

Association of the polymorphisms in the 5'-untranslated region of PTEN gene with type 2 diabetes in a Japanese population

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Abstract Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is known to act as a lipid phosphatase hydrolyzing phosphatidylinositol (PI)(3,4,5)P₃ to PI(4,5)P₂. Since the PI3-kinase product, PI(3,4,5)P₃, is an important second messenger leading to the metabolic action of insulin, PTEN functions as a potent negative regulator of insulin signaling and its gene is one of the possible candidates involved in susceptibility to the development of type 2 (non-insulin-dependent) diabetes. In the present study, we investigated the polymorphisms of the PTEN gene in Japanese patients with type 2 diabetes and non-diabetic control subjects. We identified three mutations of the gene in the type 2 diabetes patients. Among these mutations, the frequency of the substitution of C with G at position -9 (-9C→G) (SNP1), located in the untranslated region of exon 1, was significantly higher in type 2 diabetic patients than in control subjects. In addition, transfection of the PTEN gene with SNP1 resulted in a significantly higher expression level of PTEN protein compared with that of the wild-type PTEN gene in Cos1 and Rat1 cells. Furthermore, insulin-induced phosphorylation of Akt in HIRc cells was decreased more greatly by transfection of SNP1 PTEN gene than that of wild-type PTEN gene. These findings suggest that the change of C to G at position -9 of the PTEN gene is associated with the insulin resistance of type 2 diabetes due possibly to a potentiated hydrolysis of the PI3-kinase product.

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Key words: Phosphatase and tensin homolog deleted on chromosome 10; Phosphatidylinositol 3-kinase; Akt; Type 2 diabetes; Polymorphism

1. Introduction

Type 2 (non-insulin-dependent) diabetes mellitus is a polygenic disorder characterized by reduced insulin secretion and

insulin resistance [1,2]. Although the genetic background is still poorly understood, genes encoding enzymes or interacting molecules constituting the insulin signaling pathways are plausible candidates for the insulin resistance [1,2]. The activated insulin receptor phosphorylates tyrosine residues of insulin receptor substrates resulting in the association and activation of phosphatidylinositol (PI)3-kinase [1,2]. PI3-kinase functions as a lipid kinase producing PI(3,4,5)P₃ from PI(4,5)P₂ in vivo [1–3]. PI(3,4,5)P₃ plays an important role as a second lipid messenger in the performance of insulin signaling leading to the metabolic effects including glucose uptake and glycogen synthesis [1–3].

Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) was originally identified as a candidate tumor suppressor gene and shares homology with the family of protein tyrosine phosphatases [4]. Subsequent analysis demonstrated that PTEN can dephosphorylate PI(3,4,5)P₃ at position 3 on the inositol ring [5]. Studies with cells derived from PTEN-deficient mice showed increased PI(3,4,5)P₃ content and elevated activity of Akt which is one of the downstream molecules of PI3-kinase [6]. It is therefore possible that PTEN modulates insulin signaling by hydrolyzing the PI3-kinase product PI(3,4,5)P₃ to PI(4,5)P₂ via its 3'-phosphatase activity. In fact, overexpression of PTEN inhibited the activation of Akt in the metabolic action of insulin [7–9]. Insulin-induced glucose transport was decreased by expression of PTEN in 3T3-L1 adipocytes [7–9]. In addition, expression of PTEN augmented insulin-induced tyrosine phosphorylation of IRS-1, and increased the amount of IRS-2 and its association with PI3-kinase in adipose cells [9–11]. Thus, insulin signaling upstream of PI3-kinase is upregulated in response to the hydrolysis of the PI3-kinase product by PTEN. Furthermore, the specific inhibition of PTEN expression by means of an antisense oligonucleotide ameliorated insulin resistance and hyperglycemia in diabetic db/db mice [12]. Since these reports clearly indicate the involvement of PTEN in the insulin signaling system, one can consider that the PTEN gene is a plausible candidate for a causative gene of type 2 diabetes. In the present study, we examined the association of the PTEN gene with type 2 diabetes. The polymorphisms of the PTEN gene throughout the coding regions and exon-intron boundaries were closely investigated in a type 2 diabetic Japanese population.

