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Corresponding Author: Dr. Shih-Jiun Yin, PhD

Corresponding Author's Institution: National Defense Medical Center

First Author: Chien-Ping Chiang, MD

Order of Authors: Chien-Ping Chiang, MD; Shu-Wen Jao, MD; Shiao-Pieng Lee, DDS, PhD; Pei-Chi Chen, MS; Chia-Chi Chung, MS; Shou-Lun Lee, PhD; Shin Nieh, DDS; Shih-Jiun Yin, PhD

Abstract: Alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) are principal enzymes responsible for metabolism of ethanol. Functional polymorphisms of ADH1B, ADH1C, and ALDH2 genes occur among racial populations. The goal of this study was to systematically determine the functional expressions and cellular localization of ADHs and ALDHs in human rectal mucosa, the lesions of adenocarcinoma and hemorrhoid, and the genetic association of allelic variations of ADH and ALDH with large bowel disorders. Twenty one surgical specimens of rectal adenocarcinoma and the adjacent normal mucosa, including 16 paired tissues of rectal tumor, normal mucosae of rectum and sigmoid colon from the same individuals, and 18 surgical mixed hemorrhoids specimens as well as leukocyte DNA samples from 103 colorectal cancer patients, 67 hemorrhoid patients, and 545 control subjects recruited in previous study, were investigated. The isozyme/allozyme expression patterns of ADH and ALDH were identified by isoelectric focusing and the activities were assayed spectrophotometrically. The protein contents of ADH/ALDH isozymes were determined by immunoblotting using the corresponding purified class-specific antibodies; the cellular activity and protein localizations were detected by immunohistochemistry and histochemistry, respectively. Genotypes of ADH1B, ADH1C, and ALDH2 were determined by polymerase chain reaction-restriction fragment length polymorphisms. At 33 mM ethanol, pH 7.5, the activity of ADH1C*1/1 phenotypes exhibited 87% higher than that of the ADH1C*1/*2 phenotypes in normal rectal mucosa. The activity of ALDH2-active phenotypes of rectal mucosa was 33% greater than ALDH2-inactive phenotypes at 200 μM acetaldehyde. The protein contents in normal rectal mucosa were in the following order: ADH1 > ALDH2 > ADH3 = ALDH1A1, while those of ADH2, ADH4, and ALDH3A1 were fairly low. Both activity and content of ADH1were significantly decreased in rectal tumors whereas the ALDH activity remained unchanged. The ADH activity was also significantly reduced in hemorrhoids. ADH4 and ALDH3A1 were uniquely expressed in the squamous epithelium of anus at anorectal junctions. The allele frequencies of ADH1C*1 and ALDH2*2 were significantly higher in colorectal cancer and that of ALDH2*2 also significantly greater in hemorrhoids. In conclusion, ADH and ALDH isozymes are differentially expressed in mucosal cells of rectum and anus. The results suggest that acetaldehyde, an immediate metabolite of ethanol, may play an etiological role in pathogenesis of large bowel diseases.

COVER LETTER

Charles R. Goodlett, PhD
Department of Psychology, LD 124
IUPUI
402 North Blackford Street
Indianapolis, IN 46202-3275
USA

Dear Dr. Goodlett,
Please find enclosed our manuscript entitled “Expression pattern, ethanol-metabolizing activities, and cellular localization of alcohol and aldehyde dehydrogenases in human large bowel: Association of the functional polymorphisms of *ADH* and *ALDH* genes with hemorrhoids and colorectal cancer” which we wish to submit to you for publication in the Alcohol. The manuscript has been read, approved and concurred by all co-authors. All authors have made substantial contributions that meet the stated requirements for authorship and have exercised due care to ensure the integrity of the work. This work has not been published and is not currently under review for publication elsewhere. No conflict of interests exists for all authors with any commercial affiliation or consultation.

We would like to suggest following potential reviewers.

Dr. Helmut K. Seitz
Department of Medicine
University of Heidelberg
Salem Medical Center
Zeppelinstr. 11-33
D-69121 Heidelberg, Germany
Tel: +49-6221-483201
Fax: +49-6221-483494
Email: helmut_karl.seitz@urz.uni-heidelberg.de

Dr. David W. Crabb
Department of Medicine
Indiana University School of Medicine
545 Barnhill Drive
Indianapolis, IN 46202-5122

Tel: 317-274-8438
Fax: 317-274-1437
Email: dcrabb@iupui.edu

Sincerely,

Shih-Jiun Yin, Ph.D.
Professor
Department of Biochemistry
National Defense Medical Center
161 Minchuan East Road Section 6
Taipei 11490, Taiwan
Tel: +886-2-8792-3100 ext 18800
Fax: +886-2-8792-4818
E-mail: yinsj@ndmc.idv.tw

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Expression pattern, ethanol-metabolizing activities, and cellular localization of alcohol and aldehyde dehydrogenases in human large bowel: Association of the functional polymorphisms of *ADH* and *ALDH* genes with hemorrhoids and colorectal cancer

Chien-Ping Chiang[a,b], Shu-Wen Jao[c], Shiao-Pieng Lee[d], Pei-Chi Chen[e], Chia-Chi Chung[e], Shou-Lun Lee[f], Shin Nieh[g], Shih-Jiun Yin[a,e,*]

[a]Graduate Institute of Medical Sciences, National Defense Medical Center, Taipei 11490, Taiwan

[b]Department of Dermatology, Tri-Service General Hospital, Taipei 11490, Taiwan

[c]Department of Surgery, Tri-Service General Hospital, Taipei 11490, Taiwan

[d]Department of Dentistry, Tri-Service General Hospital, Taipei 11490, Taiwan

[e]Department of Biochemistry, National Defense Medical Center, Taipei 11490, Taiwan

[f]Department of Biological Science and Technology, China Medical University, Taichung 40402, Taiwan

[g]Department of Pathology, Tri-Service General Hospital, Taipei 11490, Taiwan

*Corresponding author. Department of Biochemistry, National Defense Medical Center, 161 Minchuan East Road Section 6, Taipei 11490, Taiwan. Tel.: +886-2-8792-3100 ext. 18800; fax: +886-2-8792-4818.

E-mail address: yinsj@ndmc.idv.tw (S.-J. Yin).

Abstract

Alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) are principal enzymes responsible for metabolism of ethanol. Functional polymorphisms of *ADH1B*, *ADH1C*, and *ALDH2* genes occur among racial populations. The goal of this study was to systematically determine the functional expressions and cellular localization of ADHs and ALDHs in human rectal mucosa, the lesions of adenocarcinoma and hemorrhoid, and the genetic association of allelic variations of *ADH* and *ALDH* with large bowel disorders. Twenty one surgical specimens of rectal adenocarcinoma and the adjacent normal mucosa, including 16 paired tissues of rectal tumor, normal mucosae of rectum and sigmoid colon from the same individuals, and 18 surgical mixed hemorrhoids specimens as well as leukocyte DNA samples from 103 colorectal cancer patients, 67 hemorrhoid patients, and 545 control subjects recruited in previous study, were investigated. The isozyme/allozyme expression patterns of ADH and ALDH were identified by isoelectric focusing and the activities were assayed spectrophotometrically. The protein contents of ADH/ALDH isozymes were determined by immunoblotting using the corresponding purified class-specific antibodies; the cellular activity and protein localizations were detected by immunohistochemistry and histochemistry, respectively. Genotypes of *ADH1B*, *ADH1C*, and *ALDH2* were determined by polymerase chain reaction-restriction fragment length polymorphisms. At 33 mM ethanol, pH 7.5, the activity of ADH1C*1/1 phenotypes exhibited 87% higher than that of the ADH1C*1/*2

phenotypes in normal rectal mucosa. The activity of ALDH2-active phenotypes of rectal mucosa was 33% greater than ALDH2-inactive phenotypes at 200 μM acetaldehyde. The protein contents in normal rectal mucosa were in the following order: ADH1 > ALDH2 > ADH3 ≈ ALDH1A1, while those of ADH2, ADH4, and ALDH3A1 were fairly low. Both activity and content of ADH1were significantly decreased in rectal tumors whereas the ALDH activity remained unchanged. The ADH activity was also significantly reduced in hemorrhoids. ADH4 and ALDH3A1 were uniquely expressed in the squamous epithelium of anus at anorectal junctions. The allele frequencies of *ADH1C*1* and *ALDH2*2* were significantly higher in colorectal cancer and that of *ALDH2*2* also significantly greater in hemorrhoids. In conclusion, ADH and ALDH isozymes are differentially expressed in mucosal cells of rectum and anus. The results suggest that acetaldehyde, an immediate metabolite of ethanol, may play an etiological role in pathogenesis of large bowel diseases.

