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Research paper

Novel and prevalent non-East Asian ALDH2 variants; Implications for global susceptibility to aldehydes' toxicity

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ABSTRACT

Background: Aldehyde dehydrogenase 2 (*ALDH2*) catalyzes the detoxification of aliphatic aldehydes, including acetaldehyde. About 45% of Han Chinese (East Asians), accounting for 8% of humans, carry a single point mutation in *ALDH2*2* (E504K) that leads to accumulation of toxic reactive aldehydes.
Methods: Sequencing of a small Mexican cohort and a search in the ExAC genomic database for additional *ALDH2* variants common in various ethnic groups was set to identify missense variants. These were evaluated *in vitro*, and in cultured cells expressing these new and common variants.
Findings: In a cohort of Hispanic donors, we identified 2 novel mutations in *ALDH2*. Using the ExAC genomic database, we found these identified variants and at least three other *ALDH2* variants with a single point mutation among Latino, African, South Asian, and Finnish ethnic groups, at a frequency of >5/1000. Although located in different parts of the *ALDH2* molecule, these common *ALDH2* mutants exhibited a significant reduction in activity compared with the wild type enzyme *in vitro* and in 3T3 cells overexpressing each of the variants, and a greater ethanol-induced toxicity. As Alda-1, previously identified activator, did not activate some of the new mutant *ALDH2* enzymes, we continued the screen and identified Alda-64, which is effective in correcting the loss of activity in most of these new and common *ALDH2* variants.
Interpretation: Since ~80% of the world population consumes ethanol and since acetaldehyde accumulation contributes to a variety of diseases, the identification of additional inactivating variants of *ALDH2* in different ethnic groups may help develop new 'precision medicine' for carriers of these inactive *ALDH2*.

1. Introduction

Aldehyde dehydrogenase 2 (*ALDH2*) enzymatic deficiency is one of the most common race-specific human enzymopathies in the world, affecting about 560 million East Asians, or about 8% of the world population [1, 2]. *ALDH2* deficiency was first characterized by symptoms of facial flushing, palpitation, headache, and vomiting with only moderate alcohol consumption among the East Asian carriers [3]. "Alcohol glow", "alcohol flushing", "flushing syndrome" or "alcohol intolerance" are various terminologies that describe the phenotype of this enzymatic deficiency. The unpleasant physiological responses to alcohol are caused by a rapid accumulation of acetaldehyde, due to the greatly reduced *ALDH2* function [3].

The alcohol flushing syndrome is not benign. Unequivocal epidemiological data and meta-analysis have consistently shown that alcohol consumption among *ALDH2*2* carriers leads to a significantly increased risk for several cancers, in particular, the upper aerodigestive tract cancers including oral, pharyngolaryngeal, and esophageal cancers [1, 4, 5]. For example, Yokoyama et al. reported that, amongst *ALDH2*2* allele carriers, those who were categorized as moderate or heavy alcohol drinkers had an increased odds ratio of 72.8 for esophageal cancer compared to 44.6 for those who never drink [6]. Indeed, the International Agency for Research on Cancer has classified alcoholic beverages as a human carcinogen (Group 1) [7]. Aldehyde toxicity is not limited to cancer; it has also been implicated in many other

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Research in context

Evidence before this study

A family of detoxifying enzymes called aldehyde dehydrogenases (*ALDHs*) has been studied with great interest due to its role in detoxifying aldehydes that accumulate through metabolism and to which we are exposed from the environment. Although the human genome has 19 *ALDH* genes, one ALDH emerges as a particularly important enzyme in a variety of human pathologies. This *ALDH*, *ALDH2*, is located in the mitochondrial matrix and is better known for its role in ethanol metabolism. *ALDH2* dysfunction may contribute to a variety of human diseases, including cardiovascular diseases, diabetes, neurodegenerative diseases, stroke, osteoporosis, Fanconi Anemia, cancer and in the process of aging. Furthermore, an *ALDH2* inactivating mutation (termed *ALDH2*2*) is the most common single point mutation in humans, and epidemiological studies suggest a correlation between this inactivating mutation and increased propensity for common human pathologies.

Added value of this study

We reasoned that such a common inactivating mutation must have had an evolutionary benefit and therefore may have arisen more than once. Searching the ExAC database, we now find at least 5 additional inactivating *ALDH2* mutant forms in other ethnic groups, including, African, Latino, South Asian, and Finish. We characterized these 5 mutations, which represent more than 1/500 people of a given geographical or racial population, and found that each of these single point mutations caused between 50% and 90% loss of activity and some were also associated with reduced stability. Importantly, we now demonstrate the efficacy of a better *ALDH2* activity booster, Alda-64.

Implications of all the available evidence

In the era of precision medicine, considering that *ALDH2*2* loss of activity is associated with differences in drug metabolism and in susceptibility to a variety of chronic diseases including cancer, diabetes and neurodegeneration, determining association of these newly identified common inactivating in various other populations should help with public health and patients' education regarding alcohol consumption and should be incorporated in decision making by physicians.

diseases with increased vulnerability among the *ALDH2*2* subjects [8, 9]. These diseases range from osteoporosis [10], cardiovascular disease [11], and Alzheimer's disease to rare genetic diseases, such as Fanconi Anemia [12, 13]. In addition, *ALDH2* also plays a key role, *via* its reductase activity, in the conversion of nitroglycerin to nitric oxide for vasodilation [14], thus reducing the clinical benefit of traditional nitroglycerine dosing regimens among *ALDH2*2* human subjects [15] when used to treat angina.

ALDH2 is a critical, NAD-dependent metabolic enzyme, responsible for the conversion of reactive endogenous and exogenous aldehydes to their corresponding, non-reactive, carboxylic acids [16]. Many commonly occurring aldehydes are relevant to human health, such as short-chain aliphatic aldehydes (*e.g.,* acetaldehyde arising from ethanol metabolism), aromatic aldehydes (*e.g.,* 3,4-dihydroxyphenylacetaldehyde arising from dopamine), and long-chain aliphatic aldehydes (*e.g.,* 4-hydroxy-nonenal arising from oxidative stress lipid peroxidation). Therefore, *ALDH2*, a non-P450 mitochondrial detoxifying enzyme, plays a key role in protecting all organs from damage by a variety of reactive and toxic aldehydes [2].

The *ALDH2*2* variant has thus far been characterized as an East Asian-specific polymorphism [17]. Extensive global geographic and population mapping based on data of 80,691 individuals from 366 population samples has confirmed that the *ALDH2*2* allele is highly concentrated in areas of Southeast China, Japan, Korea, Taiwan, Singapore and Vietnam [18]. Perhaps most prominently, in Taiwan the prevalence of the *ALDH2*2* carrier is as high as 49%, affecting half of the population of a single country [19].

Exhaustive literature searches on *ALDH2* have found no reports or molecular characterization of any other *ALDH2* variants (single amino acid substitutions or otherwise) affecting enzymatic function of this gene [18], except for a functional promoter SNP (−357G/A, rs886205) [20] that affects *ALDH2* gene expression level by DNA methylation in the 5′-untranscribed region [21]. With the recent compilation of several large-scale human DNA sequencing projects worldwide, such as the 1000 genomes project [22], the human Exome Aggregation Consortium (ExAC) [23], and the Genome Aggregation Database (gnomAD) [24], we sought to better understand the worldwide distribution of *ALDH2* genetic variation. Consequently, we have identified several additional common *ALDH2* single amino acid variants (missense mutations) with loss of *ALDH2* activity that are enriched within certain ethnic populations. In this era of precision medicine, mindful of the health implication of *ALDH2*2* variant in 560 million East Asians [8,25], we recognize that the potential impact on human of common variants with reduced or loss of *ALDH2* amongst other ethnic and geographical groups should be considered by policymakers, health providers, and patients.

