Published in final edited form as:
Alcohol Clin Exp Res. 2014 April ; 38(4): 921–929. doi:10.1111/acer.12340.

Single Nucleotide Polymorphisms interact to affect *ADH7* transcription

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Abstract

Background—The class IV alcohol dehydrogenase (ADH7, μ-ADH, σ-ADH) is important in the metabolism of ethanol and retinol. *ADH7* is the only *ADH* not expressed in liver, instead being expressed mainly in the upper gastro-intestinal tract. Genome wide studies have identified significant associations between single nucleotide polymorphisms (SNPs) in *ADH7* and alcoholism and cancer, but the causative variants have not been identified.

Methods—*In vitro* studies of gene expression by transient transfection into cell lines that express endogenous *ADH7* (CP-A cells) and that do not (HepG2 cells).

Results—We have identified transcriptional regulatory elements of *ADH7* and observed differences in the effects of variants on gene expression in CP-A cells and HepG2 cells. Two haplotypes of the proximal promoter that differ in a single nucleotide at rs2851028, A7P-G and A7P-A, have different transcriptional activities. There is an interaction between variants further upstream and these proximal variants: upstream regulatory sequences generally showed a greater increase or smaller reduction in activity when combined with the A7P-A promoter than with the A7P-G promoter. A sequence located 12.5 kb upstream (7P10) can function as an enhancer. In CP-A cells, both haplotypes of 7P10 increased A7P-A activity by 2.5-fold while having only 1.2-fold effect on A7P-G. In HepG2 cells, the 7P10-TTT haplotype had no effect on the A7P-A promoter but decreased A7P-G promoter activity by 50%, while the CTT haplotype increased A7P-A activity by 50% but had no effect on A7P-G.

Conclusions—These complex interactions indicate that the effects of variants in the *ADH7* regulatory elements depend on both sequence and cellular context, and should be considered in interpretation of the association of variants with alcoholism and cancer.

Keywords

Alcohol dehydrogenase 7 (*ADH7*); class IV ADH; alcoholism; gene regulation; genetic association

INTRODUCTION

The class IV alcohol dehydrogenase (ADH7, μ-ADH, σ-ADH), encoded by *ADH7* is expressed in the epithelial tissues of the upper aero-digestive tract down to the stomach (Vaglenova et al., 2003, Moreno and Parés, 1991). ADH7 efficiently metabolizes ethanol to

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acetaldehyde (Yin et al., 1990, Kedishvili et al., 1995), a known toxin and carcinogen (Salaspuro, 2009), and is particularly active at high ethanol concentrations such as those found in the upper aero-digestive tract immediately after consumption (Yin et al., 2003). ADH7 plays a role in the first-pass metabolism of alcohol (Yin et al., 1997, Han et al., 1998). ADH7 also efficiently oxidizes retinol to retinal (Yin et al., 2003, Yang et al., 1994), which is the precursor to retinoic acid, an important signaling molecule (Rhinn and Dollé, 2012) and anti-carcinogen (Siddikuzzaman et al., 2011). *Adh7*$^{-/-}$ mice show severe growth and survival defects on a vitamin-A deficient diet (Deltour et al., 1999). The potential interaction between the two substrates of ADH7 has been implicated in fetal alcohol syndrome (Duester, 1991, de Sanctis et al., 2011), cancer of the upper aerodigestive tract (Yokoyama et al., 2012, Fontanelli et al., 2013). *ADH7* expression was reduced by nearly 50% in Barrett's esophagus and esophageal adenocarcinoma specimens (Botelho et al., 2010) indicating a physiological correlation between *ADH7* and cancer.

Genome wide studies have identified associations of *ADH7* with alcoholism, drug dependence and cancer. A recombination hotspot within intron 7 of *ADH7* divides the region into two haplotype blocks (Han et al., 2005, Edenberg et al., 2006, Birley et al., 2008-a). SNPs located in the 5' haplotype block are associated with both blood alcohol and breath alcohol concentrations, and account for approximately 18% of a major quantitative trait locus within the *ADH* region influencing alcohol metabolism, or 11% of the total genetic variance (Birley et al., 2008-a, Birley et al., 2009-b). The C allele of rs1154458, located in intron 6 of *ADH7*, is linked to protection against alcoholism (Osier et al., 2004, Han et al., 2005). Two *ADH7* non-synonymous coding variants are known, rs1573496 (Ala92Gly) and rs59534319 (Lys238Glu; rare to uncommon in most populations). The GG and CG genotypes at rs1573496 confer a protective effect against cancers of the upper aero-digestive tract (Wei et al., 2010, McKay et al., 2011, Hashibe et al., 2008), where *ADH7* is expressed. There is no reported association of rs59534319 with disease, although it is only 30 bp away from the synonymous SNP rs971074, which is associated with a higher risk for drug dependence (Luo et al., 2007, Levran et al., 2009). It is likely that many of these associations reflect differences in regulation of expression, as has been found for other complex diseases.

An understanding of the *ADH7* regulatory regions and the effects of variants on regulation can help interpret the association data and point toward likely causal variants. A 232 bp proximal promoter of *ADH7* is functional in HeLa, CV-1 (monkey kidney) and H4IIE-C3 (rat liver) cell lines (Kotagiri and Edenberg, 1998). Mutation of an AP-1 transcription factor binding site significantly decreased promoter activity, indicating an important role for AP-1 in *ADH7* gene regulation (Kotagiri and Edenberg, 1998). Multiple binding sites for C/EBP transcription factors were identified. Co-expression with C/EBP-α or C/EBP-β led to decreases in promoter activity (Kotagiri and Edenberg, 1998). Since these C/EBP transcription factors are prominently expressed in the liver, the negative effect of C/EBP co-expression suggests one mechanism that contributes to *ADH7* not being expressed in the liver. Other transcription factors, including the retinoic acid receptor family, STAT family and HNF family proteins, are predicted by PROMO (Messeguer et al., 2002) to bind in the *ADH7* proximal region. The ENCODE HMR Conserved Transcription factor track (Weirauch and Raney) also predicted binding sites for the transcription factors FOXO3 and FOXJ2 in the 1.5 kb region immediately upstream of *ADH7*.

