骨粗鬆症の予防と治療ガイドライン 2011年版

編集
骨粗鬆症の予防と治療ガイドライン作成委員会
（日本骨粗鬆症学会　日本骨代謝学会　骨粗鬆症財団）
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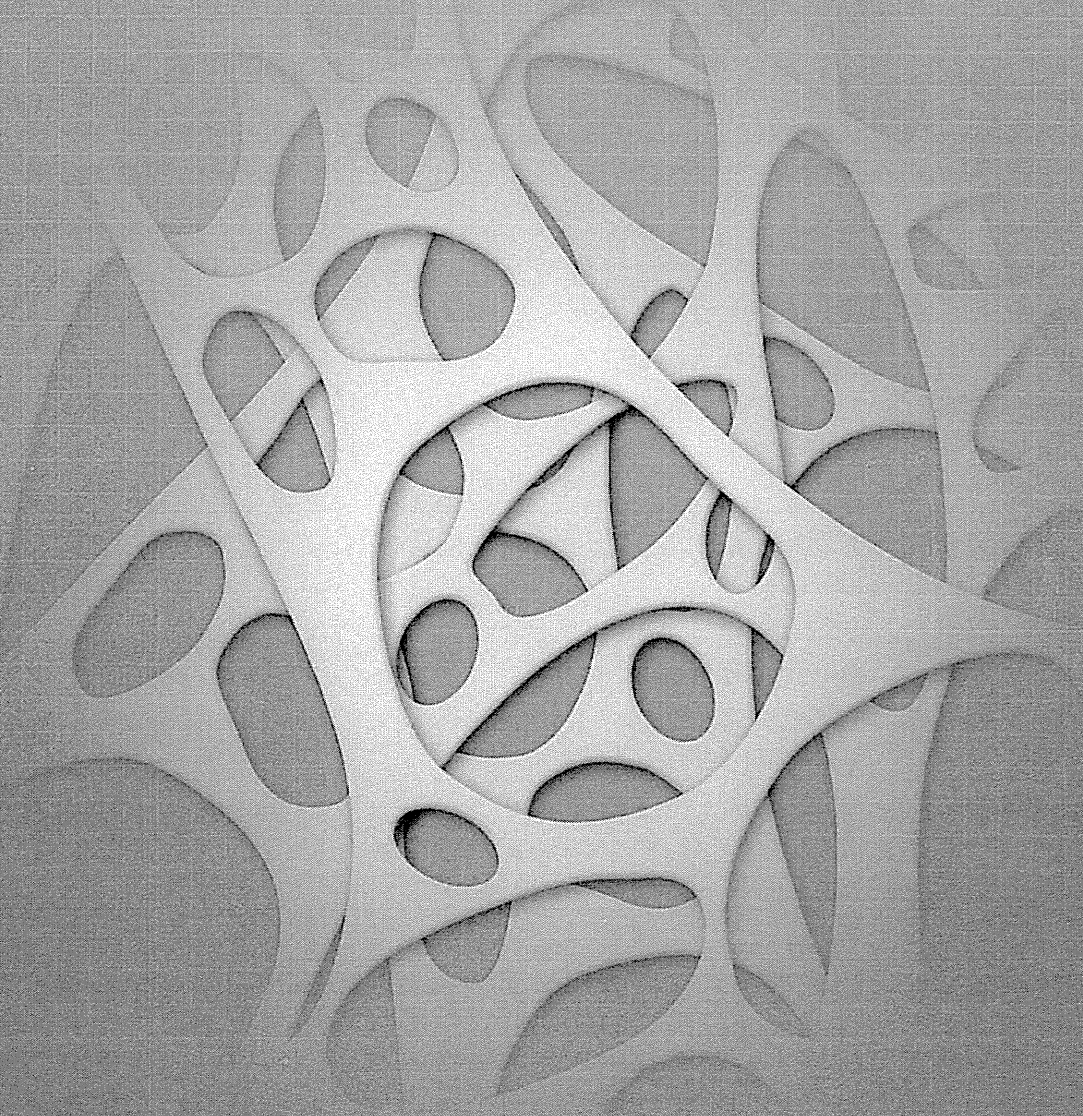

ライフサイエンス出版

表Ⅰ　エビデンスの基準(レベル)

Ⅰ	システマティックレビュー / メタアナリシス
Ⅱ	1つ以上のランダム化比較試験による
Ⅲ	非ランダム化比較試験による
Ⅳa	分析疫学的研究(コホート研究)
Ⅳb	分析疫学的研究(症例対照研究, 横断研究)
Ⅴ	記述研究(症例報告やケース・シリーズ)
Ⅵ	患者データに基づかない, 専門委員会や専門家個人の意見

(Minds診療ガイドライン作成の手引き2007)

表Ⅱ　推奨の強さの分類(グレード)

A	行うよう強く勧められる
B	行うよう勧められる
C	行うよう勧めるだけの根拠が明確でない
D	行わないよう勧められる

(福井・丹後による「診療ガイドラインの作成手順ver.4.3」2001年)

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ライフサイエンス出版株式会社(日本骨粗鬆症学会雑誌「Osteoporosis Japan」編集部)

J Nutr Sci Vitaminol, **57**, 274–279, 2011

Vitamin K_1 (Phylloquinone) or Vitamin K_2 (Menaquinone-4) Induces Intestinal Alkaline Phosphatase Gene Expression

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(Received December 20, 2010)

Summary Alkaline phosphatase (ALP) hydrolyzes a variety of monophosphate esters into inorganic acid and alcohol at a high optimum pH (pH 8–10). Previously, we identified a significant increase of intestinal ALP (IAP) activity in the rat intestine on long-term dietary vitamin K supplementation. However, it was unclear whether the induction of ALP gene expression was caused by vitamin K intake. In the present study, we examined the effects of vitamin K on IAP gene expression. A total of 21 male ICR strain mice (7 wk old) were divided into three groups: control, PK, and MK groups. Mice were orally administered a 0.1-mL solution of physiological saline in the control group, phylloquinone (3 mg/kg mouse) in the PK group, and menaquinone-4 (3 mg/kg mouse) in the MK group. Four hours after administration, we determined the ALP activity of the intestinal mucosa in three areas (duodenum, jejunum, and ileum). In the MK groups, the levels of ALP activity in the jejunum increased significantly compared with the control. Moreover, reverse transcription-polymerase chain reaction (RT-PCR) analysis using specific primers revealed that IAP mRNA expression was significantly enhanced in the jejunum in both PK and MK groups. Interestingly, vitamin K administration also increased the expression of pregnane X receptor mRNA. This is the first report concerning IAP mRNA expression induced by oral administration of vitamin K. The results support the possible involvement of vitamin K in the regulation of IAP mRNA expression as a novel pharmacological effect of vitamin K.

Key Words alkaline phosphatase, phylloquinone, menaquinone, mice, intestine

Alkaline phosphatase (ALP, EC 3.1.3.1) is an enzyme containing zinc which hydrolyzes monophosphate esters into inorganic phosphoric acid and alcohol at a high optimal pH (pH 8–10). The enzyme is distributed widely throughout the living world from bacteria to animals, excluding plants, and it exists in various tissues such as the intestine, liver, kidney, bone, placenta, stomach, and leukocytes.

