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PARP1 Val762Ala polymorphism reduces enzymatic activity ☆

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Abstract

Poly(ADP-ribose) polymerase 1 (PARP1) modifies a variety of nuclear proteins by poly(ADP-ribosyl)ation, and plays diverse roles in molecular and cellular processes. A common *PARP1* single nucleotide polymorphism (SNP) at codon 762, resulting in the substitution of alanine (Ala) for valine (Val) in the catalytic domain has been implicated in susceptibility to cancer. To characterize the functional effect of this polymorphism on PARP1, we performed *in vitro* enzymatic analysis on PARP1-Ala762 and PARP1-Val762. We found that PARP1-Ala762 displayed 57.2% of the activity of PARP1-Val762 for auto-poly(ADP-ribosyl)ation and 61.9% of the activity of PARP1-Val762 for trans-poly(ADP-ribosyl)ation of histone H1. The kinetic characterization revealed that the K_m of PARP1-Ala762 was increased to a 1.2-fold of the K_m of PARP1-Val762 for trans-poly(ADP-ribosyl)ation. Thus, the PARP1 Val762Ala polymorphism reduces the enzymatic activity of PARP1 by increasing K_m. This finding suggests that different levels of poly(ADP-ribosyl)ation by PARP1 might aid in understanding the cancer risk of carriers of the PARP1 Val762Ala polymorphism.

Keywords: PARP1; Single nucleotide polymorphism; Enzymatic activity

Poly(ADP-ribose) polymerases (PARPs, EC 2.4.2.30) catalyze the transfer of multiple ADP-ribose groups from nicotinamide-adenine dinucleotide (NAD^+) onto protein targets, thus building up a linear or branched homopolymer of repeating ADP-ribose units, i.e. poly(ADP-ribose) (PAR) (MeSH). Poly(ADP-ribosyl)ation plays diverse roles in various molecular and cellular processes, including DNA damage detection and repair, chromatin modification, transcription, cell death, insulator function, and mitotic spindle formation [1]. These processes are critical for many physiological and pathophysiological outcomes, including genome maintenance, carcinogenesis, aging, inflammation, and neuronal function [1]. More than 40 years after its discovery, poly(ADP-ribosyl)ation is recognized as a key post-translational modification, and its influence on cell's life-versus-death balance emerges from the diverse roles [2].

Human PARPs now constitute a large family of 18 proteins encoded by different genes and displaying a conserved catalytic domain, in which PARP1 is the prototypical member [3]. PARP1 has a highly conserved structural and functional organization including (1) an N-terminal double zinc finger DNA-binding domain (DBD), (2) a central auto-modification domain, and (3) a C-terminal catalytic domain [1,4,5]. The catalytic domain can be subdivided into two domains, the N-terminal regulatory domain (PARP_Reg, Pfam Accession No. PF02877) and the C-terminal domain containing the active site [6].

The targets of PARP1 include PARP1 itself, which is the primary target *in vivo*, core histones, the linker histone H1, and a variety of transcription-related factors that interact

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with PARP1 [1,4]. The auto-modification of PARP1 is termed as auto-poly(ADP-ribosyl)ation; otherwise the hetero-modification is termed as trans-poly(ADP-ribosyl)ation. PARP1's basal enzymatic activity is very low, but is stimulated dramatically in the presence of a variety of allosteric activators, including damaged DNA, some undamaged DNA structures, nucleosomes and a variety of protein-binding partners [1,4,7,8]. Several classes of PARP inhibitors have been identified and characterized. Most PARP inhibitors exhibit a competitive mode, which suggests that they block NAD^+ binding to the catalytic domain [9]. Representative compounds like 3-aminobenzamide (3-AB), nicotinamide and 1,8-dihydroxyisoquinoline have been widely used in basic research for PARP1 and poly(ADP-ribosyl)ation and their pharmacological implications in the treatment of related diseases.

Three hundred and thirty-one single nucleotide polymorphisms (SNPs), including 20 coding SNPs (cSNPs), in the *PARP1* gene have been reported, in which 11 cSNPs are nonsynonymous (http://www.ncbi.nlm.nih.gov/SNP). Current data reveal one nonsynoymous cSNP, *PARP1*: p.Val762Ala (c. 2446T>C, ref: SNP ID. rs1136410) with high heterozygosity of 35%. Its allele distribution exhibits racial difference: less than 10% in African, about 20% in Caucasian and about 45% in Asian. Residue 762 is in the catalytic domain of PARP1. The *PARP1 Val762Ala* polymorphism has been implicated in cancer susceptibility. To characterize the functional effect of this polymorphism on PARP1, we measured the enzymatic activity and kinetic parameters of PARP1-Ala762 and PARP1-Val762 *in vitro*.

