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## Human alcohol dehydrogenase 1 is an acceptor protein for polyADP-ribosylation

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### Abstract

Alcohol dehydrogenase (ADH) is important for preventing alcohol toxicity and developmental disorders, and may be involved in other diseases including neurodegenerative diseases. We found that the major acceptor protein of polyADP-ribosylation in a model organism of neurodegeneration using a *Drosophila melanogaster* mutant lacking poly(ADP-ribose) glycohydrolase, was ADH. Thus we postulated that human ADH activity might be regulated by polyADP-ribosylation, a post-translational modification. The radioactivity of [<sup>32</sup>P]NAD<sup>+</sup> was incorporated into human ADH1 by human poly(ADP-ribose) polymerase 1 *in vitro*, but was not incorporated when heat-inactivated PARP1 or a PARP inhibitor, 3-aminobenzamide, was used. The incorporated radioactivity was not released from ADH1 protein in the presence of excess amount of ADP-ribose or poly(ADP-ribose) as competitors. However, it was released by incubation with 1 M neutral NH<sub>2</sub>OH or 0.1 N NaOH, but was not with 0.1 N HCl, suggesting the bond between ADH1 and poly(ADP-ribose) is an ester linkage. When HepG2 cells, a human hepatoma cell line, were cultured in the presence of another PARP inhibitor, olaparib, ADH activity of the cell was significantly increased. These results suggest that polyADP-ribosylation could regulate ADH activity *in vivo* and might be involved in neurodegeneration.

### Keywords

ADH1; *Drosophila melanogaster*; Human hepatoma cells; Neurodegeneration; PARP1

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Declarations of interest

None.

## 1. Introduction

Alcohol dehydrogenases (ADH) can metabolize various substrates including alcohols, aldehydes, retinoids and lipid peroxidation-derived carbonyls [1]. Among 5 classes of *ADH* genes in human and rodents, most studies on ADH have focused on three classes, class I ADH (ADH1), class III ADH (ADH3) and class IV ADH (ADH4), which are highly conserved in all mammalian species [1]. ADH1 is mainly distributed in the liver and catalyzes oxidation of ethanol and retinol and also reduction of lipid peroxidation products [2–4]. ADH3 is found in every tissues and from kinetic studies it catalyzes omega-hydroxy fatty acids in addition to function as formaldehyde dehydrogenase [3]. ADH4 is present in the stomach, the esophagus and the other mucosa [2], and can also catalyze oxidation of ethanol and retinol and also reduction of the lipid peroxidation products [3,4]. ADH is important for preventing alcohol toxicity that causes human diseases [5,6]. It is reported that *Adh1* and *Adh1/4* gene knockout mice showed Parkinson's disease-like phenotypes such as reduction of locomotion, reduced sensitivity of smell, reduced monoamine levels and decreased body weight [7]. Different kinds of toxic aldehydes could be formed from peroxidation of polyunsaturated fatty acids through lipid peroxidation [4]. These aldehydes could be reduced by ADH 1 *in vivo* from enzymatic constants [3]. So if *ADH1* and *ADH1/4* gene are knocked out, accumulation of toxic aldehydes might cause deleterious effects. It is known that acetaldehyde can react with dopamine and form salsolinol [8], a neurotoxin with an effect similar to parkinsonism-inducing product 1-methyl-4-phenyl-1,2,3,6-tetrahydropyrimidine (MPTP) [9]. Thus, if the compound reaches within the brain, it can inhibit the mitochondrial respiratory chain complex I, causing degeneration of dopamine neurons in the substantia nigra [10]. A truncating mutation in *ADH1C* (G78Stop) showed significant association with Parkinson disease from a large international survey [11]. Parkinson's disease is characterized by accumulation of  $\alpha$ -synuclein as fibrils in the brain. However it is not clear how the accumulated  $\alpha$ -synuclein fibrils drives neuronal cell death. Recently Kam et al. reported that neurotoxicity induced by  $\alpha$ -synuclein preformed fibrils was dependent on polyADP-ribosylation in mouse primary cortical neuron [12].

PolyADP-ribosylation is a posttranslational modification of proteins and is suggested to be involved in regulation of various biological events including cell death through addition of ADP-ribose molecules to various acceptor proteins [13]. From recent approach using mass spectroscopy, various acceptor proteins of polyADP-ribosylation were reported including ADH3 (class III) (*ADH5* from old nomenclature) from a human breast cancer cell line [14] and Adh3 (class III) (*Adh5* from old nomenclature) from  $\beta$ -amyloid peptide-treated mouse BV2 microglial cells [15]. Thus, we hypothesized that human ADH1 protein is polyADP-ribosylated in human hepatoma cells and investigated whether the modification could regulate ADH activity *in vivo*.

## 2. Materials and methods

### 2.1. Cells and reagents

Human liver cancer cell lines, HepG2 cells (Riken, Tsukuba, Japan) and Li7 cells, were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100  $\mu$ g/ml) in a humidified incubator

with 5% CO<sub>2</sub> at 37.0 °C. The other human liver cancer cell lines, HLE cells, HuH6 cells, HuH7 cells and PLC/PRF/5 cells, were cultured in DMEM supplemented with 10% heat-inactivated FBS, penicillin (100 U/ml) and streptomycin (100 µg/ml) in a humidified incubator with 5% CO<sub>2</sub> at 37.0 °C. 3-Aminobenzamide (3AB) was purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). [<sup>32</sup>P]NAD<sup>+</sup> was purchased from PerkinElmer (Massachusetts, USA), olaparib from ChemScene LLC (New Jersey, USA), recombinant human ADH1 protein (ab123147) from Abcam (Cambridge, UK), and recombinant human PARP1 from Trevigen (Maryland, USA). Poly(ADP-ribose) was prepared as described [16]. Anti-poly(ADP-ribose) IgG column was prepared by binding of monoclonal antibody against poly(ADP-ribose) (10H) [17] to CNBr-activated Sepharose 4B (GE Healthcare).