In humans, four kinds of ALP isozyme have been identified: tissue-nonspecific ALP (liver/bone/kidney: TNSALP), intestinal ALP (IAP), placental ALP, and germ cell ALP (*1–4*). The TNSALP gene is located on chromosome 1 and consists of 12 exons and 11 introns, with the coding sequence beginning in the second exon.

A single gene for human IAP has been isolated, and the multiple forms of mRNA encoding human IAP are due to differences in polyadenylation (*2*). Although most species express a single IAP, several kinds of IAP have been identified in three species: the mouse (*5*), rat (*6, 7*), and cow (*8*).

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In rats, ALP is classified into two types: TNSALP and IAP. IAP is present in the membrane surrounding neutral fat droplets in the microvilli of the intestinal mucosa during fat absorption, and is thought to transport dietary lipids from the intestinal tract into the circulation as a component of unilamellar membranes called surfactant-like particles (SLPs) (*9*). Two different cDNA clones, IAP-I and IAP-II, for rat IAP were isolated by Lowe et al. (*6*) and Strom et al. (*7*), respectively. Strom et al. found that the expression of IAP-II mRNA was specifically enhanced by $1\alpha,25(OH)_2D_3$ administration. The two isozymes are products of two distinct genes and their cDNA sequences show 79% homology at the amino acid level. Functional differences between IAP-I and IAP-II were suggested by the differing regulation of the expression of the two mRNAs (*10*), as well as by structural and catalytic differences (*11*).

In mice, five different ALP loci have been identified: TNSALP, IAP, embryonic ALP (EAP), *Akp6*, and *Akp-ps1*. These ALP genes code for different proteins: *Akp2* encodes TNSALP, *Akp3* encodes IAP, *Akp5* encodes EAP, *Akp6* encodes a novel IAP-like isozyme expressed globally in the gut (thus called gIAP), and *Akp-ps1* encodes

the inactive pseudo-type ALP (5, 7).

Previously, we reported the enhanced effects of vitamin K on IAP activity in rats (12). Sprague-Dawley rats (6 wk old) were divided into three groups: a control (AIN-93M diet), phylloquinone (PK: 600 mg/kg diet), and menaquinone-4 (MK-4: 600 mg/kg diet) diet group. After 3 mo of feeding of vitamin K, the animals were fasted overnight. The small intestine was removed and divided into five segments. In each segment, both PK and MK-4 increased IAP activity (12).

Vitamin K acts as a cofactor for γ-glutamyl carboxylase (GGCX), and is well-known to participate in the activation of blood coagulation factors and bone mineralization (13). All forms of vitamin K have 1,4-naphthoquinone as a common ring structure, and natural vitamin K exists in two molecular forms, vitamin K_1 (phylloquinone: PK) and vitamin K_2 (menaquinone: MK-*n*). PK is abundant in green vegetables in a compound with a phytyl side chain. Vitamin K_2 is classified into MK-1–14 due to the repeat structure of the side chain, with isopren comprising the side chain. MK-4 shows marked physiological activities as a vitamin K, and is included in many animal-based foods such as meat. Recent studies have demonstrated the possibility that vitamin K regulates the expression of bone-related genes such as ALP through steroid X receptor (SXR, also termed pregnane X receptor: PXR) (14).

In the present study, we examined whether the enhancing effect of PK or MK-4 administration on IAP activity occurs via the intestinal mucosa directly, and we revealed the effects of the oral administration of PK or MK-4 on the expression of IAPs (*Akp3* and *Akp6*) and PXR in the mouse intestine.

MATERIALS AND METHODS

Experimental animals. The care and use of mice in the present study followed the guidelines of governmental legislation in Japan on the proper use of laboratory animals, and the study protocol was approved by the Institutional Review Board of Japan Women's University. A total of 21 male ICR strain mice (7 wk old) were used (31.1±0.2 g). They were fasted overnight with free access to water. On the following day, the animals were given 0.1 mL of solution via an intragastric tube: vehicle (physiological saline) for the control group (Cont.), PK (3 mg/kg mouse) for the PK group, and MK-4 (3 mg/kg mouse) for the MK group. The molecular weights of PK ($C_{31}H_{46}O_2$: MW=450.7) and MK-4 ($C_{31}H_{40}O_2$: MW=444.7) are very similar. PK and MK-4 were kindly supplied by Eisai Co., Ltd. (Tokyo, Japan).

Serum and tissue sampling. Four hours after administration, blood was collected from the abdominal aorta under ether anesthesia, and perfusion with saline was performed until the liver was blanched, in order to minimize the blood contamination of tissue samples. The small intestine was removed and divided into three regions. From the pylorus, we took the first 1 cm as the duodenum, and then separated the remaining part into the jejunum and ileum. The segments were slit longitudinally, rinsed with ice-cold saline, and scraped from the mice just after dissection. Each sample was homogenized using a Polytron homogenizer (Kinematica, Switzerland) with 10 mM Tris-buffered saline containing 1% Triton X-100 (pH 7.3) and 1 mM phenylmethylsulfonylfluoride (PMSF). The supernatant obtained after centrifugation at 7,000 ×*g* for 15 min was used as the enzyme extract.

Enzyme assay. ALP activity was determined with 10 mM *p*-nitro-phenylphosphate as a substrate in 100 mM 2-amino-2-methyl-1,3-propanediole HCl buffer containg 5 mM $MgCl_2$, pH 10.0, at 37°C, as previously reported (15). To analyze the biochemical properties of ALP, an inhibitory assay using levamisole (Lev) and L-phenylalanine (L-Phe) and a thermostability assay were performed, as previously described (15).

The enzyme activity was defined as the rate of hydrolysis of *p*-nitro-phenylphosphate and expressed in units (U=μmol *p*-nitro-phenol formed/min).

Protein concentrations were determined using BCA protein assay reagent (Pierce, Rockford, IL, USA).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Polyacrylamide gel (7.5%) electrophoresis in the presence of sodium dodecyl sulfate (SDS) was carried out according to the method of Weber et al. (16). After electrophoresis, ALP isozymes separated in the gel were stained by the coupling of β-naphthyl-phosphoric acid monosodium salt with Fast Violet B salt (17).

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR). Total RNA from the intestinal mucosa was extracted employing the acid guanidinium thiocyanate-phenol-chloroform method (18). As a template for PCR, single-strand cDNA was prepared from 1 μg of total RNA using Ready-to-go You-Prime First-Strand Beads (Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, England). PCR primers were used for *Akp3* (19), *Akp2* (20), *Akp5* (21), and PXR (22). In order to detect *Akp6*, sense (*Akp6*-up) and anti-sense (*Akp6*-down) primers were designed on the basis of the *Akp6* nucleotide sequence (GenBank: NCBI sequence data: AK008000). *Akp6*-up spans nucleotide positions 882–902 and *Akp6*-down spans 1,333–1,354 (23). The PCR conditions were as follows: 5 cycles at 94°C (1 min), 50°C (1 min), and 72°C (1 min), and 25 cycles at 94°C (30 s), 55°C (30 s), and 72°C (30 s), followed by 10 min at 72°C. Negative controls were performed with each RT-PCR reaction, omitting the template. The efficiency of reverse transcription was verified by the detection of GAPDH (glyceraldehyde-3-phosphate dehydrogenase, forward: 5′-ACC ACA GTC CAT GCC ATC AC-3′, reverse: 5′-TCC ACC ACC CTG TTG CTG TA-3′), as previously described (23).