2. Materials and methods

2.1. Blood sample collection, DNA purification and ALDH 2 genotyping and sequencing of human subjects

Blood samples for DNA extraction and *ALDH2* genotyping were collected from volunteers under the approval of IRB protocol (INF-2938-19-19-1) with written informed consent for human subject study at the blood bank from the National Institute of Medical Sciences and Nutrition Salvador Zubirán in Mexico City. The buffy coat containing peripheral blood monocyte cells were isolated by gradient centrifugation using Lymphoprep™ (STEMCELL, Germany), density medium 1.077±0.001 g/ml. The purified peripheral blood monocytes from each sample were lyzed in lysis buffer for DNA extraction by an automatic nucleic acid extraction system (NucliSENS®easyMag®, Biomerieux, France) according to the manufacturer's instructions, and eluted in 25 μl of elution buffer. *ALDH2* genotyping and DNA sequencing of all exons were carried by PCR and direct DNA sequencing of each of the exon of the entire *ALDH2* genes. PCR primer sets used for each exon are listed in Table S2.

2.2. Analysis of Exome Aggregation Consortium (ExAC) database for ALDH2 variants

High-quality variants (genotype quality ≥ 20 and depth ≥ 10) in the *ALDH2* transcript ENST00000261733, as of September, 2019, were collected from the ExAC Browser [23]. The ExAC database collected 60,706 unrelated individuals with a total of 490 variants recorded for the *ALDH2* genomic region including 5′-, 3′-untranslated regions, intron and exon regions. The dataset was exported as an Excel file and sorted by the annotation of the variants. We focused only on variants in the amino-acid coding region of *ALDH2* which comprised of 517 amino acids. The common variants were identified and defined by an allele frequency greater than 0.5% in any of the 6 major ethnic groups recorded in the database.

2.3. Cloning of ALDH2 variants

The full-length cDNA of wild type *ALDH2* gene was obtained from ATCC (ATCC clone #MGC-1806) and inserted into the pET-28a (+) vector (Novagen, MA, USA) directly following the thrombin cleavage sequence for recombinant protein production in BL21 bacterial host cells. Each of the *ALDH2* single amino acid variants was constructed from the wild type vector by site-directed mutagenesis using complimentary primer sets carrying the desired nucleotide substitution. The Pfu Turbo DNA polymerase was used for site-directed mutagenesis according to the manufacturer's guidelines (Agilent-Quick-change site-directed mutagenesis, #20522). After site-directed mutagenesis, each plasmid was transformed into BL21 competent *E. coli* (Life Science Technologies). Colonies containing the desired *ALDH2* variants were selected and confirmed by DNA sequencing. The sense strand primers used in the PCR reactions were as follows: I41V, GGCATCGTGCCATTCATTGTTCACGAAAATCTGGTTGCAGAAGAC; P92T, GCGGCGCCAGGTTGAGCCCAGCTGGAAGG; T244M, 5′-GGCCCCAGCCATGGGGCCAAATCCAGG; V304M, 5′-AGTGGGCCTGTTCCATGGCCCAATCCATAT;R338W,5′-CCGGGCAACGCTCCACTCCACAAACTCAT; E504K, 5′-TGTGACAGTTTTCACTTTAGTGTATGCCTGCAGCC.

2.4. Protein expression and purification

Recombinant *ALDH2* was expressed in LB broth (800 ml, with 50 μg/ml kanamycin) as previously reported [26]. In brief, culture was spun down (6000 g for 20 min at 4 °C), and the pellet was lysed with 20 ml of BugBuster Protein Extraction Reagent (Novagen, WI, USA) for 20 min at room temperature with slight agitation. GE His-Trap nickel affinity columns (GE, #11-0033-99) were first equilibrated at 4 °C by 10 ml of wash buffer. The bacterial cell lysate was added to the column and washed with another 10 ml of wash buffer. Recombinant *ALDH2* protein was eluted by 3 ml of elution buffer and digested by 200 units of bovine thrombin (BioPharm, # 91-030) for 14 h at 4 °C to remove the His-tag. Sterile glycerol was added to the purified enzyme for long-term storage at −80 °C.

2.5. Co-expression of ALDH2 wildtype and ALDH2 variants

pETDuet-1 plasmid vector (Novagen, MA, USA) was used for the co-expression of *ALDH2* wildtype (*ALDH2**1) and each of the E504K (*ALDH2**2), P92T (*ALDH2**4), T244M (*ALDH2**5) and R338W (*ALDH2**7). A copy of a full-length wildtype *ALDH2* cDNA was inserted into the His-tag cloning site and a copy of the corresponding *ALDH2* variant cDNA was interested into the HA-tag cloning site. Individual *ALDH2* subunits can be expressed simultaneously from two independent IPTG inducible promoters to form active *ALDH2* tetramers from the pETDuet-1 vector. Co-expression of recombinant protein was achieved by 0.5 mM IPTG induction at 30 °C using BL21 bacterial host cells. Purifications of the recombinant proteins by affinity nickel columns were carried out using a standard protocol as described above according to manufacturer's instructions (Novagen, WI, USA).

2.6. Small molecule library screening for ALDH2 activators

Small molecule library screening for *ALDH2* activators was carried out at the Stanford University High-Throughput Biosciences Center (Department of Chemical and Systems Biology). 63,500 compounds were screened using an established fluorescence coupled *ALDH2* enzymatic assay [26]. In this assay, an increased fluorescent signal emitted by resorufin (excitation/emission, 565/590 nm) from the reduction of resazurin by diaphorase was expected from the coupled redox reaction of recombinant *ALDH2* enzyme using acetaldehyde (20 mM) and NAD (10 mM) as the substrate cofactor, respectively, in the presence of an activator compound. All screening compounds were purchased from ChemBridge (San Diego, CA USA) and SPECS USA (Narragansett, RI USA) by the Stanford University High-Throughput Biosciences Center. The library compounds were dissolved in DMSO, screened and validated at 10 μM concentration. Scaled up quantifies of confirmed *ALDH2* activators of Alda-1 (N-(1,3-Benzodioxol-5-ylmethyl)-2,6-dichlorobenzamide) (MW 324.16) and Alda-64 (2-(azepane-1-carbonyl)-*N*-(2-chlorobenzyl)-2,3-dihydrobenzo (*b*) <1,4> dioxine-6-sulfonamide) (MW 464.96) were chemically synthesized by ACME Chemical (Mountain View, CA USA).

2.7. Determination of ALDH2 activity

Aldehyde dehydrogenase activity of the purified *ALDH2* enzymes was measured as conversion of NAD^+ to NADH using acetaldehyde as a substrate, as previously reported [26]. The assays were carried out at 25 °C in 50 mM sodium pyrophosphate buffer (pH 9.5) in the presence of 2.5 mM NAD^+ and 10 mM acetaldehyde. Enzyme activities were calculated, relative to that of wildtype, as the mean values ± SD.