## 2.2. *Drosophila melanogaster* mutant that has increased polyADP-ribosylation

*Drosophila melanogaster* mutant, *parg*<sup>27.1/Y</sup>, that lacks poly(ADP-ribose) glycohydrolase [18], a major enzyme to degrade poly(ADP-ribose), was established as described [19].

Extraction and purification of polyADP-ribosylated proteins was as described by Nodono et al. [17]. The purified proteins were separated with 2D gel electrophoresis. The proteins appearing as new spots on the gel after alkaline treatment of the proteins (Fig. 1, right panel) were digested with trypsin and subjected to mass spectrometer (LCMS-IT-TOF, Shimazu Co., Kyoto, Japan). The data were analyzed using LCMS solution (Shimazu Co.) and MASCOT Version 2.2 (Matrix Science K.K., Tokyo) to find out proper proteins from data base from all organism by comparing with NCBIInr and SwissProt. Significance threshold was set as  $p < 0.05$ .

## 2.3. PolyADP-ribosylation reaction with [<sup>32</sup>P]NAD<sup>+</sup>

*In vitro* polyADP-ribosylation was performed as described previously [20]. Human ADH1 protein was incubated in 50 µl polyADP-ribosylation reaction buffer (100 mM Tris-HCl, pH 8.0, 10 mM MgCl<sub>2</sub>, 1 mM DTT) with 20 U of PARP1, 1 µg of sonicated salmon sperm DNA, and 12.5 µCi (16 pmol, 10.6 ng) [<sup>32</sup>P]NAD<sup>+</sup> for 30 min at 37 °C. The reaction was stopped by adding sample buffer (0.35 M Tris-HCl, pH 6.8, 10% SDS, 0.84 M 2-mercaptoethanol, 36% glycerol, 0.03% bromophenol blue). One millimolar of 3AB was included as a negative control for polyADP-ribosylation. As another negative control, human ADH1 protein was incubated in 50 µl polyADP-ribosylation reaction buffer with 20 U of PARP1, which had been heat-inactivated for 5 min at 100 °C. The reaction products were resolved by SDS-12.5% polyacrylamide gel electrophoresis. The polyacrylamide gels were dried at 65 °C for 2 h by vacuum using Electrophoresis RapiDry (ATTO Corporation, Japan) and exposed to X-ray film to detect radioactive materials at -80 °C for 3 h in the dark.

## 2.4. Characterization of binding of poly(ADP-ribose) to ADH1

To characterize the mode of binding between ADP-ribose and ADH1 protein, the products after incubation in the polyADP-ribosylation reaction mix were incubated further with 100 µg of ADP-ribose or 300 ng of poly(ADP-ribose) as competitors, for 30 min at 37 °C before addition of sample buffer, then incubated for 5 min at 100 °C and analyzed with SDS-12.5% polyacrylamide gel electrophoresis.

The reaction products after completion of the polyADP-ribosylation reaction were treated for 1 h at 25 °C with either 1 M neutral NH<sub>2</sub>OH, 0.1 M NaOH or 0.1 M HCl. NaCl at 1 M or 0.1 M was used as negative controls. The reaction products were neutralized and analyzed by SDS-12.5% polyacrylamide gel electrophoresis. The radioactive materials were detected as described in 2.3.

## 2.5. Cell lysate preparation for ADH activity assay

HepG2 cells ( $1.0 \times 10^6$ ) were plated in 100-mm cell culture dishes. After one day incubation, cells were treated with 30  $\mu$ M olaparib or 0.15% dimethyl sulfoxide (DMSO) as a vehicle, for 30 min in a humidified incubator with 5% CO<sub>2</sub> at 37.0 °C. Cells were washed with PBS with 30  $\mu$ M olaparib or 0.15% DMSO, then harvested by treatment with a 0.025% trypsin/EDTA solution containing 30  $\mu$ M olaparib or 0.15% DMSO. After centrifugation at  $100 \times g$  for 3 min, the precipitates were washed with PBS containing 30  $\mu$ M olaparib or 0.15% DMSO. The cell pellets were collected by centrifugation at  $100 \times g$  for 3 min and dissolved in ice-cold RIPA buffer with 30  $\mu$ M olaparib or 0.15% DMSO and sonicated for 3 sec  $\times$  10 times at a setting of 2 with a HUX-XL2000 sonicator (Misonix, USA). Protein concentration was determined using BCA kit (Thermo Fisher Scientific, USA).

## 2.6. Assay for ADH activity

HepG2 cell lysates were incubated in 100  $\mu$ l of reaction mixture containing 1 M ethanol, 1 mM NAD<sup>+</sup>, and 100 mM sodium phosphate buffer (pH 8.5). ADH activity was measured for 30 min at 25.0 °C by the increase of absorbance at 340 nm following the reduction of NAD<sup>+</sup> to NADH. Reaction mixtures containing HepG2 cell lysates but without 1 M ethanol were used as a blank.

## 2.7. Statistics

Statistical significance of the difference between the control group and PARP inhibitor group was evaluated by Student's *t*-test.

