max} differences. The relative K_m differences may be due to mitochondrial changes or to genetic differences coding for ALDH2. We investigated whether there are differences in the coding regions of ALDH2 cDNA in these lines and whether the *Aldh2* genotype predicts the phenotype of alcohol consumption and the K_m of ALDH2 for NAD^+. Liver cDNA was prepared, and the *Aldh2* transcript was amplified, cloned and sequenced. Genotyping was conducted by DNA amplification and restriction enzyme digestion. When compared to $Aldh2^1$ of Sprague-Dawley, 94% of the UChA (low drinker) rats ($n = 61$), presented a mutation that changes Gln^{67} to Arg in the mature enzyme (allele referred to as $Aldh2^2$). In UChB (high drinker) rats ($n = 69$), 58% presented the $Aldh2^1$ allele, while 42% presented the Gln^{67}Arg change plus a second mutation that changed Glu^{479} to Lys (allele $Aldh2^3$). The $Aldh2^2$ allele was absent in high drinker rats. Rats of different *Aldh2* genotypes displayed marked phenotypic differences in both ethanol consumption (g/kg/day; means ± SE): ($Aldh2^1/Aldh2^1$) = 5.7 ± 0.2, ($Aldh2^2/Aldh2^2$) = 0.9 ± 0.2 and ($Aldh2^3/Aldh2^3$) = 4.6 ± 0.2; and K_ms for NAD^+ of 43 ± 3 μM, 132 ± 13 μM and 41 ± 2 μM, respectively ($Aldh2^2$ versus $Aldh2^1$ or $Aldh2^3$; $P < 0.0001$ for both phenotypes). Overall, the data show that alleles of *Aldh2* strongly segregate with the phenotype of ethanol consumption and the relative K_m for NAD^+ of ALDH2. Bases mutated suggest that non drinker $Aldh2^2$ is ancestral with regard to the coding changes in either $Aldh2^1$ or $Aldh2^3$, variants which would allow ethanol consumption and may provide an evolutionary advantage by promoting calorie intake from fermented products along with carbohydrates. *Pharmacogenetics* 13:509–515
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Introduction

A number of studies have shown that alcoholism has a genetic component in the range of 50–60% [1–4]. To unravel the genetic factors involved in alcohol intake, groups in Canada [5], Chile [6], Finland [7], Italy [8] and the USA [9] have bred rat strains for their voluntary low or high alcohol consumption. Although most groups have observed some biological differences between their low and high alcohol consuming rats, no genetic associations have been reported to account for the differences in alcohol drinking across strains. Rat lines developed at the University of Chile [6] consume ethanol at either low (0.1–2 g ethanol/kg/day; UChA line) or high (4–7 g ethanol/kg/day; UChB line) levels, when ethanol is offered as a 10% solution and water is freely available. These animals also have been shown [10] to differ in their relative K_ms for NAD^+, without changes in the V_{max} of mitochondrial aldehyde dehydrogenase (ALDH2), enzyme that metabolizes acetaldehyde generated in the oxidation of ethanol. The relative K_m for NAD^+ is five-fold higher in the UChA low-consumption animals (96–126 μM) than in the UChB high-consumption animals (21–23 μM). Such relative K_m differences could conceivably be due to mitochondrial changes following ethanol feeding (e.g. presence of endogenous substrates, inhibitors or enzyme modification in alcohol-induced megamitochondria) [11] or to genetic differences in the coding of ALDH2.

UChA and UChB animals do not display differences in blood acetaldehyde levels following ethanol administra-

tion [12]. However, the acetaldehyde determination method used may not have been sensitive enough, as marked increases in acetaldehyde levels are observed in UChA versus UChB if the animals are administered a non-competitive inhibitor of ALDH, which elevates acetaldehyde levels. In humans the (Glu[487]Lys) mutation, which renders ALDH2 virtually inactive, leads to marked elevations in blood acetaldehyde levels following ethanol intake [13,14]. Low drinker UChA rats also show a slower rate of elimination of exogenously administered acetaldehyde and a marked aversion to this metabolite compared to high drinker UChB rats, suggesting that acetaldehyde elimination in tissues with limiting NAD^+ levels [15,16], including the brain, may influence tissue acetaldehyde disposition and alcohol consumption.