2.8. Expression and protein stability of ALDH2 wildtype and variants enzymes in cultured cells

3T3 cells and human-derived fibroblasts were obtained from ATCC and Coriell Institute (AG07123, RRID:CVCL_2C18), respectively. Cells were maintained in MEM supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine and 1% (v/v) penicillin/streptomycin at 37 °C in a humidified chamber of 95% air and 5% CO_2. For experiments, cells were seeded in 96 well plates at 10,000 cells per well or 50,000 cells per well in 6 well plates for 18−24 h before transfection. Plates were transfected with 1 μg of plasmid DNA using 3 μl of Lipofectamine 2000 reagent (Life Technologies, #11668019). After 12 h, the media was replaced with fresh media to reduce toxicity of the Lipofectamine reagent. After 48 h cells were analyzed for expression and stability of the variants (under cycloheximide treatment) or treated with 50 mM ethanol for a further 48 h to determine sensitivity to oxidative stress.

2.9. ALDH2 enzyme activity in cells

Enzymatic activity of *ALDH2* was determined by measuring the conversion of NAD+ to NADH, as described previously [26]. The assays were carried out at 25 °C in 50 mM sodium pyrophosphate buffer (pH 9.5) in the presence of 2.5 mM NAD^+ and 10 mM acetaldehyde. Measurement of *ALDH2* activity was determined by directly adding 100 μg of the total lysate to the reaction mix and reading absorbance at 340 nm for 15 min. Activity was measured in the absence and presence of disulfiram (1 μM) and the difference between the two rates was attributed to the *ALDH2*.

2.10. ROS production

For cellular ROS detection, cells were incubated with 2,7-dichlorofluorescein diacetate (DCFDA) (Abcam, #ab113851) 100 μM for 30 min at 37 °C in the dark, and fluorescence was analyzed with excitation/emission at 495/529 nm, using SpectraMax M2e (Molecular devices). Fluorescence intensity was then normalized for cell number.

2.11. ATP measurements

Relative intracellular ATP levels were determined using ATP-based CellTiter-Glo Luminescent Cell Viability kit (Promega, #G7570), which causes cell lysis and generates a luminescent signal proportional to the amount of ATP present. For intracellular ATP levels, opaque-walled 96-well plates with cell lysate (50 μl) were prepared. An equal volume of the single-one-step reagent provided by the kit was added to each well and incubated for 30 min at room temperature. ATP content was measured using a luminescent plate reader SpectraMax M2e (Molecular devices).

2.12. Western blot analysis

Protein concentrations were determined using the Bradford assay (Thermo Fisher Scientific). Proteins were resuspended in Laemmli buffer containing 2−mercaptoethanol, loaded on SDS−PAGE, and transferred on to nitrocellulose membrane, 0.45 μm (Bio−Rad) as previously described [27, 28]. Cell supernatant was cleared of cellular debris by centrifugation at 1000 g for 10 min. The total lysate was resuspended in Laemmli buffer containing 2−mercaptoethanol, loaded on SDS−PAGE, and transferred on to nitrocellulose membrane, 0.45 μm (Bio−Rad). Membranes were cut at appropriate molecular weights and then probed with a mouse monoclonal antibody against β-actin (Cell Signaling Technology, # 12262, RRID: AB_2566811) and a rabbit monoclonal antibody against *ALDH2* (Abcam Cat# ab108306, RRID:AB_10862581), and visualized by ECL (0.225 mM p−coumaric acid; Sigma), 1.25 mM 3−aminophthalhydrazide (Luminol; Fluka) in 1 M Tris pH 8.5. Scanned images of the exposed X−ray film or images acquired with Azure Biosystems C600 were analyzed with ImageJ to determine relative band intensity. Quantification was performed on samples from independent cultures for each condition.

2.13. 4-HNE protein adduct measurements

4-Hydroxynonenal (4HNE) protein adducts were determined by Elisa according to manufacturer's protocol (Abcam, #ab118970). Briefly, 50 μg of protein from human-derived fibroblasts lysate were loaded onto 4-HNE conjugate coated wells, blocked using the provided blocking solution and incubated with anti-4-HNE antibody at room temperature for 1 h. After washing three times with washing buffer, HRP-peroxidase was added to each well and incubated for 1 h at room temperature with gentle shaking. Wells were washed again and incubated with the provided substrate solution. Color density was measured at 450 nm after adding stop solution and quantified by comparison with a 4-HNE-BSA standard curve.

2.14. Statistical analysis

Prism 8.0 (GraphPad Software) was used for the statistical analysis. Data shown are the mean ± s.d. with $P < 0.05$ considered statistically significant. Group differences were analyzed with one-way analysis of variance (ANOVA) followed by Fishers Least Significant Difference (LSD) test. Data distribution was assumed to be normal, but this was not formally tested. No statistical methods were used to predetermine sample sizes.

3. Results

3.1. A survey for the presence of the Latino variants of ALDH2 in a small cohort from Mexico City, Mexico

*ALDH2*2* mutation has been extensively studied for its contribution to various human diseases [1, 4, 5]. Since our publication on Alda-1[26], we received numerous correspondences from people of non-Asian heritage suffering from "alcohol flushing" like syndrome. To start determining whether this or other mutations exist in non-East Asians, we obtained 150 human DNA samples from healthy donors from a local blood bank in Mexico City, Mexico and sequenced the entire *ALDH2* gene. None had the East Asian mutation (E504K). However, we identified six subjects (4%) with a heterozygous genotype of P92T and five subjects (3.3%) with V304M (Fig. 1(A)). These results indicated that non-East Asian *ALDH2* variants could be relatively common in other human populations.

3.2. Survey of the human Exome Aggregation Consortium (ExAC) database reveals novel high-frequency ALDH2 variants

To expand our findings from the limited donor study, and determine whether these and other variants are common, we analyzed the human Exome Aggregation Consortium (ExAC) database; which provides harmonized exome DNA sequencing data from 60,706 unrelated individuals [23]. We identified 490 variants, spanning the 42 kb of the *ALDH2* genomic region. Within the 515 amino acid coding sequence, which consists of 12 exons, a total of 280 variants were identified. Amongst these, we identified 185 non-synonymous exon variants, including the *ALDH2*2* variant, 6 stop-gained mutations, and 8 frameshift mutations (Fig. 1(B), and Table S1). The data compiled in the ExAC were grouped into six different major human ethnic categories: European (non-Finnish), Finnish, Latino, African, East Asian, South Asian. The 185 non-synonymous exon variants that we identified occurred in a wide range of allele frequencies. Five missense variants were present in a frequency greater than 0.5% of the corresponding ethnic and geographical groups, which include the two we found in the small cohort from Mexico City, P92T and V304M.

