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A polymorphic variant at the Werner helicase (*WRN*) gene is associated with bone density, but not spondylosis, in postmenopausal women

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Abstract Werner syndrome (WS) is a rare autosomal recessive progeroid syndrome characterized by the premature onset of multiple age-related disorders. The gene responsible for WS has been identified as *WRN*, a member of the RecQ family of helicase genes. Based on the fact that patients with WS exhibit osteoporosis and osteoarthritis, the present study was undertaken to clarify the contribution of the *WRN* gene to the etiology of these two common age-related disorders in normal postmenopausal women. We investigated the association of a *WRN* gene polymorphism, namely c.4330 T → C leading to an amino acid substitution from Cys to Arg, with bone density and lumbar spondylosis score in unrelated Japanese postmenopausal women ($n = 377$). Genotypic frequencies of T/T, T/C, and C/C were 87.5%, 12.2%, and 0.3%, respectively. Bone density of the lumbar spine (L2–4) was significantly lower in women carrying the minor C allele than in noncarriers ($P = 0.037$). When bone density was expressed by the *Z* score after being adjusted by age and weight, carriers of the C allele showed lower values not only in the lumbar spine, but also in the total body ($P = 0.015$ and 0.042, respectively). The association study with spondylosis in postmenopausal women ($n = 221$) revealed that this polymorphism was not related to the severity of spondylosis expressed by the Kellgren–Lawrence score at any disk level of the lumbar spine (L2/3–L5/S1). These findings indicate that the *WRN* gene may be a candidate for the genetic regulation of osteoporosis, but not spondylosis, in normal Japanese postmenopausal women.

Key words aging · osteoporosis · osteoarthritis · gene

Introduction

Aging is one of the most important factors contributing to the incidence and progression of various diseases. Osteoporosis and osteoarthritis, including spinal spondylosis, are common skeletal disorders associated with age-related changes in bone and cartilage. Recent investigations have disclosed the involvement of genetic factors in these diseases [1,2].

Osteoporosis is a systemic disorder of decreased skeletal mass, as measured by bone density, and by disturbed skeletal architecture and function that results in an increased risk for bone fractures with consecutively increased morbidity and mortality. Because twin and sibling studies have revealed that the proportion of variance of bone density accounted for by genetic factors is around 50%–90% [3–5], it is clear that variation in bone density between individuals is determined largely by genetic factors. Linkage studies for whole-genome screening have defined multiple loci that regulate bone density, but the genes responsible for these effects remain to be defined [6–8]. Population-based studies and case-control studies have similarly identified polymorphisms in several candidate genes that have been associated with bone density: genes for vitamin D receptor, estrogen receptor, collagen Iα1, calcitonin receptor, peroxisome proliferator-activated receptor-γ, Heremans Schmidt-glycoprotein, and transforming growth factor (TGF)-β1 [1]. However, this is controversial and genetic components of osteoporosis are not completely understood.

Osteoarthritis is also considered a collective result of heterogeneous etiopathologic factors affecting cartilage, the most prominent of which are disorders in joint biochemistry or biomechanics [9]. Family studies have suggested that not only does osteoarthritis of the knee and hand have a strong genetic component, but so does that of the spine, spondylosis, with an increased prevalence in first-degree relatives of affected individuals [2,10]. A population-based study of twins demonstrated a clear genetic effect for radiographic osteoarthritis of the knee and hand in women, with 39%–65% of the variance being explained by genetic factors [10]. Several recent investigations have suggested mutations or poly-

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morphisms in several genes associated with either the development or severity of osteoarthritis; such genes include those encoding type II procollagen, type XI collagen, the vitamin D receptor, insulin-like growth factor (IGF)-I, and TGF-β [2]. However, the genetic susceptibility to osteoarthritis, including spondylosis, is not fully understood.

Werner syndrome (WS) is a rare autosomal recessive progeroid syndrome characterized by the premature onset of multiple age-related disorders, such as atherosclerosis, cancer, non-insulin-dependent diabetes mellitus, and ocular cataracts [11]. WS patients also show osteoporosis with impaired bone formation [12–17] and osteoarthritis in peripheral joints [12,18]. The gene responsible for WS has recently been identified at the Werner helicase locus (*WRN*) [19,20]. The *WRN* mutation involves a member of the RecQ family of helicases and may perturb DNA replication, repair, recombination, transcription, or chromosomal segregation [21]. Homozygous mutations, all of which result in the truncation of the protein, lead to WS. However, little is known about the role of *WRN* in common age-related diseases. Eight polymorphic variations of the *WRN* gene have been identified in the course of screening of *WRN* mutations: four missense and four conservative polymorphisms [22]. A single study showed that a polymorphism at amino acid 1367 Cys(TTG)/Arg(CTG) is associated with a variation in the risk of myocardial infarction among a Japanese population [23]. This polymorphism is caused by a nucleotide substitution: c.4330 T → C at the *WRN* locus that is a restriction fragment length polymorphism (RFLP) with *Pam*CI endonuclease. To examine the possible contribution of the *WRN* gene to age-related bone and cartilage disorders, we investigated the association of the RFLP with bone density and spondylotic changes in Japanese postmenopausal women.