The present studies address whether UChA and UChB rats show differences in the coding region of the cDNA for ALDH2, and whether the genotypes determined for this enzyme predict alcohol consumption and K_m values for NAD^+ in these animal lines.

Materials and methods

Experimental design

Rats of the UChA and UChB lines [6] were employed in these studies. In this continuing breeding program, low consumers and high consumers are selectively bred to generate the UChA and UChB lines, respectively. Because these rats are not inbred (Dr Ting-Kai Li, personal communication), we initially characterized rats in the UChA and UChB lines for their alcohol consumption phenotype and determined their K_ms for NAD^+ of liver ALDH2. Subsequently, liver RNA was extracted, and *Aldh2* cDNA was prepared by reverse transcriptase-polymerase chain reaction) (RT-PCR) techniques, cloned and sequenced. Cloning and sequencing of liver ALDH2 cDNA was conducted for the first 20 animals (10 UChA low consumers and 10 UChB high consumers) with knowledge of their phenotypes. Subsequently, 110 additional animals were genotyped blindly without previous knowledge or selection for a specific drinking phenotype or K_m for NAD^+ of ALDH2. Genotyping for the *Aldh2* alleles was conducted by PCR amplification and restriction enzyme digestion from tail blood genomic DNA (see below). Protocols and animal handling were approved by the Animal Ethics Committee.

Determination of alcohol consumption phenotypes and K_m for NAD^+ of ALDH2

The phenotypic assessment of alcohol consumption [6] and the determination of relative K_ms (i.e. K_m for NAD^+ of ALDH2 measured in mitochondria) [12] were performed essentially as described previously. For the determination of alcohol intake, rats were allowed free access to 10% ethanol or to water for 8 weeks, which stabilizes consumption [8]. For determination of K_m and V_{max} for NAD^+ of ALDH2, liver mitochondria were prepared by differential centrifugation of homogenates in 0.25 mM sucrose and the mitochondria (0.3 mg) were used to measure the appearance of NADH at 340 nM in a reaction mixture (3.0 ml) that contained 90 mM K_2HPO_4 (pH 7.4), 0.1 mM pyrazole, 0.1 mM acetaldehyde, 3 μM rotenone, and varying concentrations of NAD^+. In addition to the above [12], 1.8 mM $MgCl_2$ was added to the incubation mixture.

cDNA synthesis and amplification

Overall, molecular biology methods follow those previously described by Sambrook *et al.* [17]. Total RNA was prepared from approximately 400 mg of rat liver using 4 ml of Trizol reagent (Gibco-BRL, Bethesda, Maryland, USA) (five rounds of homogenization of 30 s with an Ultra Turrax T25 homogenizer (Janke & Kinkel, IKA Labortechnik, Staufen, Germany) with an ice incubation of 1 min between rounds); cDNA was obtained from 4 μg of RNA by reverse transcription using the primer 5′-TAA GCA CTG AGG GTG GAA CC-3′ (3 μM) and 200 U of MMLV reverse transcriptase (Promega, Madison, Wisconsin, USA). Following a 5-min joint denaturation of the RNA (4 μg) and the primer (0.5 μg) in water at 70°C, reactions were performed in a suitable buffer in 25 μl total volume for 60 min at 42°C. Second strand synthesis was accomplished by PCR using 8 μl of the reverse transcription reaction as template and primers: 5′-CCC GCC GTG GGC CAC GCC TGA-3′ (forward) and 5′-TAA GCA CTG AGG GTG GAA CC-3′ (reverse). Reaction conditions were: 50 μl total volume, 200 μM each dNTP (Promega), 100 pmol each primer, 1.6 mM $MgCl_2$, 2 U Taq DNA polymerase (Promega). Thermal cycling program: 3 min at 94°C; 35 cycles of 1 min at 94°C, 2 min at 64°C, 2 min at 72°C; 10 min at 72°C. The 1.8 kb cDNA amplicon was purified from 1.5% agarose gels (low melting point) (Gibco-BRL) using the Wizard PCR-Preps Purification System (Promega), and was sometimes followed by a phenol extraction step.