Keywords: Alcohol dehehydrogenase and aldehyde dehydrogenase; Human rectum and anus; Colorectal cancer and hemorrhoid; Isozyme and allozyme; Activity and cellular localization; Genetic polymorphism

Introduction

Diarrhea and flatulence are the most frequently reported gastrointestinal symptoms after prolonged period of excessive alcohol consumption, and marked histopathologic changes have been observed in rectal mucosa of heavy drinkers (Salaspuro, 2003; Egerer et al., 2005). Epidemiological studies showed a dose-response relationship between alcohol intake and the risk of colorectal cancer (Cho et al., 2004; Mizoue et al., 2008; Moskal et al., 2006; Pedersen et al., 2003), and an increased risk for rectal than for colon cancer by beer consumption (Kune and Vitetta, 1992). Genetic polymorphisms of alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) may contribute to susceptibility to colorectal carcinogenesis (Homann et al., 2009; Matsuo et al., 2002; Yokoyama et al., 1998).

The metabolic, pharmacological, and toxicological effects of ethanol depend on the duration of exposure and the concentrations of ethanol and its metabolite acetaldehyde in body fluids and tissue (Chen et al., 2009b; Zakhari and Li, 2007). During ethanol consumption, colonic ethanol concentrations are equal to those in the blood (Halstedt et al., 1973). However, colorectal mucosa and the luminal contents may exhibit significantly higher levels of acetaldehyde, produced by intracolonic bacteria, than that in blood (Jokelainen et al., 1994; Seitz et al., 1990). Acetaldehyde has been incriminated as an etiological factor in pathogenesis of the lesions and tumor of large bowel (Salaspuro, 2003; Seitz and Stickel,

2007).

ADH and ALDH are the principal enzymes responsible for ethanol metabolism in humans (Lee et al., 2006a; Yin and Agarwal, 2001). Human ADH family comprises class I ADH1A (also denoted αα), ADH1B (ββ) and ADH1C (γγ); class II ADH2 (ππ); class III ADH3 (χχ); and class IV ADH4 (μμ or σσ) (Duester et al., 1999). ADH1B and ADH1C exhibit functional polymorphisms among racial populations with allozymes ADH1B1, ADH1B2 and ADH1B3, and ADH1C1 and ADH1C2, respectively (Yin et al., 2006). In human ALDH family, mitochondrial ALDH2 and cytosolic ALDH1A1 are the major forms responsible for metabolism of acetaldehyde (Peng and Yin, 2009). Approximately 40% of the East Asians lack ALDH2 activity due to a point mutation of the variant allele *ALDH2*2*. The allelic variations of both ADH1B and ALDH2 have been documented to influence drinking behavior and risk for alcohol dependence (Chen et al., 2009a).

ADH and/or ALDH activities in human colon or rectum were reported (Jelski et al., 2004; Seitz et al., 1996; Yin et al., 1994) but to date they have not been systematically investigated. Distribution of class I ADH was examined in human alimentary tract using polyclonal antibodies in the presence of interclass cross-reactivities (Lee et al., 2006b; Pestalozzi et al., 1983). To investigate relative contributions of multiple ADH and ALDH forms to ethanol

metabolism in human large bowel and the potential implications in pathogenesis, we report here a comprehensive, from phenotype to genotype study of the expression pattern and protein contents of ADH and ALDH isozymes/allozymes, the ethanol- and acetaldehyde-oxidizing activities, and the cellular activity and protein localizations of ADH/ALDH isozymes in human large bowel in conjunction with and the genetic association of functional polymorphisms of *ADH* and *ALDH* genes with vulnerability to colorectal disorders.

Materials and methods

Human tissue and blood specimens

Colorectal and hemorrhoid tissues from adult Han Chinese patients were obtained during routine operations for therapeutic treatment of primary large bowel carcinomas and mixed hemorrhoids, respectively. All patients gave their informed consent. The colorectal specimens were from 21 patients (11 men and 10 women; age range 40–83 years; mean age ± S.D., 67 ± 12 years), and hemorrhoid specimens from 18 patients (12 men and 6 women; age 33–78 years; 50 ± 13 years); none of the patients had a history of high alcohol consumption nor were they taking histamine H_2-receptor antagonists 1 week before surgery. Rectal tumor tissue and the adjacent normal mucosal portions, or hemorrhoid tissue, were dissected and stored at −70°C within 30 minutes after resection. The tissue ADH and ALDH activities were stable at

this storage temperature for at least 6 months. Formalin-fixed paraffin-embedded normal and lesion tissue blocks were from 16 patients with rectal tumor and 12 patients with mixed hemorrhoids. Blood specimens were obtained from patients with colorectal adenocarcinoma and with mixed hemorrhoids, confirmed by histopathologic examinations; all patients gave their informed consent. Blood specimens were from 103 colorectal cancer patients (57 men and 46 women; age range 20–89 years; mean age ± S.D., 62 ± 16 years) and 67 mixed hemorrhoid patients (29 men and 38 women; age 21–73 years; 46 ± 13 years); all the patients were occasional drinkers or nondrinkers and none of them had a history of habitual or heavy drinking. The studies in this report were approved by the Institutional Review Board of the Tri-Service General Hospital.

Preparation of homogenate supernatants

Colorectal and hemorrhoid tissue specimens (0.2–0.3 g) were homogenized in 2 volumes (vol/wt) of ice-cold 10 mM sodium phosphate at pH 7.5, with a Polytron homogenizer (Kinematica AG, Littau, Switzerland). The resulting homogenate was centrifuged at 100,000*g* for 1 h at 4°C. The supernatants were kept in an ice bath for enzymatic studies. The ADH and ALDH activities in the supernatant were stable at least 6 h in the ice bath.

Isoelectric Focusing

ADH and ALDH isozymes/allozymes were identified by the agarose isoelectric focusing procedures as described previously (Yin et al., 1997). The phenotypes of ADH and ALDH

were identified by staining for enzyme activity at 120 mM ethanol and 130 mM propionaldehyde, respectively, in 50 mM sodium pyrophosphate, pH 8.5, containing 1 mM NAD^+, 0.3 mM thiazolyl blue tetrazolium bromide, and 0.05 mM Meldola blue (Yin et al., 1997).

Genotyping

Genomic DNA was extracted from leukocytes as described previously (Chen et al., 1999) and from surgical tissues using a Miniprep Kit (Viogene-bioteck Corp., Sunnyvale, CA). Determination of the single-nucleotide polymorphic sites at exon 3 of the *ADH1B* gene, exon 8 of the *ADH1C* gene, and exon 12 of the *ALDH2* gene was carried out using polymerase chain reaction–restriction fragment length polymorphism as described previously (Chen et al., 1999).

Protein Determination

Protein concentration was determined using the method by Lowry and colleagues (1951) using bovine serum albumin (BSA) as the standard.

Activity Assays

ADH activity was determined spectrophotometrically at 30°C in 0.1 M sodium phosphate, pH 7.5, containing 33 mM ethanol, 2.4 mM NAD^+, and 1 mM semicarbazide. ALDH activity was assayed spectrophotometrically at 30°C and pH 7.5 in 0.1 M sodium phosphate, containing 200 μM acetaldehyde, 2.4 mM NAD^+, 1 mM ethylenediaminetetraacetate, and 10

mM 4-methylpyrazole. Acetaldehyde was redistilled before use. A 5-minute assay in the absence of ethanol or acetaldehyde was subtracted as blank. One milliunit (mU) of the enzyme activity of ADH and ALDH corresponds to 1 nanomole NADH produced per minute, based on an extinction coefficient of 6.22 $mM^{-1}cm^{-1}$ for NADH at 340 nm.