Materials and methods

Patients

Genotype analysis by RFLP was performed using genomic DNA extracted from peripheral blood samples obtained from 377 healthy postmenopausal Japanese women living in Nagano Prefecture (age range 41–91 years; mean (±SD) age 65.6 ± 9.3 years). Exclusion criteria included endocrinological disorders (e.g., hyperthyroidism, hyperparathyroidism, diabetes mellitus), liver or renal diseases, use of medications known to affect bone metabolism (e.g., estrogen, bisphosphonates, vitamin D, calcium supplements, corticosteroids, anticonvulsants, heparin), and unusual gynecological history. All patients were unrelated volunteers who gave informed consent before inclusion in the study. The study was approved by the committee for ethics in the Research Institute and Practice for Involutional Diseases. For the association study with spondylotic changes in the lumbar spine, 221 women (age range 45–91 years; mean age 63.5 ± 8.2 years) of the 377 women enrolled in the study, for whom anteroposterior and lateral X-rays of the lumbar spine were available, were analyzed.

Measurements of phenotypes

Bone mineral density (BMD; mg/cm^2) of the second through to the fourth lumbar spine (L2–L4) and of the total body was measured by dual-energy X-ray absorptiometry (DPX-Lp; Lunar, Madison, WI, USA). This parameter was also expressed as a *Z* score that is a deviation from the weight-adjusted average BMD of each age based on the data of 20000 Japanese women installed in Lunar DPX-L. The severity of spondylotic changes at L4/5 and L5/the first sacrum (S1) was graded on a five-point scale (0–4) according to the Kellgren–Lawrence scoring system [24] on a lateral radiograph of the lumbar spine under standardized conditions.

The following biochemical parameters were measured: serum concentrations of calcium (Ca), phosphate (P), alkaline phosphatase (ALP), intact osteocalcin (I-OC; Elisa, Teijin, Tokyo, Japan), N-fragment osteocalcin (N-OC; Teijin), parathyroid hormone (PTH), and calcitonin (CT). The Ca/creatinine ratio, P/creatinine ratio, pyridinoline (Pyr; HPLC method), and deoxypyridinoline (Dpyr; HPLC method) in the urine were also measured.

Genomic DNA analysis

Genomic DNA samples were extracted from peripheral leukocytes using standard procedures and samples subjected to polymerase chain reaction (PCR) amplification. Genomic DNA (0.1 μg) was amplified using *Taq* DNA polymerase (PE Biosystem, CA, USA) with the primer 5′-GCCTAATCAGAATGTTAGTT-3′ and antisense primer 5′-TCAGTATTGATGCCTACCTC-3′. PCR was performed through 30 cycles by the following steps: denaturation at 94°C for 30 s, annealing at 54°C for 30 s, and extension at 72°C for 90 s (PE-9700; PE Biosystem). The PCR product was digested with *Pma*CI (Takara, Shiga, Japan), separated on 3.0% agarose gel, and visualized by ethidium bromide staining. Genotype was determined by the presence or absence of the *Pma*CI site in the PCR product amplified from the genomic DNA. The *Pma*CI site was present in the C allele (coding for Arg), but not in the T allele (coding for Cys). Therefore, the C allele yielded 101-bp and 92-bp fragments, while the T allele yielded a single 193-bp fragment.