The goal of this study is to identify elements regulating *ADH7* transcription and examine how genetic variants affect regulation. Two human cell lines with contrasting *ADH7* expression were chosen to study *ADH7* gene regulation. CP-A epithelial cells, derived from a non-dysplastic Barrett's esophageal sample immortalized by hTERT (Palanca-Wessels et al., 1998) express *ADH7* endogenously. HepG2 human hepatoma cells (Knowles et al.,

1980) do not express *ADH7*. We identified regulatory elements, including some with cell-specificity, and detected interactions among them.

MATERIALS AND METHODS

Bioinformatics

The mammalian conservation track in the UCSC genome browser was used to identify the regions of conservation between human and other vertebrates in the sequence upstream of *ADH7*. Published literature and online databases including ALFRED (Rajeevan et al., 2012) and HGMD (Stenson et al., 2009) were used to identify disease associated SNPs. Linkage Disequilibrium (LD) data from the NCBI Hapmap database (The International HapMap Consortium, 2010; Data Rel 27, Feb 09) and the 1000 genome database (The 1000 Genomes Project Consortium, 2012; based on Ensembl v69, Oct 2012) were used to identify LD between SNPs in the vicinity of *ADH7* and disease-associated SNPs. The Hapmap and dbSNP databases were used to identify naturally occurring haplotypes and the samples to use as templates for obtaining them.

Cloning of test fragments and their variants

Two naturally occurring haplotypes of an 841 bp *ADH7* proximal promoter (A7P-G/A; −19 to −859 bp relative to *ADH7* translation start site in GenBank AAC51351.1) were generated by PCR amplification from an anonymous human DNA sample using Invitrogen Platinum Pfx polymerase (Life Technologies, Grand Island, NY; catalog 11708-013). The two promoter haplotypes, A7P-A and A7P-G were cloned into HindIII and BglII sites in the multiple cloning site of the pXP2 luciferase vector (Nordeen, 1988), oriented so that the promoters drive luciferase expression. Restriction sites for NcoI, Acc65I, NotI and XhoI were included in the forward primer of A7P to facilitate further subcloning. Fragments of approximately 1 kb each extending12.5 kb upstream of the *ADH7* gene were amplified from human DNA using Invitrogen Platinum Pfx polymerase. DNA samples used as templates in PCR amplification of naturally occurring haplotypes of 7P5, 7P6, 7P8 and 7P10 (Table 1) were obtained from the Coriell Institute for Medical Research (Camden, New Jersey, USA). Fragments 7P2 to 7P10 (except 7P6) were cloned into Acc65I and XhoI sites of pXP2 upstream of promoters A7P-G and A7P-A, and tested for effect on each promoter activity. 7P6 was cloned into NcoI and XhoI sites of pXP2 upstream of A7P-G and A7P-A. 7P10 sub-fragments were cloned into HindIII and XhoI sites upstream of A7P-A. 7P10 sub-fragment E2 was cloned into HindIII and XhoI sites upstream of A7P-A in the reverse orientation to make E2flip, and into ApaI and SwaI sites to make E2far. E2 was cloned into HindIII and XhoI sites upstream of luciferase gene to make E2prom.

Cell culture and Transient transfections

HepG2 human hepatoma cells (HB-8065; ATCC, Manassas, VA) were cultured in MEM (ATCC) with 10% fetal bovine serum (Invitrogen, Carlsbad, CA), 4 mM glutamine (Thermo Scientific Hyclone, Waltham, MA) and 1× Penicillin and Streptomycin (Thermo Scientific Hyclone) on cell bind surface plates (Corning Inc., Tewksbury, MA; CLS3296) at 37°C. Transient transfections in HepG2 cells were done by seeding 0.8×10^5 cells per well in Corning cell bind surface 12-well plates (Corning Inc., Tewksbury, MA; CLS3336). 24 h after seeding, medium was changed and cells were transfected with 1.52 pmoles of test DNA, 15 ng of pCMV β-galactosidase plasmid (Clontech, Mountain View, CA) and enough pUC19 DNA to get a total DNA amount of 1 μg per well. 2 μl per well Fugene HD (Roche, Indianapolis, IN) was used as the transfection reagent.

CP-A cells (ATCC, CRL-4027) are an hTERT immortalized cell line obtained from a non-dysplastic Barrett's Esophagus tissue. CP-A cells were cultured in Keratinocyte-SFM

(Invitrogen, Carlsbad, CA; 17005-042, with each 500 ml supplemented with 25 mg Bovine pituitary extract and 2.5 μg human recombinant epidermal growth factor supplied with the medium) plus 10% FBS and 1% Penicillin-Streptomycin (ATCC, 30-2300) at 37 °C. For transient transfections, CP-A cells were seeded at 3.75×10^5 cells per well in 12-well cell bind plates (Corning Inc., Tewksbury, MA; CLS3336). Transfections were done 24 h after seeding as described above.

Reporter gene assays

HepG2 cells were collected in ice cold 1× PBS (made from 10 × PBS solution; Fisher Bioreagents, Hampton, New Hampshire; BP3994) 30 h after transfection. Cells were centrifuged and the pellet was resuspended in 100 μl of 1× Reporter Lysis buffer (Promega, Madison, WI; E3971). Cell lysates were prepared by repeated freeze-thaw cycles in dry ice and water. Lysates were centrifuged to pellet cell debris and the supernatants transferred to new tubes. Luciferase activity was measured on a Spectramax LS (Molecular Devices, Sunnyvale, CA) using the Luciferase assay system (Promega, Madison, WI; E1501), with 20 μl of the extract. β-galactosidase assays were carried out with 5 μl extract using the Galacto-Light System (Tropix, Bedford, MA).

CP-A cells were harvested 24 h after transfections by washing once with 1× PBS, and scraping into 250 μl 1× Reporter lysis buffer. Lysates were then freeze-thawed repeatedly and centrifuged to pellet down cell debris, as described above. 20 μl and 10 μl of CP-A cell supernatant was used for luciferase and β-galactosidase assays respectively.