Cloning

Amplicons were cloned in pGEMT-Easy (Promega) by direct TA cloning using 50 ng of vector, 150 ng of amplicon and 3 U of T4 DNA ligase in a total volume of 10–20 μl; ligations were performed at 4°C overnight. *Escherichia coli DH5α* competent cells were transformed and plated on LB agar plates containing ampicillin (100 μg/ml), X-gal (40 μg/ml) and IPTG (0.5 mM). Candidate clones were screened by plasmid size and confirmed by digestion with *Eco*RI which cuts the 1.8-kb *Aldh2* insert once and additionally releases it from the vector.

Sequencing

Plasmids were prepared using the Wizard Plasmid

Miniprep DNA Purification System (Promega), precipitated once with sodium acetate and ethanol and resuspended in water. Sequencing was carried out using the 'Dyedeoxy Fluorescent DNA Sequencing Kit' (Applied Biosystems-Perkin Elmer, Foster City, California, USA), various gene and vector specific primers and performed at the DNA Synthesis and Sequencing Facility of Universidad de Chile on an ABI 373A sequencer or at the Ecology Department of Universidad Católica de Chile on an ABI Prism 3100 sequencer.

Genotyping of animals for *Aldh2*

Genomic DNA was initially prepared from 100–200 mg of liver tissue homogenized in DNazol (Gibco-BRL) (1 ml per 100 mg tissue) and subjected to a phenol extraction step. The genotype at the *Aldh2* locus was determined from genomic DNA by PCR amplification of exon 13 followed by digestion with *Hga*I. In 20 out of 20 rats examined, the restriction pattern of exon 13 fully predicted the coding sequences, including those coding for amino acids 67 and 479. Thus, in further studies with 110 rats, genotyping from exon 13 was conducted from genomic DNA prepared from fresh blood (100 μl) using 1 ml of DNAzol. Exon 13 (GenBank accession #AY034137) was amplified by PCR from 100 ng of gDNA using 2 U of Taq DNA polymerase (Promega) and 2 μM each of forward (5′-GCG TGC AGG CTT CCT CAG C-3′) and reverse (5′-TAA GCA CTG AGG GTG GAA CC-3′) primers in 1.2 mM $MgCl_2$, 0.8 μg/μl BSA, Tris-HCl 10 mM pH 9.0 (at 25°C), 50 mM KCl and 0.1% Triton X-100 in a volume of 50 μl and using the same program indicated above but with an annealing temperature of 65°C. Amplicons (2 μg) were digested overnight at 37°C with 2 U of *Hga*I (New England Biolabs, Beverly, Massachusetts, USA) in 10 mM Tris-HCl pH 7.0, 10 mM $MgCl_2$ and 1 mM DTT. The digestion products were run on a non-denaturing 12% polyacrylamide gel at 5 W, stained with ethidium bromide and visualized under ultraviolet light.

Statistical analyses

Data are presented as means ± SE, with the number of animals indicated in parenthesis. Both the chi-square test and the *t*-test were conducted using the SISA statistical program (http://home.clara.net/sisa/).

Results

Phenotyping for high or low voluntary alcohol consumption was conducted by allowing the selectively bred UChA (low drinker) and UChB (high drinker) rats free access to a choice of 10% ethanol or water for 8 weeks. This was followed by liver mitochondria preparation and determination of K_m for NAD^+ of ALDH2. Mean alcohol consumption of 10 UChA and 10 UChB animals from which the ALDH2 cDNAs were sequenced were UchA = 0.24 ± 0.06 g/kg/day and UCHB = 5.1 ± 0.20 g/kg/day (means ± SE), and their K_ms for NAD^+ of ALDH2 were 187 ± 20 μM and 39 ± 0.7 μM, respectively.