The amplified samples were analyzed using 5.25% polyacrylamide gel electrophoresis (PAGE). The gels were stained with ethidium bromide and observed under UV light. The band intensity on PCR photographs was quantified by densitometry (AE6920M, ATTO, Tokyo, Japan). The PCR product was normalized to the intensity of the band for the house-keeping gene GAPDH, and is expressed as a ratio of the relative band intensity.

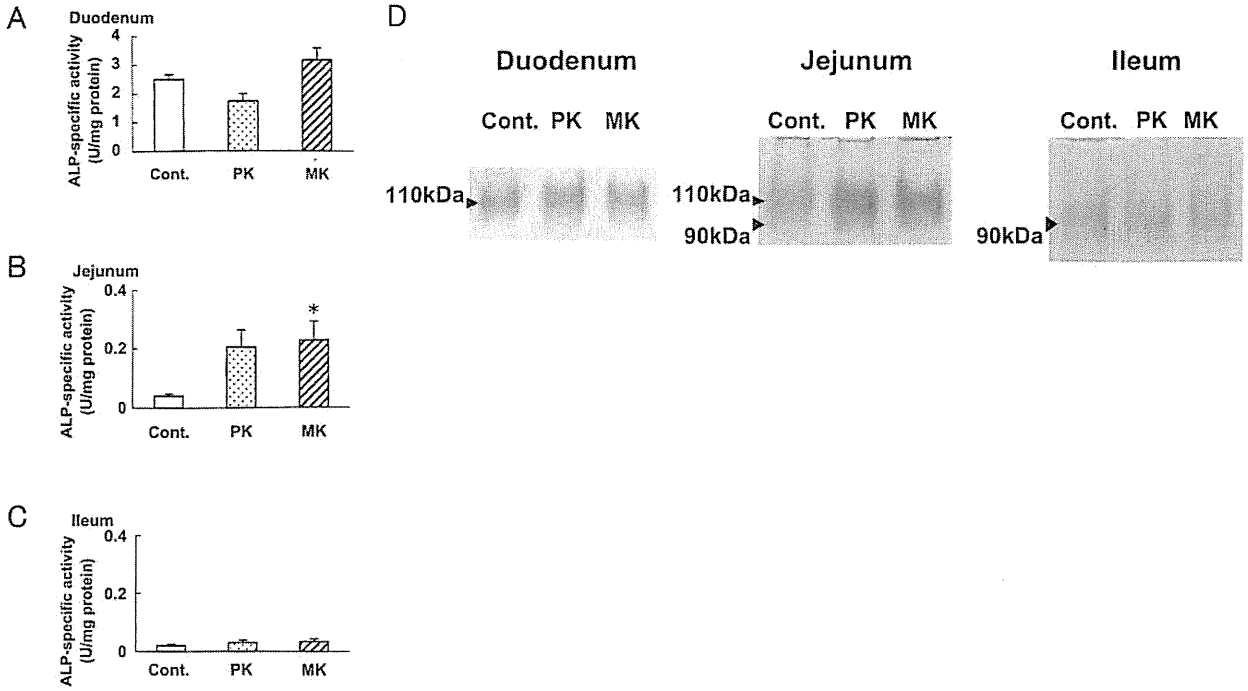

Fig. 1. ALP-specific activities of the duodenum (A), jejunum (B), and ileum (C). Results are the mean±SE of 7 animals. Significant difference between the MK and control groups (*$p<0.05$). D: Mouse intestinal ALP isozymes separated by polyacrylamide gel electrophoresis. The gels was stained for ALP activity with a β-naphtyl-phosphoric acid monosodium salt, Fast violet B salt. Cont.: control, PK: phylloquione, MK: menaquinone-4.

Statistical analyses. Values are shown as the mean± standard error (SE).

Dunnet's multiple comparison test was used after ANOVA to compare the significance of differences among the control and PK or MK. Differences were considered significant at $p<0.05$. Analysis was conducted using SPSS 18.0J (SPSS, Inc., Chicago, IL, USA).

RESULTS

ALP activity

To examine whether IAP was secreted from the intestinal mucosa on the oral administration of PK or MK, we measured the levels of serum ALP activity (mU/mL) in the control, PK, and MK groups, being 13.7±1.2, 10.3±2.0, and 14.1±1.8 (mean±SE), respectively, showing no significant differences among these groups.

ALP-specific activities in the intestine are shown in Fig. 1. There were no significant differences in ALP activities among these groups in the duodenum (Fig. 1A) and ileum (Fig. 1C). As presented in Fig. 1B, ALP activity of the MK group in the jejunum was significantly higher compared with the control group ($p<0.05$).

Molecular weight determination by SDS-PAGE

The molecular weights of ALPs of each intestinal segment were estimated employing SDS-PAGE analysis. As shown in Fig. 1D, the 110-kDa band of the major ALP isozyme was detected in the duodenum among these groups. In the jejunum, ALP enzymes were separated into two bands of 110 and 90 kDa, and the intensity of their enzymatic activity increased markedly in both PK and MK groups, similarly to the results regarding the specific ALP activity in the jejunum. In the distal part of the intestine (ileum), the ALP isozyme showed a main band of 90 kDa among these groups. No additional band was observed in any intestinal samples among the groups.

Table 1. Inhibitory effects of levamisole, L-phenylalanine and heat inactivation of ALP preparations of the jejunum.

Groups	Relative activity(%)		
	Levamisole (1 mM)	L-Phenylalanine (20 mM)	Heat inactivation (60°C 10 min)
Cont.	95.2±1.6	23.0±1.0	42.4±3.4
PK	94.2±2.1	23.5±1.4	46.1±2.3
MK	95.5±1.6	24.2±2.0	45.9±2.5

Each value represents mean±SE ($n=7$).
The ALP activity was assayed based on the rate of *p*-NPP hydrolysis. The effect of the inhibitor was determined in the presence of 5 mM $MgCl_2$ in the assay mixture. Remaining ALP activity with inhibitors or after heat treatment is expressed as a percent of non-treated controls. Results are the mean±SE of 7 animals.