Expression and purification of ADH and ALDH

The expression and purification of recombinant human ADH1C1, ADH2, ADH3, ADH4, ALDH1A1, ALDH2, and ALDH3A1were as described previously (Chiang et al., 2009; Lee et al., 2006b). The isolated recombinant ADH1,ADH2, ADH3, ADH4, ALDH1A1, ALDH2, and ALDH3A1, exhibited a single Coomassie blue-staining band with the molecular mass of 40 kDa for ADHs, 55 kDa (ALDH1A1/2), and 54 kDa (ALDH3A1), on sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Generation and purification of antibodies

Rabbit antisera against human ADH1C1, ADH2, ADH3 and ADH4, and that against human ALDH1A1, ALDH2 and ALDH3A1were generated as described previously (Chiang et al., 2009; Lee et al., 2006b). Class cross-reactivity of the polyclonal antisera was eliminated using affinity chromatography with sepharose covalently linked to the respective non-immunogen isozymes, i.e., the ADH or ALDH family members that were not used for immunization, according to the manufacturer's procedure. The yielding class-specific antibodies were then further purified by affinity chromatography using the antigen isozymes, i.e., ADH1C1, ADH2,

ADH3 or ADH4 for ADH family, and ALDH1A1, ALDH2 or ALDH3A1 for ALDH family immobilized on the sepharose resins. The affinity-purified antibodies in phosphate-buffered saline containing 5% BSA remained stable for several months when stored in aliquots at −70°C. Concentration of the affinity-purified antibodies was assessed by enzyme-linked immunosorbent assay using commercially available rabbit IgG as the standard as described previously (Chiang et al, 2009; Lee et al., 2006b).

Immunoblot analysis

Immunoblotting of tissue homogenate-supernatants were performed by a PhastSystem according to the manufacturer's procedure (Amersham Biosciences, Little Chalfont, UK). Immnodetection was carried out using goat anti-rabbit IgG conjugated with alkaline phosphatase and 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium for inspection of class cross-reactivity of the affinity-purified antibodies, or using goat anti-rabbit IgG conjugated with horseradish peroxidase and the western lightning chemiluminescence reagent (PerkinElmer Life Sciences, Boston, MA) for determination of tissue ADH/ALDH isozyme contents. The immunoreactive bands were evaluated by densitometric analysis using the Chemigenius 2 Chemiluminescent Image System (Synoptics Ltd, Cambridge, UK). Local background was subtracted for each band by using a separate nearby reference area.

Immunohistochemistry

Surgical tissue sample was embedded in paraffin and cut into 4-μm sections. The tissue

section was incubated with affinity-purified class-specific antibodies, followed by detection using the super sensitive non-biotin horseradish peroxidase system (BioGenex Laboratories, San Ramon, CA), and then slightly counterstained with hematoxylin. The expressions of ADH and ALDH isozymes in rectal tumors and the normal tissues were compared by running under the same batch of experiments for tissue sectioning, and the corresponding immunostaining and imaging procedures. Preimmune antisera were used as a control and failed to elicit specific signals in tissues examined by immunohistochemistry in this study.

Histochemistry

Frozen unfixed tissue was cut into 20-μm sections using a cryostat at −15°C. The staining reaction mixture for class I ADHs contained 5 mM ethanol or 400 μM 5β-androstan-3β-ol-17-one (a specific substrate for ADH1C) (McEvily et al., 1988), 2.4 mM NAD^+, 11 mM sodium pyruvate, 0.5 mM cyanamide, 0.33 mM phenazine methosulfate, and 3.4 mM nitrotetrazolium blue chloride in 50 mM sodium phosphate at pH 7.5 and 37°C. The staining mixture for ALDH1A1 and ALDH2 contained 5 mM acetaldehyde, 1 mM NAD^+, 11 mM sodium pyruvate, 0.33 mM phenazine methosulfate, and 3.4 mM nitrotetrazolium blue chloride in the 50 mM phosphate buffer. Control sections were performed in the presence of 5 mM 4-methylpyrazole as an ADH inhibitor, or 0.5 mM cyanamide as an ALDH inhibitor, in the respective staining mixtures. The inhibitors were preincubated with tissue sections in the phosphate buffer for 20 minutes in a shaking bath to facilitate diffusion into the cell before

addition of the activity staining mixture. The expressions of ADH and ALDH isozymes in rectal tumors and the normal tissues were compared by running under the same batch of experiments for tissue sectioning, and the corresponding activity staining and imaging procedures.

Statistics

Results are expressed as mean ± S.E.M. Statistical significance of differences between multiple groups of ADH or ALDH activities and the protein contents was evaluated by the Scheffe's test after one-way analysis of variance (ANOVA). Differences in *ADH/ALDH* genotypes and alleles at the polymorphic sites were calculated by direct counting with the Pearson's χ^2 test. Fisher's exact test was used for correction of small sample sizes ($n < 5$) in some genotypic groups. All analyses were conducted using PASW Statistics 18.0 (SPSS Inc., Chicago, IL). Haplotype frequencies and linkage-disequilibrium coefficients of *ADH1B* and *ADH1C* genes were estimated by use of the ARLEQUIN program kindly provided by Schneider et al. (http://anthropologie.unige.ch/software/arleguin). The haplotype method developed by Valdes and Thomson (1997) was used to evaluate the relative importance of the two polymorphic sites in determining susceptibility to colorectal cancer.

Results

Phenotype and activities of ADH and ALDH

Phenotypic patterns of ADH and ALDH isozymes in human rectal adenocarcinoma and hemorrhoids were identified by agarose isoelectric focusing (Fig. 1). Class I ADH1C was the predominant isozyme expressed in rectal tumor and the adjacent normal mucosal tissue as well as in the paired normal sigmoid colon mucosa (Fig. 1a). The staining intensity of ADH1C appeared weaker in 81% (13/16) of rectal tumor tissues than that of the adjacent normal mucosae studied. Faint ADH1B activity bands were also detectable in some of the rectal and colonic mucosal samples studied, which may be due to the presence of smooth muscle tissues. This is consistent with that ADH1B was the sole isozyme form detected in the muscle layers of human colon (Yin et al, 1994). In addition to class I isozymes, class IV ADH4 was detected in 94% (17/18), with varying degrees of intensity, of the mixed hemorrhoid samples studied. Class III ADH3 was undetectable in the tissues due to its nearly unsaturation with ethanol (Lee et al., 2003). ALDH1A1 and ALDH2 were expressed in all samples of the rectum, colon and hemorrhoids studied (Fig. 1b). Weak ALDH3A1 activity bands were detected in the majority of the above tissues studied. ALDH4A1 was undetectable in the bowel tissues.

Ethanol-oxidizing activities in human rectum and hemorrhoids at 33 mM ethanol, an upper blood alcohol level for social drinking, according to ADH1C phenotypes are shown in Table 1. Normal rectal mucosa with the homozygous ADH1C*1/*1 phenotype exhibited 52–87%

higher activity, in terms of milliunits per g tissue or per mg tissue protein, than that of the heterozygous ADH1C*1/*2 phenotype ($P < .05$ by Student's *t* test). This is compatible with that ADH1C1 isozyme exhibits a greater V_{max} than that of ADH1C2 (Bosron et al., 1983). Similar trend of activities was observed for hemorrhoids with different ADH1C phenotypes. The ADH activities of hemorrhoid tissues were significantly lower than that of the rectal mucosae. The acetaldehyde-oxidizing activity in hemorrhoids with ALDH2-active phenotype was significantly higher than that of the ALDH2-inactive phenotypes. ADH and ALDH activities in rectal adenocarcinoma, normal mucosae of rectum and sigmoid colon of the same individuals were compared in Table 2. The rectal tumor ADH activity was 7-fold lower than that of the adjacent normal rectum mucosa. The ADH activity in normal colon mucosa was 28% lower than that of the rectal mucosa ($P < .05$ by paired Student's *t* test). In contrast, the ALDH activity in rectal tumors was not significantly different from that of the normal mucosae of rectum and colon. The concentrations of total cytosolic proteins were found similar in rectal tumor, the normal rectal mucosa, and the normal colon mucosa, i.e., 41.8 ± 2.3, 40.8 ± 2.3, and 41.1 ± 1.7 mg/g tissue (means ± S.E.M.), respectively.