The RT-PCR product of the *Aldh2* liver mRNA yielded an amplicon that contained the complete reading frame for the mature ALDH2 (89–1591) plus 33 bases of the 5′ destination peptide and 284 bases of the 3′ untranslated region (tsp = 1; A in ATG = 32). Thus, the PCR product covered bases 57–1874 of *Aldh2* cDNA. The sequence obtained from UChA and UChB animals was compared to that for the Sprague-Dawley rat *Aldh2* cDNA first described by Weiner *et al.* [18]. The coding sequence of the Sprague-Dawley rat is identical to that subsequently reported by us for the Wistar-derived Lewis rat (GenBank accession #AF529165). Three distinct alleles were detected in the University of Chile lines (Table 1). The first one, termed *Aldh2¹* for historical reasons, is identical to the Sprague-Dawley rat allele [18] both in its coding and untranslated regions. A second allele, referred to as *Aldh2²*, showed a substitution in nucleotide 288A>G (CAG to CGG), thus changing the deduced amino acid sequence of the enzyme from Gln^{67}Arg (in this context, the direction of the change refers to differences with respect to *Aldh2¹*, but does not denote which mutation appeared first in evolution; see discussion). Allele *Aldh2²* presented additional base differences that do not change the amino acid coded (547C>T; 736T>C and 1507C>T) and a large number of substitutions, insertions and deletions in the 3′ untranslated region. The existence in *Aldh2²* of a direct tandem repeat of the sequence 5′-CTGCCAAGAGAAACCCCTTCACCAAAGC-3′ found in positions 1671–1699 of the

Table 1 Differences in *Aldh2* cDNA in three alleles

Base number	*Aldh2¹*	*Aldh2²*	*Aldh2³*
288	A (Gln^{67})	G (Arg^{67})	G (Arg^{67})
547	C	T	C
736	T	C	T
1507	C	T	T
1523	G (Glu^{479})	G (Glu^{479})	A (Lys^{479})
1597	C	T	C
1623	A	G	G
*Ins*** 1699–1700	No	Yes	no
1765	A	G	G
1805	T	C	T
Ins 1808/09 (C)	No	Yes	No
1811	G	A	G
Del 1823–24 (AC)	No	Yes	No
Del 1831 (T)	No	Yes	No

The double line divides the coding (top) and non-coding (bottom) regions of *Aldh2* cDNA. Bases were numbered according to the *Aldh2* cDNA of the Sprague-Dawley rat with tsp = 1 (GenBank: X14977) incorporating the two one-nucleotide insertion corrections (1769–1770 ins C and 1854–1855 ins G) (Sapag *et al.*, unpublished observations). Amino acid numbering is according to the mature enzyme. *Del*, Deletion; *Ins*, insertion. *Ins*** corresponds to a tandem repeat insertion of the same 29 bases (5′-CTG CCA AGA GAA AAC CCC TTC ACC AAA GC-3′) that exist between nucleotides 1671 and 1699 of the Sprague-Dawley rat cDNA.

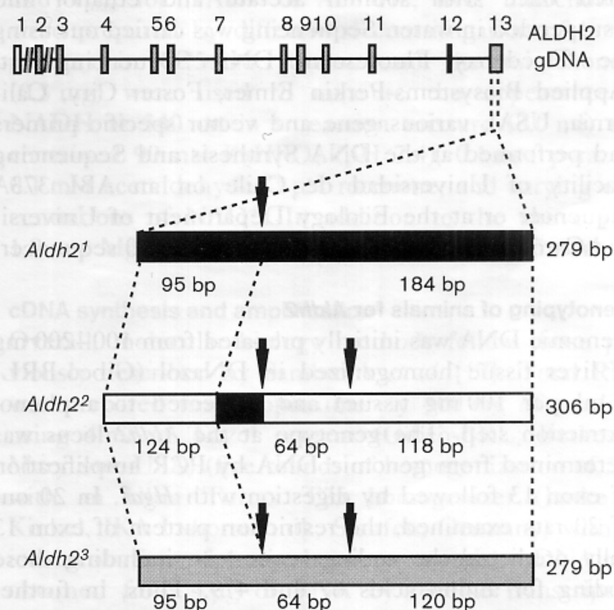
Sprague-Dawley rat is noteworthy. In the *Aldh2²* allele, such a sequence is present a second time between nucleotides 1699–1700 (numbering according to the cDNA of Sprague-Dawley), thus resulting in a longer allele. The third allele, *Aldh2³*, presented changes coding for two amino acids: $Gln^{67}Arg$ (CAG to CGG mutation) and $Glu^{479}Lys$ (GAG to AAG). The *Aldh2³* allele did not present the tandem repeat. A number of differences in the 3′ untranslated region characterize *Aldh2¹*, *Aldh2²* and *Aldh2³*. Table 1 shows the similarities and differences between the three alleles.

