SUPPLEMENTARY MATERIAL

Amplification of bisulfite-converted genomic DNA and subsequent methylation analysis of ALDH2 promoter region

Amplification of ALDH2 target sequences of the purified bisulfite-converted DNA was done through a (semi-) nested touch-down polymerase chain reaction (PCR) approach. This approach included two consecutive PCR amplification steps that yielded the target region of 254 bp. Sequences of oligonucleotides used as bisulfite-primers can be found in supplementary table S1. Amplification products of the second PCR were purified using the Agencourt® AMPure® XP magnetic beads (Beckman Coulter, Krefeld, Germany). Sequencing of the target fragment was performed by using a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and an Applied Biosystems/HITACHI 3500xl Genetic Analyzer (Applied Biosystems) according to the manufacturer's instructions.

The bisulfite-primer ALDH2-Bis-F1 was used for forward sequencing of the ALDH2 gene. Products of the sequencing PCR were purified using the Agencourt® CleanSeq® XP magnetic beads (Beckman Coulter, Krefeld, Germany) and then used for sequencing. Electropherograms and sequences respectively, detected by the Genetic Analyzer, were analyzed using the specialized Epigenetic Sequencing Methylation analysis Software (ESME) to determine methylation rates for every CpG-site. All polymerase chain reactions were performed in a C1000TM Thermal Cycler (BIO-RAD, Hercules, CA, USA) using touch-down PCR. A Biomek® NXP (Beckman Coulter, Krefeld, Germany) was used for pipetting, transferring steps and purification of DNA and amplified oligonucleotides.

PCR protocols for target amplification and methylation analysis of ALDH2 promoter region

(Semi-) nested reaction components

0.4 µL (20 pmol) of the regarding forward primer (F1/F2)

0.4 µL (20 pmol) of the regarding forward primer (RC1/RC2)

1 µL DNA

3.2 µL H_2O

5 µL HotStarTaq® Master Mix Kit (QIAGEN, Hilden, Germany)

Σ 10µL Total volume

PCR protocol

1. Taq activation: 15 min at 95°C

2. Denaturation: 30 sec at 95°C

3. Primer annealing: 45 sec at T_m + 10°C,

4. Elongation: 1 min at 68°C

Steps 2–4 are repeated 15 times.

The annealing temperature was decreased by 1°C per cycle, until -5° below the T_m of the primers.

5. Denaturation: 30 sec at 95°C

6. Primer annealing: 45 sec at T_m - 5°C

7. Elongation: 1 min at 68°C

Steps 5–7 are repeated 20 times.

8. Elongation: 5 min at 65°C

9. Hold: 12°C

The T_m of the bisulfite-primers was set 50°C for the amplification of the ALDH2 target in the first and second round of the semi-nested polymerase chain reaction.

Genotyping of rs886205

Amplification of rs886205 target region by touch-down PCR

The rs886205 target fragment of the ALDH2 promoter covered 224 bp (-508 to -284 before the ATG codon) and was amplified by touchdown PCR using sequences of oligonucleotides used as primers listed in supplementary table S2.

The PCR product (224 bp) was then subjected to restriction enzyme digestion using HpyAV (New England BioLabs, Ipswich, Massachusetts, USA) that specifically recognizes the motif CCTTC/GGAAG and cuts the respective strands at 5' CCTTC$(N)_6$ 3' and 3' GGAAG$(N)_5$ 5' respectively. This CCTTC motif is located -423 bp and -356 bp before the ATG codon.

The latter is the polymorphic motif, having either an "A" or a "G" -360 before the start codon.

Reaction components

0.4 µL (20 pmol) of the regarding forward primer (F1/F2)

0.4 µL (20 pmol) of the regarding forward primer (RC1/RC2)

1 µL DNA

3.2 µL H_2O

5 µL HotStarTaq® Master Mix Kit (QIAGEN, Hilden, Germany)

Σ 10µL Total volume

PCR protocol

1. Taq activation: 15 min at 95°C

2. Denaturation: 30 sec at 95°C

3. Primer annealing: 45 sec at T_m + 10°C,

4. Elongation: 1 min at 68°C

Steps 2–4 are repeated 15 times.