Materials and methods

Expression vector and site-directed mutagenesis. The human PARP1 expression vector pSG9M-PARP1 has been described previously [10]. A point mutation (c. 2446C>T) was introduced into codon 762 of human *PARP1* cDNA with a QuikChange® Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA), resulting in an amino acid substitution of valine for alanine. Mutagenesis was performed according to the manufacturer's recommendation with a sense primer 5′-GTGTGCAGGCCA AGGTGGAAATGCTTGACAACC-3′ and an anti-sense primer 5′-GGTTGTCAAGCATTTCCACCTTGGCCTGCACAC-3′. The point mutation was confirmed by sequencing.

Parp1$^{-/-}$ mouse embryonic fibroblasts (MEFs) culture and transient transfection. Parp1$^{-/-}$ MEFs [11] were maintained in Dulbecco's modified Eagle's medium (Gibco-Invitrogen) with 10% FBS (Hyclone, Logan, UT, USA) and antibiotics (Gibco-Invitrogen). We transfected Parp1$^{-/-}$ MEFs (A12) with the PARP1-Ala762 or PARP1-Val762 expression vector by Lipofectamine2000 (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions.

PARP activity blotting for auto-poly(ADP-ribosyl)ation. A cell pellet (1×10^6 cells) was frozen in liquid nitrogen for 3 min, suspended in 100 μl 1× SDS–PAGE loading buffer, and sonicated. Cell extracts without heating were subjected to SDS–PAGE (8%) and transferred to nitrocellulose membrane. Auto-poly(ADP-ribosyl)ation of PARP1 was measured by activity blotting [12] for the incorporation of ^{32}P-NAD^+ (Perkin-Elmer, Boston, MA, USA) into the poly(ADP-ribose) polymer primed at PARP1 on membrane, in the presence of activated DNA (Sigma–Aldrich, St. Louis, MO, USA). After autoradiography with Super RX film, the activity blot was subjected to immunodetection with the polyclonal antibody against PARP1 (1:1000, Roche, Mannheim, Germany). The blots were analyzed with Bio Imaging System and software GeneTools version 3.06.02 (SynGene, Cambridge, UK).

Chemiluminescent PARP assay for trans-poly(ADP-ribosyl)ation of Histone H1. Cell extracts were prepared with the Universal Chemiluminescent PARP Assay Kit (Trevigen, Gaithersburg, MD, USA). According to manufacturer's instructions, trans-poly(ADP-ribosyl)ation of histone H1 was measured by the chemiluminescent assay for the incorporation of biotinylated NAD^+ (Trevigen) into the poly(ADP-ribose) polymer primed at solid phase immobilized histone H1 (Trevigen), in the presence of activated DNA (Trevigen). Cell extracts corresponding to 0.1 U PARP-HAS (Trevigen) in Western blot was loaded into each well. Reactions were performed with 34 μM total NAD^+ for 60 min at 25 °C in triplicates. Chemiluminescence was read with the FLUOstar OPTIMA microplate reader and software version 1.30 (BMG Labtechnologies, Offenburg, Germany). In the parallel wells, PARP1 activity was measured after the 3-aminobenzamide (Trevigen) treatment at the same concentration of NAD^+ (34 μM).

Kinetic characterization for trans-poly(ADP-ribosyl)ation of histone H1 by chemiluminecent PARP assay. Different time points (5–60 min) of the chemiluminecent PARP assay were tested for the steady range of reaction rate in the presence of activated DNA. The final concentration of total NAD^+, mixed with biotinylated NAD^+ (Trevigen) and NAD^+ (Sigma–Aldrich) (ratio 1:67), ranged from 25 to 400 μM. Reactions were carried out for 30 min at 25 °C in triplicates. K_m was calculated from the Lineweaver–Burk plot.

Results

Reduced enzymatic activity of PARP1-Ala762 for auto-poly(ADP-ribosyl)ation

To confirm the sequence of human *PARP1* cDNA, the expression vector was sequenced. Alignment of the cDNA sequence with the Ensembl transcript (ENST00000272146) of *PARP1* revealed three variants. Two are synonymous at codon 192 and 352, and one is non-synonymous at codon 762 resulting in the substitution of alanine for valine. To generate the PARP1–Val762, we introduced a point mutation (c. 2446C>T) at codon 762 of the *PARP1* cDNA to substitute valine for alanine in the expression vector.