Because the 3′ region in each of the three alleles was unique and indicative of the total cDNA sequence, it was possible to genotype from this region. In order to determine whether the genotypes *Aldh2¹*, *Aldh2²* and *Aldh2³* would predict the phenotypes of ethanol consumption and the K_ms for NAD^+ of ALDH2, we obtained (from tail blood DNA) PCR products corresponding to positions 1596–1874 of *Aldh2* cDNA. These are found in exon 13 (GenBank accession #AY034137). A single restriction enzyme (*Hga*I) provided digestion patterns that were specific for each allele. The strategy is presented in Fig. 1, showing that the fragments obtained by cleavage with *Hga*I were markedly different for each allele: (i) *Aldh2¹* yields fragments of 95 and 184 bp; (ii) *Aldh2²* yields fragments of 64, 118 and 124 bp; and (iii) *Aldh2³* yields fragments of 64, 95 and 120 bp. Figure 2 presents the actual digestion bands obtained for homozygous and heterozygous mixtures of cDNA amplicons, whose allelic nature had been previously determined by cloning and complete sequencing.

Table 2 presents the allelic frequency and genotypes of 61 UChA and 69 UChB rats. In UChA (low drinker) rats, 94% had an *Aldh2²* allele and 92% were *Aldh2²*/*Aldh2²* homozygous. The probability of such prevalence in only one of the two phenotype groups is $P < 0.0001$. UChB (high drinker) animals had two alleles: *Aldh2¹* = 58% and *Aldh2³* = 42% (UChB and either allele 1 or 3; $P < 0.0001$). Genotypic frequencies in UChB were *Aldh2¹*/*Aldh2¹* = 46%; *Aldh2³*/*Aldh2³* = 30%; and *Aldh2¹*/*Aldh2³* = 23%. Notably, there were no *Aldh2²* alleles in UChB rats nor *Aldh2³* alleles in UChA rats. Conversely, data show that the *Aldh2²* allele is not the only cause of an aversion to ethanol in these lines because 3% of the low drinker UChA animals did not carry this allele. Thus, it is not possible to conclude that the *Aldh2²* allele is dominant in the low drinker *Aldh2¹*/*Aldh2²* animals.

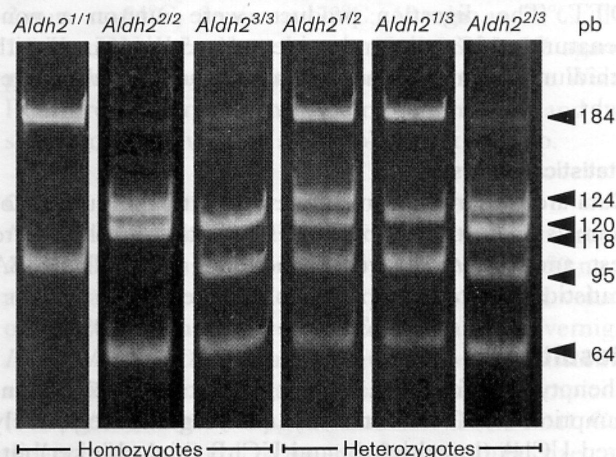
The possibility of genotyping from tail blood allowed us to select animals with well characterized genotypes to determine if these predicted their alcohol consumption and their K_m phenotypes. Genotypes highly predicted the phenotypic differences for both ethanol