The annealing temperature was decreased by 1°C per cycle, until -5° below the T_m of the primers.

5. Denaturation: 30 sec at 95°C

6. Primer annealing: 45 sec at T_m - 5°C

7. Elongation: 1 min at 65°C

Steps 5–7 are repeated 25 times.

8. Elongation: 5 min at 65°C

9. Hold: 12°C

The T_m of the primers was set 60°C for the amplification of the rs886205 target.

Allele-specific digestion of the ALDH2 promoter target sequence containing the rs886205 polymorphism

Subsequently, 1µL of 6x loading dye were added to 5µL of digested PCR product and separated by 2% agarose gel electrophoresis that was stained with ethidium bromide, then visualized and photographed using GelDoc™ XR^+ (Bio-Rad Laboratories GmbH, München, Germany, see supplementary figure S1). Digestion of the amplified ALDH2 promoter fragments by HpyAV yields 91, 52, 83 bp fragments for A/A genotypes, 91, 52, 83 and 134 bp fragments for A/G, 91 and 134 bp fragments for G/G genotypes.

Thus, the yield of 134 bp fragments after HpyAV digestion of promoter fragments distinguishes G-allele carriers from homozygous A-allele carriers. In order to distinguish between homo- and heterozygous G-allele carriers, subsequent DNA sequence analysis was performed for all fragments yielding 134 bp bands.

Digestion Mix

0.1 µL BSA (100x)

0.5 µL HpyAV (2 Units)

0.9 µL NEB-4 buffer (10x)

3 µL DNA

5.5 µL H_2O

Σ 10µL Total volume

Digestion of PCR products was accomplished by incubation of reaction samples for 1 hour at 37°C. 1µL of amplified product of the first PCR was used as template for the second PCR.

Analysis of ALDH2 protein expression in blood samples from alcohol dependent patients and healthy controls with and without the G-allele of rs886205

Analysis of ALDH2 protein expression in blood samples from alcohol dependent patients on day 1, 7 and 14 and healthy controls with and without the G-allele of rs886205 was performed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent detection of human ALDH2 by western blot analysis. Proteine lysates of frozen blood samples were generated according to the modified Aqua Preserve™ Blood Protein Extraction Protocol (Multi Target Pharmaceuticals, USA):

1. Lysis of blood cells:

100µL of frozen blood were incubated with 100µL of Aqua Preserve and vortexed for 1 min.

2. Pelleting of proteins:

1.2 ml of acetone were added to lysed blood cells, vortexed for 1 min, and subsequently centrifuged at 3.500 g at room temperature to pellet proteins.

3. Solubilization of proteins:

After removing supernatant from protein pellet, 0.5 ml of ProMelt solution was added, vortexed for 1 min and incubated at room temperature for 3x 5 min rolling was completely removed.

4. Protein lysates were divided into 2x 200µL aliquots and 1x 20µL for following determination of protein concentration by the Bradford protein assay.

spective standard curve.

Western blotting of blood lysates

100 µg of total protein were then loaded on a 10 % SDS-polyacrylamide gel, 25 µg of HeLa Whole Cell Lysate (Santa Cruz Biotechnology, USA) were loaded as a positive control for both ALDH2 and vinculin detection by consecutive western blotting technique. After sample loading, samples were separated for 2.5 h at constant 100 V using running buffer containing following constituents:

3.0 g Tris base

14.4 g Glycine

20 % SDS (g/ml)

Then, gels were blotted on nitrocellulose membranes (GE Healthcare, England) for 1.5 h at 400 mA constant using blotting buffer containing following constituents:

1400 ml H_2O

200 ml 10x Running Buffer

400 ml Methanol

Blotted membranes were then blocked with 5 % non fat dry milk (NFDM) in Tris-buffered saline (TBS) supplemented with 0.1 % Tween-20 for 1 h at 4 °C. After blocking, membranes were incubated at 4°C overnight with a primary polyclonal rabbit anti human ALDH2 antibody (Proteintech, England) in a concentration of 1/1000 in 2.5%-NFDM/TBST or a primary polyclonal goat anti human vinculin antibody (Santa Cruz Biotechnology, USA), respectively in a concentration of 1/500 in 2.5%-NFDM/TBST. Subsequently, membranes were washed 3 times with 2.5 NFDM/TBST for 5 minutes at 4°C and incubated with a goat anti rabbit IgG Horseradish

peroxidase (HRP)-linked antibody (Cell Signaling Technology, USA) for detection of bound primary ALDH2 antibody and a mouse anti goat IgG HRP-linked antibody (Santa Cruz Biotechnology, USA) for detection of bound primary vinculin antibody, respectively. Both goat anti rabbit and mouse anti goat secondary antibodies were incubated at a concentration of 1/20.000 in 2.5% NFDM/TBS-T for 1 h at room temperature with the respective membranes.

Membranes were washed 3 times with 0.1 % TBS-T and 3 times TBS for 5 min at 4 °C before detection was performed using a Versa Doc™ Imaging System (BioRad, USA).

Validation of specific detection of human ALDH2 protein by polyclonal rabbit anti human ALDH2 antibody

Before detecting ALDH2 in blood protein lysates with the referring polyclonal rabbit anti human ALDH2 antibody, we performed western blot analysis also on protein lysates of peripheral blood monocytes, neuronal cells and erythrocytes that led to detection of different bands detected by ALDH2 depending on the respective cell type. In order to confirm the validity of ALDH2 protein (55 kd) detection in protein lysates generated from frozen whole blood samples as described above, liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) of proteins immunoprecipated (IP) by the utilised polyclonal rabbit anti human ALDH2 was performed.

For that, we incubated 20 µL of Protein A/G Plus-Agarose Immunoprecipitation Reagent (Santa Cruz Biotechnology, USA) with 10 µL of polyclonal rabbit anti human ALDH2 antibody (Proteintech, England) rolling for 2 h at 4°C. After incubation with antibody, A/G Plus-Agarose Immunoprecipitation Reagent was washed 5 times with NP40 lysis buffer containing constituents listed below:

366 ml H_2O

6 ml Igepal

60 ml Glycerol

60 ml $Na_4P_2O_7$

60 ml NaF

18 ml NaCl

30 ml HEPES pH 7.6

After washing steps, 20 µL of A/G Plus-Agarose IP Reagent were then added 800 µL blood protein lysate and incubated rolling over night at 4°C. A/G Plus-Agarose IP beads were then centrifuged, supernatant was removed and beads were resuspended in 15 µL of Laemmli loading buffer containing:

9.6 ml 1 M Tris HCl pH 6.8

12 ml 20 % SDS

12 ml Glycerol 100%

6.4 ml Beta-Mercaptoethanol

0.024 g Bromophenol blue

Resuspended beads with bound protein were heated up to 105 °C for 5 min, added 2 µL acrylamide, centrifuged again and subjected to SDS-PAGE. We also loaded purified ALDH2 protein (Santa Cruz Biotechnology, USA) and IP of this positive control by polyclonal rabbit anti human ALDH2 antibody unto the gel.

After SDS-PAGE of IPs and positive controls, lanes containing IPs of blood lysates were Coomassie Brilliant blue stained, whereas those lanes containing positive controls were cut from the rest and subjected to western blot analysis as described before. Detection of ALDH2 protein was done using a monoclonal mouse anti human ALDH2 antibody (Santa Cruz Biotechnology, USA). We then cut out protein bands of the stained gel that corresponded to the ALDH2 bands

detected by western blot analysis in positive controls at 55 kD and subjected these to LC-MS/MS analysis. LC-MS/MS analysis of the 55 kD IP-gel band revealed 5 unique peptide sequences of human aldehyde dehydrogenase 2 with an overall mascot score of 264.55 and a sequence coverage of 12.38 % when searching human entries of the uniprot databases for matching peptide sequences. Thus, we could confirm specific detection of human ALDH2 from blood protein lysates after SDS-PAGE and western blotting analysis at 55 kD.

All chemicals used for western blot analysis and validation of human ALDH2 protein detection by polyclonal anti human ALDH2 antibody were purchased from Sigma-Aldrich (USA).