Expression and contents of ADH *and* ALDH

The expression of ADH and ALDH family members in human normal rectal mucosa was probed by the corresponding class-specific affinity-purified antibodies on immunoblotting (Fig. 2). No cross-reactivity of the purified antibodies against non-immunogen class members

of the same family was detected as described previously (Chiang et al., 2009). The molecular masses of rectal ADH1/2/3/4, ALDH1A1/2, and ALDH3A1 were corresponded to the respective purified recombinant ADH/ALDH isozyme standards. No other minor band on immunoblots for the homogenate supernatants of rectal mucosae was detected by the class-specific affinity-purified antibodies. ADH1 exhibited highest protein content, followed by ALDH2, ADH3 and ALDH1A1 (the latter two showed similar tissue contents) (Fig. 3b). The ADH1 contents in rectal adenocarcinoma and the adjacent normal rectal mucosa were determined to be 26.8 ± 3.5 and 64.9 ± 5.4 mg/g tissue (means ± S.E.M., $n = 6$; $P < .01$ by paired Student's *t* test), respectively. The protein contents of ADH2, ADH4, and ALDH3A1 were extremely low. This is consistent, wherein very faint band or no appreciable activity of these isozymes were detected on isoelecric focusing gels. The immunoquantification was validated by the reasonably precise isozyme standards on immunoblots ($R^2 > .98$) (Fig. 3a) as well as that the tissue isozyme concentrations were diluted and measured within linear range of the corresponding purified protein standards.

Cellular localization of ADH *and* ALDH

Cellular distribution of ADH and ALDH isozymes in human rectal adenocarcinoma and the adjacent normal mucosa was examined by the corresponding class-specific purified antibodies at comparable antibody concentrations (Fig. 4). Class I ADHs predominantly expressed in the absorptive columnar epithelial cells and to a much lesser extent in the

cytoplasm of goblet cells (undetectable in the mucin globules) and muscularis mucosae. ADH1 was nearly absent in the submucosa. ADH3 was ubiquitously expressed and appeared higher in the lumen surface epithelial cells. Like ADH1, ALDH1A1 and ALDH2 were predominantly localized in the absorptive epithelia but less intense in the muscularis mucosae. Interestingly, a rarity of cells showing strikingly higher staining intensity of ALDH1A1 were sporadically detected in the normal rectal crypt (Fig. 4i, inset to the right). It may stand for mucosal stem cells (Balber, 2011) but requires further confirmation of the identity. In contrast to cytosolic ALDH1A1, ALDH2 exhibited a punctate cytoplasmic pattern, suggesting its mitochondrial localization. The cellular expressions of ADH2, ADH4, and ALDH3A1 were very low using comparable concentrations of the corresponding class-specific antibodies (data not shown). This is in agreement with the extremely low protein contents of ADH2, ADH4, and ALDH3A1 in normal rectal mucosa. ADH1, ADH3, ALDH1A1, and ALDH2 were detected in disorganized malignant epithelial cells. Their immunostaining intensity was much lower in the adjacent stromal tissues. Of the 16 rectal adenocarcinoma samples studied, 12 were graded as moderately differentiated, 2 well and 2 poorly differentiated. Eighty-eight percent (14/16) of the tumor tissues examined showed a striking reduction in ADH1 intensity compared with that of the adjacent normal mucosae. By contrast, the majority of ADH3 (94%), ALDH1A1 (75%), and ALDH2 (88%) remained unchanged for cancerous tissues versus the normal tissues. The expressions of ADH2, ADH4 and ALDH3A1 were fairly low

in rectal tumor tissue secions (data not shown). Compared with that of adjacent normal mucosa, the intensity of ALDH3A1 activity band on isoelectric focusing in rectal tumors appeared inconsistently varied, i.e., 31% (5/16) increased, 19% (3/16) decreased, and 50% (8/16) unchanged.

Cellular localizations of ADH and ALDH activities in human rectal tumor and the normal mucosa are shown in Fig. 5. Class I ADHs were detected in the absorptive epithelium and muscularis mucosae of normal rectal mucosa. The marked staining intensity in muscularis mucosae may be due to the presence of the high-activity ADH1B2 allozyme in the examined tissue (identified with homozygous *ADH1B*2/*2* genotype) at cosubstrate concentrations of 5 mM ethanol and 2.4 mM NAD^+ (Yin et al., 1984, 1994). This explanation was supported by that ADH1C isozyme was virtually absent in muscularis mucosae in the studied tissue with *ADH1B*1/*1* genotype although it was prominent in lumen surface epithelium using 5β-androstan-3β-ol-17-one, a specific substrate for ADH1C (Marschall et al., 2000; McEvily et al., 1988), for activity staining. The control section staining with ethanol showed a completely blank background in the presence of 5 mM 4-methylpyrzaole, a specific class I ADH inhibitor, indicating that the activity staining can be attributed to the class I ADHs. However, the control section using 5β-androstan-3β-ol-17-one as substrate still showed some background staining in the presence of 4-methylpyrazole, suggesting that other

3β-hydroxy-5β-steroid active dehydrogenases that are insensitive to the inhibition may exist in rectal mucosa and the tumor tissues. The activity distribution of ALDH1 and ALDH2 was more prominent in the lumen surface epithelium and muscularis mucosae. The control section showed that the low-K_m ALDH activities could be completely blocked by pretreatment with the inhibitor cyanamide. Class I ADHs and ALDH1/2 were detected in tumor cells of the disorganized glandular tissue. The staining intensity of ADH1 using ethanol as substrate was found appreciably decreased in all of the tumor tissues examined (16/16) as compared to that of the normal mucosa whereas ALDH1/2 activity appeared to remain unchanged with most of the tissues studied (15/16).

Figure 6 shows cellular distribution of ADH and ALDH isozymes in human mixed hemorrhoids probed by the corresponding class-specific purified antibodies at comparable antibody concentrations. A total of 12 mixed hemorrhoid samples, diagnosed as grade III or IV, were examined. ADH1 expressed higher in columnar epithelial cells of rectum than that in the stratified squamous epithelial cells of anus at anorectal junctions. The expression of ADH1 was barely detectable in the lesional portions consisting of submucosal varices and hypertrophic smooth muscle beneath the anal and rectal mucosae. ADH3 was expressed in both types of the epithelia and also detectable in smooth muscles. In contrast, ADH4 exhibited a restricted expression in the stratified squamous epithelium of anus, showing

highest intensity at the basal layer cells of the epithelium at anorectal junctions. ALDH1A1 appeared to be equally intensely stained in both types of epithelia at the junctions. The expression of ALDH2 was higher in the columnar epithelium of rectum and also detectable in the perivascular smooth muscle tissue, whereas ALDH3A1 was higher in the stratified squamous epithelium of anus with highest intensity at the basal layer cells.

Functional polymorphisms of ADH/ALDH genes and bowel diseases

Table 3 shows genotype and allele distributions of *ADH1B*, *ADH1C*, and *ALDH2* in 103 patients with colorectal adenocarcinomas (colon cancer, 60; rectal cancer, 43) and 67 mixed hemorrhoid patients. Normal individuals (n = 545) from our previous study (Chen et al., 1999) were used as control group, which may represent a general population of Han Chinese in Taiwan. The allele frequency of *ADH1C*1* was significantly higher in colorectal cancer patients than that of the controls while *ADH1B* polymorphism exhibited similar genotype and allele frequencies between the cancer patients and controls. Hemorrhoid patients displayed similar *ADH1B* and *ADH1C* allelic frequencies to those of controls. Since the gene loci of *ADH1B* and *ADH1C* are in linkage disequilibrium (Chen et al., 1999) and it may influence disease susceptibility, the haplotype frequencies of *ADH1C*1–ADH1B*1*, *ADH1C*1–ADH1B*2*, *ADH1C*2–ADH1B*1*, and *ADH1C*2–ADH1B*2* in colorectal patients were further analyzed and calculated to be 0.194 ± 0.028, 0.762 ± 0.030, 0.039 ± 0.013, and 0.005 ± 0.005 (means ± S.D.), respectively. The standardized linkage

disequilibrium coefficient was 0.855 ($P < 10^{-6}$), indicating a strong linkage between the two gene loci. This strong linkage has been observed in controls as described previously (Chen et al., 1999). Relative haplotype analyses indicated that controlled for either *ADH1B*1* or *ADH1B*2*, frequency ratios of *ADH1C*1* to *ADH1C*2* in cancer patient group were still significantly higher than that in control group ($P < .01$); whereas that controlled for either *ADH1C*1* or *ADH1C*2*, frequency ratios of *ADH1B*1* to *ADH1B*2* remained insignificant difference between the two groups. This confirmed that *ADH1C* polymorphism, rather than *ADH1B* polymorphism, was associated with vulnerability to colorectal cancer. The frequencies of variant allele ALDH2*2 in patients with colorectal cancer and hemorrhoids were significantly higher than that of controls (Table 3).