Fig. 1



Digestion diagram for genotyping of *Aldh2* alleles found in UChA (low drinker) and UChB (high drinker) rats with the restriction enzyme *Hga*I. Amplicons of exon-13 DNA were obtained by the polymerase chain reaction with the forward primer 5′-GCG TGC AGG CTT CCT CAG C-3′ and the reverse primer 5′-TAA GCA CTG AGG GTG GAA CC-3′ (nucleotides 13537–13555 and 13815–13796 of the gDNA corresponding to nucleotides 1596–1614 and 1874–1855, respectively, of cDNA). Rectangles represent exons. Exon 1 (white rectangle) and introns 1 and 2 (interrupted lines) have not been sequenced. Rat *Aldh2* gene structure according to GenBank accession AY034137. Bars represent the different *Aldh2* alleles; arrows indicate the restriction enzyme (*Hga* I) hydrolysis site, and the size of each fragment is shown below each allele. The solid rectangle in allele *Aldh2²* indicates the tandem repeat of the 29-bp insert.

Fig. 2



Bands of allelic *Aldh2* cDNA mixtures digested with *Hga* I following electrophoresis on 12% polyacrylamide. The polymerase chain reaction amplicons subjected to digestion were obtained from different *Aldh2* alleles, with forward (1596–1614: 5′-GCG TGC AGG CTT CCT CAG C-3′) and reverse (1874–1855: 5′-TAA GCA CTG AGG GTG GAA CC-3′) primers. The size of the fragments (bp) is shown.

Table 2 *Aldh2* **allele frequencies and genotype frequencies**

	Allele frequency			*Aldh2* genotype frequency					
Group	*Aldh2*[1]	*Aldh2*[2]	*Aldh2*[3]	*1/1*	*2/2*	*3/3*	*1/2*	*1/3*	*2/3*
UChA ($n = 61$)	0.06	0.94[a]	0	0.03	0.92	0	0.05	0	0
UChB ($n = 69$)	0.58	0[b]	0.42	0.46	0	0.30	0	0.23	0

[a]*UChA*: *Aldh2*[2] frequency versus *Aldh2*[1] and *Aldh2*[3] ($P < 0.0001$). [b]*Aldh2*[2] frequency in UChA versus *Aldh2*[2] in UChB ($P < 0.0001$).

consumption (g/kg/day; means ± SE): $Aldh2^1/Aldh2^1 = 5.7 \pm 0.2$, $Aldh2^2/Aldh2^2 = 0.9 \pm 0.2$ and $Aldh2^3/Aldh2^3 = 4.6 \pm 0.2$ ($P < 0.01$ versus $Aldh2^1/Aldh2^1$); and the K_ms for NAD^+ of 43 ± 3 μM, 132 ± 13 μM and 41 ± 2 μM, respectively ($Aldh2^2$ versus $Aldh2^1$ or $Aldh2^3$, $P < 0.001$ for both phenotypes). There were no significant phenotypic differences between the heterozygous $Aldh2^1/Aldh2^3$ (alcohol consumption $= 5.3 \pm 0.7$ g/kg/day, $K_m = 41 \pm 0.6$ μM) and the homozygous $Aldh2^1/Aldh2^1$ or $Aldh2^3/Aldh2^3$ animals. Phenotypes were not associated with changes in V_{max} for ALDH2, measured under conditions in which both substrates are in excess: $Aldh2^1/Aldh2^1 = 36 \pm 2$, $Aldh2^2/Aldh2^2 = 28 \pm 1$ and $Aldh2^3/Aldh2^3 = 27 \pm 2$ nmol NADH/mg protein/min.