All test fragments were transfected in at least 3 independent experiments, with each experiment having at least 3 replicates. Relative activity of a test construct was calculated by normalizing each luciferase activity to the internal control β-galactosidase activity, to correct for the transfection efficiency, and then calculating the ratio of normalized luciferase activity of test fragment to that of the promoter haplotype driving the luciferase expression in the construct. P-values were calculated using two-tailed t-tests of the normalized values.

RNA extraction and purification, real time PCR

HepG2 and CP-A RNA were extracted using Trizol (Life Technologies, Carlsbad) and purified using the Qiagen RNeasy mini kit (Qiagen, Germantown, MD) following the manufacturer's protocol. DNA concentrations were determined by absorbance at 260 nm using a Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA). Superscript III First-strand synthesis system (Invitrogen, Carlsbad, CA; 18080-051) was used to synthesize cDNA from 1 μg RNA following the manufacturer's protocol. qPCR assays were performed using 10 μl of 2× Power SYBR Green mastermix (Life technologies, Carlsbad, CA; 4367659), 2 μl of 3 μM primers, 3 μl of 2× diluted cDNA and water in the StepOnePlus Real-time PCR system (Life Technologies, Carlsbad, CA; 4376598). No RT and no DNA controls were included in each experiment.

RESULTS

ADH7 proximal promoter variant rs2851028 and its effect on activity

There are two haplotypes of the 841 bp fragment that extends from −19 bp to −859 bp with respect to the *ADH7* translation start site, A7P-A and A7P-G, that differ at a single SNP: rs2851028, at −628 bp (Figure 1). Both haplotypes were tested for promoter activity by transient transfections in CP-A esophageal cells that express *ADH7* and in HepG2 hepatoma cells that do not (Table 2). These cells represent the complementary pattern of *ADH7* expression seen in humans (Westerlund et al., 2007, Vaglenova et al., 2003). Both haplotypes of the promoter fragment were active in both cell types (Figure 2). The promoter

construct with the minor G allele was 1.6 - to 2-fold more active than the promoter with the A allele. The empty vector p×P2 had no activity in either cell type.

Upstream regulatory regions

Conservation extended approximately 12.5 kb upstream of *ADH7*, with only patches of conservation seen in the rest of the intergenic sequence between *ADH7* and *C4orf17*, which flanks the ADH gene cluster on one end. We therefore focused our studies on this 12.5 kb region. Fragments of approximately 1 kb (Figure 1) were cloned upstream of the A7P-G and A7P-A promoters and tested for effects on promoter activity by transient transfections.

Monomorphic fragments

Fragments 7P2, 7P3, and 7P4, together spanning from −798 bp to −3515 bp, did not have any reported SNPs, nor did fragments 7P7 and 7P9 (Figure 1). These monomorphic fragments showed different activity depending on which promoter haplotype they were combined with and which cells they were tested in. Fragment 7P2 reduced activity of both promoters by 10–15% in CP-A cells (Figure 3A) and reduced the activity of the A7P-A promoter by a similar 20% in HepG2 cells, but had a significantly larger effect on A7P-G activity in HepG2 cells (55% reduction) (Figure 3B). 7P3 significantly increased both promoter activities in CP-A cells but had little effect on the activity of A7P-A in HepG2 cells, and significantly reduced activity of A7P-G (by 38%) in HepG2 cells. 7P4 had stronger effects on the A7P-G promoter in both cells, but in different directions: it increased A7P-A and A7P-G promoter activities in CP-A cells (by 15% and 40% respectively), while decreasing both activities in HepG2 cells (by 20% and 35% respectively). In CP-A cells, 7P7 increased A7P-A activity but decreased A7P-G activity to a similar extent, whereas in HepG2 cells it decreased both promoters, with a much stronger effect on A7P-G (Figure 3B). 7P9 had little effect on A7P-A activity in CP-A cells, but strongly decreased A7P-G activity. In HepG2 cells, 7P9 decreased activity of both promoters, with a much stronger effect on A7P-G.

Polymorphic fragments

Fragments 7P5, 7P6, 7P8 and 7P10 contain SNPs (Figure 1) in linkage disequilibrium with variants having significant disease associations (Table 3) (Wei et al., 2010, Osier et al., 2004, McKay et al., 2011, Levran et al., 2009, Hashibe et al., 2008, Han et al., 2005, Cadoni et al., 2012, Birley et al., 2008-a, Birley et al., 2009-b). Transient transfections were done with two naturally occurring haplotypes of each of these fragments cloned upstream of both A7P-A and A7P-G promoters. 7P5-ATAC had no effect on either promoter activity in CP-A cells (Figure 4A) while strongly reducing both promoter activities (about 50%) in HepG2 cells (Figure 4B). 7P5-GCGT showed both promoter-specific and cell-specific function: it had no effect on A7P-A but decreased A7P-G activity by 20% in CP-A cells; in HepG2 cells, it increased A7P-A activity by 20% but decreased A7P-G activity by 40%.

Both 7P6 haplotypes decreased both promoter activities by 36–47% in CP-A cells (Figure 4A). In HepG2 cells (Figure 4B), 7P6-A reduced A7P-A activity by 20% with no significant effect of 7P6-G. Both 7P6 haplotypes showed a dramatic 85% reduction of A7P-G activity. The 7P8 haplotypes 7P8-GC and 7P8-AG increased A7P-A activity in CP-A cells by 40% and 20% respectively but had nearly no effect on A7P-G (Figure 4A). In HepG2 cells (Figure 4B), both 7P8 variants decreased A7P-A activity by 20% and A7P-G activity by approximately 60%.

The 7P10 variants had the greatest positive impact on promoter activity. In CP-A cells, both 7P10 haplotypes increased the A7P-A promoter activity 2.3–2.4 fold (Figure 4A) while having significantly smaller effects on A7P-G activity (1.1–1.3 fold). In HepG2 cells, 7P10-

CTT increased A7P-A activity by 40% with no effect on A7P-G, and 7P10-TTT had no effect on A7P-A but decreased A7P-G activity by 40% (Figure 4B).