Discussion

This comprehensive report has integrated the isozyme patterns, allozyme phenotypes, protein contents, ethanol-metabolizing activities at a near physiological pH and pharmacologically attainable concentrations, and cellular localizations of ADHs and ALDHs in human large bowel. ADH1 exhibits the highest protein content by immunotitration, followed by ALDH2, ALDH1A1, and ADH3, suggesting that class I ADH and class I/II ALDHs are the major isozyme forms responsible for metabolism of ethanol in rectal mucosa. ADH1C is the predominant class I isozyme expressed in rectal mucosa, as evidenced by agarose isoelectric

focusing, and it is most intensely localized in the surface epithelium by histochemistry using the specific substrate 5β-androstan-3β-ol-17-one and by immunohistochemistry with the class-specific antibodies. The cellular distribution of class I ADH in human rectum is in general similar to that reported for rodents detected by immmunostaining or oligonucleotide in situ hybridization (Haselbeck and Duester, 1997; Vaglenova et al., 2003; Westerlund et al., 2007). It is worth noting that there is a striking transition of expressions of ADH and ALDH at the anorectal junction. Both ADH4 and ALDH3A1 are detected in the squamous epithelium of anus, showing highest intensity at the basal layer cells, but they are essentially absent in the columnar epithelium of rectum. This is compatible with the isozyme patterns of ADH and ALDH in mixed hemorrhoids revealed on agarose isoelectric focusing. ADH4 and ALDH3A1 are also expressed in the mucosae of human mouth (Dong et al., 1996), esophagus (Yin et al., 1993), and stomach (Yin et al., 1997) whereas they are missing in the mucosae of duodenum and jejunum (Yin et al., 1997) except trace ALDH3A1 activity band detectable in rectal and colonic mucosae (Yin et al., 1994).

The ethanol-oxidizing activities in colorectal mucosa are 5 to 8-fold higher than that of the acetaldehyde-oxidizing activities. The higher ADH versus ALDH activity appears to be a general trend, with the exception of pancreas, in human digestive tract and the associated organs. At pH 7.5 in the presence of 33 mM ethanol or 200 μM acetaldehyde, the mean ADH

and ALDH activities in human surgical tissues are as follows (in decreasing order of ADH activity): liver (ADH1B*/1*1 phenotype, 2900 mU/g; ALDH2-active phenotype, 1060 mU/g) (Yao et al., 1997); esophageal mucosa (ADH, 605 mU/g; ALDH, 29.9 mU/g) (Yin et al., 1993); rectal mucosa (ADH1C*1/*1, 305 mU/g; ALDH2 active, 41.8 mU/g; this study) ; gastric mucosa (ADH1C*1/*1, 238 mU/g; ALDH2 active, 132 mU/g) (Yin et al., 1997); colonic mucosa (ADH1C*1/*1, 183 mU/g; ALDH2 active, 40.2 mU/g) (Yin et al., 1994); gingiva (ADH, 90.0 mU/g; ALDH1/2 activity too low to be reliably measured) (Dong et al., 1996); pancreas (ADH1B*1/*1, 64 mU/g; ALDH2 active, 213 mU/g) (Chiang et al., 2009); tongue (ADH, 50.6 mU/g; ALDH1/2 activity too low to be reliably measured) (Dong et al., 1996). All of the above studies have been determined under the same assay conditions for ADH and ALDH. No significant difference of ADH or ALDH activities in the segments of ascending, transverse, descending and sigmoid colon mucosae was found (Seitz et al., 1996; Yin et al., 1994). There were no significant effects of gender and age on ADH or ALDH activities in human stomach and colon (Lai et al., 2000; Yin et al., 1994, 1997). The higher ADH activity in rectum versus that of colon is in agreement with previous reports with human (Seitz et al., 1996) and rats (Boleda et al., 1989; Pronko et al., 2002). A higher ADH activity in human rectum suggests that it could result in increased acetaldehyde levels after alcohol consumption. It is widely accepted that acetaldehyde, a chemically reactive and cytotoxic metabolite, contributes in part to pathogenesis of colorectal disorders (Salaspuro, 2003; Seitz

and Stickel, 2007).

ADH1C, a major form of class I ADHs, exhibits a significant reduction in rectal adenocarcinomas compared with that of the normal tissues at both enzyme activity and the protein contents. This observation is consistent with results of the corresponding isozyme on isoelectric focusing and in immunohistochemical and histochemical examinations. This is also in general agreement with previous reports, e.g., 43–57% decreased expression of ADH1C mRNA in colorectal cancer (Groene et al., 2006; Croner et al., 2005), but in contradiction with a significant increase of ADH1 activity in both tumor tissues and sera of colorectal cancer patients (Jelski et al., 2004, 2010). The reports by Jelski and colleagues used a fluorogenic substrate, 4-methoxy-1-naphthaldehyde, for activity measurement which lacked a systematic verification of the claimed specificity of the methodology. Unlike ADH1, the ADLH1/2 activities remain unchanged for tumor tissues and this is supported by observations with the isoelectric focusing, histochemistry and immunohistochemistry. The isoelectric focusing reveals varied outcomes of ALDH3A1 activity band, i.e., 31% increased, 19% decreased, and 50% unchanged, in 16 paired rectal cancer and normal mucosal tissues studied. Consistent with this finding, immunohistochemical study of ADLH3A1 shows no marked distinctions between the tumor and normal tissues. Marselos and Michalopoulos (1987) reported a 2.8-fold rise of class III ALDH activity in human colonic adenocarcinoma versus

normal mucosa using benzaldehyde and $NADP^+$ as cosubstrates. The discrepancy may be in part due to that the enzyme activity determination used was not specific for ALDH3A1.

The allele *ADH1C*1*, rather than *ADH1B*2*, is associated with a higher risk for colorectal cancer as revealed by genetic association and relative haplotype analyses. *ADH1C*1* /*1 homozygosity has recently been associated with increased risk of colorectal neoplasms in heavy drinkers (Homann et al., 2009). Since the patients recruited in the present study were occasional or non-drinkers without a habitual or heavy drinking history, our results suggest that *ADH1C* polymorphism may be an independent risk factor for colorectal cancer etiology. The *ALDH2*2* is a risk allele for both colorectal cancer and mixed hemorrhoids. This variant allele has been associated with colorectal tumors with high alcohol consumption (Matsuo et al., 2002; Yokoyama et al, 1998) and also identified as an independent risk factor, irrespective of drinking and smoking, for esophageal squamous cell carcinoma (Cui et al., 2009). Deficiency of low-K_m ALDH2 activity can impair removal of locally produced acetaldehyde by intracolonic bacteria (Jokelainen et al., 1994; Seitz et al., 1990) and causes oxidative stress-induced cell injury (Chen et al., 2008). The described pathogenetic mechanisms may contribute, at least in part, to etiology of colorectal disorders. It requires further studies of large sample numbers with age and gender matched controls to confirm association of the functional polymorphisms of *ADH* and *ALDH* with colorectal cancer and hemorrhoids in

relation to environmental factors such as drinking and dietary habits, and to elucidate potential interactions between them.