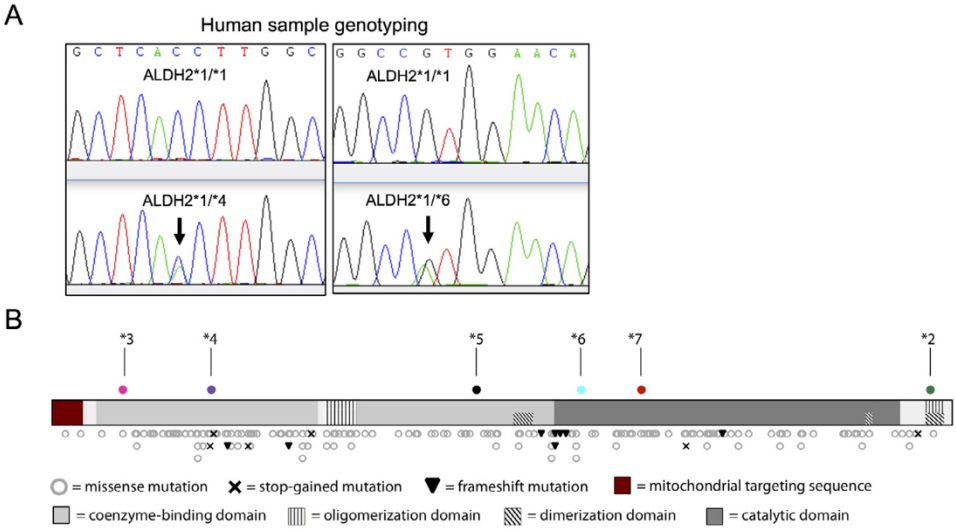
Following the established nomenclature (*ALDH2*1* for the common active, so called wildtype, variant and *ALDH2*2* for the common East Asian E504K mutant variant) we designated these new variants as *ALDH2*3* (I41V), *ALDH2*4* (P92T), *ALDH2*5* (T244M), *ALDH2*6* (V304M), and *ALDH2*7* (R338W) (Fig. 1(C)). As expected, the *ALDH2*2* (E504K) variant is the most prevalent genetic variant worldwide, with an allele frequency of 26.6% among East Asians, which is 10 times higher than the next most two common variants, *ALDH2*4* (P92T) and *ALDH2*6* (V304M), with allele frequencies of 2.5% and 2.4%, respectively, among Latinos (Fig. 1(B)). As identified in the ExAC database, other high-frequency variants are also present in Finnish (*ALDH2*7*), South Asian (*ALDH2*5*) and African (*ALDH2*3*) cohorts.

3.3. Recombinantly expressed ALDH2 variants have diminished activity in vitro

The *ALDH2* tetramer functions as a dimer of dimers, where each of the four monomeric units contains a dinucleotide (NAD^+) binding domain and a catalytic domain featuring three consecutive cysteine residues and a glutamate, which is required for the catalysis at the active site. Dimerization occurs *via* key contacts between two monomers, and subsequent tetramerization is assisted by contacts between three-stranded β-sheets in each monomer. The common variants span the full length of the *ALDH2* primary structure and are interspersed throughout the dimerization, oligomerization, coenzyme-binding domain, and catalytic domain [29] (Fig. 1(C) and (D)). Based on location alone, the functional consequences of these common variants are not clear. Therefore, we studied the *in vitro* enzymatic activity of these new variants.

We focused on the five variants with relatively high allele-frequency (>0.5% in their population) and those that present with homozygotic individuals in the genomic datasets (Fig. 1(C), Table S1). In addition to *ALDH2*2*, these five unreported *ALDH2* variants had a significant reduction in activity as compared with the *ALDH2*1* variant (referred to as wild type from here on; Fig. 2(A)). The most active variant of those studied, *ALDH2*3* (I41V), which was identified in the African population, had a mean activity of only 60% relative to the untreated wild type enzyme, even though this variant results from only a single-carbon change in the amino acid sidechain.

The two Latino variants, *ALDH2*4* (P92T) and *ALDH2*6* (V304M) had only 32% and 11% activity of wildtype, respectively (Fig. 2(A)), and together potentially represent about 40 million people in the world [30]. Of importance from an epidemiological perspective is also the Finnish variant *ALDH2*7* (R338W), with an activity 23% of wildtype (Fig. 2(A)). Although the population represented by this allele is much smaller than the African, Latino, or Asian counterparts, the ability to harness the unprecedented data available from the



C

Mutation	ALDH2 Designation	No. of Allele Found	No. of Allele Examined	Allele Frequencies (%)	Major Ethnicity
NONE	WT (ALDH2*1)				
E504K	ALDH2*2	1878	6882	26.6	East Asian
I41V	ALDH2*3	62	10248	0.6	African
P92T	ALDH2*4	284	11526	2.5	Latino
T244M	ALDH2*5	115	15785	0.7	South Asian
V304M	ALDH2*6	258	10352	2.4	Latino
R338W	ALDH2*7	21	1682	1.2	Finnish

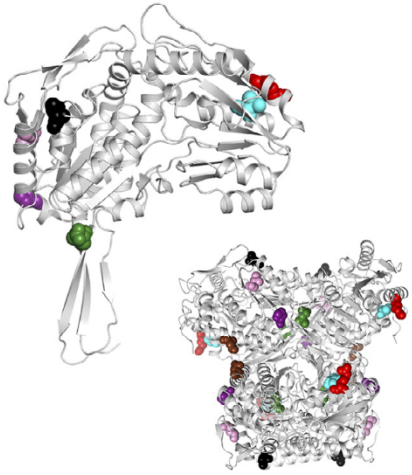


Fig. 1. Unstudied, high-frequency variants of *ALDH2* have been extracted from the ExAC database (A). Sequence analysis from human samples showing examples of the Latino *ALDH2**4 (P92T) and *ALDH2**6 (V304M) variants. **(B)** All mutations identified in the Exome Aggregation Consortium (ExAC) database are shown on a linearized map of the *ALDH2* protein structure. **(C)** Previously uncharacterized mutations in *ALDH2* were identified using the ExAC database. Those variants with allele frequency percentages greater than 0.1% in a given ethnic population are shown in Table S1, along with the previously studied mutant E504K. Mutation numbering refers to the amino acid sequence of the mature protein with the 17-amino acid mitochondrial targeting sequence. Color code assignments are used consistently in the reporting of this work. Ethnicity assignments were made based off of available information in the ExAC database and might not represent all affected populations. **(D)** The mutated residues are shown with their respective color codes on the crystal structure (PDB: 1O05) of the *ALDH2* monomer (top) and tetramer (bottom, inset).

FinnGen initiative will allow us to search out direct correlations between this Finnish variant and health outcomes. Finally, although the *ALDH2**5 variant (T244M) has 38% of wildtype activity (Fig. 2(A)), this mutation, like the Latino variants, represents a significant global population of potentially 14 million South Asians (0.7% of the total 2 billion South Asians) [31].

3.4. ALDH2 *isozyme-specific activators, Alda-1 and Alda-64, and their effects on* ALDH2 *variants*

The first *ALDH2*-specific enzyme activator, Alda-1, was identified previously by a high throughput screening of 65,000 compounds, using purified recombinant *ALDH2*2* [26]. Since then, an additional 65,000 small-molecule compounds were added to the library, and an additional screen of that library identified 380 hits that increased wildtype *ALDH2* activity by 12–160% at 20 μM. Of these, we confirmed 21 new *ALDH2* activator compounds. Racemic Alda-64 (2-(azepane-1-carbonyl)-*N*-(2-chlorobenzyl)-2,3-dihydrobenzo (*b*) <1,4> dioxine-6-sulfonamide) (MW 464.96; Fig. 2(B)) was the most potent activator of *ALDH2* and was therefore characterized here further.

At 20 μM, Alda-64 increased *ALDH2* catalytic activity by 80% as compared to 55% by Alda-1. Similar to Alda-1, Alda-64 exhibited specificity for the *ALDH2* family; it did not activate ALDH1, ALDH3A1 or ALDH5A1 (Fig. 2(A), (C) and [26]). Alda-1 increased the activity of the E504K variant (*ALDH2*2* homo-tetramer) by ~14-fold and Alda-64 activated by ~11-fold (Fig. 2(A) and (B)), but this activity was still only about ~20% of absolute wild type activity. In contrast, Alda-64 brought the activity of *ALDH2**3 (I41V) and *ALDH2**5 (T244M) to wildtype levels, effects that were substantially greater than the effect of Alda-1 on these variants. There was >2-fold activation of *ALDH2**7 (R338W), *ALDH2**6 (V304M) by Alda-64 and a ~70% increase in activity of *ALDH2**4 (P92T; Fig. 2(A) and (B)). Surprisingly, only the Latino variant P92T (*ALDH2**4) and South Asian variant T244M (*ALDH2**5) exhibited a significant activation by Alda-1 (~70% increase each; Fig. 2(B)); there was no significant effect of Alda-1 on the activity of the other three new variants tested, suggesting that the reduced enzyme activity caused by these mutations might cause a structural change in a way that is fundamentally different from that of E504K and the Alda-1-responsive P92T and T244M variants.