Properties of ALP in the intestine

The enzymatic properties of ALP preparations of the jejunum which increased significantly on PK or MK administration were investigated employing an inhibition experiment with levamisole (Lev) and L-phenylalanine (L-Phe) and through a thermo-stability test. It is well known that IAP activity is not inhibited by Lev and is more stable to L-Phe and more heat-stable than TNSALP. As shown in Table 1, there was no significant

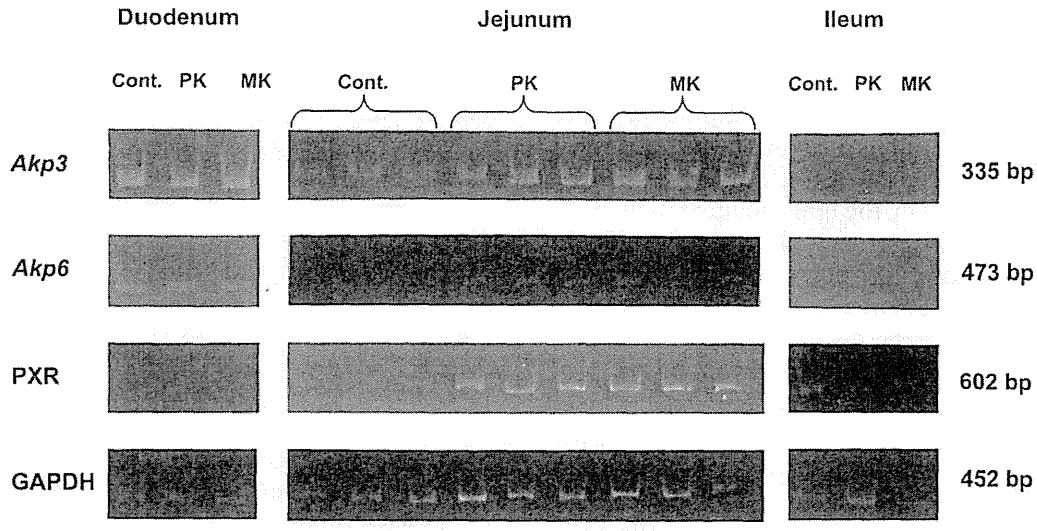

Fig. 2. Detection by RT-PCR of mRNAs for *Akp3*, *Akp6*, PXR, and GAPDH in the duodenum, jejunum, and ileum. PCR products were electrophoresed in a 5.25% polyacrylamide gel. Cont.: control, PK: phylloquinone, MK: menaquinone-4.

difference in the relative activities among these groups. We confirmed that these jejunum ALP preparations were effectively inhibited by L-Phe but not by Lev, and were heat-stable (60°C, 10 min), corresponding to the property of other mammalian intestinal type ALPs.

RT-PCR analysis of ALP mRNA expression

RT-PCR-based detections of mRNA for *Akp3*, *Akp6*, and PXR in the duodenum, jejunum, and ileum are shown in Fig. 2. The PCR products of *Akp3* (335 bp) were detected in the duodenum and jejunum. The PCR products of *Akp6* (473 bp) were detected in the duodenum, jejunum, and ileum. The PCR products of PXR (602 bp) were detected in the duodenum, jejunum, and ileum. The intensities of mRNA expression of *Akp3* and *Akp6* were very similar both in the duodenum and ileum among the three groups (Cont., PK, and MK groups). The intensities of mRNA expression of *Akp3*, *Akp6*, and PXR were enhanced in both the PK and MK groups compared with the control group in the jejunum.

PCR products of *Akp2* (198 bp) and *Akp5* (500 bp) were not detected in any of these intestinal samples.

In order to compare these intensities of mRNA expression in the jejunum, we determined the relative density of the PCR products of the mouse jejunum. As shown in Fig. 3A, the intensities of *Akp3* expression increased significantly in the PK group compared with the control group ($p<0.01$). Furthermore, the intensities of *Akp6* expression were also increased in both the PK and MK groups compared with the control group ($p<0.01$, $p<0.05$, respectively) (Fig. 3B). Interestingly, the intensities of PXR expression in both the PK and MK groups were significantly higher than in the control group ($p<0.05$, $p<0.01$, respectively) (Fig. 3C).

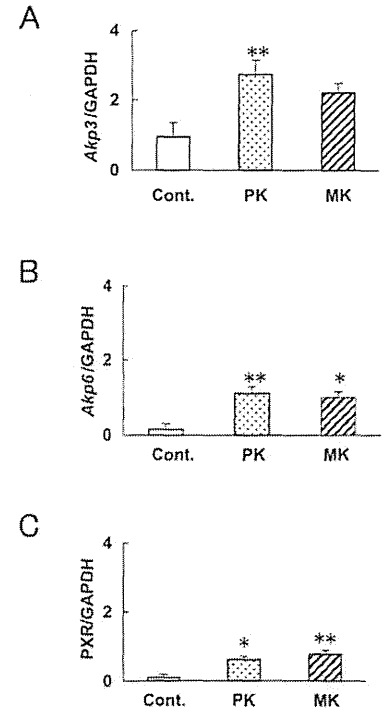

Fig. 3. The relative density of PCR products in the mouse jejunum. The diagrams show the relative density of the PCR products. A: The relative density of PCR products for *Akp3*. B: The relative density of PCR products for *Akp6*. C: The relative density of PCR products for PXR. Results are the mean±SE of 4 animals. Significant difference between the PK or MK and control groups (*$p<0.05$, **$p<0.01$). Cont.: control, PK: phylloquinone, MK: menaquinone-4.

DISCUSSION

Previously, we reported that several dietary factors such as fat-feeding, vitamin K, and lactose increased IAP activities in rats (*12*, *15*, *24*). The high-level activity of IAP, which localizes at the brush border of intestinal epithelium cells, suggests the participation of this enzyme in the transport of nutrients.

Recently, we reported that both long-term dietary PK and MK-4 supplementations enhance IAP activity in rats (*12*). After 3 mo of feeding, we measured IAP activity by dividing it into five segments. In each segment, both PK and MK-4 increased IAP, and the level of IAP activity in the proximal jejunum was significantly

higher than that in the control group ($p<0.05$) (*12*). To examine whether the enhancing effect of PK or MK-4 on IAP activity occurs via the intestinal mucosa directly, we performed an oral administration of PK or MK-4 using mice. In the present study, we discovered that the levels of mouse jejunum ALP activity were also significantly increased by the oral administration of PK or MK compared with the control group (Fig. 1B). In addition, we confirmed that the increased ALP isozymes induced by the oral administration of PK or MK showed similar biochemical properties to the typical intestinal type ALP, with no significant differences among these groups (Table 1).

By SDS-PAGE analysis, we detected a 110-kDa ALP enzyme in the duodenum and 90-kDa ALP enzyme in the ileum (Fig. 1D). Both the 110- and 90-kDa ALP enzymes were detected in the jejunum, and the enzymatic activities of these bands were enhanced by the oral administration of PK or MK. The product of the *Akp3* gene was expressed specifically in the duodenum, and the product of *Akp6* was expressed through the small intestine (*5*). Therefore, we considered that the 110- and 90-kDa ALP enzymes may correspond to the IAPs encoding *Akp3* and *Akp6*, respectively.

We then performed RT-PCR analysis in order to examine the expression of IAPs (*Akp3* and *Akp6*) in the mouse jejunum. PCR products for *Akp3* and *Akp6* mRNAs in the jejunum were detected, and a significant increase in the PCR products of *Akp3* due to the oral administration of PK was observed (Fig. 3A). Moreover, a significant increase in the PCR products of *Akp6* due to the oral administration of PK or MK-4 was also observed (Fig. 3B). These results suggest that the induction of *Akp3* and *Akp6* may be regulated by PK or MK-4.

As the results of RT-PCR, the expression of mRNA for PXR was detected in the duodenum, jejunum and ileum, and it was enhanced significantly in both the PK and MK groups in the jejunum compared with the control group (Fig. 3C). Interestingly, the intestinal segment where the expression of mRNA for PXR by vitamin K had been enhanced corresponded to a similar segment where the expression of mRNA for IAP was enhanced.