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2. Materials and methods

2.1. Subjects

107 unrelated subjects with type 2 diabetes (57 men and 50 women, aged 60.5 ± 1.2 years, duration of hyperglycemia 12.5 ± 5.2 years, BMI 23.6 ± 2.5 kg/m², HbA_{1c} $7.4 \pm 1.1\%$) and 100 non-diabetic control subjects (48 men and 52 women, aged 59.9 ± 1.7 years, BMI 22.8 ± 2.1 kg/m², HbA_{1c} $5.2 \pm 0.3\%$) were recruited from the outpatient clinic of the First Department of Internal Medicine, Toyama Medical and Pharmaceutical University Hospital, Toyama, Japan. Of the diabetic patients, 13% were treated with diet, 56% with oral hypoglycemic agents and 31% with insulin. All the subjects enrolled in this study were ethnic Japanese. Type 2 diabetes was diagnosed according to the criteria of the World Health Organization [13]. Before participation, the purpose and risks of the study were carefully explained both verbally and in writing, and written informed consent was obtained from all the participants. The protocol was approved by the ethics committee of Toyama Medical and Pharmaceutical University.

2.2. Materials

Human crystal insulin was provided by Novo Nordisk Pharma Ltd. (Copenhagen, Denmark). A monoclonal anti-FLAG antibody (M2) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). A monoclonal anti-Erk2 antibody was from Transduction Laboratories (Lexington, KY, USA). A polyclonal anti-Akt antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). A polyclonal anti-Ser⁴⁷³ phosphospecific Akt antibody and a polyclonal anti-Thr³⁰⁸ phosphospecific Akt antibody were from New England Biolabs, Inc. (Beverly, MA, USA). All other reagents were of analytical grade and purchased from Sigma or Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

2.3. Genetic analyses

Blood samples were obtained from each subject and genomic DNA was extracted from peripheral blood leukocytes using a Wizard genomic DNA purification kit (Promega, Madison, WI, USA). Mutation analysis of the PTEN gene was conducted using the polymerase chain reaction-single-stranded conformational polymorphism (PCR-SSCP) technique and heteroduplex analysis [14]. Primers were designed to include intron–exon boundaries to clarify possible mutations in splicing sites, based on the sequence reported in DDBJ/EMBL/GenBank (accession number AF067844). In the design, exons 8 and 9 were divided into two segments. 11 sets of biotinylated primers for PCR amplification, which were also used for sequencing, and the cycling conditions are shown in Table 1. After PCR amplification, DNA fragments were electrophoresed at 4 or 25°C without or with 5% glycerol in 0.5×MDE gel (FMC BioProducts, Rockland, ME, USA), and visualized with the biotin/streptavidin–alkaline phosphatase/PPD system using an Imaging High-Chemilumi-kit (Toyobo, Osaka, Japan). All SSCP variants were investigated by direct sequencing using the dGTP BigDye terminator cycle sequence FS ready reaction kit and an ABI Prism 377 DNA sequencer (PE Applied Bio-

systems, Foster City, CA, USA) after purification of the PCR products using a MinElute PCR purification kit (Qiagen, K.K., Tokyo, Japan).

2.4. Plasmid construction

C-terminally FLAG-tagged wild-type PTEN and SNPI PTEN expression vectors were constructed. In brief, a fragment of PTEN cDNA was prepared with a 5'-primer containing a *Hind*III restriction site (underlined): 5'-AAGCTTTTCTTCAGCCACAGGCTCCC-AGACATG-3' and a 3'-primer containing a *Bam*HI restriction site (underlined): 5'-GGATCCAAGACTTTTGTAAATTTGTGTATGCTGATCTTC-3' based on the sequence reported in DDBJ (accession number U93051) and subcloned into the *Hind*III/*Bam*HI site of a FLAG-tag containing mammalian expression vector pFLAG-CMV-5b (Sigma Chemical Co.) (pWild-PTEN-FLAG). SNPI was generated using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) with two primers: 5'-TTCTTCAGCCACAGGGTCCCAGACATGACAGCC-3' (sense) and 5'-GGCTGTACATGTC-TGGGACCCTGTGGCTGAAGAA-3' (antisense) (pSNPI-PTEN-FLAG). The appropriate construction of these plasmids was confirmed by DNA sequencing.