Table 1. Background parameters and serum/urine markers of carriers and non-carriers of the minor C allele

	Subjects for bone density (n = 377)		Subjects for spondylosis (n = 221)	
	C (−)	C (+)	C (−)	C (+)
Number (frequency) of women	330 (87.5%)	47 (12.5%)	189 (85.5%)	32 (14.5%)
Age (years)	62.8 ± 0.61	61.2 ± 1.90	66.4 ± 0.65	63.9 ± 1.72
Years after menopause	14.6 ± 0.58	13.4 ± 1.50	17.1 ± 0.69	14.7 ± 1.66
Body weight (kg)	50.9 ± 0.46	53.5 ± 1.18	50.0 ± 0.57	53.5 ± 1.59
Height (cm)	150.7 ± 0.34	151.5 ± 1.14	149.9 ± 0.43	151.4 ± 1.41
Ca (mg/dl)	9.12 ± 0.02	9.15 ± 0.06	9.14 ± 0.03	9.16 ± 0.07
P (mg/dl)	3.47 ± 0.03	3.61 ± 0.06	3.49 ± 0.04	3.63 ± 0.07
ALP (IU/l)	168.8 ± 3.20	173.2 ± 9.73	171.8 ± 3.94	171.0 ± 9.4
Intact osteocalcin (ng/ml)	7.37 ± 0.25	7.35 ± 0.53	7.65 ± 0.30	7.33 ± 0.61
N-fragment osteocalcin (ng/ml)	14.6 ± 0.44	15.1 ± 1.01	14.8 ± 0.51	14.8 ± 1.11
Intact PTH (pg/ml)	37.7 ± 1.04	34.6 ± 1.82	36.3 ± 1.03	33.7 ± 2.13
Calcitonin (pg/ml)	23.4 ± 0.70	24.3 ± 1.71	23.1 ± 0.76	23.3 ± 2.25
$1,25(OH)_2D_3$ (pg/ml)	33.3 ± 0.72	35.1 ± 1.76	33.5 ± 0.89	35.0 ± 1.98
$25(OH)D_3$ (ng/ml)	21.1 ± 0.36	19.9 ± 0.86	21.6 ± 0.49	19.6 ± 1.06
Pyridinoline (pmol/μmol Cre)	33.7 ± 0.73	31.3 ± 1.35	35.4 ± 0.89	32.8 ± 1.40
Deoxypyridinoline (pmol/μmol Cre)	7.19 ± 0.16	6.83 ± 0.31	7.40 ± 0.18	7.00 ± 0.38

Data are the mean ± SD
Ca, calcium; P, phosphate; ALP, alkaline phosphatase; PTH, parathyroid hormone; Cre, creatinine

Statistical analysis

Comparisons of BMD, the Z score, biochemical markers, and the Kellgren–Lawrence score between carriers and non-carriers of the minor C allele were performed using non-parametric analysis (Student's t-test; StatView-J 4.5, SAS, Cary, NC, USA). $P < 0.05$ was considered statistically significant.

Results

Genotypic frequency of the WRN polymorphism, and its association with background data and serum/urine markers

For the association study with bone density, 377 unrelated Japanese postmenopausal women were genotyped by RFLP at the *WRN* locus. The number (frequency) of women of each of the genotypes T/T, T/C, and C/C, was 330 (87.5%), 46 (12.2%), and 1 (0.3%), respectively. For the association study with spondylotic changes, 221 of 377 women for whom lumbar spine X-rays for Kellgren–Lawrence scoring were available were analyzed. In this population, the number (frequency) of each of the genotypes above was 189 (85.5%), 31 (14.0%), and 1 (0.5%), respectively. The genotypic distribution was not different between the two populations, and was similar to that reported previously in a Japanese population [23]. Because only one woman showed a homozygous CC genotype, statistical analyses were performed by comparing carriers of the minor C allele (heterozygous CT genotype and homozygous CC genotype) and non-carriers (homozygous TT genotype; Table 1). Background parameters, such as age, years after menopause, body weight and height, serum markers, such as Ca, P, ALP, intact osteocalcin, N-OC, intact PTH, CT, $1,25(OH)_2D_3$, $25(OH)_2D_3$, and urine markers, such as Pyr and Dpyr, did not differ between carriers and non-carriers of the C allele. All values for the woman with the homozygous CC genotype were within the mean ± 1SD values in both populations.

Association of the WRN polymorphism with bone density of the lumbar spine and total body

To determine the possible association of the *WRN* gene with osteoporosis, bone density of the lumbar spine (L2–4) and the total body was compared between carriers and non-carriers of the C allele (Fig. 1). Bone density was expressed as the raw BMD and the Z score after being adjusted by age and weight. A significant difference was seen in the lumbar spine BMD, and the C allele was associated with low BMD ($P = 0.037$). This association was stronger when bone density was expressed by the Z score ($P = 0.015$). In analysis of the bone density of the total body, the C allele was also associated with the Z score ($P = 0.042$), although not with the raw BMD ($P = 0.203$). The Z score of the woman with the homozygous CC genotype was −0.392 in the lumbar spine and 0.153 in the total body, both values being less than the mean values of women carrying the C allele. These results indicate that the *WRN* RFLP is correlated with bone density in postmenopausal women.