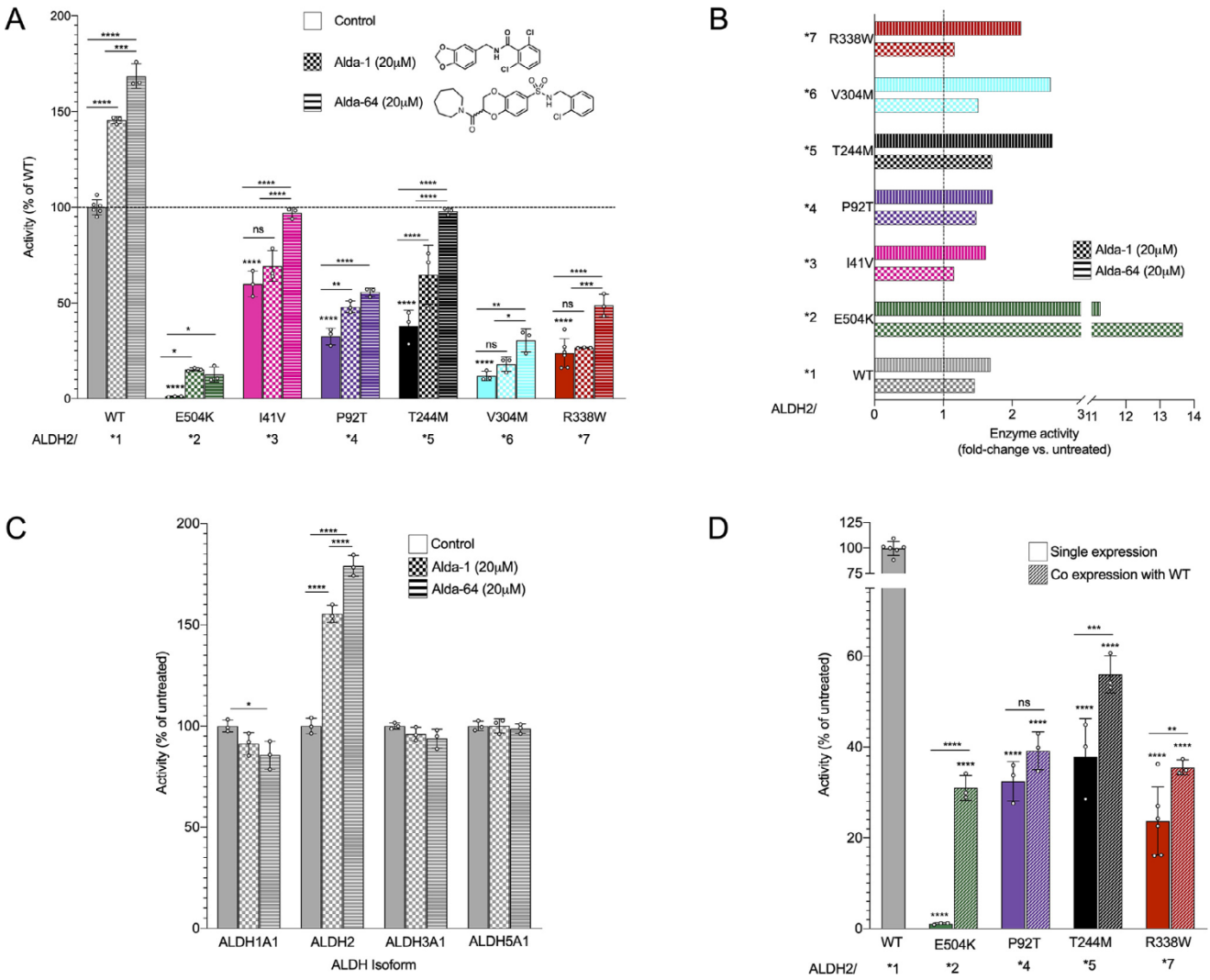


Fig. 2. Newly characterized *ALDH2* mutants show decreased activity *in vitro* compared to wildtype (WT) enzyme. (A) *ALDH2* activity of the recombinant mutants upon acetaldehyde treatment was measured using the conversion of NAD+ to NADH, without treatment (solid bars) and with the *ALDH2* activators Alda-1 and Alda-64 (checkered and striped bars, respectively). $n = 3–6$; Mean, standard deviation, probability by one–way ANOVA (with Fischer's LSD post hoc test) **** p-value <0.0001; *** p-value <0.001; ** p-value <0.01; * p-value <0.05. **(B)** Fold change of *ALDH2* activity improvement in each individual *ALDH2* variants by Alda-1 and Alda-64. **(C)** Dehydrogenase activity measured in other ALDH isoforms after treatment with Alda-1 and Alda-64. $n = 3$; Mean, standard deviation, probability by one–way ANOVA (with Fischer's LSD post hoc test) **** p-value <0.0001; * p-value <0.05. **(D)** *ALDH2* activity measured in enzyme produced in a co-expression system of WT and *ALDH2* variants to mimic heterozygous carriers. $n = 3$; Mean, standard deviation, probability by one–way ANOVA (with Fischer's LSD post hoc test) **** p-value <0.0001; ** p-value <0.01; * p-value <0.05.

3.5. *Dominant negative effect of the* ALDH2 *variants*

As discussed, *ALDH2* is a tetramer, consisting of four monomeric subunits of 55 kDa encoded by the *ALDH2* gene [16]. The *ALDH2*2* (E504K) subunit exerts a dominant-negative effect on the wildtype *ALDH2*1* subunit in the heterotetramer, due to structural changes in the NAD co-factor binding site [9, 32]. The *ALDH2* activity of human carriers of the heterozygous *ALDH2*1/*2* genotype is 25–40% of wild type enzyme, which explains the alcohol flushing reaction in *ALDH2* heterozygote carriers [33]. It is therefore important to determine whether human carriers of a heterozygous genotype of any of the newly identified *ALDH2* variants also have reduced *ALDH2* catalytic activity, as is the case of *ALDH2*1/*2* heterozygous East Asians. Therefore, we next co-expressed wild type and each the four *ALDH2* variants, E504K, P92T, T244M and R338W, on a bacterial dual expression vector, pETDuet-1, to evaluate the dominant-negative effect of the new variants on the wild type subunit. Co-expressed wild type/E504K (*ALDH2*1/*2*) heterotetramer had 31% of the enzyme activity relative to the wild type/wild type (*ALDH2*1/*1*) activity, corroborating previously published co-expression results [26], and reflecting, for example, the dominant negative effect in human liver tissue [33, 34]. The P92T, T244M and R338W all appear to have a greater degree of dominant-negative effect on the enzymatic activity of *ALDH2*, relative to *ALDH2*2* (Fig. 2(D)). The activity of homozygote-equivalent mutants was indistinguishable from the activity of the heterozygotes, *ALDH2*1/*4* (wildtype/P92T) and only slightly elevated for *ALDH2*1/*5* (wild type/T244M) and *ALDH2*1/*7* (wild type/R338M); the activity of all the *ALDH2* heterozygotic variants ranged from 30% to 55% of the WT activity (Fig. 2(D)).