Recent studies have revealed that vitamin K functions as a ligand for nuclear steroid and xenobiotic receptor (SXR), as well as a cofactor for γ-carboxylase (*25*). SXR is expressed predominantly in the liver and intestine, and it regulates transcription such as of cytochrome P450 (CYP) 3A4, which is an enzyme involved in drug metabolism, and MDR1 (multidrug resistance protein 1) which is activated by a diverse array of pharmaceutical agents including taxol, rifampisin, and clotrimazole (*26, 27*). Ichikawa et al. identified novel SXR target bone-related genes that were regulated by MK-4 in osteoblastic cells using microarray analysis (*28*). Among extracellular matrix-related genes, they demonstrated that a small leucin-rich repeat proteoglycan, tsukushi, contributes to collagen accumulation (*28*).

We demonstrated for the first time that the oral administration of vitamin K (both PK and MK-4) enhanced the level of IAP mRNA expression in the mouse intestine, and PXR mRNA expression also increased. Further studies on the physiological functions of ALP and transcriptional regulation of ALP induction will provide useful data on the novel effect of vitamin K.

Acknowledgments

This work was partially supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (No. 20500725).

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Biochem Genet (2012) 50:389–396
DOI 10.1007/s10528-011-9483-z

Association of *CYP19* Gene Polymorphism with Vertebral Fractures in Japanese Postmenopausal Women

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Received: 18 March 2011 / Accepted: 17 November 2011 / Published online: 9 December 2011

Abstract This study investigates aromatase gene polymorphism, which might influence bone strength in terms of mineral density and quality. We explored the relationship between *CYP19* polymorphisms and vertebral fractures in postmenopausal Japanese women. In addition, we compared estrogen and testosterone levels in Japanese postmenopausal women with and without fractures. Osteoporotic postmenopausal women showed higher incidences of vertebral fractures than osteopenic women or women with normal lumbar bone mineral density (L2-4 BMD). Estrogen concentrations in postmenopausal women were associated with BMD; however, no association was found between sex hormone levels and the presence of fractures. The C allele rs2470152 was significantly associated with increased risk of vertebral fractures ($P = 0.04$), whereas none of the *CYP19* polymorphisms showed differences in sex steroid levels between subjects with and without fractures. Allelic variants of aromatase genes appear to interact to influence the risk of vertebral fractures in postmenopausal Japanese women.

Keywords Aromatase gene polymorphisms · Vertebral fractures · Postmenopausal women

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Introduction

Osteoporosis is caused by multiple factors, including environmental factors (such as calcium intake), exercise, and estrogen levels. The main source of estrogen in postmenopausal women is the aromatization of androgenic precursors, a reaction catalyzed by the cytochrome P450-(CYP) aromatase enzyme, encoded by *CYP19* located on chromosome 15q21.1. It has recently been reported that estrogen levels are genetically determined by aromatase activity (Olson et al. 2007; Haiman et al. 2007; Sowers et al. 2006). In addition, allelic variants of the aromatase gene have been associated with bone mineral density (BMD) and bone fractures (Hong et al. 2007; Masi et al. 2001; Somner et al. 2004). A/G polymorphisms in the 3′ untranslated region (UTR) and the I.2 promoter (rs10046 and rs1062033; Rinancho et al. 2005) and an A/G polymorphism in the I.6 promoter rs4775936 (Enjuanes et al. 2006) of the aromatase gene have been studied in relation to osteoporosis and BMD, but the results remain controversial. In addition, an rs2470152 polymorphism in the aromatase gene has been shown to affect serum estrogen levels in Swedish men (Eriksson et al. 2009). Therefore, in order to analyze the association with the risk of vertebral fractures in postmenopausal women, we conducted a cross-sectional study of the interaction between *CYP19* gene polymorphisms and sex steroid hormone levels or risk of vertebral fractures in Japanese postmenopausal women. In this study, we focused on four markers (rs2470152, rs4775936, rs1062033, and rs10046) to clarify the association between polymorphisms in aromatase genes and vertebral fractures.

Materials and Methods

Study Subjects

Three hundred sets of genomic DNA and serum samples were provided from the collected samples of the Institute of Medical Sciences, Tokyo University, obtained for tailor-made medicine realization projects. These samples were collected from the various institutions that were members of these projects following the approval of the individual ethics committees. Ethical approval was obtained from the Ethics Committee of the Leading Project for Personalized Medicine in the Institute of Medical Science, University of Tokyo, and the Tokyo Metropolitan Geriatric Hospital. Another 300 DNA samples were collected from women for the purpose of analyzing the relationship between polymorphisms and the etiology of disease in the Japanese population. The samples were provided by the Leading Project for Personalized Medicine of the Ministry of Education, Culture, Sports, Science and Technology, Japan.

The samples were divided into three categories according to the *T* score of the measurement of lumbar spine BMD (L2-4 BMD) by dual energy X-ray absorptiometry (DXA) as defined by the World Health Organization: *T* scores of −1.0 and above were classified as normal BMD, scores of −2.5 to −1.0 were considered osteopenia, and scores below −2.5 were considered osteoporotic.

For assessment of vertebral fractures, anteroposterior and lateral X-ray examinations of the thoracic and lumbar spine were performed. Morphometrically,

a vertebral fracture was defined in terms of the ratio of the anterior height of vertebral body to the posterior height (below 0.75) or the ratio of the center height to the anterior or posterior height (below 0.8). In all cases, the vertebral fractures were evaluated by two groups of radiologists and geriatricians in each institute.

Sex Steroid Assay

The serum levels of testosterone (T) and estradiol (E_2) were measured by mass spectrophotometry (LC–MS/MS). Bioavailable testosterone and estrogen, which includes the free form and the albumin-binding form, were measured by LC–MS/MS (Arai et al. 2010). Serum samples were stored at −70°C until analyzed. For statistical analysis, the values were transformed into logarithmic form, since the values are exponential and the distributions of T and E_2 levels were skewed using the raw data.

Genotype Analysis

We examined four polymorphisms of *CYP19*: rs1062033, a G/C SNP located at around exon 1.2 (at position chromosome 15, 49335230); rs10046, a T/C SNP located in the 3′ UTR (at position chr.15, 49290276); rs4775936, a C/T SNP located in the vicinity of exon 1.6 (at position chr.15, 49323314); and rs2470152, a T/C SNP located in intron 1 (at position chr.15, 49382254). These SNPs were identified by searching the National Center for Biotechnology Information (NCBI) database because they are analyzable by the readily available TaqMan assays used for disease association studies (Applied Biosystems). Polymorphisms in genomic DNA were measured by the TaqMan assay. Age, body mass index, and years since menopause were examined in three SNP genotypes among four *CYP19* markers.

Statistical Analyses

Chi-square analysis was used to compare the numbers of osteoporosis, osteopenia, and normal patients by T scores of L2-4 BMD with and without fractures. Similarly, each parameter was compared among the three genotypes in four *CYP19* markers using ANOVA. The correlation between estradiol levels and L2-4 BMD was shown using Pearson's coefficients. The associations between aromatase gene polymorphisms and vertebral fracture risk were compared by Chi-square analysis using SPSS software.