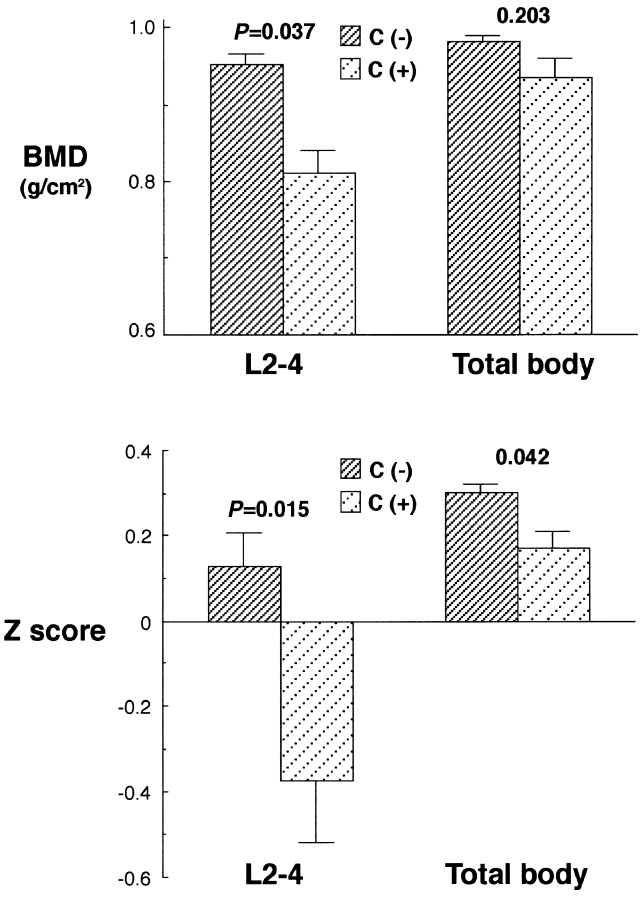

Fig. 1. Association of the *WRN* polymorphism with bone density in postmenopausal women (n = 377). Bone density of the lumbar spine (*L2–L4*) and the total body was compared between women carrying the minor C allele (*C(+)*; heterozygous CT genotype and homozygous CC genotype, n = 47) and non-carriers (*C(−)*; homozygous TT genotype, n = 330). Bone mineral density (*BMD*) was measured by dual-energy X-ray absorptiometry, and was also expressed as a Z score, which is a deviation from the weight-adjusted average BMD of each age. Data are expressed as the mean ± SEM. P values for the differences between carriers and non-carriers are given

Association of the WRN polymorphism with lumbar spondylosis

We further investigated the association of the *WRN* RFLP with the severity of spondylotic changes at each disc level (L2/3–L5/S1) of the lumbar spine, expressed as the Kellgren–Lawrence score, in 221 postmenopausal women (Fig. 2). No significant difference in spondylosis score was seen at any disc level between carriers and non-carriers of the C allele. The scores of the woman with the homozygous CC genotype were within the mean ± 1SD values at any disc level. These results indicate that the *WRN* RFLP is not correlated with lumbar spondylosis in postmenopausal women.

Discussion

Based on the fact that patients with WS exhibit osteoporosis and osteoarthritis, the present study investigated the possible contribution of the *WRN* gene to the etiology of these two common age-related disorders in normal postmenopausal women. A missense polymorphism, namely c.4330 T → C of the *WRN* gene, was associated with bone density of the lumbar spine and the total body; however, it was not related to lumbar spondylosis. These results provide the first evidence indicating that the *WRN* gene may be involved in the etiology of an age-related bone disorder in normal subjects.

Of the polymorphic variations of the *WRN* gene [22], the present study focused on the c.4330 T → C for two reasons. First, this polymorphism is the only one that has been reported to be associated with a common disease, myocardial infarction, in a general population [23]. Second, this polymorphic site is close (by three amino acids) to the nuclear localization signal motif in the C-terminal region of the WRN protein. It has been shown that the loss of this C-terminal domain precludes the localization of the protein to the nucleus [25]. All known *WRN* mutations result in truncated gene products with loss of this C-terminal domain. This provides a satisfactory explanation for the fact that all known mutations act as null alleles and appear not to result in individually distinct phenotypes, despite the observation that individual mutations are found throughout *WRN*. Therefore, we speculated that the substitution of an amino acid (1367 Cys → Arg) due to the nucleotide variation in this region may alter the strength of the nuclear localization signal, thus providing a change of the potential of transport to the nucleus in response to various physiologic and pathologic challenges.