To compare the auto-poly(ADP-ribosyl)ation of PARP1-Ala762 and PARP1-Val762, the two expressing vectors were transiently transfected into Parp1$^{-/-}$ MEFs, respectively, and the cell extracts were analyzed by activity blotting and Western blotting (Fig. 1A). The activity of PARP1-Ala762 and PARP1-Val762 was compared according to normalized protein loading by immunodetection. PARP1-Ala762 displayed 57.2% activity of PARP1-Val762 for auto-poly(ADP-ribosyl)ation in the presence of activated DNA (Fig. 1B).

Reduced enzymatic activity of PARP1–Ala762 for trans-poly(ADP-ribosyl)ation of histone H1

To compare the trans-poly(ADP-ribosyl)ation by PARP1-Ala762 and PARP1-Val762, cell extracts of PARP1-Ala762 or PARP1-Val762 transient expression in Parp1$^{-/-}$ MEFs were subjected to the chemiluminescent PARP assay. The activity of PARP1-Ala762 and PARP1-Val762 was compared based on the equal protein loading semi-quantified by Western blot. PARP1-Ala762 displayed

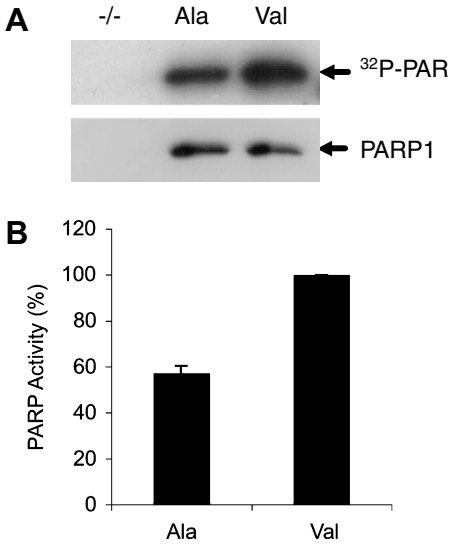


Fig. 1. Reduced auto-poly(ADP-ribosyl)ation of PARP1-Ala762. (A) Activity blot of PARP1-Ala762 and PARP1-Val762. ^{32}P-PAR, the incorporation of ^{32}P-NAD$^+$ into the poly(ADP-ribose)polymer primed at PARP1 in the presence of activated DNA, is shown in the upper panel. Immunodetection of PARP1 is shown below as a reference for protein loading. Parp1$^{-/-}$ MEFs extract is shown as negative control. (B) Enzymatic activity of PARP1-Ala762 and PARP1-Val762. The activity of PARP1-Val762 is set as 100%. Data are means ± standard deviations for three experiments.

61.9% activity of PARP1-Val762 for trans-poly(ADP-ribosyl)ation of histone H1 in the presence of activated DNA (Fig. 2).

To compare the inhibition of PARP1-Ala762 and PARP1-Val762 by PARP inhibitor, the trans-poly(ADP-ribosyl)ation of histone H1 by PARP1-Ala762 and PARP1-Val762 was measured after the PARP inhibitor 3-AB treatment at the same concentration of substrate NAD$^+$ (34 μM). 85.7% activity of PARP1-Ala762 and 81.5% activity of PARP1-Val762 was inhibited by 3-AB (Fig. 2). With 3-AB inhibition, PARP1-Ala762 displayed

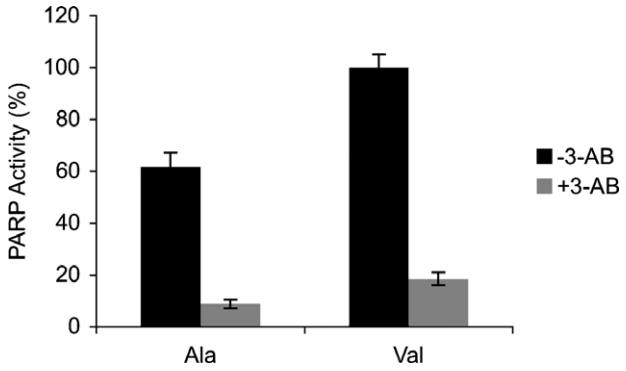
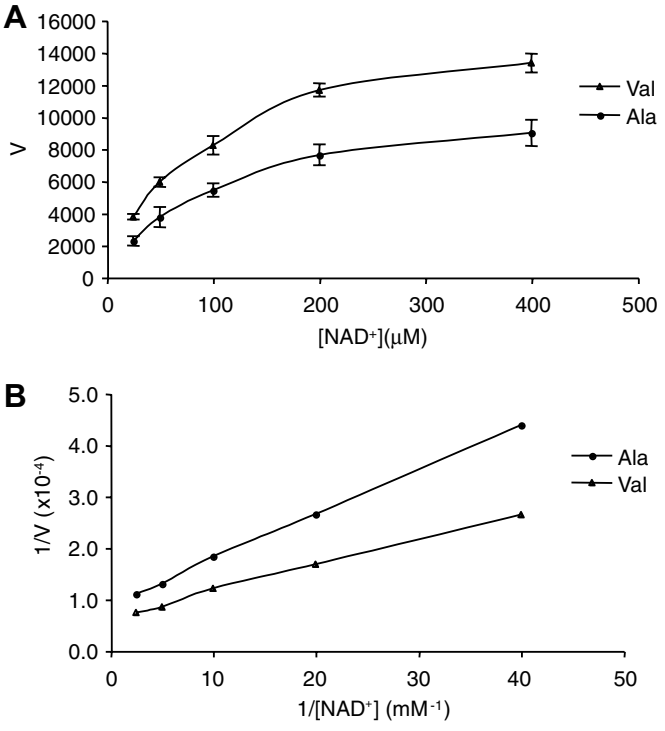