In conclusion, this study presents a systematic correlation of the functional expressions of ADH and ALDH family members in human rectum and the lesions of large bowel. Preliminary genetic association studies suggest that allelic variations of *ADH1C* and/or *ALDH2* may involve in etiology of colorectal tumor and hemorrhoids despite in the absence of heavy alcohol consumption.

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Figure legends

Fig. 1. Agarose isoelectric focusing of (a) ADH and (b) ALDH isozymes from surgical specimens of rectum and hemorrhoid. Gels were stained for the enzyme activity. (a) Lane 1 shows a surgical liver specimen with ADH1B*2/*2 and ADH1C*1/*1 phenotype for comparison; lanes 2, 4, and 6 shows normal rectum mucosae, and lanes 3, 5, and 7, the paired rectal adenocarcinoma tissues respectively; lane 8 shows an additional paired normal sigmoid colon mucosa to that of the lanes 6 and 7 from the same patient; lanes 9 and 10 show two hemorrhoid tissues, respectively. Lanes 2–10 are ADH1C*1/*1 phenotype. μμ, ADH4; ππ, ADH2; αα, ADH1A; $\beta_2\beta_2$, ADH1B2; $\gamma_1\gamma_1$, ADH1C1; $\alpha\beta_2$ and $\beta_2\gamma_1$, heterodimers of the subunits of ADH1A and ADH1B2 and of ADH1B2 and ADH1C1, respectively. (b) For lanes of the tissue specimens, see (a). Lane 1, a liver with ALDH2-active phenotype for comparison; lanes 2–5, and 9 are ALDH2-active phenotype, and lanes 6–8 and 10, ALDH2-inactive phenotype. Numerals 1A1, 2, 3A1, and 4A1 represent ALDH1A1, ALDH2, ALDH3A1, and ALDH4A1, respectively. Hb, hemoglobin.

Fig. 2. Western blot analysis for expression of ADHs and ALDHs in rectal mucosa. Immunodetection by the class-specific affinity-purified antibodies to ADH1, ADH2, ADH3, ADH4, ALDH1A1, ALDH2, and ALDH3A1, respectively. In upper panels, lane 1 (10 ng) and

lane 2 (20 ng) are the corresponding antigen standards ADH1, ADH2, ADH3, and ADH4, respectively; lanes 3 and lane 4 are two normal rectal samples. In lower panels, lane 1 (10 ng) and lane 2 (20 ng) are the antigen standards of ALDH1A1, ALDH2, and ALDH3A1, respectively; lane 3 and lane 4 are the same two rectal samples shown in above panel. The concentrations of the antibodies used for detection were 0.03–0.04 μg/ml.

Fig. 3. Comparison of protein contents of ADHs and ALDHs in rectal mucosa. (a) Standard curves through the origin for densitometric determination. The observed densitometry unit for 20 ng of each antigen isozyme is set at 200. (b) Tissue contents of ADH and ALDH family members. The ADH⁄ALDH standards and tissue extracts were run on the same gel for quantification. Nine randomly selected normal rectum mucosal samples with ALDH2-active phenotype were used for determination. Bars represent means ± S.E.M. ADH1, ADH2, ADH3, and ADH4 were 69.7 ± 4.1, 4.0 ± 0.9, 15.4 ± 4.2, and 2.1 ± 0.9 μg⁄g tissue, respectively; ALDH1A1, ALDH2, and ALDH3A1 were 15.0 ± 2.6, 24.2 ± 5.3, and 1.7 ± 0.4 μg⁄g tissue, respectively. Statistical significance of differences between multigroup comparisons was evaluated by ANOVA. $^{*}P < .001$ versus ADH1; $^{**}P < .01$ versus ALDH2.

Fig. 4. Immunohistochemical detection of ADHs and ALDHs in normal and adenocarcinoma rectal tissue. Reference sections of (a) normal rectum and (b) rectal tumor staining with

hematoxylin and eosin. Representative immunohistochemical control sections of (c) normal rectum and (d) rectal tumor using a preimmune serum for detection. Cellular localizations of (e, f) ADH1, (g, h) ADH3, (i, j) ALDH1A1, and (k, l) ALDH2 in normal rectum and the rectal tumor, respectively, using the corresponding class-specific antibodies for detection. The lower left insets in panels a, e, g, i, and k show higher magnification views of normal rectal columnar absorptive epithelia; the lower right insets in panels i and k show crypt and muscularis mucosae of normal rectum; and the lower left insets in panels b, f, h, j, and l show disorganized glands of rectal tumor. The concentrations of the antibodies used in detection for ADHs and ALDHs were 0.05–0.06 μg/ml. ×100, a–l; ×400, lower left insets in panels a, b, e–l and lower right inset in panel k except the lower right inset in panel i (×1000). Scale bar in panels with magnification ×100 denotes 200 μm and that in insets with ×400 and ×1000 denotes 50 and 20 μm, respectively.

Fig. 5. Histochemical detection of ADHs and ALDHs in normal rectum and rectal adenocarcinoma. Panels a, b; e, f; and i, j are representative histochemical sections of normal rectum and panels c, d; g, h; and k, l are representative histochemical sections of rectal tumor. Class I ADHs were stained for enzyme activity at 5 mM ethanol in the absence (a, c) and the presence (b, d) of inhibitor of 5 mM 4-methylpyrazole. ADH1C isozyme was stained for activity at 400 μM 5β-androstan-3β-ol-17-one in the absence (e, g) and the presence (f, h) of

5 mM 4-methylpyrazole. ALDH1 and ALDH2 were stained for enzyme activity at 5 mM acetaldehyde in the absence (i, k) and the presence (j, l) of inhibitor of 0.5 mM cyanamide. The tissue sample for panels a–d was the *ADH1B*2/*2* genotype and ADH1C*1/*1 phenotype, that for panels e–h was the *ADH1B*1/*1* genotype and ADH1C*1/*1 phenotype, and that for panels i–l was the ALDH2-active phenotype. The insets in panels a, e, and i show higher power views of normal rectal columnar absorptive epithelia; and the insets in panels c, g, and k show disorganized glands of rectal tumor. ×100, panels a–l; ×400, all insets. Scale bar in panels denotes 200 μm and that in insets, 50 μm.

Fig. 6. Immunohistochemical detection of ADHs and ALDHs in anorectal junction of mixed hemorrhoids. The stratified epithelium and the columnar epithelium are shown on the left side and the right side of anorectal junction, respectively. (a) Reference section staining with hematoxylin and eosin. (b) Representative immunohistochemical control section using a preimmune serum for detection. Cellular localizations of (c) ADH1, (d) ADH3, (e) ADH4, (f) ALDH1A1, (g) ALDH2, and (h) ALDH3A1, using the corresponding class-specific antibodies for detection. The lower left insets in panels a, c, d, and f show higher magnification views of squamous epithelial cells; the lower right insets in panels c, d, f, and g show columnar epithelial cells; the lower left insets in panels e and h show basal layer of squamous epithelium; and the lower left inset in panel g shows perivascular smooth muscle

tissue in hemorrhoids. The concentrations of the antibodies used in detection for ADHs and ALDHs were 0.05–0.06 μg/ml. ×40, a–h; ×400, all insets. Scale bar in panels with magnification ×40 denotes 500 μm and that in sets, 50 μm.