2.5. DNA transfection and Western blotting

Cos1 cells and Rat1 fibroblasts overexpressing insulin receptors (HIRC) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). The cells were transiently transfected with each plasmid utilizing Transit-LT1 transfection reagent according to the manufacturer's instructions (Pan Vera Co., Madison, WI, USA). 48 h after transfection, the cells were serum-starved for 16 h. The cells were treated with or without 10 nM insulin at 37°C for 5 min. The cells were lysed in a buffer containing 30 mM Tris, 150 mM NaCl, 10 mM ethylenediamine tetraacetic acid (EDTA), 0.5% sodium deoxycholate, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na₃VO₄, 160 mM sodium fluoride, 10 µg of aprotinin per ml, 10 µM leupeptin (pH 7.4) for 15 min at 4°C. The cell lysates were centrifuged to remove insoluble materials. The supernatants were separated by 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and electrically transferred onto polyvinylidene difluoride membranes. After incubation with the specified antibody, enhanced chemiluminescence detection was performed according to the manufacturer's instructions (Amersham Pharmacia Biotech, Buckinghamshire, UK) [15].

2.6. Statistical analysis

The data are represented as the mean ± S.E.M. *P*-values were determined by chi-square test (Fisher's exact test) or Student's *t*-test, and *P* < 0.05 was considered statistically significant.

3. Results and discussion

We conducted PCR-SSCP analysis and direct sequencing of

Table 1
Nucleotide sequence of DNA primers used for PCR amplification of nine exons of PTEN

	Forward primer	Reverse primer	<i>T_m</i> (°C)	Fragment size (bp)
Exon 1	5'-CTTCTGCCATCTCTCTCCTC-3'	5'-ACTACGGACATTTTCGCATC-3'	58	212
Exon 2	5'-CTGCATATTTTCAGATATTTCTTT-3'	5'-CTGTGGCGGAGAAATCTTTTC-3'	58	197
Exon 3	5'-GGTGGCTTTTGTGTTTGTG-3'	5'-TTAATCGGTTTGAAGATACAA-3'	54	209
Exon 4	5'-ATTCAAGCAATGTTTGTAGT-3'	5'-TCGATAATCTGGATGACTCA-3'	56	210
Exon 5	5'-GCAACATTTCTAAAGTTACCTA-3'	5'-CTATTTTCAATAAATCTCA-3'	54	385
Exon 6	5'-CATAGCAATTTAGTGAAATAACT-3'	5'-GATATGGTTAAGAAACTGTTTC-3'	58	274
Exon 7	5'-CCTGTGAAATAATCTGGTATG-3'	5'-CTCCCAATGAAAGTAAAGTACA-3'	60	229
Exon 8-1	5'-AGGTGACAGATTTCTTTTCTTA-3'	5'-ACTAGATATTCCTTGTCATTAT-3'	56	238
Exon 8-2	5'-GAAGTCTATGTGATCAAGAAA-3'	5'-CAGCTGTACGCCTAGAATTA-3'	56	227
Exon 9-1	5'-TCATATTTGTGGGTTTTCATT-3'	5'-GTGTCAGAAATCTATAATGAT-3'	56	159
Exon 9-2	5'-TCTGTAAACACAGATGTTAG-3'	5'-TTTTCATGGTGTGTTTATCCCT-3'	56	160

Primers for the analyses of the PTEN gene are shown. Exons 8 and 9 were divided into two segments. PCR was carried out in a 50-µl reaction mixture, containing 200 ng of template DNA, 1.0 mmol/l of MgCl₂, 200 µmol/l of each dNTP, 200 nmol/l of each primer, and 1.0 U of KOD plus DNA polymerase with anti-KOD monoclonal antibodies in the buffer supplied by the manufacturer (Toyobo, Osaka, Japan). The PCR conditions were as follows: initial denaturation at 94°C for 2 min, then 30 cycles of denaturation at 94°C for 15 s, annealing at *T_m*–5°C for 30 s, and elongation at 68°C for 40 s.

Table 2
Single nucleotide polymorphisms of the PTEN gene in the Japanese population

SNP	Location	Designation ^a	Change	Prevalence (%)		P-value
				Diabetic patients	Control subjects	
SNP1	5'-UTR ^b	c. -9C→G	non-coding	15/107 (14.0)	5/100 (5.0)	0.034
SNP2	intron 4	IVS 5-11T→G	—	1/107 (1.0)	0/100 (0.0)	N.S.
SNP3	exon 8	c. 859T→C	Ser287Pro	1/107 (1.0)	0/100 (0.0)	N.S.

The c. preceded the nucleotide number of the reference cDNA sequence (DDBJ accession number U93051), denoting the A of the ATG of the initiator Met codon as +1. Intronic polymorphism was designated by the intron (IVS: intronic variant sequence) number, a negative number starting from the G of the acceptor site invariant AG. P-value was determined by chi-square test (Fisher's exact test).