Discussion

The current data indicate that rats selectively bred for their high or low ethanol consumption present marked differences in their *Aldh2* genotypes. UChA (low drinker) rats display a mutation in which amino acid 67 in the mature ALDH2 is arginine ($Aldh2^2$) rather than glutamine ($Aldh2^1$). We further demonstrate that the $Gln^{67}Arg$ change increases four- to five-fold the K_m for NAD^+ of ALDH2. The $Aldh2^2$ allele also segregates strongly with low ethanol consumption. The kinetic changes in ALDH2 may constitute the mechanism for avoiding consumption of ethanol in a free choice situation. The tissue(s) in which these genotypic changes is/are expressed should still be determined because the concentration of NAD^+ (and the NAD/NADH ratio) in the mitochondria in each tissue will depend on many factors, including the shuttling of reducing equivalents, the use of NAD^+ by other reactions and the capacity of the respiratory chain to oxidize NADH. An overall slower metabolism of exogenously administered acetaldehyde has been observed in UChA animals [15]. Given that the associations observed between the *Aldh2* genotype and phenotypes are high, causality is highly probable. However, we prefer to use the term allele 'segregation' to describe the correlations observed because direct proof of causality would require showing that a transgenic rat in which the $Gln^{67}Arg$ change is incorporated into the ALDH2 of an inbred drinker rat reduces alcohol consumption. However, there are additional aspects suggesting that the segregation might be causal rather than spurious: (i) the protein mutated metabolizes acetaldehyde, a product of ethanol metabolism rather than being a protein unrelated to ethanol effects; (ii) the changes in K_m are consistent with the changes in consumption; and (iii) the UChA and UChB lines are not inbred, but rather display marked differences in six loci (Dr Ting-Kai Li, personal communication). These considerations strengthen the probability of a causal association between the genotype and the phenotype studied.

A recent study [19] reported that *Aldh2*(–/–) knockout mice show blood acetaldehyde levels after ethanol access that were not different from those in *Aldh2*(+/+). This may be due to the fact that in the mouse, unlike the rat [20], acetaldehyde metabolism is mainly cytosolic [21] because of a high activity aldehyde dehydrogenase with a K_m for acetaldehyde of 10 μM (compared to a cytosolic aldehyde dehydrogenase with a K_m of 180 μM in man) [22]. Thus, an inactivating mutation of ALDH2 in humans (such as the *ALDH2*2* mutation) is expected to yield higher blood steady-state levels of acetaldehyde than in the mouse. Nevertheless, the *Aldh2*(–/–) knockout did show a reduced voluntary ethanol consumption versus the wild-type *Aldh2*(+/+) mice, suggesting that blood acetaldehyde, generated mainly in the liver, is not the only factor determining avoidance of ethanol. The investigators suggested that brain acetaldehyde levels (generated endogenously) may be important in determining alcohol preference, although an effect in many peripheral organs with retrograde innervation to the brain cannot be ruled out. Other groups have also suggested that brain ALDH2 activity may influence ethanol volition [23,24]. Data reported earlier for low drinker UChA rats, in which blood acetaldehyde levels after alcohol administration (in the absence of ALDH inhibitors) did not differ from levels in UChB (high drinker) rats [12], are consistent with the knockout studies [22]. In the UChA line, a higher K_m of ALDH2 for NAD^+ may lead to higher acetaldehyde levels in peripheral or brain tissues with low NAD^+ levels. Nevertheless, it should also be noted that the methods used in the earlier studies [12] may not have been able to detect relevant changes in circulating acetaldehyde.

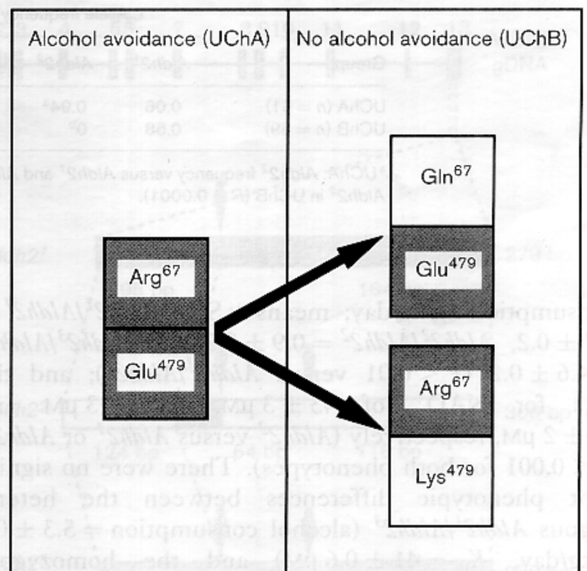
In the Indianapolis-bred strains of rats, Carr *et al.* [25] reported that the homozygous Arg^{67} genotype was seen in 39% of the low drinking animals (NP strain) and in only 5% of the high drinking animals (P strain), which is consistent with our findings. However, it is clear that a low ethanol preference in the NP animals appears be more strongly associated to factors other than Arg^{67}. Furthermore, our data show that a second change in the cDNA reading frame, Glu^{479}Lys in the presence of Arg^{67}, obliterates the effect of Arg^{67} in relation to both the K_m for NAD$^+$ of ALDH2 and alcohol consumption. This area of the cDNA was not genotyped in the Indianapolis animals.

