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ORIGINAL ARTICLE

Association of the formiminotransferase N-terminal sub-domain containing gene and thrombospondin, type 1, domain-containing 7A gene with the prevalence of vertebral fracture in 2427 consecutive autopsy cases

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We previously reported 2 osteoporosis-susceptibility genes—formiminotransferase N-terminal sub-domain containing gene (*FONG*) and thrombospondin, type 1, domain-containing 7A (*THSD7A*)—in which we identified two common single-nucleotide polymorphisms, rs7605378 (*FONG*) and rs12673692 (*THSD7A*). The former was associated with a predisposition to osteoporosis and the latter with bone mineral density. To further elucidate the importance of these polymorphisms in the pathogenesis of osteoporosis, we examined their association with the incidence of vertebral fracture. DNA extracted from the renal cortex of 2427 consecutive Japanese autopsies (1331 men, mean age: 79 years; 1096 women, mean age: 82 years) were examined in this study. The presence or absence of vertebral fracture during each subject's lifetime was determined by a thorough examination of the clinical records, as well as autopsy reports. After adjustments for sex and age at autopsy, logistic regression analysis revealed that homozygotes for the risk alleles of rs7605378 (A-allele) or rs12673629 (A-allele) possess an increased risk of vertebral fracture. The subjects simultaneously homozygous for both the risk alleles of rs7605378 (AA genotype) and rs12673629 (AA genotype) showed significantly higher risk of vertebral fracture (odds ratio 2.401, 95% confidence interval 1.305–4.416, $P=0.0048$) than those who had at least one non-risk allele of either rs7605378 (AC/CC genotypes) or rs12673629 (AG/GG genotypes). The results suggest that Japanese subjects homozygous for the risk alleles of rs7605378 and rs12673629 have a higher risk of vertebral fracture.
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INTRODUCTION

Osteoporosis is one of the most common skeletal diseases in the world, affecting more than 200 million individuals. With the increasing age of the population in developed countries, its prevalence is estimated to be markedly increasing.[1] Osteoporosis is clinically characterized by reduced bone mass and compromised bone strength, leading to an increased risk of fracture. Its diagnostic criteria of Japanese Osteoporosis Society includes reduced bone mineral density (BMD) $<70\%$ of young adult mean and/or the presence of fragility fracture.[2] As the BMD value does not necessarily represent bone strength and there is no convenient method to evaluate bone quality, the presence of fragility fracture is considered to be the hallmark of osteoporosis.

Like many other common diseases, multiple factors, including genetic variations, determine the predisposition for the onset or progression of osteoporosis, as has been indicated by genetic–epidemiological studies.[3,4] Numerous studies on genetic risks for osteoporosis have been performed to date, mainly using association studies and linkage analysis for assessing BMD as a quantitative trait.[5,6] Although BMD is an important predictor of fracture, knowledge of the history of fracture during a person's lifetime provides more direct and convincing evidence regarding bone

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fragility; therefore, a genetic association study of the incidence of fracture as an object variable is valuable.

Previously, in a genome-wide association study, we found a single-nucleotide polymorphism (SNP), rs7605378 on chromosome 2q33.1 that showed significant association with susceptibility to osteoporosis.[7] The SNP lies within a previously unknown gene, which we named formiminotransferase N-terminal sub-domain containing gene (*FONG*). We have also reported that an SNP (rs12673692) located in the gene thrombospondin, type 1, domain-containing 7A (*THSD7A*) displayed significant association with lumbar and femoral BMD.[8] However, the association between these genetic variations and fractures remains unclear. In the present study, we further confirmed the clinical significance of these SNPs in determining bone strength by examining their association with the incidence of vertebral fracture in 2427 consecutive Japanese elderly autopsy cases, in which complete patient history, as well as pathological data at autopsy were available.

MATERIALS AND METHODS

Subjects

The study protocol was approved by the ethics committee of Tokyo Metropolitan Geriatric Hospital. Written informed consent, including consent to use extracted DNA in medical and genetic studies, was obtained from the bereaved family of each subject before autopsy.

The study group comprised 2427 consecutive Japanese autopsy cases (1331 men, mean age 79 years; 1096 women, mean age 82 years), performed at Tokyo Metropolitan Geriatric Hospital between 1995 and 2011. None of the female subjects had been treated with hormone-replacement therapy. The presence or absence of any disease was determined by a thorough examination of the autopsy report. Most major diseases were included in the database.