3.6. *Expression and stability of* ALDH2 *variants in cultured cells*

To understand the effects on protein activity and stability of these new variants, we transiently transfected 3T3 cells. Surprisingly, when transfected with the same amount of DNA, the expression of all the mutants was significantly reduced as compared to the overexpressed WT (*ALDH2*1*) (Fig. 3(A)). Interestingly, the stability of protein, when measured following a 12-h cycloheximide treatment, showed that only ALDH*2, *6 and *7 (E504K, V304M and R338W), had reduced protein stability whereas *ALDH2*3*, *4 and *5 (I41V, P92T, T244)

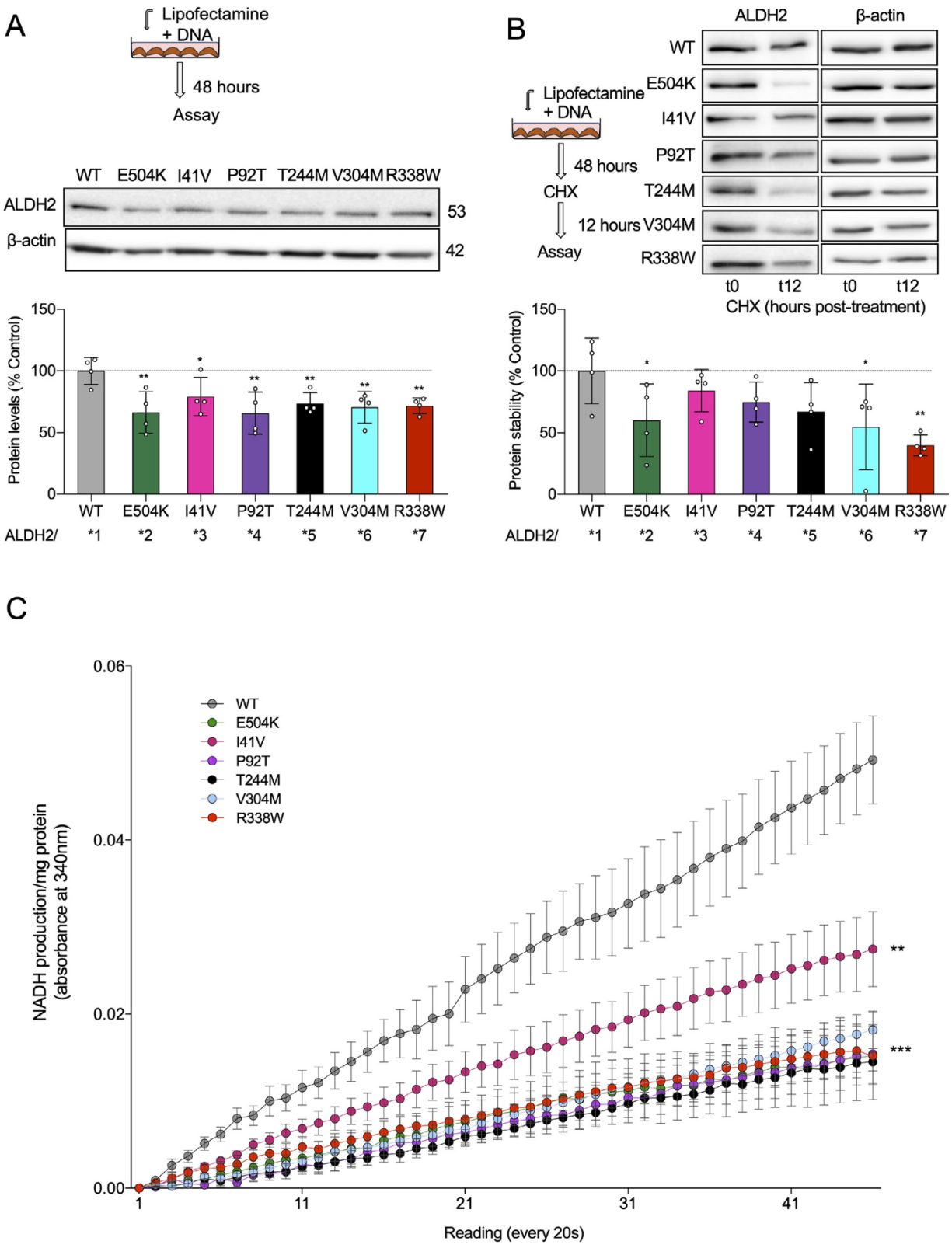


Fig 3. Novel *ALDH2* variants show lower stability and activity in transiently transfected cells. (A) Experimental paradigm; *ALDH2* protein levels were determined in transiently transfected 3T3 cells by immunoblotting of total cell lysate. Levels were quantified and presented as ratio *vs* β–actin (loading control). $n = 4$; Mean, standard deviation, probability by one–way ANOVA (with Fischer's LSD post hoc test) ** p-value <0.01; * p-value <0.05. **(B)** Experimental paradigm; *ALDH2* protein stability was determined in transiently transfected 3T3 cells by immunoblotting of total cell lysate after 100 μm cycloheximide for 12 h. Levels were quantified and presented as ratio *vs* β-actin (loading control). $n = 4$; Mean, standard deviation, probability by one–way ANOVA (with Fischer's LSD post hoc test) ** p-value <0.01; * p-value <0.05. **(C)** *ALDH2* specific activity was determined in transiently transfected 3T3 cells. $n = 4$; Mean, standard deviation, probability by one–way ANOVA (with Fischer's LSD post hoc test) **** p-value <0.0001 *vs* other groups.

variants had similar stability to that of WT (Fig. 3(B)). We also measured the effect on *ALDH2* activity and found similarly low activity in all the 3T3 lines expressing the inactive variants, with the *ALDH2**3 overexpressing cell line showing the highest activity and stability (Fig. 3(B) and (C)).

We next measured the cellular ATP levels, cell ROS, and cell death in the 3T3 cell lines overexpressing WT, as well as in each of the *ALDH2* variants. We observed a mild reduction in ATP 25–75% as well as an increase in cellular ROS accumulation across all the mutants similar to the defects in E504K (Fig. 4(A) and (B)). However, only three mutations, *ALDH2*2,* *3 and *4 (E504K, I41V, P92T), showed a significantly increased cell death under basal levels (Fig. 4 (C)). We observed a similar response of increased cell death along with accumulation of 4-HNE protein adducts in human-derived fibroblasts expressing *ALDH2*2,* *3 and *4 (E504K, I41V, P92T) variants relative to cells expressing WT (Figs. S1A and S1B). 4-HNE is an aldehyde generated during lipid peroxidation and cleared by *ALDH2*.

Since *ALDH2* activity is required for alcohol detoxification, we assessed the consequence of ethanol injury in 3T3 cells expressing the various *ALDH2* variants. Exposure to ethanol (50 mM) for 48 h significantly reduced cellular ATP levels and increased ROS levels in each of the cell lines expressing mutant *ALDH2* variants relative to the cell line expressing WT *ALDH2* (Fig. 4(D) and (E)). There was also a corresponding increase in ethanol-induced cell death in both 3T3 cells and human-derived fibroblasts individually expressing *ALDH2* variants relative to the cell line expressing WT *ALDH2* (Figs. 4(E), (F) and S1C). Both Alda-1 and Alda-64 rescued the defects significantly, but the effect of Alda-64 appeared to be superior in terms of restoring cellular ATP levels, reducing ROS, and importantly, reducing cell death as measured by LDH release (Fig. 4(D)–(F)), especially in the case of *ALDH2**3 and *ALDH2**5. Alda-1 and Alda-64 treatment also reduced the levels of 4-HNE protein adducts (Fig. 4(G)) and cell death (Fig. S1C) in human-derived fibroblasts expressing the different *ALDH2* variants exposed to ethanol (50 mM) for 48 h, with exception of *ALDH2**3 and *6 (I41V, V304M) variants, which did not improve survival upon Alda1 treatment.