Results

Bone Density Data

There were significantly more women with fractures than without among patients with osteoporosis ($T < -2.5$; $P < 0.05$), and there was no significant increase in fractures among normal patients or those with osteopenia. There were no differences

in the log estradiol (Log E_2) or log testosterone (Log T) values between women with fractures and those without fractures (Table 1).

Relationship Between L2-4 BMD and Estrogen level

Log E_2 levels in postmenopausal women were significantly associated with L2-4 BMD ($r = 0.21$, $p = 0.03$; Fig. 1), whereas log T levels showed no association (data not shown).

Genotype Analysis

When we examined the correlation between the four polymorphisms (rs2470152, rs1062033, rs4775963, and rs10046) and vertebral fractures in postmenopausal women, we found a significant correlation for rs2470152 ($P = 0.04$) but not for the

Table 1 Bone mineral density of postmenopausal women with and without fractures

T score[a] & sex steroids	Women without fractures (137)	Women with fractures (138)	P
$T < -2.5$	18	37	0.015
$-2.5 \leq T < 1.0$	12	13	NS
$-1.0 \leq T$	4	5	NS
Log E_2 (pg/ml)	0.335 ± 0.383	0.327 ± 0.330	NS
Log T (ng/dl)	2.033 ± 0.367	2.067 ± 0.247	NS

[a] Bone mineral density was measured in 89 of the 275 subjects

NS not significant

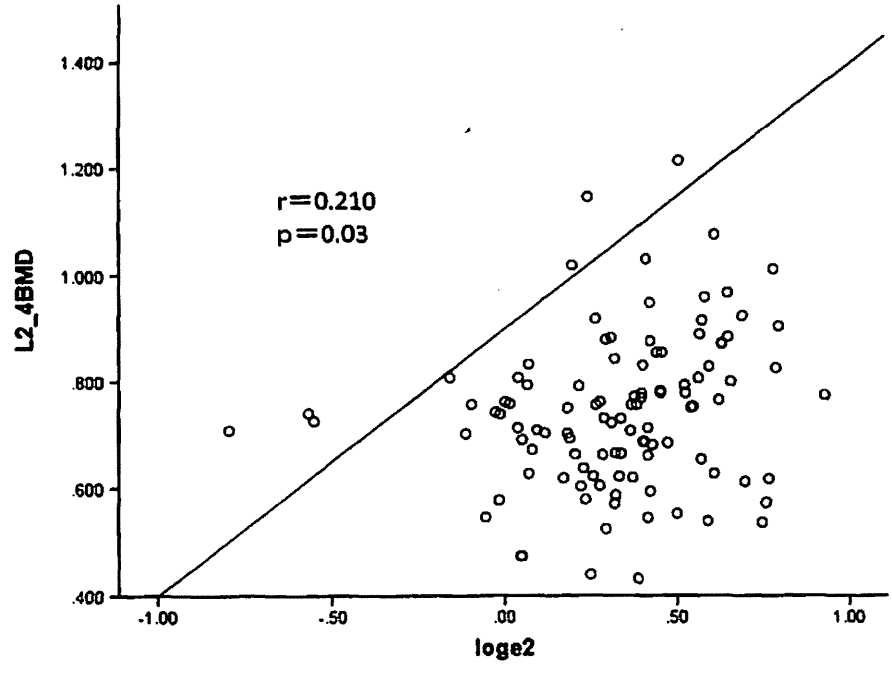

Fig. 1 Correlation between log E_2 and L2-4 BMD in postmenopausal women. Estrogen levels were significantly correlated with L2-4 BMD ($r = 0.21$, $p = 0.03$)

Table 2 Correlation of four *CYP19* SNPs with vertebral fractures

SNP	Genotype	Premenopause		*P*	Postmenopause		*P*
		Control ($n = 19$)	Case ($n = 6$)		Control ($n = 136$)	Case ($n = 138$)	
rs2470152	CC	9	1	0.408	44	47	0.040[a]
	TC	8	4		56	71	
	TT	2	1		36	20	
rs4775936	CC	5	1	0.712	48	59	0.416
	CT	9	4		68	59	
	TT	5	1		20	20	
rs1062033	CC	4	1	0.693	47	56	0.507
	CG	9	4		64	62	
	GG	6	1		25	20	
rs10046	TT	7	1	0.550	31	26	0.324
	TC	8	4		67	62	
	CC	4	1		38	50	

[a] Only rs2740152 polymorphisms of the aromatase gene showed a significant correlation with vertebral fractures ($P = 0.04$)

Table 3 Characteristics of postmenopausal Japanese women and three SNPs of rs2470152

Characteristic	Genotype			*P*
	CC	CT	TT	
Age (years)	72.8 ± 9.3	73.6 ± 8.3	74.3 ± 6.8	NS
Body mass index (kg/m^2)	22.4 ± 4.6	21.8 ± 4.5	21.1 ± 4.3	NS
Years since menopause	24.2 ± 9.5	24.3 ± 10.3	25.4 ± 11.6	NS
Log E_2 (pg/ml)	0.302 ± 0.319	0.330 ± 0.280	0.307 ± 0.357	NS
Log T (ng/dl)	2.001 ± 0.347	2.067 ± 0.269	2.050 ± 0.313	NS
L2-4BMD (110)	0.753 ± 0.135	0.739 ± 0.157	0.729 ± 0.118	NS
LT score (89)	−2.6 ± 1.2	−2.6 ± 1.3	−3.3 ± 0.8	NS

NS not significant

other three polymorphisms (Table 2). There were no differences in age, body mass index, or years since menopause among the three SNP types in the four *CYP19* markers (Table 3).

Discussion

We examined the relationship between aromatase-related genes and vertebral fractures by analyzing *CYP19* gene polymorphisms in Japanese women. Among four markers, no differences were found in serum T and E_2 concentrations in the Japanese postmenopausal women. It is possible that local E_2 concentrations are

more important in local tissues rather than serum levels. Bone cells are able to express aromatase and other enzymes required for estrogen synthesis locally (Janssen et al. 1999; Shouzu and Simpson 1998; Watanabe et al. 2004), and aromatase activity in cultured osteoblasts is quantitatively similar to that in adipose stromal cells (Shouzu and Simpson 1998). Thus, estrogen synthesized in bone cells might be important in postmenopausal bone metabolism.

Eriksson et al. (2009) found that genetic variants of rs2470152 in aromatase are associated with E_2 levels, showing that G alleles were correlated with higher serum E_2 levels and BMD in Swedish men than other alleles. Our results, however, showed that the C allele of rs2470152 is associated with vertebral fractures, a finding that suggests that ethnicity, race, and sex differences might influence the results of SNP studies in osteoporosis. The SNP rs2470152 is located in the region of the I.4 promoter (Bulun and Simpson 1994), and it is interesting that the G→A transition of rs2470152 is likely to alter a potential binding site for the binding protein of the transcription factor cAMP response element. The major reason for the discrepancy between our results and those of the Swedish study may be gender differences. The Swedish study focused only on male cohorts. We could not detect any disequilibrium between rs2470152 and the other three markers viewed in HapMap.