# 3. Results

## 3.1. The major acceptor protein of polyADP-ribosylation in a *Drosophila* mutant was ADH

A *Drosophila* mutant, which is defective in poly(ADP-ribose) glycohydrolase (PARG) [18] that degrades poly(ADP-ribose), was used. They were processed as described [17]. The acceptor proteins of polyADP-ribosylation were isolated from the extract of *Drosophila* mutant and were purified with affinity chromatography using an anti-poly(ADP-ribose) IgG column. Since the acceptor proteins are bound with poly (ADP-ribose) of varying chain length, they could not be detected as distinct bands in gel electrophoresis, but could appear as converged bands after cleaving off poly(ADP-ribose) chain. Therefore, the purified acceptor proteins were treated without or with 1 M NaOH for 15 min on ice to cleave the ester bond between the acceptor proteins and poly (ADP-ribose). Then, they were separately resolved with 2D gel electrophoresis (Fig. 1). 2D gel electrophoresis was repeated 8 times and the newly appeared spots (arrowhead) derived from 28,800 *Drosophila* mutant flies were collected and were subjected to mass spectrometry as described in Materials and Methods. The most prominent spot as shown in Fig. 1 (right panel) was identified as *Drosophila* ADH

protein (Uni-ProtKB/SwissProt – P00334 (ADH\_DROME); Score 458, Mass 27 kDa; pI = 7.74; coverage 51%). We conclude that the major acceptor protein of polyADP-ribosylation in *Drosophila melanogaster* is ADH.

### 3.2. PolyADP-ribosylation of human ADH1 by human PARP1 *in vitro*

Radioactivity from [<sup>32</sup>P]NAD<sup>+</sup> was incorporated mostly into poly (ADP-ribose) polymerase 1 (PARP1), which is the canonical PARP among PARP family members [21], as known as automodification of PARP1 [22,23] (Fig. 2). But significant incorporation was observed in human ADH1 (Fig. 2). The incorporation into ADH1 was not observed when heat-inactivated PARP1 or a PARP inhibitor, 3AB, was used (Fig. 2). Histone H1, known to be polyADP-ribosylated with short chain of poly(ADP-ribose) [24], was used as a positive control. Thus, it appeared that human ADH1 was ADP-ribosylated by PARP1. In addition, the incorporated radioactivity was not removed from ADH1 protein in the presence of excess amount of ADP-ribose (100 µg, 180 nmol, 3.6 mM) or poly(ADP-ribose) (300 ng, 0.55 nmol as ADP-ribose residues, 11 µM) as competitors (Fig. 3). These results further indicated that this linkage was covalent, as was also shown with histone H1 as a positive control.

### 3.3. The major chemical bond between ADH1 and poly(ADP-ribose) is an ester linkage

To know the nature of the chemical bond between the acceptor amino acid and poly(ADP-ribose), the chemical stability of the bond was analyzed. The bond between ADH1 and poly(ADP-ribose) was mostly cleaved with either 1 M neutral NH<sub>2</sub>OH or 0.1 M NaOH for 1 h at 25 °C, but not with 0.1 M HCl, suggesting the presence of an ester bond as the major linkage [25,26] (Figs. 4 and 5). The chain length of incorporated ADP-ribose into ADH did not appear to be sufficiently long to change the electrophoretic mobility, similar to the findings with the histone H1 acceptor *in vitro*, suggesting that it might be < 10 ADP-ribose moieties. Thus, we conclude that human ADH1 could be polyADP-ribosylated *in vitro* with an ester bond as the major linkage.

### 3.4. PARP inhibitor increased the enzymatic activity of ADH1 in HepG2 cells

To know how ADP-ribosylation affects ADH1 activity *in vivo*, we measured ADH1 activity in human liver cell lines using PARP inhibitor. First, we checked the expression levels of ADH1, PARP1 and PARG proteins. ADH1 and PARP1 protein levels were similar in human liver cell lines except for HuH-6 and HuH-7 cells. PARG protein level was similar in human liver cell lines except for Li7 (Fig. 6). We chose HepG2 cells, which expressed a significant amount of ADH1, PARP1 and PARG proteins for determination of ADH1 activity. When HepG2 cells were cultured in the presence of olaparib, a potent inhibitor of polyADP-ribosylation, there was no significant cytotoxicity below 50 µM within 72 h (Fig. 7). It was expected that if ADP-ribosylation of ADH1 inhibited its enzymatic activity, then the PARP inhibitor would increase ADH activity. In fact, when olaparib was added to HepG2 cell culture at 30 µM for 30 min, ADH activity of the cell lysate (1 mg) was significantly increased as compared to the control (Fig. 8). Therefore, we conclude that ADH activity of a human hepatoma cell line, HepG2 cells, could be regulated through polyADP-ribosylation.

## 4. Discussion

Here we found that human ADH1 is polyADP-ribosylated *in vitro* by showing the incorporation of radioactivity from [<sup>32</sup>P]NAD<sup>+</sup> with human PARP1. The chemical bond between ADH1 and poly(ADP-ribose) suggests the presence of mainly ester linkages; the acceptor amino acid could be either glutamic acid or aspartic acid as was already reported for histone H1 [25]. About 30% reduction of the radioactivity after HCl treatment of ADH might be due to the presence of a bond between serine residues of ADH1 [27] and a small amount of the radioactivity remaining after NH<sub>2</sub>OH or NaOH treatment of ADH1 might be explained by the presence of a bond between lysine residues of ADH1 [28]. Of note, when PARP inhibitor was added to cultured HepG2 cells, a human hepatoma cell line, ADH activity of the cells was significantly increased, suggesting that ADH activity is regulated by polyADP-ribosylation *in vivo*. In the liver, since alcohol is primarily metabolized through ADH, how liver diseases induced by alcohol are regulated through polyADP-ribosylation would be an interesting question to be studied in the future.