7P10, which had the greatest effect on activity of the A7P-A promoter, was fragmented into three partially overlapping sub-sequences (Figure 5A) to better localize the regulatory element(s). Transient transfections in CP-A cells showed that one of the sub-fragments, 7P10-E2 had the strongest effect on promoter activity (Figure 5B), twice that of the whole fragment. The other sub-fragments 7P10-E1 and 7P10-E3 had some activity, but less than the whole fragment.

Given its location and relatively strong effect on promoter activity, 7P10-E2 was tested to determine if it functioned as an enhancer (Figure 6A). It did not function alone as a promoter (7P10-E2prom; Figure 6B). It did function nearly as well in either orientation (E2flip vs E2), and when moved further away from the promoter, fulfilling characteristics of an enhancer (Figure 6B).

DISCUSSION

We have identified elements regulating *ADH7* promoter activity in physiologically relevant cell systems, and observed functional differences in the activities of naturally occurring haplotypes of both the promoter and upstream regulatory elements. There are cell-specific differences, and also differences in how the upstream sequences affect the promoter with the A allele at rs2851028 vs. the G allele. In CP-A cells, which express *ADH7* endogenously, some fragments increase activity of the promoter with the A allele more than they do the promoter with the G allele, and others increase the A-promoter while decreasing activity of the G-promoter. Most of the upstream fragments reduce promoter activity in HepG2 cells, which don't express endogenous *ADH7*, with a stronger effect on the promoter with the G allele. Thus the effects of a particular regulatory polymorphism are complex, and depend upon the sequence context of other polymorphisms and on cell type. This can complicate interpretation of association data.

The pattern of linkage disequilibrium among the upstream variants and those associated with diseases shows three distinct LD blocks (Table 3). Variants in block 1, which contains the A7P promoter with rs2851028 and the upstream 7P5 fragment with rs1154473, are in strong LD ($r^2 \geq 0.8$, D' ≥ 0.9) with variant rs1154458, associated with alcoholism (Osier et al., 2004, Han et al., 2005), rs2654849, associated with age of onset of regular alcohol use (van Beek JH, 2010) and rs1154460, associated with upper aero-digestive tract (UAT) cancers (Oze et al., 2009, Hakenewerth et al., 2011). The more active allele of the promoter, rs2851028-G, is in LD with the C allele of rs1154458, which protects against alcoholism (Osier et al., 2004, Han et al., 2005). This is analogous to the faster-metabolizing isoforms of *ADH1B* and *ADH1C*, which have protective effects on risk for alcoholism and excessive drinking (Thomasson et al., 1993, Hurley and Edenberg, 2012, Edenberg and Foroud, 2013, Chen et al., 1999, Bierut et al., 2012). Though the 7P5 haplotypes have similar effects relative to each promoter in CP-A cells, there is a potential synergistic interaction between the two 7P5-GCGT haplotype and rs2851028-A in HepG2 cells.

rs1154460, also in block 1, is associated with upper aerodigestive cancers, the risk increasing with increasing alcohol consumption (Hakenewerth et al., 2011). In this case, it is the less active A allele of rs2851028 that is in LD with the risk allele for cancer, rs1154460-A; the cancer risk increased with increasing alcohol consumption (Oze et al., 2009, Hakenewerth et al., 2011). The direction of affect is similar to the association of the less active *ADH1C*2* with cancer, with the risk increasing additively with alcohol consumption (Xue et al., 2012, Peters et al., 2005, Bongaerts et al., 2011). Retinoic acid and its precursor

retinol are anti-carcinogens (Siddikuzzaman et al., 2011) and signaling molecules (Rhinn and Dollé, 2012) that are important for the maintenance of epithelial tissues (Osanai et al., 2007) including those expressing *ADH7*. The two ADH7 substrates use the same active site (Yin et al., 1997, Yang et al., 1994, Kedishvili et al., 1995, Yokoyama et al., 2012, Han et al., 1998), so there is potential competition between them that could reduce the oxidation of retinol in the presence of ethanol. Although there are other retinol dehydrogenases in the super family that includes the medium chain enzymes (ADHs) and the short chain dehydrogenase (SDR) family, ADH7 has the greatest efficiency for oxidation of retinol to retinaldehyde (Parés et al., 2008). The importance of ADH7 has been shown both by morphological changes including inflammation and intestinal metaplasia in the gastric mucosa when ADH7 activity is reduced (Matsumoto et al., 2005) and by the severe growth and survival defects when $Adh7^{-/-}$ mice are raised on a vitamin-A deficient diet (Deltour et al., 1999).

Block 2 contains 6 upstream SNPs in fragments 7P5, 7P6, 7P8 and 7P10, located between −4667 bp to –12578 bp. These upstream fragments affect regulatory function in a cell- and promoter haplotype-dependent manner. The minor alleles of these SNPs are in LD with the minor alleles of SNPs associated with the early stages of alcohol metabolism (Table 3) (Birley et al., 2008-a, Birley et al., 2009-b).

The third LD block comprises the 7P10 enhancer SNP rs17589306, in LD with two SNPs associated with cancer (Table 3) (Wei et al., 2010, McKay et al., 2011, Hashibe et al., 2008, Cadoni et al., 2012) and drug dependence (Luo et al., 2007, Levran et al., 2009) but not, thus far, with alcoholism or alcohol metabolism (Birley et al., 2008-a). One of these, rs1573496, is a coding SNP associated with upper aerodigestive cancer (Wei et al., 2010, McKay et al., 2011, Hashibe et al., 2008, Cadoni et al., 2012); it is in complete LD with the enhancer SNP rs17589306. rs971074, a synonymous SNP, is associated with drug dependence (Luo et al., 2007, Levran et al., 2009, Hakenewerth et al., 2011). The other non-synonymous coding polymorphism rs59534319, 30 bp away from rs971074, is uncommon to rare in most populations and no associations have yet been reported; it is, however, possible that it contributes to the reported associations in this linkage block.