Figure 1

Fig. 1

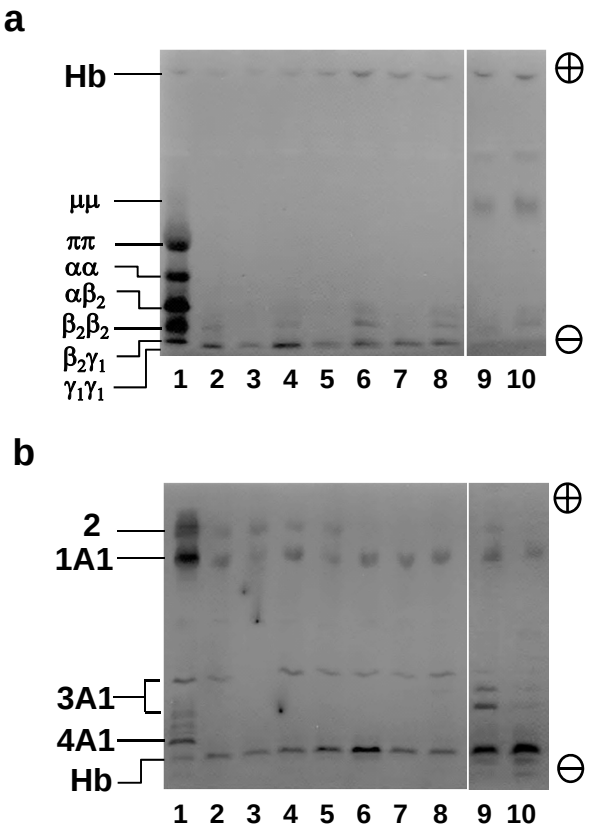

Figure 2

Fig. 2

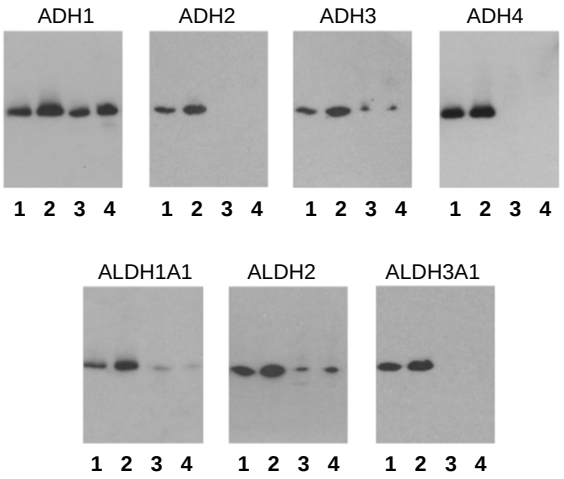

Figure 3

Fig. 3

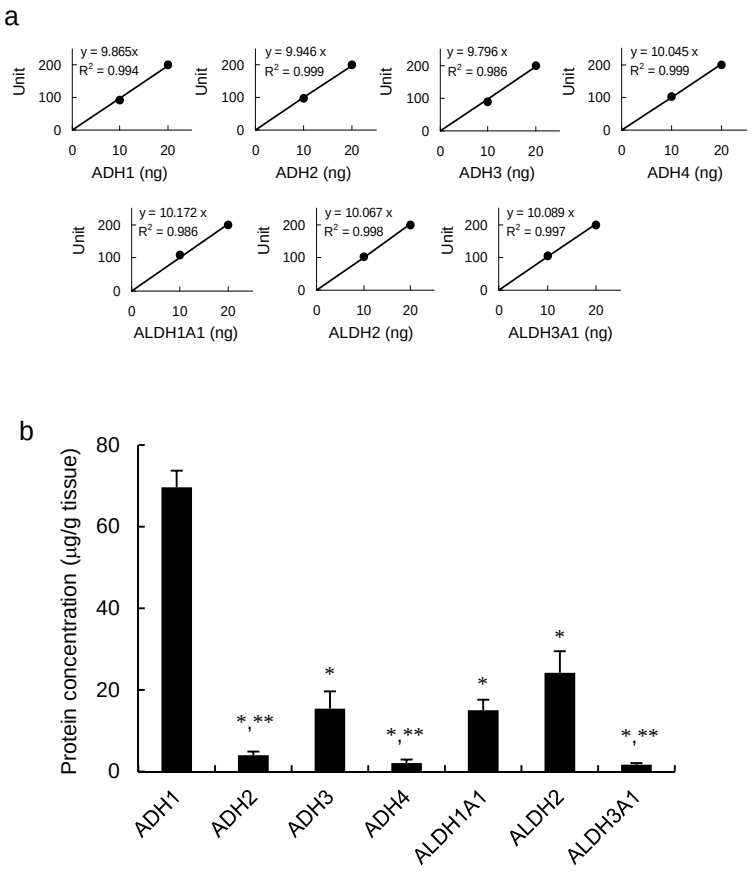

Figure 4

Fig. 4

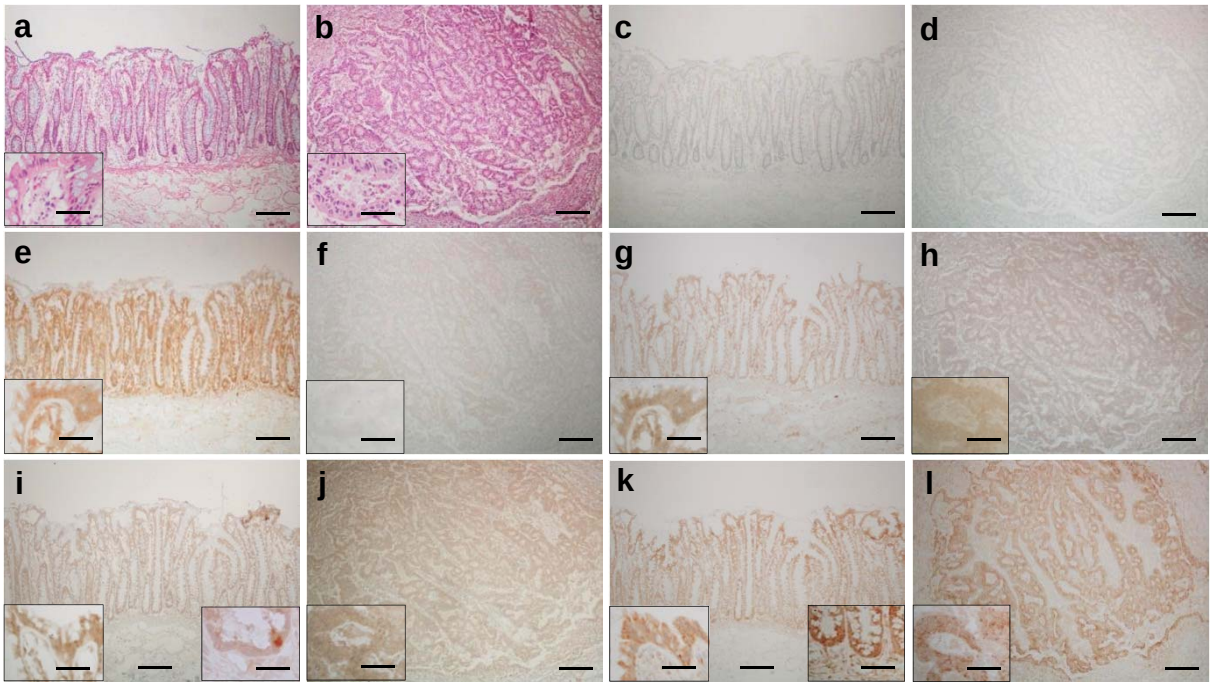

Figure 5

Fig. 5

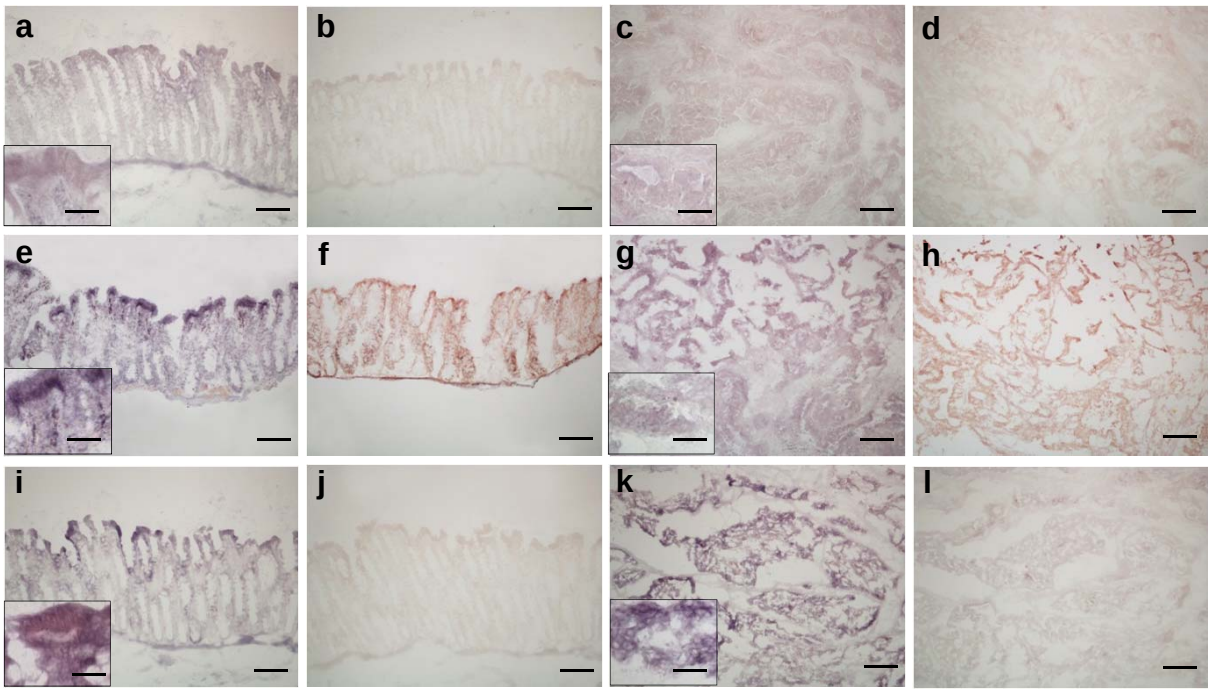

Figure 6

Fig. 6

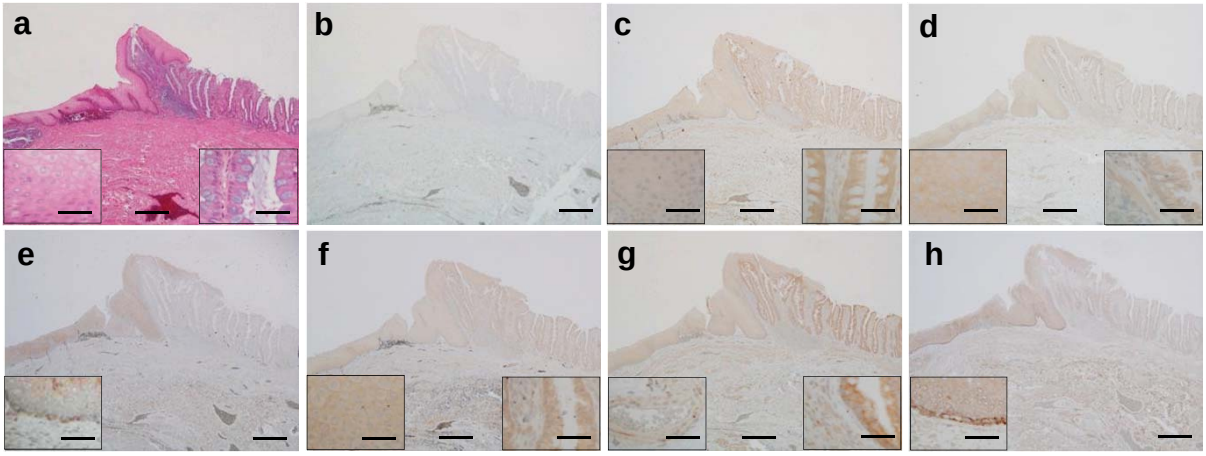

Table 1

Rectal mucosa and hemorrhoid ADH and ALDH activities of different phenotypes

Tissue	Phenotype	*n*	Specific activity	
			mU/g tissue	mU/mg protein
			Ethanol (33 mM)	
Rectal mucosa	ADH1C*1/*1	17	305 ± 28	7.81 ± 0.65
Rectal mucosa	ADH1C*1/*2	4	163 ± 32	5.13 ± 1.27
Hemorrhoid	ADH1C*1/*1	14	187 ± 32[*]	4.20 ± 0.87[*]
Hemorrhoid	ADH1C*1/*2	4	102 ± 26[*]	1.41 ± 0.36[**]
			Acetaldehyde (200 μM)	
Rectal mucosa	ALDH2 active	12	41.8 ± 4.6	1.15 ± 0.15
Rectal mucosa	ALDH2 inactive	9	31.4 ± 5.2	0.88 ± 0.19
Hemorrhoid	ALDH2 active	7	33.0 ± 7.4	0.81 ± 0.30
Hemorrhoid	ALDH2 inactive	11	16.0 ± 1.1[***]	0.29 ± 0.03[***]

ADH and ALDH activities were determined in 0.1 M sodium phosphate, pH 7.5, at 30°C, containing 33 mM ethanol, 2.4 mM NAD^+, and 1 mM semicarbazide for the ADH assay, or containing 200 μM acetaldehyde, 2.4 mM NAD^+, 1 mM EDTA, and 10 mM 4-methylpyrazole for the ALDH assay. Statistical significance of differences between multigroup comparisons was evaluated by ANOVA. Values are mean ± S.E.M.

[*]$P < .05$ versus rectal mucosa ADH1C*1/*1 phenotype.

[**]$P < .01$ versus rectal mucosa ADH1C*1/*1 phenotype.

[***]$P < .01$ versus rectal mucosa ALDH2-active phenotype.

Table 2

ADH and ALDH activities in rectal cancer and paired colon tissue

Tissue	*n*	Specific activity	
		mU/g tissue	mU/mg protein
		Ethanol (33 mM)	
rectal mucosa	16	312 ± 28	7.94 ± 0.70
rectal adenocarcinoma	16	44 ± 9[*,**]	1.14 ± 0.26[*,**]