Fig. 2. Reduced trans-poly(ADP-ribosyl)ation of histone H1 by PARP1-Ala762. Enzymatic activity of PARP1-Ala762 and PARP1-Val762 for trans-poly(ADP-ribosyl)ation of histone H1 in response to activated DNA is shown, with or without treatment of 3-AB at the same concentration of NAD$^+$ (34 μM). The activity of PARP1-Val762 without 3-AB treatment is set as 100%. Data are means ± standard deviations for triplicates.

47.8% activity of PARP1-Val762 for trans-poly(ADP-ribosyl)ation of histone H1 (Fig. 2). The enzymatic activity decrease of PARP1-Ala762 is consistent with or without 3-AB inhibition.

Increased K_m of PARP1-Ala762 for trans-poly (ADP-ribosyl)ation of histone H1

To understand the enzymatic activity decrease of PARP1-Ala762, we characterized the kinetics of PARP1-Ala762 and PARP1-Val762 for trans-poly(ADP-ribosyl)ation of histone H1 in the presence of activated DNA. The time range of steady reaction rate was tested and the 30 min time point was chosen. With NAD$^+$ at total concentrations varied from 25 to 400 μM, the reaction rates presented in chemiluminescence readings were plotted versus total NAD$^+$ concentrations (Fig. 3A), and in the



C

PARP1	Km (μM)
Ala762	96.4
Val762	78.1

Fig. 3. Kinetic characterization of PARP1-Ala762 and PARP1-Val762 for trans-poly(ADP-ribosyl)ation of histone H1. (A) Rates of trans-poly (ADP-ribosyl)ation of histone H1 in the presence of activated DNA as a function of NAD$^+$ concentrations. The reaction rate is presented in chemiluminescence reading. Reactions were performed in triplicates. (B) Lineweaver–Burk plot of trans-poly(ADP-ribosyl)ation of histone H1. (C) K_m of PARP1-Ala762 and PARP1-Val762. K_m was calculated from the Lineweaver–Burk plot.

Lineweaver–Burk coordinates (Fig. 3B). K_m was determined from the Lineweaver–Burk plot (Fig. 3C). The K_m of PARP1-Ala762 was increased to 1.2-fold of the K_m of PARP1-Val762 for trans-poly(ADP-ribosyl)ation of histone H1. These results thus support the reduced catalytic activity of PARP1-Ala762.

Discussion

In this study, we investigated the functional effect of the *PARP1 Val762Ala* polymorphism on PARP1. We have shown that this polymorphism reduces the enzymatic activity of PARP1 by increasing K_m. In our experiments PARP1-Ala762 displayed 57.2% activity of PARP1-Val762 for auto-poly(ADP-ribosyl)ation, and 61.9% activity of PARP1-Val762 for trans-poly(ADP-ribosyl)ation of histone H1, in the presence of activated DNA. For the modification of PARP1 itself and histone H1, the activity decrease of PARP1-Ala762 is consistent. These *in vitro* results are also consistent with the *in vivo* study of freshly isolated peripheral lymphocytes, in which AA genotype showed 65.5% activity of VV genotype for ^{3}H-NAD$^+$ incorporation into cells [13]. Furthermore, the kinetic characterization revealed that the K_m of PARP1-Ala762 was increased to 1.2-fold of the K_m of PARP1-Val762 for trans-poly(ADP-ribosyl)ation of histone H1, which could explain the reduced catalytic activity of PARP1-Ala762.