Data regarding vertebral fracture

The diagnosis of vertebral fracture during each subject's lifetime was made based on the clinical records and, when the radiographic examination was not performed, the presence or absence of the fracture was confirmed by autopsy reports. Cases of traumatic fractures due to traffic accidents, as well as apparent pathological fractures caused by metastatic cancers were not included. In total, 193 cases of vertebral fractures were identified (male, 75 patients; female, 118 patients). It should be noted here that we could not thoroughly confirm that the observed vertebral fractures were truly fragility fractures due to osteoporosis since the measurement of vertebral BMD was not performed in most patients.

Genotyping

Total genomic DNA was extracted from the renal cortex by the phenol/chloroform method according to the standard protocol. Genotyping for rs7605378 in *FONG* and rs12673692 in *THSD7A* was carried out using a real-time PCR System (StepOnePlus, Applied Biosystems, Foster City, CA, USA) with the TaqMan SNP Genotyping Assay method, as previously described.[9] The SNP genotyping assay IDs C_11510522_10 and C_2560761_10 (Applied Biosystems) were used for rs7605378 and rs12673692, respectively. The genotyping success rate was 100% for rs7605378 and 99.8% for rs12673629 in the present study.

Statistical analyses

The allele frequency was calculated, and a χ^2-test was used to determine the deviation of the genotype distribution from the Hardy–Weinberg equilibrium. Multiple logistic regression analysis was conducted to estimate the odds ratio (OR) and 95% confidence interval (CI) for the association between vertebral fracture risk and the genotypes, sex and age. All statistical analyses were performed with SAS ver.9.2 (SAS Institute Inc., Cary, NC, USA). A two-sided *P*-value of <0.05 was considered statistically significant.

RESULTS

In this study, the percentage frequency distribution of the AA, AC and CC genotypes at rs7605378 was 31.4%, 50.3% and 18.3%, respectively, and that of AA, AG and GG genotypes at rs12673629 was 12.1%, 44.3% and 43.6%, respectively. The minor allele frequencies of rs7605378 (C-allele) and rs12673629 (A-allele) were 0.40 and 0.36 for subjects with vertebral fracture, and 0.43 and 0.34 for those without it, respectively. The genotype frequency distributions for both SNPs were in Hardy–Weinberg equilibrium.

As shown in Table 1, the prevalence of vertebral fracture was found to be higher in subjects homozygous for A-allele of rs7605378 as compared with those who bear at least one C-allele (AC and CC) in both sexes. Likewise, A-allele homozygote for rs12673629 showed higher prevalence of the fracture than the other genotype carriers (AG and GG) in both sexes.

As the effect of rs7605378 and rs12673629 on the prevalence of vertebral fracture was apparently the A-recessive genetic model, the fracture data for AA genotype were compared with those for the other genotypes using multiple logistic regression analysis considering sex and age at autopsy. As shown in Table 2, the OR for the risk of vertebral fracture in subjects homozygous for the risk allele of rs7605378 (A-allele) was 1.364 (95% CI: 1.002–1.858, $P<0.05$) as compared with those who had at least 1 C-allele (AC/CC). This indicated that A-allele homozygosity for rs7605378 was independently associated with the increased risk of vertebral fracture. Likewise, homozygous carriers of the risk allele of rs12673629 (A-allele) also showed higher OR for the risk of vertebral fracture than the other genotype carriers did (AA vs AG/GG, OR 1.452, 95% CI: 0.963–2.189, $P=0.0753$), although which was not statistically significant. As expected, the prevalence of vertebral fracture was higher in women than in men and it also increased as age at autopsy increased (Table 2).