There is only a single ADH gene in *Drosophila melanogaster* in contrast to 5 classes of genes in man and rodents. Fruit flies feed on decaying fruit, which contains ethanol concentrations as high as 6–7% (v/v). When larvae of fruit flies are exposed to ethanol, they maintain high concentrations of ethanol in their body to protect against parasitoid wasp infection, since high ethanol concentrations exterminate larvae of parasitoid wasp [29]. Indeed, ADH might be one of the essential factors for survival from alcohol intoxication [30]. When larvae of fruit flies were exposed to medium containing 10% ethanol, about 60–70% of wild-type larvae reached adulthood, while 100% of ADH-negative larvae did not [31]. The *Parg* knockout *Drosophila melanogaster* mutant, which showed accumulation of poly(ADP-ribose), revealed neurodegenerative disease-like phenotypes including reduced locomotion, inability to fly and became moribund and died within 10 days [19]. Additionally, we found that the major acceptor protein of polyADP-ribosylation was ADH by mass spectroscopy after purification of the extract from the *Drosophila* mutant. The results suggest polyADP-ribosylation of ADH could be one of the mechanisms of neurodegeneration in *Drosophila*. Concerning ADP-ribosylation of ADH, Fuentes et al. previously reported that the ADH from *Entamoeba histolytica* was mono-ADP-ribosylated, implying the involvement of a different enzyme with respect to PARP-induced polyADP-ribosylation, and that the mono-ADP-ribosylation of ADH did not have any effect on enzymatic activity in *Entamoeba histolytica* [32].

The involvement of polyADP-ribosylation in the neurodegeneration of neuronal cells was recently reported [12]. Preformed  $\alpha$ -synuclein fibrils induced PARP1 activation in mouse primary cortical neurons, leading to cell death. Intrastriatal injection of preformed  $\alpha$ -synuclein fibrils induced loss of dopamine neurons and these pathological changes were reduced by addition of inhibitors of PARP or in PARP1 knocked out mice [12]. They also found the elevated poly(ADP-ribose) level was observed in cerebrospinal fluid of the patients with Parkinson's disease [12]. It is not clear at present whether PARP inhibitors could be used to prevent or treat the neurodegenerative diseases in humans. Further studies are needed to clarify the involvement of ADH in neurodegeneration and the effect of PARP inhibitors for clinical use.

In conclusion, human ADH1 was polyADP-ribosylated *in vitro* by human PARP1. The chemical bond between human ADH1 and poly (ADP-ribose) suggests the presence of mainly ester linkages. When HepG2 cells, derived from human hepatoma, were cultured in the presence of a PARP inhibitor, ADH activity of the cells was significantly increased, suggesting that polyADP-ribosylation could regulate ADH activity *in vivo*.

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## Abbreviations:

<b>3AB</b>	3-aminobenzamide
<b>DMSO</b>	dimethyl sulfoxide
<b>DTT</b>	dithiothreitol
<b>FBS</b>	fetal bovine serum
<b>NAD<sup>+</sup></b>	nicotinamide adenine dinucleotide
<b>NADH</b>	reduced nicotinamide adenine dinucleotide
<b>PARPs</b>	poly(ADP-ribose) polymerases
<b>PARG</b>	poly(ADP-ribose) glycohydrolase
<b>PBS</b>	phosphate-buffered saline
<b>SDS</b>	sodium dodecyl sulfate