^aDesignation for each SNP was based on the recommendations by the Nomenclature Working Group [24].

^b5'-UTR, 5'-untranslated region.

the gene encoding the PTEN protein in 107 patients with type 2 diabetes and 100 control subjects, and identified three polymorphisms. The first is a substitution of C with G at position -9 (SNP1) which lies in the 5'-untranslated region of exon 1. The second is a substitution of T with G in intron 5 (SNP2). The third is a substitution of T with C at position 859 (SNP3) which lies in exon 8 and causes a missense mutation from Ser to Pro at position 287 (Table 2). Among these polymorphisms, SNP1 was found in 15 (14.0%) diabetic patients and in five (5.0%) control subjects, showing a significant difference in the frequency of individuals with the mutant allele between the two groups ($P < 0.05$). Diabetic patients had both heterozygous and homozygous SNP1 variants, whereas control subjects had the heterozygous but not homozygous variant. The allele frequencies of the type 2 diabetic patients and non-dia-

betic control subjects were 7.9 and 2.5%, respectively (Table 3). The odds ratio for the frequency of the mutant genotype in the diabetic group in relation to the control group was 3.10 (the 95% confidence interval; 1.08–8.87) ($P < 0.05$). Since the allele frequency and the odds ratio for the frequency of mutant genotype were significantly higher in the diabetic group than in the control group, the mutation appears to be associated with type 2 diabetes in the Japanese population.

The untranslated region from position -9 to -7 is adjacent to the Kozak sequence (gccA/gccATGg) and gcc at -9 to -7 is also considered part of the consensus sequence for the initiation of translation [16]. Furthermore, position -9 is included in the repetition of G at positions -3, -6 and -9 of the consensus sequence, and the periodicity of G residues is reported to help ribosomes stay in frame during translation [17]. Although this 5'-untranslated consensus sequence appears to be important for translation initiation, the sequence at this site in wild-type PTEN, ctcccagacATGa, is not typically optimal [16,17]. By substituting the sequence comprising this site resulting in a recovery of the periodical occurrence of the G residue (gtcccagacATGa), SNP1 seems to be more closely matched to the consensus sequence for the greater expression of PTEN [16,17]. To examine whether SNP1 affects the translation of the PTEN mRNA, we constructed a PTEN cDNA containing the SNP1 substitution. The same amounts of wild-type PTEN and SNP1 PTEN cDNA were transiently transfected into Cos1 cells. The expression of PTEN protein was studied by immunoblot analysis of cell lysates with anti-FLAG antibody. Interestingly, a greater amount of PTEN was expressed by transfecting PTEN cDNA bearing SNP1 rather than wild-type PTEN cDNA. By densitometric analysis, the expression of SNP1 PTEN was found to be increased by $37.2 \pm 8.9\%$ compared to that of wild-type PTEN (Fig. 1). The increased expression of PTEN was not limited to Cos1