Residue 762 might be implicated in the binding of substrate NAD$^+$. Crystal structure of the catalytic domain of human PARP1 [14] reveals that Val762 is located in the fifth helix of the N-terminal PARP_Reg sub-domain, facing the pocket of active site (Fig. 4). The loss of two methyl groups from valine to alanine increases the distance between residue 762 and glycine888, which is the closest neighbour of residue 762 in the active site [6,15]. This steric change may loose the binding of NAD$^+$ in the pocket and impair the enzymatic activity. The role of residue 762 in PARP1 activity suggests the structure–function relationship in the catalytic domain, between the N-terminal regulatory sub-domain and the C-terminal sub-domain holding the active site.

The *PARP1 Val762Ala* polymorphism, causing about 40% decrease of PARP1 activity, is common in Caucasian and Asian, which suggests that the level of poly(ADP-ribosyl)ation by PARP1 is different in population. The *PARP1 Val762Ala* polymorphism has been implicated in cancer susceptibility. This polymorphism is associated with increased risk of esophageal squamous cell carcinoma, smoking-related lung cancer and gastric cardia cancer in Chinese populations [16–18]. In addition, it is associated with increased risk of prostate cancer in a Caucasian population and deficient enzyme function [13]. These associations all show an allele dosage-dependent manner. The AA genotype is associated with a higher cancer risk (odds ratio around 2 referring to VV genotype), and the VA genotype shows a slightly but not significantly increased cancer risk [13].

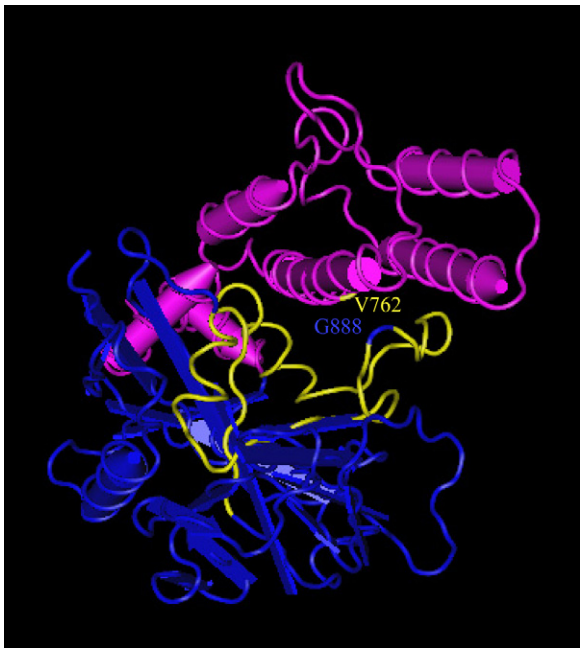


Fig. 4. Crystal structure of PARP1 catalytic domain. With a Cn3D 4.1 software (a 3-dimensional structure viewer, http://www.ncbi.nlm.nih.gov/Structure/CN3D/cn3d.shtml), the crystal structure of PARP1 catalytic domain (PDB: 1WOK_A, MMDB ID. 32358) is shown. Pink domain, N-terminal regulatory domain (PARP_Reg). Blue domain, C-terminal domain holding the active site (yellow).

Carcinogenesis is a multistep process involving aberrations in a variety of cellular processes including genome maintenance, cell-cycle control, proliferation, differentiation, and cell death. PARP1 and poly(ADP-ribosyl)ation have been implicated in all of these processes, suggesting possible connections between PARP1 dysfunction and carcinogenesis [1,19,20]. Parp1 knockout mice have shown increased incidence of spontaneous hepatocellular carcinoma, cancer induced by certain alkylating agents and cancer in combination with deficiency of other genome maintenance factors [1,20,21]. Reduced poly(ADP-ribosyl)ation has been found in lymphocytes of laryngeal cancer patients [22]. Therefore, the deficiency of poly(ADP-ribosyl)ation by PARP1 and the degree of the deficiency might be implicated in carcinogenesis of the *PARP1 Val762Ala* polymorphism carriers.

We have shown that the enzymatic activity decrease of PARP1-Ala762 is consistent with or without inhibition. Several inhibitors have entered human clinical trials, for example INO-1001 (Inotek) in Phase II development for myocardial infraction [23]. A PARP inhibitory dose (PID) of AG014699 (Pfizer GRD) of 12 mg/m^2/d was established by treating patients with advanced solid tumors [24]. However, the PID for treatment might change according to *PARP1* genotype of patients. The distribution of the *PARP1 Val762Ala* polymorphism in patients and the relevant low PARP1 activity should be taken into account for the PARP inhibitor clinical trials and the future therapy.

Acknowledgments

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