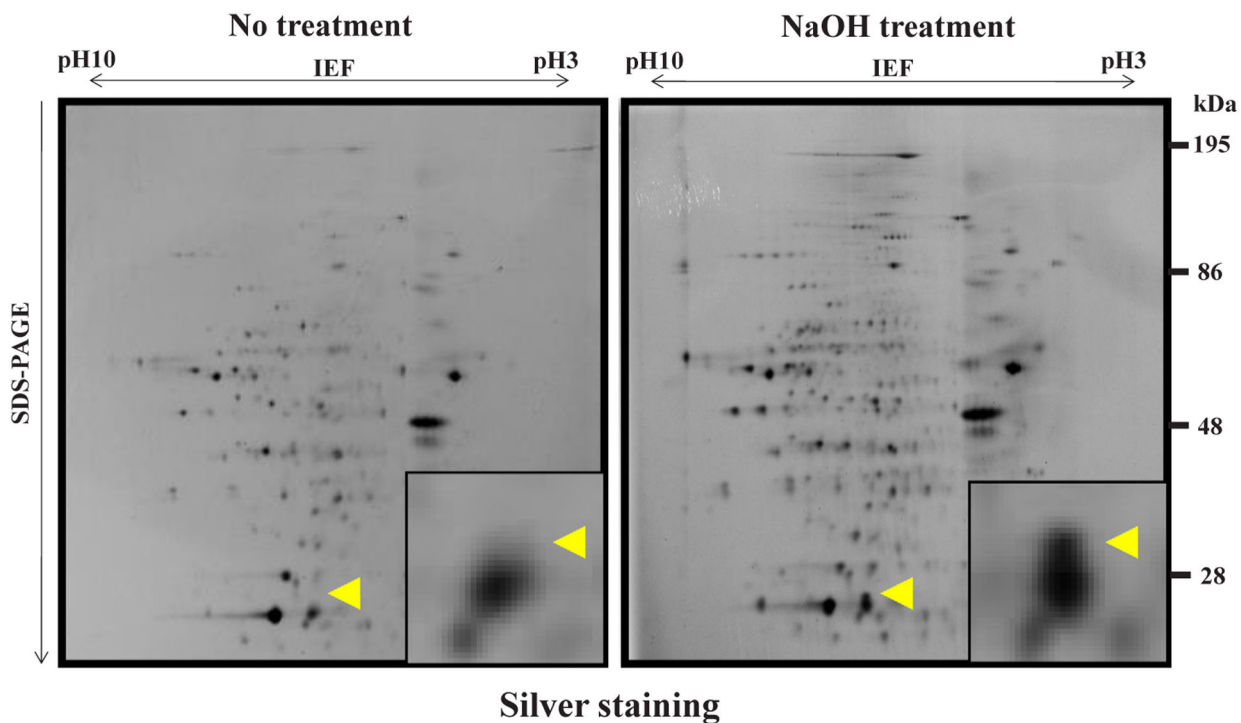
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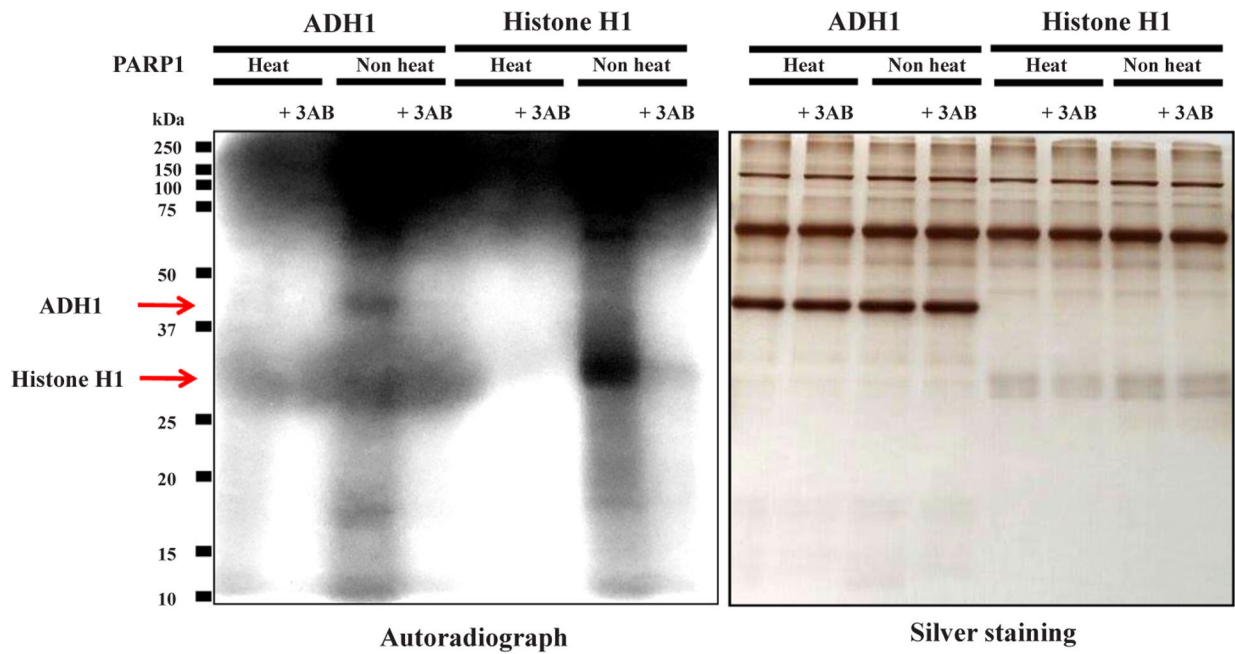
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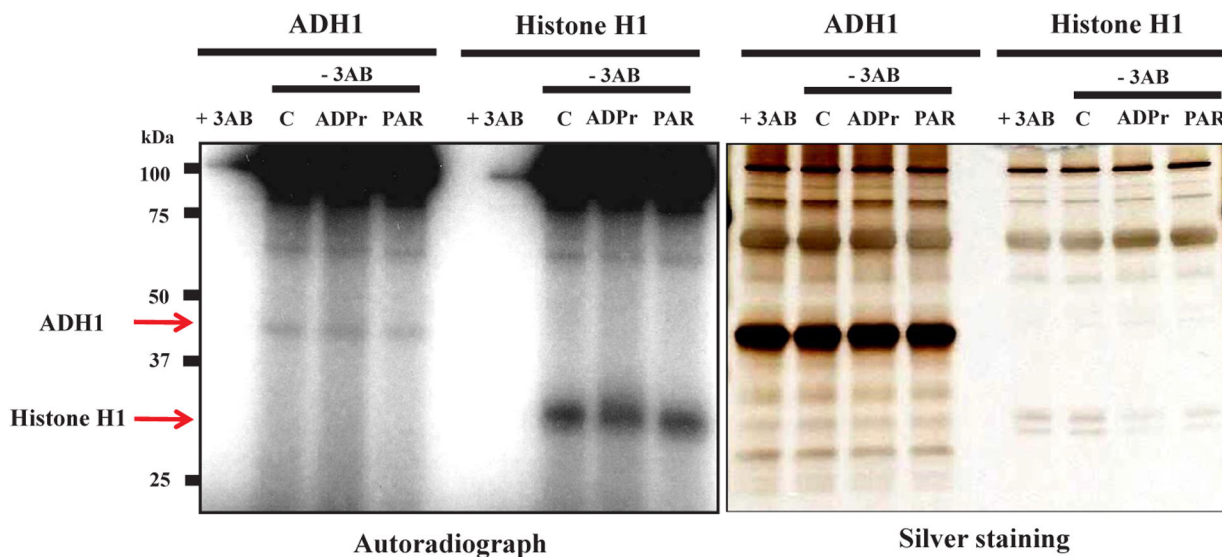
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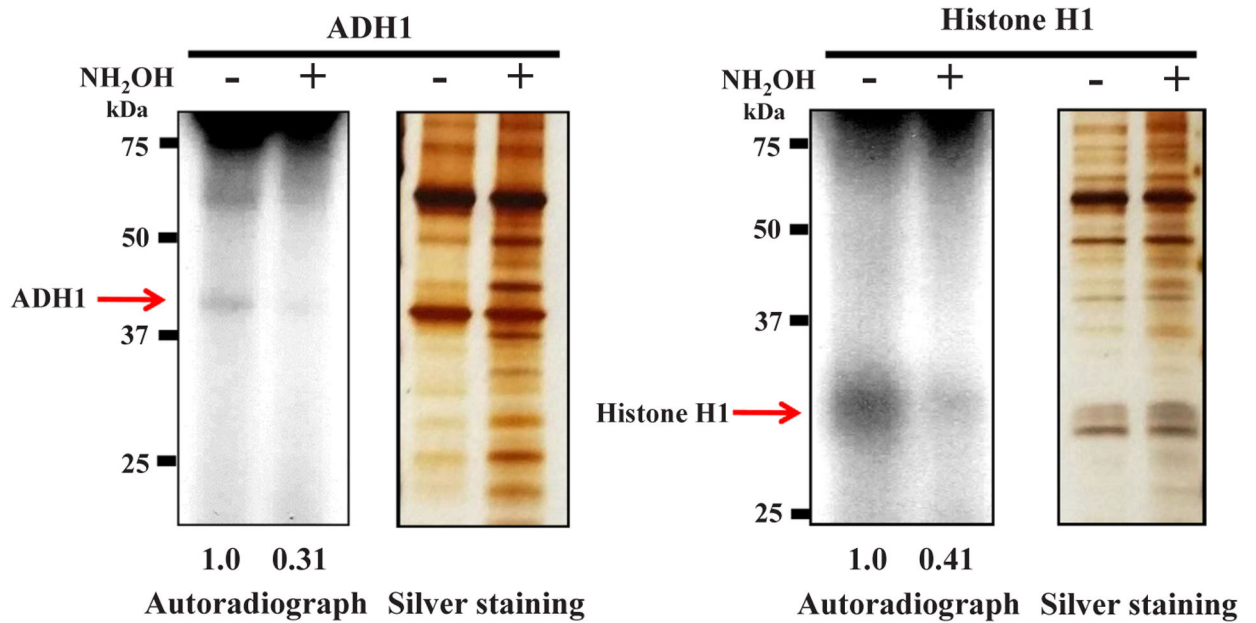
**Fig. 1.** Separation on 2-D gel electrophoresis of affinity purified proteins without or with alkaline treatment Silver staining of 2-D gel electropherograms, each derived from fifteen thousand of *Drosophila* mutant flies. The most prominent protein spot, which appeared after NaOH treatment (right panel) but not without NaOH treatment (left panel), is marked with an arrowhead. The insets represent magnified vision. The representative data from two independent experiments are presented [reproduced from ref. 17 with the permission from Springer Nature, copyright 2011].