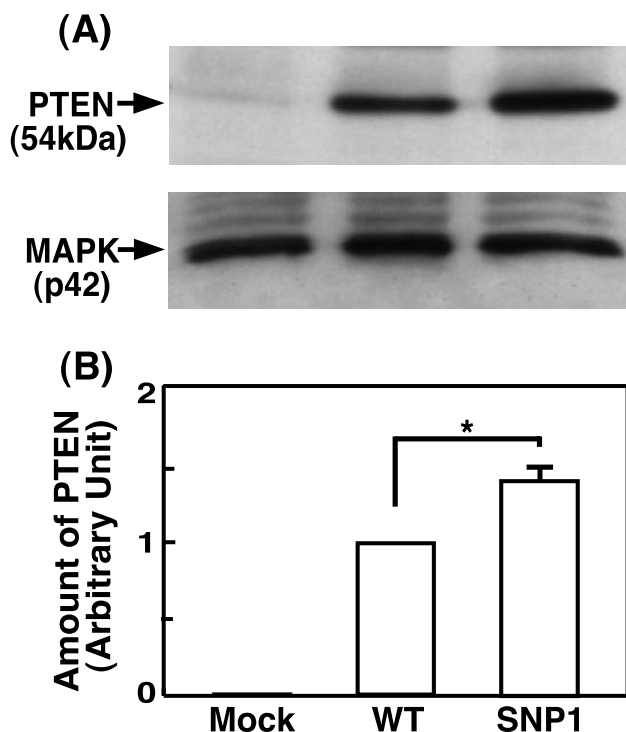


Fig. 1. Immunoblot analysis of PTEN protein transiently expressed in Cos1 cells. A: Cos1 cells were transfected with vacant vector, pWild-PTEN-FLAG, or pSNP1-PTEN-FLAG. The cell lysates were separated by 7.5% SDS-PAGE, and immunoblotted with anti-FLAG antibody or anti-Erk2 antibody. Representative results are shown. B: The amount of expressed PTEN protein corrected for the loaded protein amount was quantitated by densitometry. Results are the mean $\pm$ S.E.M. of three separate experiments. * $P < 0.05$ vs. wild-type (WT)-PTEN expression in Cos1 cells by Student's *t*-test.

Table 3
Prevalence of SNP1 in type 2 diabetic patients and non-diabetic control subjects

	Type 2 diabetic patients	Control subjects	P-value
Number of subjects	107	100	
Allele			
C/C (WT)	92	95	
C/G (hetero)	13	5	
G/G (homo)	2	0	
Allele frequency (%)	7.9	2.5	0.015

Allele frequency of guanine was calculated by the following formula: allele frequency = ((G/G(homo allele) $\times$ 2) + (C/G(hetero allele))) / number $\times$ 2. P-value was determined by chi-square test (Fisher's exact test).

cells, because the enhanced expression of PTEN by the SNP1 substitution was also seen in Rat1 fibroblasts overexpressing insulin receptors (HIRc cells) (Fig. 2A).

PTEN is known to act as a negative regulator of insulin signaling by hydrolyzing the PI3-kinase product PI(3,4,5)P₃ to PI(4,5)P₂, enhanced expression of PTEN by the SNP1 substitution may lead to diminished insulin signaling distal to PI3-kinase [7,8]. Akt is a downstream target molecule of PI3-kinase important for the metabolic action of insulin [7,8]. Since Akt is primarily activated as a result of its phosphorylation, we examined the effect of wild-type and SNP1 PTEN cDNA transfection on insulin-induced phosphorylation of Akt [7,8]. Insulin induced phosphorylation of Akt at Ser⁴⁷³ in LacZ-transfected control HIRc cells. Transfection with wild-type PTEN cDNA decreased insulin-induced phosphorylation of Akt by $40.5 \pm 4.3\%$ in HIRc cells. Importantly, the phosphorylation of Akt was more profoundly reduced by $59.4 \pm 3.1\%$ in HIRc cells transfected with SNP1 PTEN cDNA (Fig. 2B). The amount of protein loaded among the samples was confirmed to be equal by immunoblot analysis with anti-Akt antibody (Fig. 2C). In addition, expression of SNP1 PTEN reduced insulin-induced phosphorylation of Akt at Thr³⁰⁸ and activation of Akt significantly greater than expression of wild-type PTEN in HIRc cells (data not shown). These results indicate that insulin resistance may occur, at least in part, at a step distal to the PI3-kinase in human possessing SNP1 substitution of PTEN. In fact, insulin resistance occurring at the level of insulin signaling distal to PI3-kinase in human and animal models of type 2 diabetes has been reported, although the mechanism involved is not precisely investigated [18–21].

SNP2 and SNP3 were not found in control subjects and each mutation was only found in one patient of the diabetic group. It is not clear whether these variants are associated with type 2 diabetes in the Japanese population. Concerning the SNP3 (Ser287Pro) variant, PTEN can be phosphorylated at the Ser/Thr residues of its C-terminal region by the protein kinase CK2 [22]. The phosphorylation is known to be important for the stability of PTEN against its proteasome-mediated degradation [22]. Although the Ser at residue 287 of PTEN (SNP3) is not found to be critical in the previous reports, it is still possible that the level of expression and/or the function of PTEN can also be affected by this substitution.

Variants of the coding region in PTEN gene including SNP1–3 identified in the present study were not seen in Danish Caucasian patients with type 2 diabetes [23]. Conversely, four intronic polymorphisms identified in the Danish patients were not found in the current Japanese diabetic patients [23]. Although it would be necessary to examine on a larger scale of Japanese subjects whether these intronic polymorphisms of PTEN gene are in fact not seen in Japanese populations, these differences may arise from a different genetic background between the two ethnic groups. Thus, Japanese type 2 diabetic patients are not particularly obese as shown in the current study compared to Danish Caucasian diabetic patients [23].