In a genome-wide study of alcohol dependence in Han Chinese, *ADH7* was reported to be hypomethylated in alcoholics, although the sites were not shown (Zhang et al., 2013). The UCSC Genome Browser shows two sites of methylation in the proximal promoter region (at bp −148 and −787) and one site in the enhancer region (at −12059, in 7P10-E2); these are partially methylated in many cell types, including HepG2. None are at polymorphic sites, and their effect on gene expression is not known.

Our study demonstrates that variants affecting *ADH7* gene regulation show combinatorial and cell-specific interactions. This highlights the complexity of interpreting the effects of individual SNPs, because they are dependent upon other SNPs that are in *cis*. Detailed analyses of haplotypes that are associated with alcohol-related traits will be needed to identify which SNPs are functional and the consequences of different combinations of SNPs.

Acknowledgments

We thank Jun Wang and Ronald E. Jerome for technical assistance.

This research was supported by a grant from the National Institute on Alcohol Abuse and Alcoholism, R37 AA006460.

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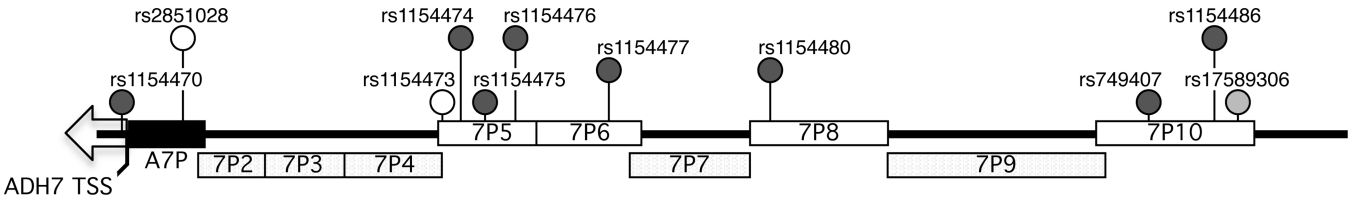

Figure 1.

Map of the *ADH7* promoter and upstream fragments in chromosomal orientation, drawn to scale, with promoter (black solid), monomorphic fragments (patterned) and polymorphic fragments (not shaded). Transcription occurs right to left (arrow) from the translation start site (TSS). SNPs are shown, with the three LD blocks represented as solid black circles, plain circles or grey circle.

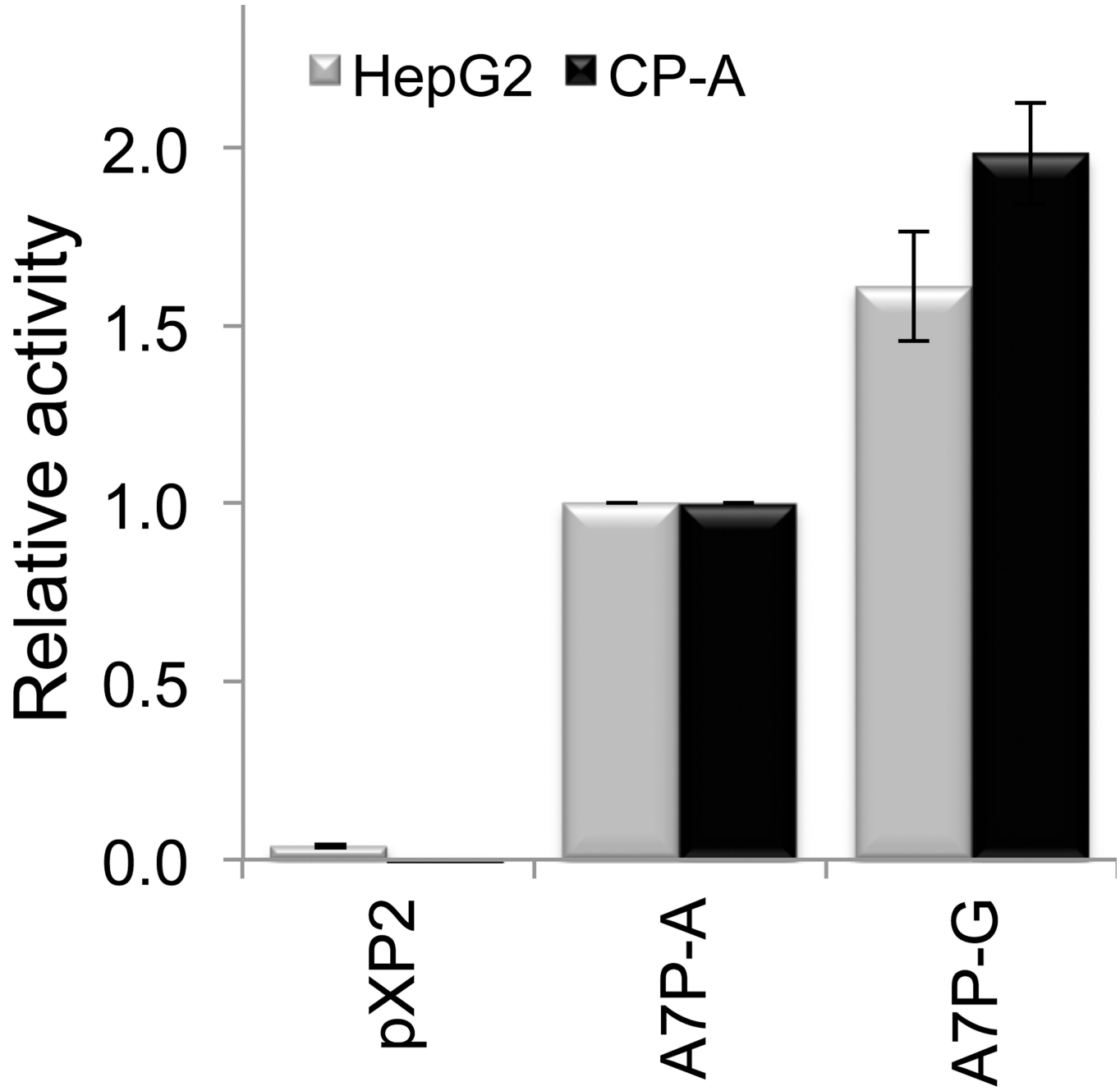

Figure 2.
ADH7 promoter haplotypes are active in both CP-A and HepG2 cells. The relative activity is the ratio of promoter activity of the G construct (A7P-G) to the A construct (A7P-A) within each cell line. Standard errors of mean are shown (n≥12). P-value was 3×10^{-5} (HepG2 cells) and 1×10^{-6} (CP-A cells).