As shown in Table 3, multiple logistic regression analysis, after adjustment for sex and age at autopsy, also revealed that the subjects simultaneously homozygous for both the risk alleles of rs7605378 (AA) and rs12673629 (AA) showed a significantly higher risk of vertebral fracture (OR 2.401, 95% CI: 1.305–4.416, $P=0.0048$) as

Table 1 Prevalence of vertebral fracture in subjects with different genotypes

			Vertebral fracture	n
rs7605378	Total	AA	72 (9.5)	761
		AC	87 (7.1)	1222
		CC	34 (7.7)	444
	Men	AA	30 (7.2)	418
		AC	36 (5.4)	665
		CC	9 (3.6)	248
	Women	AA	42 (12.2)	343
		AC	51 (9.2)	557
		CC	25 (12.8)	196
rs12673629	Total	AA	31 (10.6)	292
		AG	75 (7.0)	1070
		GG	87 (8.2)	1055
	Men	AA	15 (9.4)	160
		AG	25 (4.2)	589
		GG	35 (6.1)	575
	Women	AA	16 (12.1)	132
		AG	50 (10.4)	481
		GG	52 (10.8)	480

Number (percent) of subjects is presented.

Table 2 Multiple logistic regression analysis of the association between the prevalence of vertebral fracture and genotypes, sex and age

Factors	*OR (95% CI)*	P-*value*
rs7605378, AA vs AC/CC	1.364 (1.002–1.858)	<0.05*
rs12673629, AA vs AG/GG	1.452 (0.963–2.189)	0.0753
Sex, women vs men	1.729 (1.269–2.356)	<0.001*
Age, 1 year older	1.048 (1.029–1.067)	<0.001*

Abbreviations: CI, confidence interval; OR, odds ratio.
*$P<0.05$ (significant).

Table 3 Multiple logistic regression analysis of the association between the prevalence of vertebral fracture and the status of homozygosity for the risk alleles rs7605378 (A-allele) and rs12673629 (A-allele)

rs7605378	*rs12673629*	*OR (95% CI)*	P-*value*	n
AC/CC	AG/GG	1.000		1464
AA	AG/GG	1.272 (0.906–1.785)	0.1645	661
AC/CC	AA	1.218 (0.708–2.096)	0.4768	193
AA	AA	2.401 (1.305–4.416)	0.0048	99

Abbreviations: CI, confidence interval; OR, odds ratio.
Data were adjusted for sex and age at autopsy.

compared with those who had at least one non-risk allele of either rs7605378 (AC/CC) or rs12673629 (AG/GG).

The prevalence of vertebral fracture was negatively associated with that of ischemic heart disease ($P=0.0004$), myocardial infarction ($P=0.0039$), acute leukemia ($P=0.0055$), myelogenous leukemia ($P=0.0015$), and positively with that of Parkinson's disease ($P=0.0051$), valvular disease of the heart ($P=0.0171$) and multiple myeloma ($P=0.0006$); however, none of these diseases was associated with the genetic polymorphisms of *FONG* and *THSD7A*. The fracture prevalence was also found to be negatively associated with body height ($P<0.0001$) and body weight ($P=0.001$); both of which were not associated with the genetic polymorphisms. There was no association between vertebral fracture and renal/parathyroidal diseases, which sometimes affect calcium metabolism.

DISCUSSION

The present study has shown, for the first time, that SNPs in the genes *FONG* (s7605378) and *THSD7A* (rs12673692), which are novel osteoporosis-susceptibility genes that we have previously reported,[7,8] are associated with the prevalence of vertebral fracture in 2427 consecutive Japanese elderly autopsy cases. Multiple logistic regression analyses that included sex and age at autopsy, which are established risk factors for vertebral fracture, revealed that homozygotes for the risk alleles of rs7605378 (A-allele) or rs12673629 (A-allele) possess an increased risk of vertebral fracture (Table 2). Furthermore, the subjects simultaneously homozygous for both the risk alleles of rs7605378 and rs12673629 were at a much higher risk of vertebral fracture (OR 2.401, 95% CI: 1.305–4.416, $P=0.0048$) as compared with those who had at least one non-risk allele of either rs7605378 or rs12673629 (Table 3).

The diagnosis of vertebral fracture was made based on clinical records and autopsy findings. With regard to apparent clinical fractures, their occurrences were easily and convincingly established from the clinical records, as well as the autopsy reports. However, the presence of asymptomatic morphometric fractures often needed to be confirmed by the autopsy reports, as radiographic examination of the spine was not necessarily performed in each patient. Contrary to radiographic examination, autopsy examination is subjective, and mild cases of vertebral fracture that would be easily detected by radiography are often overlooked. Therefore, it should be noted that the frequency of vertebral fracture may be underestimated in the present study. In fact, <80% of vertebral fractures in the present study were clinically detected and further confirmed by the autopsy. About 5% of the fractures were morphologically detected by the radiographic examination and further confirmed by the autopsy. About 5% of the fractures were morphologically detected by the radiographic examination, but the autopsy records did not depict them. The other fractures were not clinically noticed, thus the radiographic examination was not performed, and were finally found at autopsy.