In summary, we have shown an association between the $-9C \rightarrow G$ polymorphism of the PTEN gene and type 2 diabetes in the Japanese population, providing, at least in part, new insight into the pathogenesis of type 2 diabetes in Japan. Studies which cover a large number of subjects and other ethnic populations will be necessary to evaluate the contribution of SNP1 ($-9C \rightarrow G$) and SNP3 (Ser287Pro) to the pathogenesis of insulin resistance and type 2 diabetes.

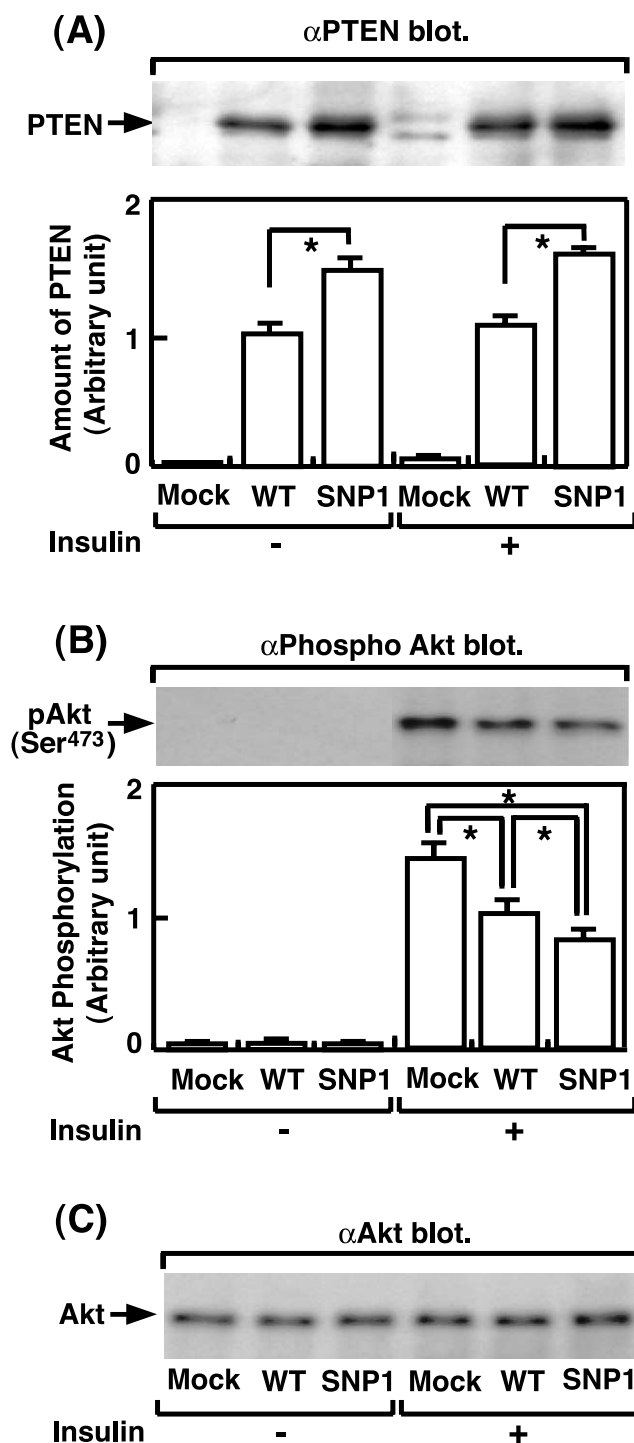


Fig. 2. Effect of PTEN expression on insulin-induced phosphorylation of Akt in HIRc cells. HIRc cells were transfected with vacant vector, pWild-PTEN-FLAG, or pSNP1-PTEN-FLAG. The cells were serum-starved for 16 h, and treated with or without 10 nM insulin for 5 min at 37°C. The cell lysates were separated by 7.5% SDS-PAGE, and immunoblotted with anti-FLAG antibody (A), anti-phosphospecific Akt antibody (B), or anti-Akt antibody (C). Representative results are shown. The amount of expressed PTEN and phosphorylated Akt corrected for the loaded protein amount was quantitated by densitometry. Results are the mean $\pm$ S.E.M. of five separate experiments. * $P < 0.05$ vs. wild-type (WT)-PTEN expression (A) and * $P < 0.05$ (B), compared by Student's *t*-test.

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