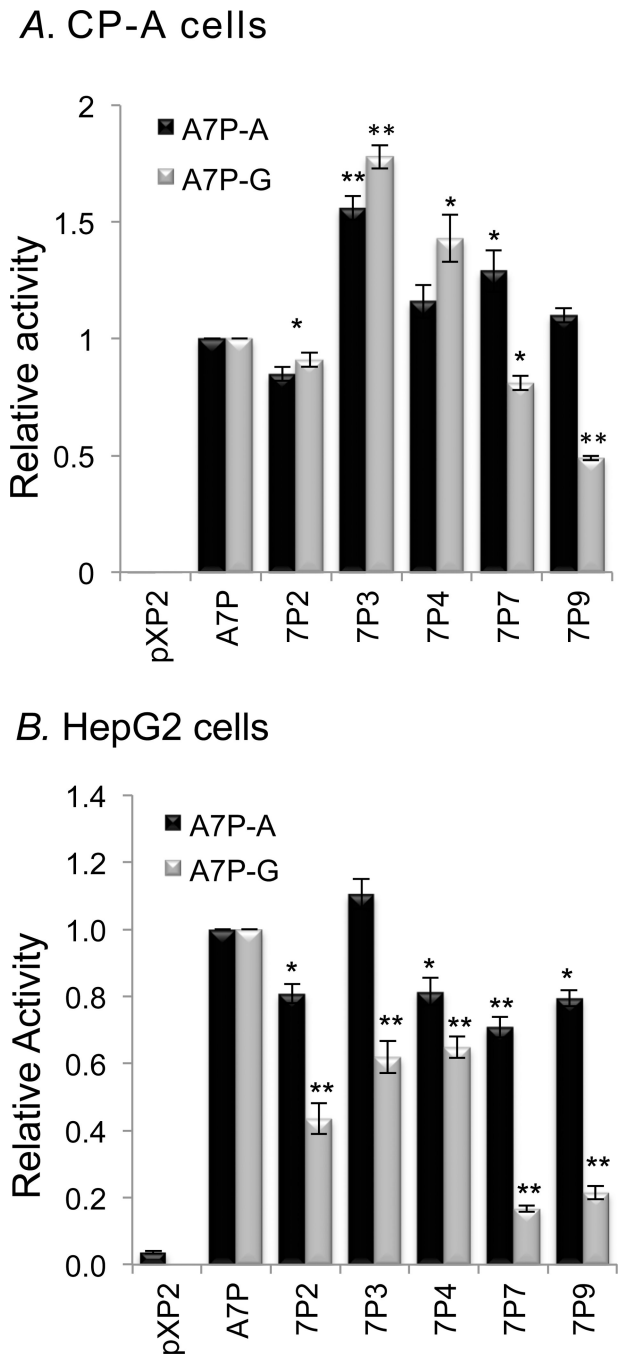

Figure 3.

Activity of monomorphic fragments on the two promoter haplotypes. Transient transfections of the monomorphic upstream sequences 7P2, 7P3, 7P4, 7P7 and 7P9 were done in **A)** CP-A cells and **B)** HepG2 cells. Relative activity of each construct represents the ratio of normalized luciferase activity of the test fragment to the A7P-A (black) promoter or A7P-G (grey shaded). Scales of vertical axes are different. Error bars represent standard errors of mean. * indicate p-values ≤ 0.01; ** indicate p-values ≤ 1×10^{-5}.

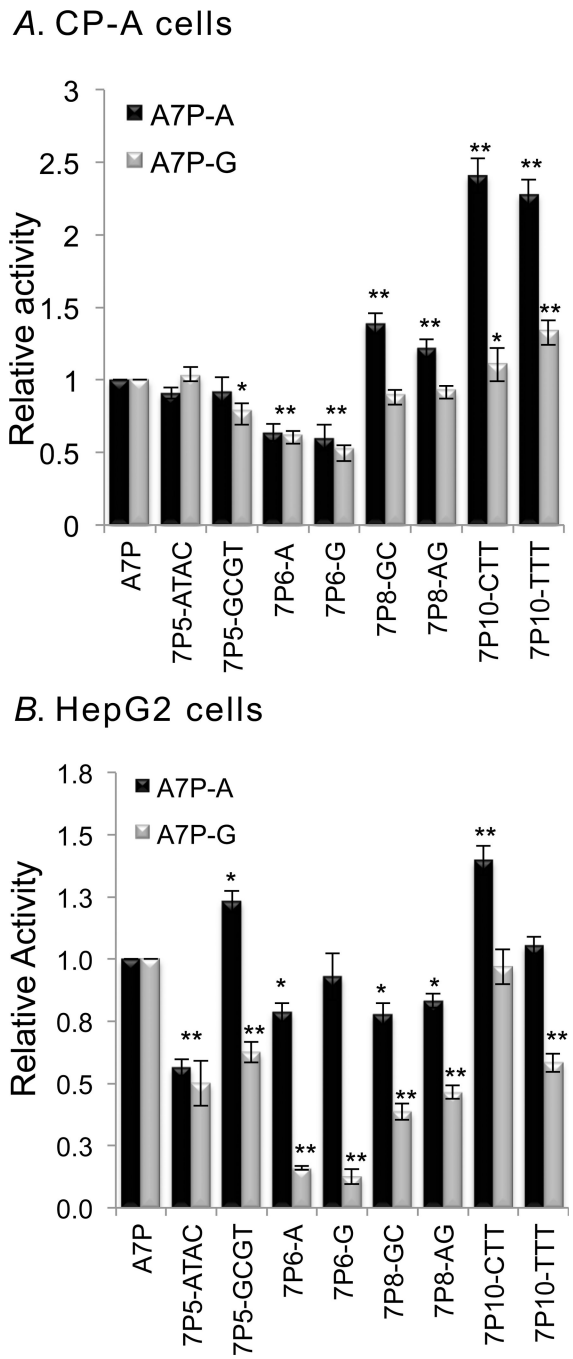

Figure 4.