In a study of rat lines bred in Finland (drinker rats: AA and non drinker rats: ANA) [26], no association was found between the presence of Arg^{67} and the drinking phenotype because Arg^{67} was present in a large proportion (74–75%) in both the drinker and non drinker lines. Such a finding was interpreted as an indication that there was no correlation between genotype and phenotype. However, because the investigators did not sequence the full *Aldh2* cDNA, it is unknown whether their drinker animals had both Arg^{67} and Lys^{479} in their ALDH2, as found in the *Aldh2^3* allele in our drinker line (UChB), or other amino acid changes. From their data [25], a two-amino acid change may have existed because the isoelectric focusing of their homozygous Arg^{67}/Arg^{67} ALDH2 showed two activity bands with different isoelectric points.

As indicated by Carr *et al.* [25] the presence of Arg^{67} (CGG) in ALDH2 is likely ancestral, thus preceding the base change that yielded Gln^{67} (CAG), because changes from C>T, with correction of G>A in the opposite strand, often result from the mutagenic effect of the deamination of 5′-methylcytosine which yields thymine [27]. The base change responsible for the change in amino acid coding from Glu^{479} (GAG) to Lys^{479} (AAG) is of the same type, which suggests that Glu is also ancestral versus Lys. However, the Aldh2^3 did not present the tandem motif seen in Aldh2^2.

We suggest that in the rat, a one-point mutation (Fig. 3) along with evolution at either one of two different sites in the *Aldh2* gene to yield either Arg^{67}Gln or Glu^{479}Lys gave these early mammals an evolutionary advantage by allowing alcohol consumption from fermented fruits, along with the carbohydrates from which ethanol was derived [28]. Such a view is also in agreement with the finding that the point mutation yielding the substitution Glu^{479}Lys is only present in alleles coding for Arg^{67}. While the mechanism by which the Glu^{479}Lys substitution in the rat lowers the K_m in the Arg^{67} ALDH2 is unknown, studies by Wei *et al.* [29] have shown that the K_m for NAD$^+$ of ALDH2 (a tetrameric enzyme) can be markedly altered by changes in amino acids (e.g. in positions 487 and 475) that are far from Glu^{268} and Cys^{302} at the active site. The data obtained provide information to guide new site-directed mutagenesis studies.

Fig. 3



Amino acid differences between the ALDH2 of animals that show ethanol avoidance and animals that do not show ethanol avoidance. The direction of the amino acid changes from ethanol avoidance to no avoidance are proposed on the basis of the nucleotide differences found in the codons for the mature ALDH2 of UChA (low drinker) and UChB (high drinker) animals.

In conclusion, we have shown that mutations in *Aldh2* strongly segregate with the phenotypes of low and high alcohol consumption and with kinetic differences in ALDH2, suggesting that pharmacogenetic differences affect voluntary alcohol consumption. Bases mutated in the coding region suggest that *Aldh2^2* in low drinkers is ancestral to the coding changes of either *Aldh2^1* or *Aldh2^3*, which segregate with higher ethanol consumption. The latter alleles may have an evolutionary advantage, allowing lower mammals calorie intake from fermented products containing volatile ethanol, along with the carbohydrates present therein.

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