CYP19 SNPs (rs10046) were found to be associated with differences in E_2 levels in the European Prospective Investigation of Cancer-Norfolk (EPIC-Norfolk) cohort study (Dunning et al. 2004). SNP rs10046 explains 1.6% of the variance in the E_2:T ratio; however, this SNP is not associated with breast cancer risk (Dunning et al. 2004). The rs10046, a T/C SNP located in the 3′ UTR, 19 nucleotides downstream from the translation terminus, has been reported to be associated with increased levels of aromatase mRNA expression in tumors (Gruber et al. 2002). In our study, rs10046 was correlated with neither serum E_2 levels nor vertebral fractures. The *CYP19* genotypes demonstrated higher mRNA levels at the rs1062033 locus in postmenopausal osteoporosis. *CYP19* is regulated in a different manner and in different tissues by a hormonally controlled promoter or adipose stromal cell promoter (Mahendroo et al. 1993; Harada et al. 1993). Genetic polymorphisms of *CYP19* might be involved in other processes, such as mRNA stabilization, transcription enhancement, or the post-translational regulation of expression. Neither SNP 1062033 nor rs4775936 was significantly correlated with either serum E_2 levels or vertebral fractures.

We could not detect lower levels of bioavailable serum E_2 by LC–MS/MS in rs2470152; however, another group has shown differences in E_2 levels as measured by RIA according to *CYP19* genotype in a study that included both premenopausal and postmenopausal women (Somner et al. 2004). The discrepancy between the two studies seems to be due to the assay systems used. Bioavailable estrogen levels in postmenopausal women are more relevant than total estrogen levels, which include E_2 bound by sex hormone-binding globulin (SHBG), for bone metabolism. Despite the absence of differences in estrogen levels among the various genotypes, we found that vertebral fracture rates are associated with the *CYP19* genotype in postmenopausal Japanese women in this study. There is much evidence for the role of aromatase activity in bone homeostasis (Miyaura et al. 2001; Oz et al. 2000), and, as previously described, the pharmacological inhibition of aromatase is also associated

with a decrease in BMD and increased risk of fractures (Eastell and Hannon 2005). This indicates that aromatase in local tissues plays roles, both physiologically and pathologically, in bone metabolism.

In conclusion, we provide statistical evidence that the C allele in rs2470152 of the *CYP19* gene is associated with an increased risk of vertebral fractures in postmenopausal Japanese women. Further studies are necessary to detect functional SNPs that induce differences in bone metabolism. Furthermore, we need more participants to detect differences in E_2 levels based on the *CYP19* SNPs of aromatase genes.

Acknowledgment This work was supported by the Japanese Osteoporotic Foundation with funds donated by Eli Lilly, Japan, in 2008.

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Effectiveness of the Salivary Occult Blood Test as a Screening Method for Periodontal Status

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Background: Community-based periodontal examinations are not popular despite the high prevalence of periodontal disease among adults. This study examines the effectiveness of a novel salivary occult blood test (SOBT) as a screening method for periodontal status.

Methods: Comprehensive health examinations were conducted in adult residents aged ≥40 years in Hisayama, Fukuoka, Japan; 1,998 subjects, each with ≥20 teeth, were analyzed. A paper test strip was used to perform the SOBT and was followed by a periodontal examination. Results were ranked as negative or positive. Subjects with ≥15% of teeth with bleeding on probing (BOP) or ≥1 tooth with a probing depth (PD) ≥4 mm were defined as having a poor periodontal status. The relationship between the results of the SOBT and periodontal parameters and among other variables was examined.

Results: The sensitivity and specificity of the SOBT in screening for poor periodontal status were 0.72 and 0.52, respectively. In a multivariate logistic regression analysis, the results of the SOBT were significantly associated with the proportion of teeth with BOP and the proportion of teeth with PD ≥4 mm, independent of age, sex, use of antihypertensive medication, use of antidiabetic medication or insulin therapy, and the number of decayed or filled teeth.

Conclusion: The SOBT may offer a simple screening method for periodontal status when a thorough periodontal examination is not possible, although it is not sufficiently specific to be a reasonable substitute for a periodontal examination. *J Periodontol 2011;82:581-587.*

KEY WORDS

Epidemiology; mass screening; periodontal disease; saliva.

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Periodontal disease is prevalent in the adult population.[1] Regular periodontal maintenance therapy had been reported to prevent the progression of tissue destruction in patients with chronic periodontitis, including in patients who smoke.[2] However, in 2004, only 32.7% of Japanese adults aged ≥20 years had received a dental checkup.[3] A screening test for periodontal status may trigger more frequent dental visits.

A clinical diagnosis of periodontitis requires an evaluation by a trained examiner and evidence of gingival inflammation, loss of connective tissue surrounding the teeth measured by a clinical examination using a periodontal probe, and bone loss detected by radiography.[4] However, it may be difficult to introduce such thorough periodontal examinations in health-maintenance procedures in large-scale populations. A more readily applied periodontal screening method is desirable.

Salivary occult blood tests (SOBTs) have been evaluated primarily as a screening method for gingivitis.[5-9] However, a recently developed SOBT was reported to discriminate subjects with a poor periodontal condition, which was defined as bleeding on probing (BOP) in ≥20% of teeth or a probing depth (PD) ≥6 mm plus BOP in ≥1 teeth.[10] Because this SOBT uses a paper strip coated with anti-human hemoglobin

doi: 10.1902/jop.2010.100304

monoclonal antibody, the dietary effects of hemoglobin from other animal species can be avoided.[11]

The aim of the present study is to examine the effectiveness of the new SOBT in screening for periodontal status in a large-scale population.

MATERIALS AND METHODS

Study Population

Study participants were recruited in 2007 in the town of Hisayama, which is a suburb of the Fukuoka metropolitan area in southern Japan. The town registry listed 3,810 residents, aged 40 to 79 years; 2,861 (75.1%) of these individuals provided written consent to participate in the study and underwent a comprehensive examination. Dental and medical examinations were performed on 2,669 subjects, including edentulous individuals. There were no pregnant females among the subjects. All subjects who had ≥20 teeth were included in the study; 671 subjects who had <20 teeth or who were not able to provide sufficient data were excluded because of the inherent difficulties of properly assessing the periodontal health of these individuals; data on individuals with fewer teeth indicated that they are susceptible to poor periodontal conditions.[12] Thus, the final study sample consisted of 1,998 subjects (888 males and 1,110 females).

The ethics committee of Kyushu University Faculty of Dental Science approved the study design, data collection methods, and procedure for obtaining informed consent (approval 20B-1).

SOBT

The SOBT[§] was carried out in all subjects between 8:00 am and 11:00 am before the oral health examination. Each subject had refrained from eating and drinking since the previous night but toothbrushing status before saliva collection could not be confirmed. The SOBT immunologically detects human hemoglobin in saliva by using a colloidal gold-labeled anti-human hemoglobin monoclonal antibody. The SOBT was carried out in the sitting position in an examination room. Each subject rinsed his or her mouth for 10 seconds with 3 ml distilled water, which was then spat into a small paper cup. The lower end of the paper test strip containing the colloidal gold-labeled antibody was dipped into the sample. The colloidal gold-labeled antibody dissolved in the solution where it reacted with human hemoglobin in the sample to form an immune complex. The immune complex moved along the test strip by capillary action until it was trapped by an anti-human hemoglobin monoclonal capture antibody immobilized on the upper portion of the strip and resulted in a magenta line. This type of immunochromatography assay is often used as a point-of-care test.[13,14] After 5 minutes, an examiner (YS, SA, TT, and YS and Miki Kawada, Noriaki Kamio, Masaki Yasui, Nao Fukui, and Mikiko Tomioka, Faculty of Dental Science, Kyushu University, Fukuoka, Japan) visually ranked the magenta-stained band according to a color chart as follows: no visible band = negative (manufacturer's reference concentration: <2 μg/ml human hemoglobin), and a visible magenta band = positive (manufacturer's reference concentration: ≥2 μg/ml human hemoglobin). The procedure and criterion of the SOBT were explained to each examiner beforehand, although the examiner reliability of the test was not verified.