Activity of polymorphic fragments on the two promoter haplotypes. Transient transfections of two naturally occurring haplotypes of each polymorphic fragment (7P5, 7P6, 7P8 and 7P10) were done in **A)** CP-A cells and **B)** HepG2 cells. Each sequence was tested for effect on both promoters A7P-G and A7P-A. Relative activity of each construct represents the ratio of normalized luciferase activity to the promoter on which it was tested, A7P-G (grey shaded) or A7P-A (black). Error bars represent standard errors of mean. Scales of vertical axes are different. Relative activities are shown with * indicating p-values ≤ 0.008; ** indicate p-values ≤ 1×10^{-4}

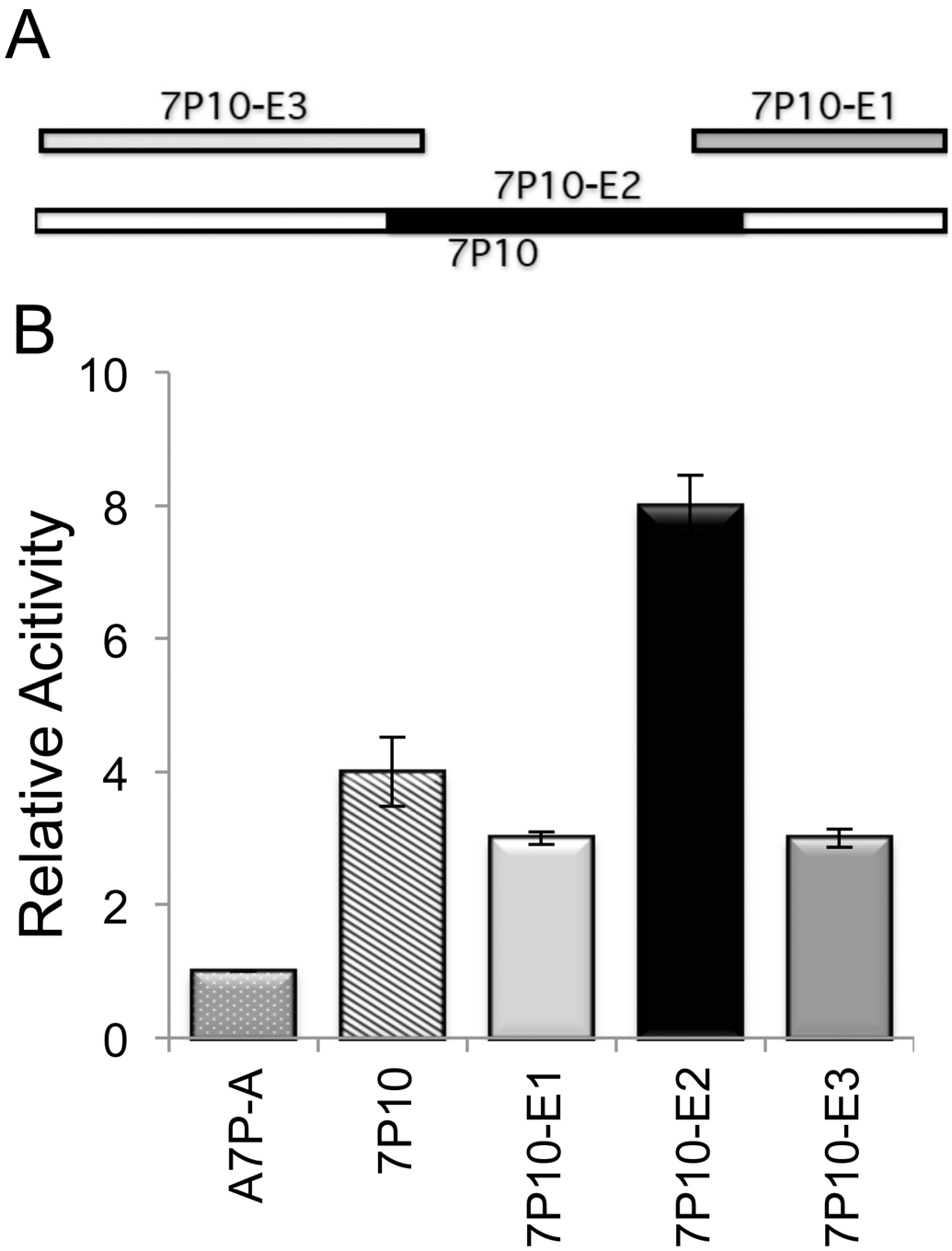

Figure 5.
Localization of regulatory elements in fragment 7P10. ***A)*** 7P10 sub-fragments approximately 500 bp in size are shown as oriented on the chromosome, with E1 being the farthest upstream from *ADH7*. ***B)*** Activity of sub-fragments of 7P10 in CP-A cells.