4. Discussion

560 million people, or 8% of the world population, are affected by the enzymopathy of *ALDH2* deficiency. Despite affecting such a large percentage of the world population, all current *ALDH2* deficient subjects are reported to carry one identical single amino acid variant (*ALDH2*2*, rs671), which appears to have originated from a single founder in East Asia 2000–3000 years ago [35]. No other *ALDH2* single amino acid polymorphism has been reported or characterized thus far. In contrast, another very common human enzymopathy, glucose-6-phosphate dehydrogenase (G6PD) deficiency (also known as favism), which affects 400 million people or 5.7% of the world population, has been well-documented with more than 160 single amino acid variants [36]. Carriers of many of these variants have reduced G6PD enzyme activity and are susceptibility to anemia, newborn jaundice, or acute hemolysis induced by oxidative stress [36]. Both *ALDH2* and G6PD are multimeric metabolic enzymes, consisting of similarly sized protein subunits of 517 and 515 amino acids, respectively. We reasoned that both enzymopathies have, at some point in time, provided some health benefits to humans, perhaps in the presence of bacterial, viral or parasite-induced diseases [37]. Regardless of the potential evolutionary advantage for these common enzymopathies in humans, we predicted that other inactivating mutations in *ALDH2* may also be common among other ethnic and geographical groups.

In this study, we confirmed the presence of at least five new common variants that cause a substantial loss of *ALDH2* activity. As all of these mutants are dominant (Fig. 2(D)), and given the allele frequencies and 2019 world population, we estimate that 80 million Latinos (10% of the world 800 million Latinos carrying the P92T and V304M variants), 28 million South Asians (1.4% of the 2 billion South Asians carrying the T224M variant) and 14 million Africans (1.2% of the 1.2 billion Africans carrying the I41V variant) may be affected by *ALDH2* enzyme deficiency. Thus, there could be an additional ~120 million individuals who are *ALDH2* enzyme deficient worldwide, in addition to the 560 million East Asians carrying the *ALDH2*2* mutation. Of particular consequence to human health, the two very common Latino variants, *ALDH2**4 (P92T) and *ALDH2**6 (V304M), together affecting ~5% of Latinos, had only 32% and 11% activity of wildtype, respectively (Fig. 2(A)).

Relative to WT *ALDH2* (*ALDH2**1), the impairment in *ALDH2* activity *in vitro* was much greater for the *ALDH2*2* variant as compared with the new *ALDH2* mutant variants (Fig. 2(A)). When overexpressed in 3T3 cells, except for the African variant, *ALDH2**3, the loss of *ALDH2* activity for all the variants were similar, showing about 30% lower protein levels, with *ALDH2**3 showing the least effect on levels or stability (Fig. 3(A) and (B)). All the variants show about 60% lower *ALDH2* activity, again with *ALDH2**3 being the least impaired. Whether these data reflect the stability and activity of the variants in patient-derived cells remains to be determined. However, these data are quite consistent with the relative impairment of *ALDH2* activity in the *in vitro* assays.

Our studies comparing the resistance of cells overexpressing each of the mutants to ethanol-induced toxicity (Figs. 4 and S1) suggest that the *in vivo* consequences of these variants might be comparable. We found similar increases in cell death (~50%), an increase in cellular ROS levels (~50%) and reduced cellular ATP levels (~35%) following exposure to ethanol (Fig. 4(D)–(F)). Even under basal conditions, increase in cell ROS and cellular ATP levels were similar (Fig. 4(A) and (B)). Moreover, we observed a pronounced accumulation of 4-hydroxynonenal (4-HNE)-protein adducts in human-derived fibroblasts overexpressing *ALDH2* variants (which have reduced *ALDH2* activity) compared with cells overexpressing *ALDH2* WT. These findings suggest an impaired aldehyde detoxification capacity in cells overexpressing *ALDH2* variants.

The difference between the effect of the two small molecule activators of *ALDH2*, Alda-1 and Alda-64, on the various variants suggests a different molecular mechanism for their activation. Alda-1 was not as effective as Alda-64 in compensating for the loss of activity on cellular ATP levels, cellular ROS levels and accumulation of 4-HNE protein adducts in the presence of ethanol for *ALDH2**3 and *ALDH2**6, translating to less protection from ethanol-induced cell death in both 3T3 cells and human-derived fibroblasts overexpressing these variants *versus* the other *ALDH2* variants (Fig. 4(D)–(G)). These data underscore the opportunity for further exploration of both the structural origin of the new variants' dysfunction and also for developing of new, more efficacious classes of *ALDH2* activators. Moreover, *ALDH2* activation using either Alda-1 or Alda-64 was sufficient to counteract the excessive 4-HNE accumulation and protecting cells against aldehydic load. These new findings along with our previous data demonstrate the critical role of *ALDH2* in metabolizing toxic aldehydes.

If the functional deficits of *ALDH2**3, *4, *5, *6 and *7 are confirmed in cells derived from individual carriers of these variants, there are new implications to our findings for these common variants in specific ethnic and geographic populations; our very small sample of 150 Latinos in Mexico City, identifying six subjects carrier of *ALDH2**4 and five carriers of *ALDH2**6 (Fig. 4(G)), supports the need for larger genotyping studies in the corresponding populations to confirm the prevalence of these variants. As discussed, epidemiological studies suggest an association between *ALDH2*2* deficiency, alcohol consumption, and certain common and rare diseases [9]. Previous genome-wide association studies have implied that several SNPs near the *ALDH2* chromosome region (12q24) might be associate with variation in alcohol consumption [38]. However, a more recent and thorough genome-wide study examining 86,627 individuals from 4 different major ethnic groups (non-Hispanic white, Hispanic/Latino,