colon mucosa	16	224 ± 31	5.69 ± 0.84
		Acetaldehyde (200 μM)	
rectal mucosa	16	39.2 ± 4.4	1.04 ± 0.15
rectal adenocarcinoma	16	41.5 ± 16.8	0.94 ± 0.39
colon mucosa	16	45.4 ± 3.0	1.15 ± 0.11

For assay conditions, see Table 1. Rectal tumor tissue and the adjacent normal rectum mucosa as well as the paired normal sigmoid colon mucosa were from the same patients. Statistical significance of differences between multigroup comparisons was evaluated by ANOVA. Values are mean ± S.E.M.

[*]$P < .001$ versus rectal mucosa.

[**]$P < .001$ versus colon mucosa.

Table 3

Table 3

Genotype and allele distributions of *ADH1B*, *ADH1C* and *ALDH2* in patients with colorectal cancer and hemorrhoids

Gene	Group	Subject number	Genotype number (Frequency)**			*P* value***	Allele number (Frequency)		*P* value***
			**1/*1*	**1/*2*	**2/*2*		**1*	**2*	
ADH1B	Controls*	545	43 (0.08)	205 (0.38)	297 (0.54)		291 (0.27)	799 (0.73)	
	Colorectal ca.	103	7 (0.07)	34 (0.33)	62 (0.60)	0.565	48 (0.23)	158 (0.77)	0.309
	Hemorrhoids	67	7 (0.10)	24 (0.36)	36 (0.54)	0.765	38 (0.28)	96 (0.72)	0.682
ADH1C	Controls*	545	448 (0.82)	93 (0.17)	4 (0.01)		989 (0.91)	101 (0.09)	
	Colorectal ca.	103	94 (0.91)	9 (0.09)	0 (0.00)	0.067	197 (0.96)	9 (0.04)	0.021
	Hemorrhoids	67	59 (0.88)	8 (0.12)	0 (0.00)	0.613	126 (0.94)	8 (0.06)	0.206
ALDH2	Controls*	545	304 (0.56)	218 (0.40)	23 (0.04)		826 (0.76)	264 (0.24)	
	Colorectal ca.	103	43 (0.42)	53 (0.51)	7 (0.07)	0.025	139 (0.67)	67 (0.33)	0.012
	Hemorrhoids	67	29 (0.43)	31 (0.46)	7 (0.10)	0.032	89 (0.66)	45 (0.34)	0.019

*Data for control group were from Chen et al. (1999).

**Because they are rounded to two significant figures, frequencies may not sum to 1.00.

***To increase statistical power, genotype number and allele number, instead of the frequency, were used in comparison.