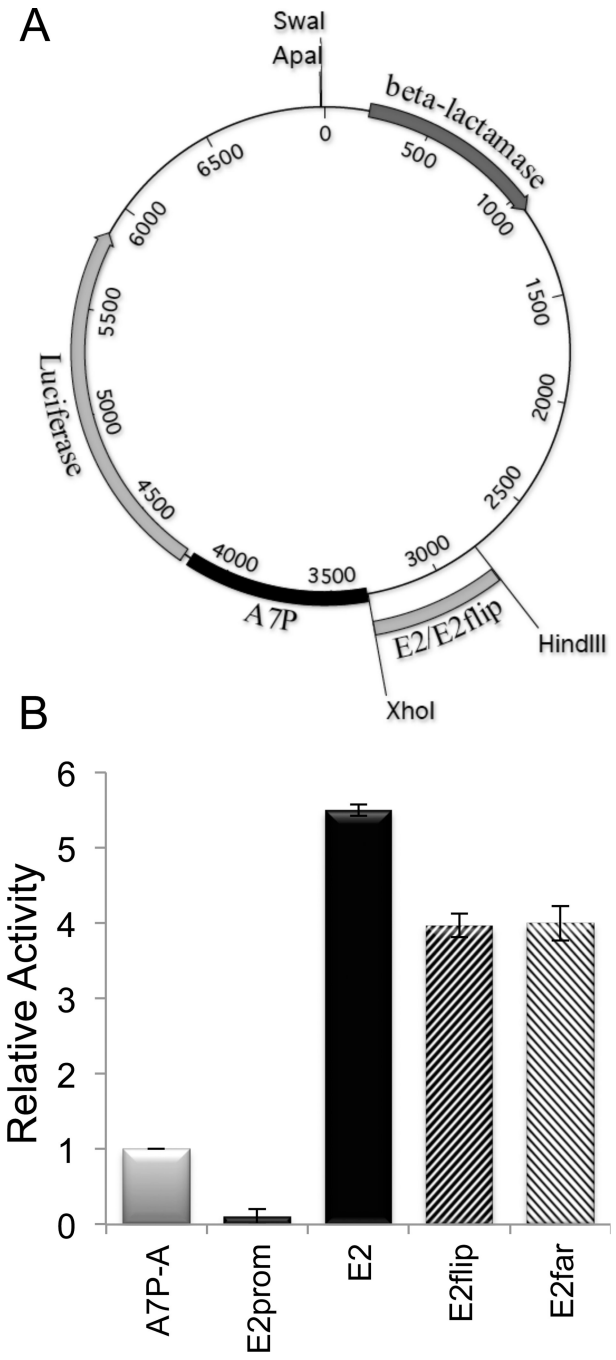

Figure 6.

Fragment 7P10 acts as an enhancer. ***A)*** Map of the p×P2 vector construct with E2 or E2flip (E2 in reverse orientation) cloned into the HindIII and XhoI sites immediately upstream of A7P drawn to scale. E2far contains E2 cloned into the ApaI and SwaI sites 3.5 kb away from the promoter to test for position effects. ***B)*** 7P10-E2 tested for promoter activity (E2prom) and enhancer properties, orientation independence (E2flip) and position independence (E2far), by transient transfections in CP-A cells. Activity was measured relative to A7P-A. Error bars represent standard error of means. All p-values $\leq 1 \times 10^{-8}$

Table 1

Upstream fragments and their tested variants

Test fragment location is with respect to the *ADH7* translation start site. DNA source: Samples from Coriell Institute (NA number) or anonymous DNA used to amplify test fragments of different haplotypes. Haplotypes tested are listed in order of the SNPs. Haplotype frequencies in three populations obtained from the Hapmap (The International HapMap, 2005) and 1000 genome (THE 1000 GENOMES PROJECT, 2012) databases; CEU, Northern and Western Europeans from Utah; CHB+JPT, East Asian Han Chinese and Japanese and YRI, Yoruba from Nigeria).

Test fragment (Location)	dbSNP	DNA Source	Haplotypes tested	Haplotype frequency		
				CEU	YRI	CHB+ JPT
A7P (−19 to −859)	rs2851028	anonymous	G A	0.18 0.83	0 1	0.58 0.42
7P5 (−3475 to −4667)	rs1154473, rs1154474, rs1154475, rs1154476	NA12057 NA12751	ATAC GCGT	0.41 0.30	0.51 0	0.57 0.24
7P6 (−4571 to −5741)	rs1154477 rs1442477	NA12057 NA12751	GG AG	0.69 0.31	0.92 0.08	0.76 0.24
7P8 (−6954 to −8492)	rs1154480, rs1154481	NA07000	GC AG	0.05 0.36	- -	0.23 0
7P10 (−10815 to −12578)	rs749407, rs1154486 rs17589306	anonymous	CTT TTT	0.48 0.36	0.78 0.08	0.78 0.21

Table 2
ADH expression in HepG2 and CP-A cells

Expression was tested using RT-PCR and the results depicted as a "+" for gene expressed or "−" for no gene expression.

Gene	CP-A	HepG2
ADH1A	−	−
ADH1B	−	−
ADH1C	+	−
ADH4	−	+
ADH5	+	+
ADH6	+	+
ADH7	+	−

Table 3
Upstream SNPs in LD with disease associated SNPs

Upstream SNPs in LD with disease associated SNPs and the test fragments containing them are listed. SNPs with $r^2 \geq 0.8$, D' ≥ 0.9 are defined as within an LD block. The disease phenotypes associated with each SNP are listed, along with the references.

LD block	SNPs in tested fragments	Tested Fragments	Disease phenotype and associated SNPs
1	rs2851028 rs1154473	A7P 7P5	**rs1154458**- Protection against alcoholism (Han et al., 2005, Osier et al., 2004) **rs2654849**- Age at onset of regular alcohol use (van Beek JH, 2010) **rs1154460**- UAT cancers (Oze et al., 2009, Hakenewerth et al., 2011)
2	rs1154474 rs1154475 rs1154476 rs1154477 rs1154480 rs749407	7P5 7P5 7P5 7P6 7P8 7P10	**rs1154461**- Alcohol metabolism (Birley et al., 2009, Birley et al., 2008) **rs1154468**- Alcohol metabolism **rs894363**- Alcohol metabolism **rs1154470**- Alcohol metabolism; Extraversion and conscientiousness in Substance-dependent subjects (Luo et al., 2008)
3	rs17589306	7P10	**rs971074**- Drug dependence & heroin addiction (Luo et al., 2007, Levran et al., 2009), **rs1573496**- UAT cancer (Hashibe et al., 2008, McKay et al., 2011, Wei et al., 2010, Cadoni and Pandolfini, 2012)