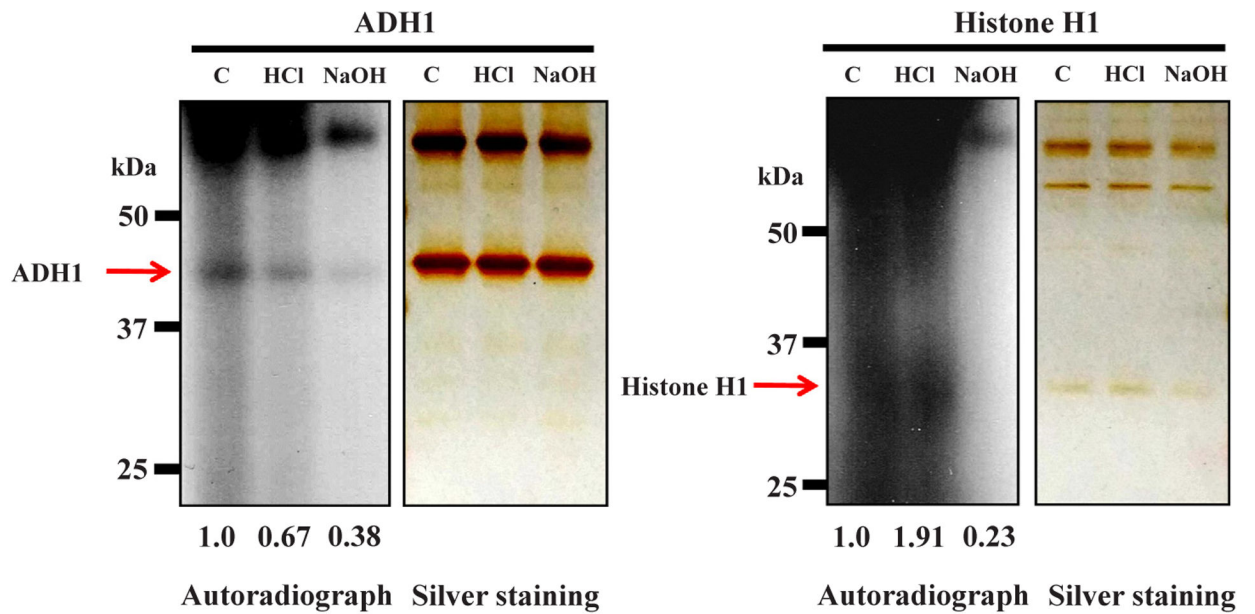
**Fig. 2.** PolyADP-ribosylation of human ADH1 with [ $^{32}\text{P}$ ]NAD $^{+}$  *in vitro* Human ADH1 protein was incubated with [ $^{32}\text{P}$ ]NAD $^{+}$  under polyADP-ribosylation reaction conditions; with native or heat-inactivated PARP1 without or with 3AB. These samples were subjected to SDS-12.5% polyacrylamide gel electrophoresis followed by autoradiography (left panel) and silver staining (right panel). Histone H1 is a positive control for polyADP-ribosylation. Data are representative of three experiments.