The mechanism whereby this polymorphism at the *WRN* gene affects bone density remains to be clarified. WS patients are known to show osteoporosis with impaired osteoblastic bone formation but normal osteoclastic bone resorption [14,15], which is different from bone changes with normal aging in the general population. In fact, Shiraki et al. reported that of the serum/urine markers, only the serum intact osteocalcin level was significantly lower in WS patients than in age-matched controls [16]. However, in the present study, the *WRN* polymorphism was not associated with serum/urine markers of bone turnover, including intact osteocalcin. It is speculated that the *WRN* gene may possibly have a stronger effect on other definite factors of bone density in the postmenopausal generation than on bone turnover, such as peak bone density and bone loss by menopause through the imbalance of formation and resorption. Although evidence for the expression and function of the WRN protein in fibroblasts has been

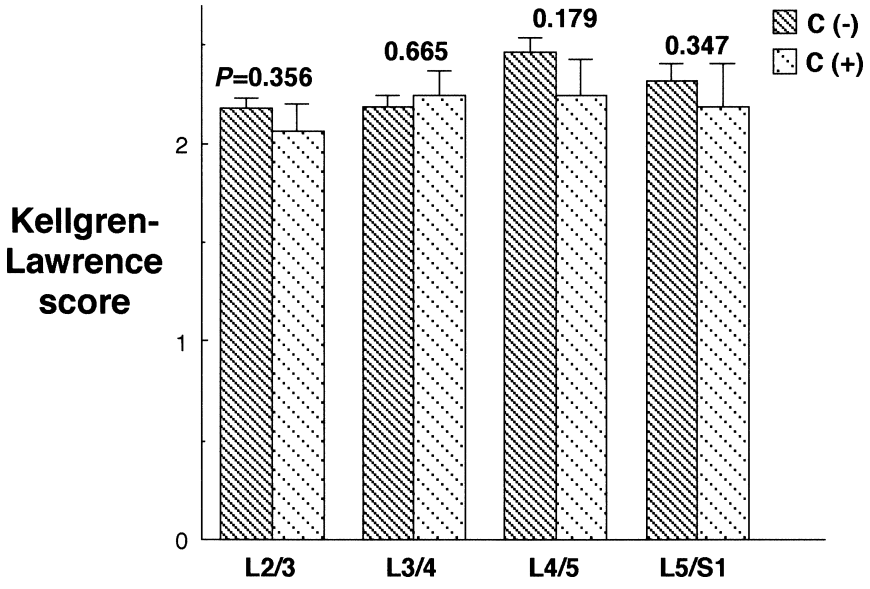

Fig. 2. Association of the *WRN* polymorphism with lumbar spondylosis in postmenopausal women (n = 221). Spondylotic change of the lumbar spine (*L2/3–L5/S1*) was compared between carriers (n = 32) and non-carriers (n = 189) of the minor C allele. The severity of spondylotic change at each disc level was graded on a five-point scale (0–4) according to the Kellgren–Lawrence scoring on a lateral radiograph of the lumbar spine. Data are expressed as the mean ± SEM. P values for the differences between carriers and non-carriers are given

accumulated [26,27], the expression and function of the WRN protein in bone cells, including osteoblastic cells, remain unknown. A report showing that treatment with recombinant human IGF-I increased bone volume in a patient with WS, osteoporosis, and a low serum IGF-I level suggests the participation of other factor(s), like IGF-I, in the signaling of the WRN protein in bone tissues [28]. Further knowledge of the role of the WRN protein in bone cells will help in the understanding of the direct and indirect mechanisms whereby the WRN polymorphism affects bone density.

Osteoporosis is a catabolic disease for bone metabolism, whereas osteoarthritis includes an anabolic aspect, such as subchondral sclerosis and osteophyte formation, implying the existence of some common genetic background causing these opposite phenotypes. In fact, several epidemiologic studies have shown that bone density in patients with osteoarthritis is greater than in controls [29–31]. However, in the present study, an allele that was associated with bone density was not associated with spondylotic change. This result suggests that the effect of the *WRN* gene on the development of one disease is, at least in part, independent of the other. Recent reports also support independent genetic backgrounds for these two diseases, although some common environmental factors, such as body weight, are known to adversely affect them [32].

It will be interesting, in any future studies of the role of *WRN* polymorphisms in health and disease, to evaluate haplotypes, rather than single-site polymorphisms. It would also be desirable to couple such studies with functional analyses of the various alleles. Such studies may have to wait for more information on the nature of the function of the WRN protein.

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