Yoshimura *et al.*[10] reported that the prevalence of vertebral fracture detected by radiography for participants of the Miyama study in their 50, 60, 70 and 80s was 2.9%, 10.3%, 13.2%, and 25.0% for men, and 2.1%, 9.1%, 20.5%, and 54.2% for women, respectively. Corresponding figures in the present study were 0%, 2%, 4%, and 8% for men, and 0%, 3%, 10%, and 12% for women, respectively. If the radiographic examination of the spine had been performed for all the patients in our study, the number of the morphometric fractures would be much increased. However, most of the clinical fractures and severe morphometric fractures confirmed by autopsy were thoroughly included in our present study and, therefore, the prevalence of vertebral fracture in our study population obviously reflects, at least to some degree, the bone fragility.

It is important to know whether the higher risk of vertebral fracture in subjects carrying the risk alleles of the SNPs is due to the direct effect of the polymorphisms on bone or an indirect effect of disorders of other organs or tissues. For that purpose, we examined previous histories and pathological findings of the subjects, and analyzed their association with the genetic polymorphisms as well as the prevalence of vertebral fracture. However, we could not identify any disease that may mediate the genotype effect on the fracture prevalence, suggesting the direct association between the polymorphisms and bone fragility. Interestingly, the prevalence of vertebral fracture was negatively associated with that of ischemic heart disease and myocardial infarction. The observations seem to be contradictory to our expectation considering a possible linkage between vascular systems and bone metabolism. Sedentary life style due to impaired cardiac function might have decreased the risk of fall and subsequent fractures.

Further analysis using a protein motif analysis program (http://www.ebi.ac.uk/Tools/InterProScan/) revealed that FONG contains a formiminotransferase cyclodeaminase domain in its deduced amino acid sequence.[7] Formiminotransferase cyclodeaminase is a mammalian metabolic enzyme that is involved in the conversion of histidine to glutamic acid, and formiminotransferase cyclodeaminase has a transferase activity that transfers a formimino group from *N*-formimino-L-glutamic acid to tetrahydrofolate to generate glutamic acid and 5-formiminotetrahydrofolate.[11] Glutamate signaling is considered to have an important role in bone homeostasis. For instance, L-glutamic acid is secreted by osteoclasts, and knockout of the glutamate transporter 1 in mice causes the animals to develop osteoporosis.[12] These lines of evidence suggest that FONG can potentially regulate bone metabolism.

Proteins encoded by the thrombospondin, type 1, domain-containing gene family possess a unique sequence called thrombospondin type 1 repeats. The type 1 repeats in thrombospondin-1 bind and activate transforming growth factor-beta, a cytokine involved in a variety of cellular processes, including bone matrix formation.[13] Transforming

growth factor-beta, which is abundant in bone matrix, is known to be an important regulator of osteoblast proliferation and differentiation, and has been shown to directly affect bone formation *in vivo*.[14] Therefore, it should be further investigated whether THSD7A has an important role in the pathogenesis of osteoporosis, possibly by modulating the activity of transforming growth factor-beta.

A limitation of this study is that the mechanism underlying the association between the genetic polymorphisms and vertebral fracture remains to be clarified. In the future, not only functional studies of FONG and THSD7A in the context of bone metabolism but also genetic analyses exploring the role of the SNPs in exerting the functions of FONG and THSD7A are required to elucidate the precise mechanism responsible for the association between the polymorphisms and the fracture.

In conclusion, our results suggest that Japanese subjects homozygous for the risk alleles of rs7605378 in *FONG* and rs12673629 in *THSD7A* have a significantly higher risk of vertebral fracture. Although the exact molecular mechanism of the role of SNPs in fracture risk is still unclear, these polymorphic markers may be useful for identifying at-risk individuals and implementing preventive measures against future vertebral fracture.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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