Oral Health Examination

Each subject received an oral health examination after the SOBT in a supine position under sufficient artificial light on a normal dental chair or a portable dental chair. Based on the method of the Third National Health and Nutrition Examination Survey,[15] one of nine dentists (YS, SA, TT, and YS and Miki Kawada, Noriaki Kamio, Masaki Yasui, Nao Fukui, and Mikiko Tomioka, Faculty of Dental Science, Kyushu University, Fukuoka, Japan) performed a periodontal examination, based on the standardized probing technique, by using a periodontal probe[‖] and examined PDs and clinical attachment loss (AL) on the mesiobuccal and mid-buccal sites of all retained teeth, except for third molars because partially impacted third molars frequently have pseudopockets. Examiner reliability for the PD assessment was verified by an interexaminer calibration of volunteers who had similar characteristics to the study population; Cohen κ value was >0.8, which indicated very good interexaminer agreement. The presence of BOP was defined as teeth exhibiting gingival bleeding within a few minutes after probing periodontal pockets. Because the relation to the SOBT was similar in PDs and clinical AL but slightly stronger in PDs than in clinical AL, this study used PD and BOP as periodontal parameters to examine the relationship between the SOBT and periodontal inflammatory findings.

General Examination

A blood sample was collected from the antecubital vein in the morning after an overnight fast and analyzed for fasting plasma glucose concentrations. Each participant completed a self-administered questionnaire in advance that included smoking habit (never, former, or current) and medication use; the questionnaire was checked by trained nurses (Miki Inenaga, kanako Mochimatsu and Terumi Kakumori, Hisayama, Fukuoka, Japan). A former smoker was defined as a subject who quit smoking >1 year before the day of the examination.

§ Perioscreen Sunstar, Osaka, Japan.
‖ PCP11, Hu-Friedy, Chicago, IL.

Statistical Analyses

The percentage of teeth with BOP (%BOP) and the percentage of teeth with PD ≥4 mm (%PD) were divided into three categories from the distribution of data. The relationship between both periodontal parameters was then examined. Differences were evaluated with the Pearson χ^2 test, and linearity was evaluated with the Mantel-Haenszel χ^2 test. Periodontal status was defined based on the following periodontal parameters: poor = %BOP ≥15% or %PD >0%, and healthy = %BOP <15% and %PD = 0%. The relationship between SOBT and periodontal status was examined, and the sensitivity, specificity, and positive and negative predictive values of the SOBT were calculated.

Logistic regression analyses were performed to determine the effects of %BOP and %PD and other variables on the SOBT level, calculating the odds ratio and 95% confidence interval. Age, sex, smoking habit, number of teeth, number of decayed or filled (DF) teeth, fasting plasma glucose level, use of antihypertensive medication, use of lipid-lowering medication, and use of antidiabetic agent or insulin therapy were included as confounding variables in a step-wise multivariate logistic regression analysis. P <0.05 indicated statistical significance. Statistical analyses were performed using a software program.¶

RESULTS

For 51.9% of subjects, the %PD was 0%. The remaining subjects were divided in one-half: >0% PD <10% and %PD ≥10% (24.1%). The %BOP was <15% in 59.7% of subjects, and the rest of subjects were divided in one-half: ≥15% BOP <30% (20.9%) and ≥30% (19.4%). Table 1 shows the relationship between the %BOP and %PD. The periodontal parameters were positively associated with each other.

Table 2 shows the relationship between SOBT and periodontal status. Using negative or positive as a cutoff, the sensitivity for detecting poor periodontal status was 0.72, and the specificity value was 0.52. The positive and negative predictive values were 0.69 and 0.55, respectively.

Table 3 shows the effects of periodontal parameters and other variables on SOBT. The %BOP, %PD, age, sex, smoking habit, fasting plasma glucose level, use of antihypertensive medication, use of lipid-lowering medication, use of antidiabetic agent or insulin therapy, number of teeth, and number of DF teeth were significantly associated with SOBT by simple logistic regression analyses (Table 3). In a step-wise multivariate logistic regression analysis, %BOP, %PD, age, sex, use of antihypertensive medication, use of antidiabetic agent or insulin therapy, and number of DF teeth were each independently associated with SOBT (Table 3).

DISCUSSION

This study examined the effectiveness of a recently developed SOBT that uses an anti-human hemoglobin monoclonal antibody as a simple screening method for periodontal status. The test showed a reasonable level of sensitivity in the identification of subjects with gingival bleeding and/or deep periodontal pockets.

Several screening tests without a direct examination of periodontal tissue have been explored. One of these methods, the validity of self-reported periodontal status, was demonstrated.[16-19] Although this method can be easily and inexpensively applied, a single questionnaire item has not been shown to be effective in detecting poor periodontal status.[16,17] Increased efficacy was achieved with a combination of multiple questions and the assessment of conventional risk indicators,[18,19] but cutoffs are difficult to determine, and cross-population generalizations are problematic.

Saliva can be readily and non-invasively obtained, and there are significant opportunities to advance the development of salivary biomarkers for periodontal disease.[20] In a study[21] that examined the level of β-glucuronidase activity in saliva, the sum of sensitivity and specificity to screen the subjects with four or more sites with PD ≥5 mm was ≈120%. In another study,[22] of the various biochemical markers in saliva examined, the salivary lactate dehydrogenase (LDH) level showed the highest sensitivity (0.66) and specificity (0.67) for the presence of BOP or PD ≥4 mm. Finally, an examination[23] in pregnant women of a combination of LDH, alkaline phosphatase, and a different SOBT from the one used in the present study showed that the sensitivity and specificity in screening subjects with Community Periodontal Index (CPI) codes 3 or 4 were 89% and 62%, respectively. However, when SOBT was used alone,[23] the sensitivity and specificity were 37% and 91%, respectively. Methods examining various salivary biomarkers are applicable to large populations because they are rapid, and sample collection does not require a dental specialist. However, the instruments and reagents needed for analyses introduce cost and time requirements.

The SOBT used in the present study is an existing saliva test and has some advantages. It can be used on large numbers of subjects without the assistance of dental professionals. The results are available quickly, and the cost is low (the present SOBT would cost ≈US$1.3 per capita). In previous studies, a different type of SOBT showed significant correlation with the gingival index[5,6] and with crevicular fluid flow,[6] and another SOBT showed a significant correlation with the PMA index in adolescents[7,8] but not in adults

¶ SPSS version 17.0, IBM, Tokyo, Japan.