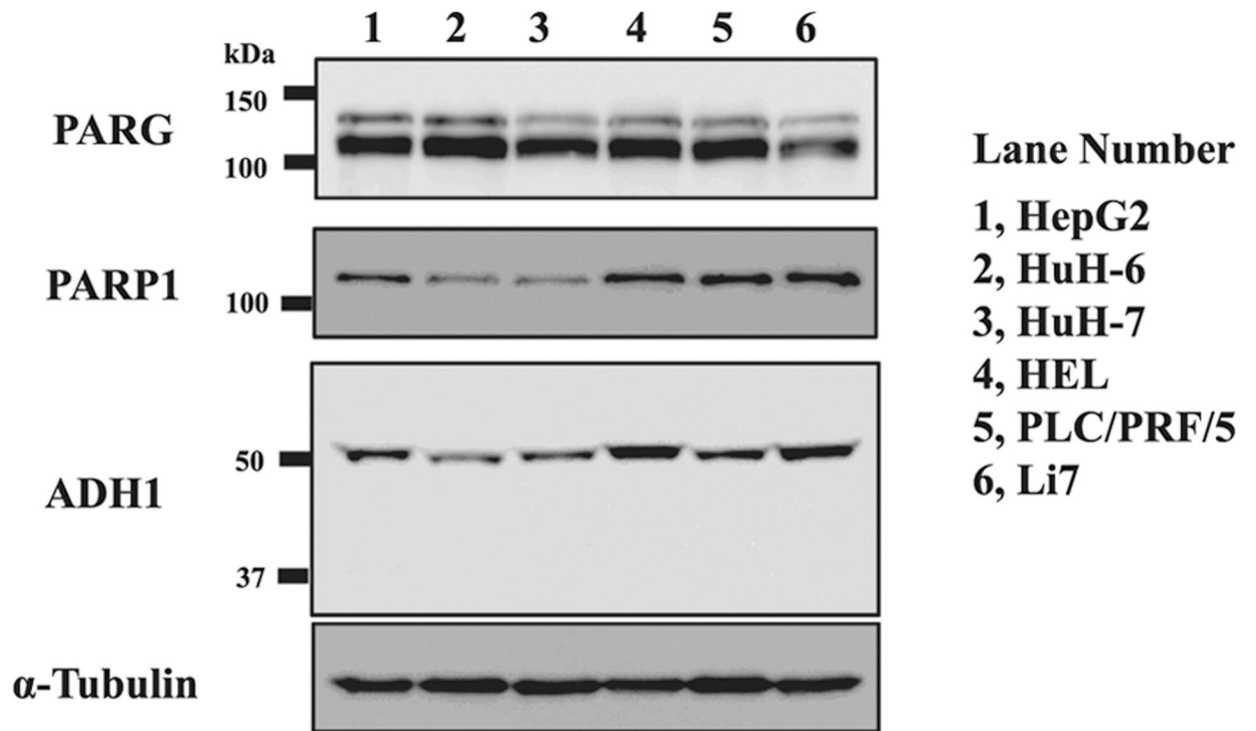
**Fig. 3.** Covalent linkage between human ADH1 and poly(ADP-ribose) *in vitro* Human ADH1 protein was incubated with [<sup>32</sup>P]NAD<sup>+</sup> (12.5 μCi, 16 pmol, 10.6 ng) under polyADP-ribosylation reaction conditions. The reaction was stopped by addition of 1 mM 3AB and then distilled water (Control, C), 100 μg (180 nmol) ADP-ribose (ADPr) or 300 ng (0.55 nmol as ADP-ribose residues) poly(ADP-ribose) (PAR). These samples were subjected to SDS-12.5% polyacrylamide gel electrophoresis followed by autoradiography (left panel) and silver staining (right panel). Histone H1 is a positive control for polyADP-ribosylation. Data are representative of three experiments.

**Fig. 4.**

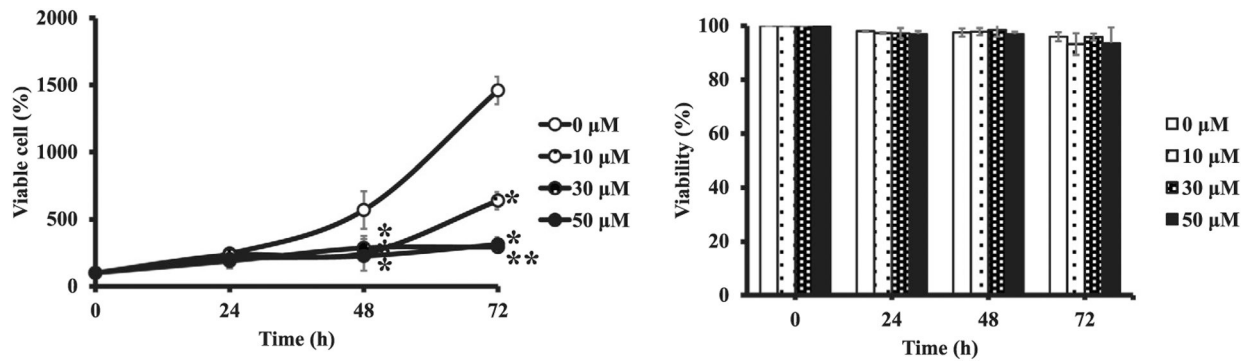
Chemical susceptibility to  $\text{NH}_2\text{OH}$  of the bond between human ADH1 and poly(ADP-ribose) PolyADP-ribosylated ADH1 was treated with 1 M  $\text{NH}_2\text{OH}$  (pH 7.5) for 1 h at 25 °C. The sample was subjected to SDS-12.5% polyacrylamide gel electrophoresis followed by autoradiography and silver staining (left panel). Histone H1 is a positive control for polyADP-ribosylation (right panel). Data are representative of three experiments. The levels of radiolabeled ADH1 and histone H1 proteins were also calculated by Image-J and were digitized.