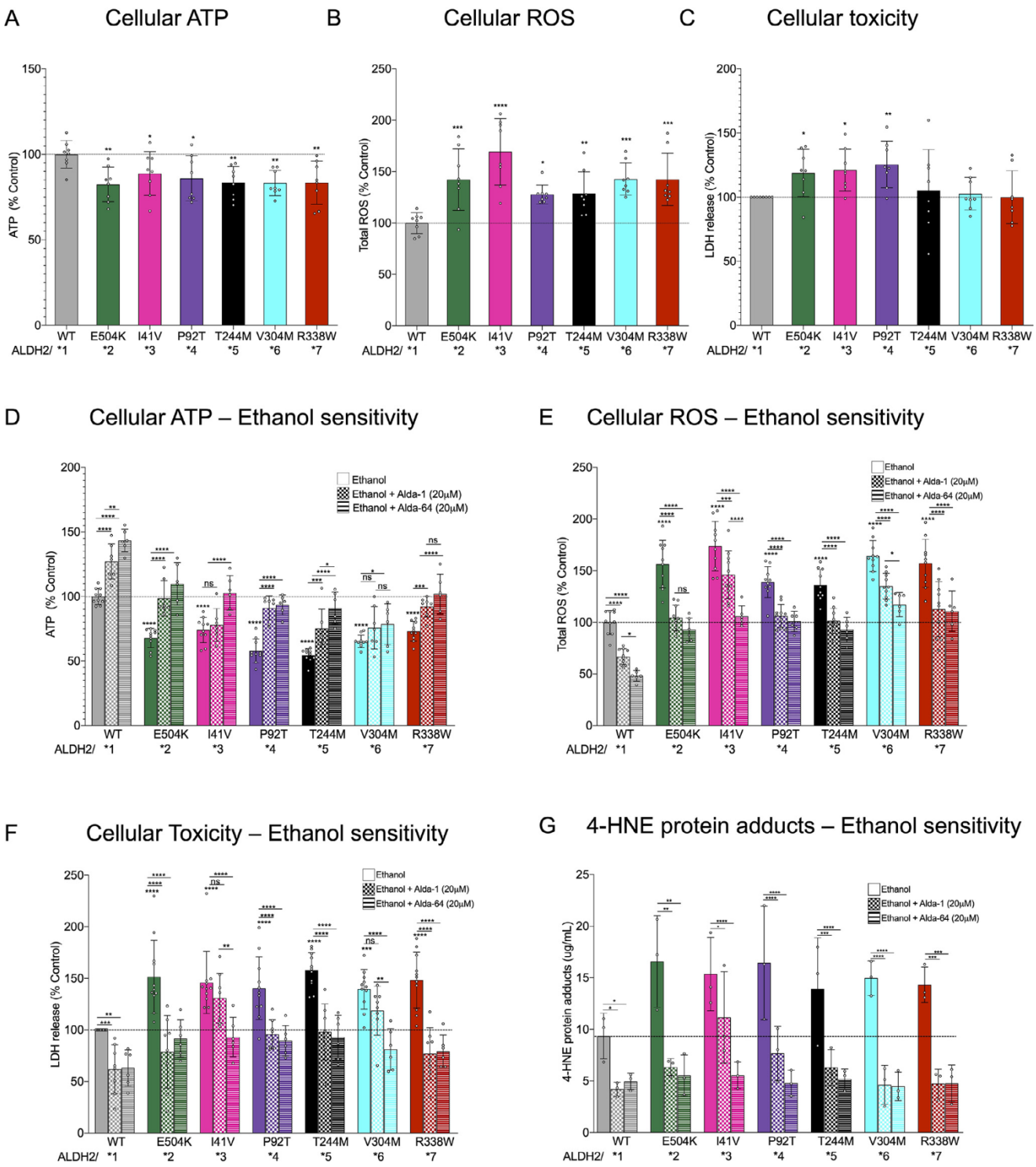


Fig 4. Novel *ALDH2* mutant are sensitive to ethanol toxicity. (A) Cellular ATP levels were measured in transiently transfected 3T3 cells using ATP-based CellTiter-Glo Luminescent Cell Viability kit. $n = 8$; Mean, standard deviation, probability by one–way ANOVA (with Fischer's LSD post hoc test) ** p-value <0.01; * p-value <0.05. **(B)** Total cellular ROS levels were measured with 2,7 dichloro- fluorescein diacetate in cells treated as in (A). $n = 8$; Mean, standard deviation, probability by one–way ANOVA (with Fischer's LSD post hoc test) ** p-value <0.01; * p-value <0.05. **(C)** Lactate dehydrogenase activity levels in supernatant in cells treated as in (A). $n = 8$; Mean, standard deviation, probability by one–way ANOVA (with Fischer's LSD post hoc test) ** p-value <0.01; * p-value <0.05. **(D)** Cellular ATP levels were measured in transiently transfected 3T3 cells in the presence or absence of Alda-1/Alda-64 (20 μM/48 h; 50 mM Ethanol) using ATP-based CellTiter-Glo Luminescent Cell Viability kit. $n = 8$; Mean, standard deviation, probability by one–way ANOVA (with Fischer's LSD post hoc test) **** p-value <0.0001; ** p-value <0.01; * p-value <0.05. **(E)** Total cellular ROS levels were measured with 2,7 dichloro- fluorescein diacetate in 3T3 cells treated as in (D). $n = 8$; Mean, standard deviation, probability by one–way ANOVA (with Fischer's LSD post hoc test) **** p-value <0.0001; ** p-value <0.01; * p-value <0.05. **(F)** Lactate dehydrogenase activity levels in supernatant in 3T3 cells treated as in (D). $n = 8$; Mean, standard deviation, probability by one–way ANOVA (with Fischer's LSD post hoc test) **** p-value <0.0001; ** p-value <0.01; * p-value <0.05. **(G)** 4-HNE protein adducts in transiently transfected human-derived fibroblasts treated as in (D). $n = 3$; Mean, standard deviation, probability by one–way ANOVA (with Fischer's LSD post hoc test) **** p-value <0.0001; *** p-value <0.001; ** p-value <0.01; * p-value <0.05.

East Asian and African Americans) ruled out that any of these nearby SNPs in the non-coding sequence of *ALDH2* were causal or contributed to variation in alcohol consumption [39]. New consideration should be given to educating people at risk of these diseases regarding the consumption of alcoholic beverages.

Our study provides the first molecular evidence, based on a human genomic database search together with *in vitro* enzymatic assay and cellular characterization, that there are at least five more *ALDH2* deficiencies in humans, caused by mutations other than the common and well-characterized E504K substitution (*ALDH2*2*). We also showed that it is possible to correct these enzymopathies with small molecules, Alda-1 and Alda-64, identified in a small molecule screen. Confirmation of the health consequences of the *ALDH2* variants in humans is required, as are epidemiological studies to determine the extent of their impact on global populations. The contribution of other rarer *ALDH2* variants should also be considered. Our current research efforts focus on enzyme structural studies, and drug development using new chemotypes, to identify compounds that specifically target each *ALDH2* variant more effectively. Finally, considering that at least 80% of the world's population consumes alcoholic beverages and that even moderate consumption poses a health risk, as has been reported for increased risk of upper aerodigestive tract cancer and other diseases in *ALDH2*2* East Asians,[1,5,8,9] the health impact of the newly identified common *ALDH2* mutations in other populations should be considered. In the era of precision medicine, the health risks for carriers of the common *ALDH2* variants in each of the ethnic groups should, therefore, be evaluated.

5. Caveat and limitations

The novel and prevalent non-East Asian *ALDH2* variants identified and characterized in this manuscript are limited to the database collection by ExAC. Although the ExAC database has included the six major ethnic groups worldwide, it still represents only a small fraction of the world population and does not exclude the possibility that other distinct, common and undescribed *ALDH2* variants may exist in other races and subethnic groups in different regions of the world. Our survey of the Latino *ALDH2* variant is based on 150 human samples collected from Mexico. This is a small number of sample size from a single location for the Latino ethnic group. Future and more extensive collections and characterizations are needed to confirm our observations and the frequencies reported by the human genomic database. Finally, clinical studies to correlate the genotype of the carriers of the new *ALDH2* variant with reduced *ALDH2* activity and their phenotype of alcohol flushing reactions are needed.

6. Author contributions

C-HC and DM-R conceived the research idea and designed the research plan. C-HC, JCBF, AUJ, MCS, SJL, JH-MH, NDF and RM performed the experiments. PRC, DDM, FLB and GHRQ prepared IRB approval, collected human blood samples in Mexico and participated in manuscript discussion. C-HC, JCBF, AUJ and DM-R wrote the manuscript. All authors read and approved the manuscript.

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Declaration of Competing Interest

Daria Mochly-Rosen and Che-Hong Chen hold patents related to Alda-1 activation of *ALDH2*1* and *ALDH2*2*. One of the patents is licensed to Foresee Pharmaceuticals, a company that DM-R consults. However, these authors do not own stocks of the company and none of this research is supported by the company.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ebiom.2020.102753.

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