**Fig. 5.** Chemical susceptibility of the bond between human ADH1 and poly(ADP-ribose) to NaOH but not to HCl. PolyADP-ribosylated ADH1 was treated with 0.1 M NaCl (Control, C), 0.1 M HCl or 0.1 M NaOH for 1 h at 25 °C. The sample was subjected to SDS-12.5% polyacrylamide gel electrophoresis followed by autoradiography and silver staining (left panel). Histone H1 is a positive control for polyADP-ribosylation (right panel). Data are representative of three experiments. The levels of radiolabeled ADH1 and histone H1 proteins were also calculated by Image-J and were digitized.



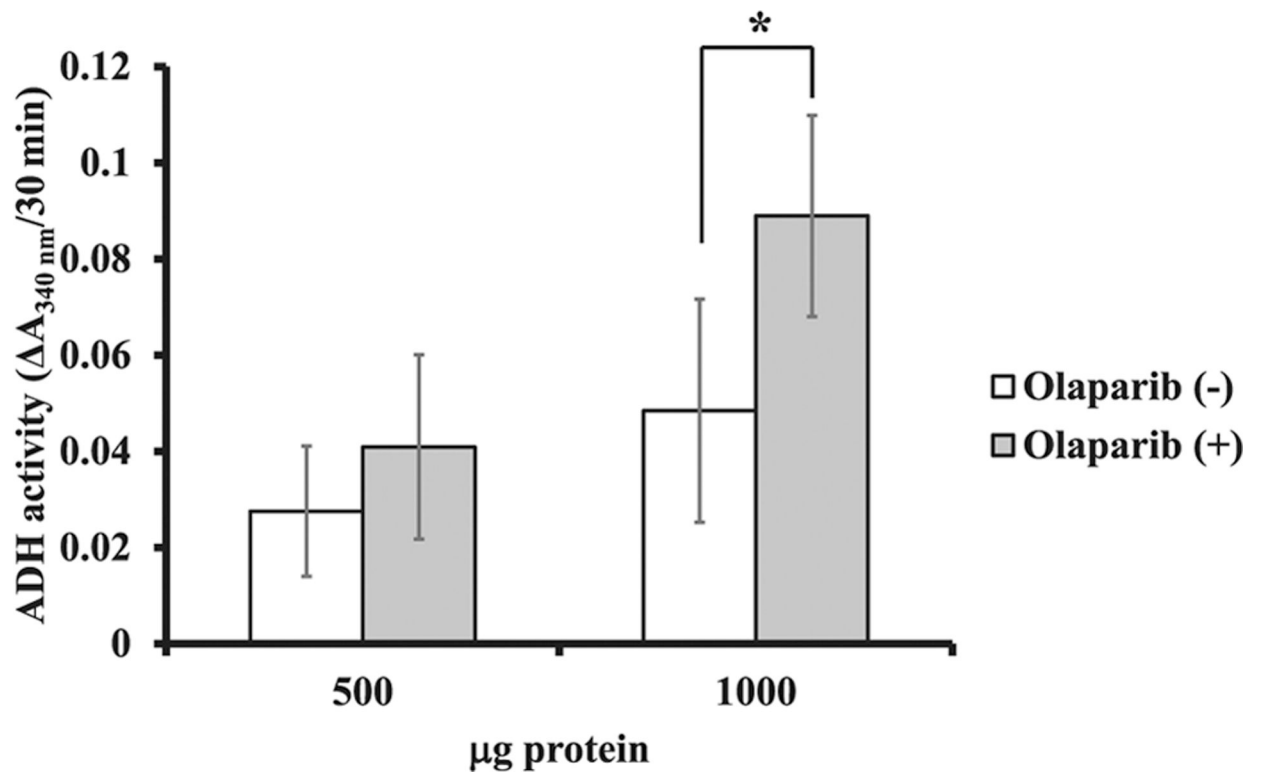
**Fig. 6.** ADH1 protein levels in human liver cell lines Lysates from proliferating human liver cell lines were subjected to SDS-10% polyacrylamide gel electrophoresis followed by Western blotting using antibodies against ADH1, PARG, PARP1 and  $\alpha$ -tubulin.



**Fig. 7.**

Effect of PARP inhibitor (olaparib) on proliferation and viability of HepG2 cells (A) Proliferation of HepG2 cells was determined at 24 h, 48 h and 72 h without or with olaparib. (B) Cell viability was calculated by dividing the number of cells that did not stain with trypan blue by the total number of cells. Cells were counted with hemocytometer. The result is the means  $\pm$  standard deviations from two independent experiments. Start;  $1.0 \times 10^5$  cells/3.5 cm dish (represented as 100%). Significances were 0  $\mu$ M vs. 10  $\mu$ M olaparib, 0  $\mu$ M vs. 30  $\mu$ M olaparib, 0  $\mu$ M vs. 50  $\mu$ M olaparib. \* $p < 0.05$ , \*\* $p < 0.01$ .





**Fig. 8.**

Effect of PARP inhibitor on ADH1 activity ADH1 activity was assayed as described in “Materials and Methods”. The result is the means  $\pm$  standard deviations from three independent experiments. Cell lysates containing 500  $\mu\text{g}$  and 1000  $\mu\text{g}$  protein were used. Significances were olaparib (-) vs. olaparib (+). \* $p < 0.05$ .