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Nonassociation of Estrogen Receptor Genotypes with Bone Mineral Density and Estrogen Responsiveness to Hormone Replacement Therapy in Korean Postmenopausal Women

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ABSTRACT

Hormone replacement therapy (HRT) prevents bone loss in postmenopausal women, but some women are resistant to therapy. A recently reported case of severe estrogen resistance caused by a germline mutation at the estrogen receptor (ER) gene locus suggests the possibility that other variants of the ER gene could be responsible for resistance to HRT and could also be an answer to the heritable components of bone density. Three restriction fragment length polymorphisms (RFLPs) at the ER gene locus, represented as *Bst*UI (or B variant), *Pvu*II, and *Xba*I, and their relationship to bone mineral density (BMD) and estrogen responsiveness to HRT were examined in 248 healthy postmenopausal women, aged 41–68 yr (mean ± SD, 52.0 ± 4.6 yr) in Korea. The *Bst*UI restriction site was not found in Korean women. The distribution of the *Pvu*II and *Xba*I RFLPs was as follows: PP, 35 (14.1%); Pp, 136 (54.8%); pp, 77 (31.1%); and XX, 18 (7.3%); Xx, 72 (29.0%); and xx, 158 (63.7%), respectively (capital letters signify the absence of and lower case letters signify the presence of the restriction site of each RFLP). There was no significant relation between ER genotypes and z score values of lumbar spine BMD. Also, no significant genotypic differences were found in the change in lumbar spine BMD and those in biochemical markers before and after 1 yr of HRT. These data indicate no significant effects of ER genotypes on BMD and estrogen responsiveness after HRT. (*J Clin Endocrinol Metab* **82:** 991–995, 1997)

IN 1994, the case study by Smith *et al.* was the first to describe a man with complete estrogen deficiency caused by a germ-line mutation of estrogen receptor (ER) genomic DNA (Arg^{157}stop) (1). A 28-yr-old man with severe estrogen resistance had incomplete epiphyseal closure and severe osteoporosis with biochemical evidence of increased bone resorption. This case offers a great deal of information about estrogen's effect on bone. Estrogen is critical for bone maturation and normal bone development even in males, in whom testosterone cannot be substituted. From this case, we could infer that any other variants of the ER gene might also have an influence on bone and cause estrogen resistance to hormone replacement therapy (HRT).

Recently, two studies have reported that the *Pvu*II and *Xba*I restriction fragment length polymorphisms (RFLPs) at the ER gene locus had a significant effect on bone. The study reported by Kobayashi *et al.* (2) showed that the PPxx genotype of the two combined RFLPs was associated with low bone mineral density (BMD), whereas the report by Qi *et al.* (3) revealed that the pp genotype of the *Pvu*II RFLP was related to low BMD. The contradictory conclusions of both groups about the association of BMD with the ER RFLPs demand further investigation.

The Christiansen group reported that about 1.2% of early healthy postmenopausal women who received HRT over 2 yr had lost more than 1% of forearm bone mineral content per yr (4). That is, they demonstrated that nonresponders to HRT existed. However, there have been no reported studies about the mechanism of estrogen resistance occurring in some women despite good drug compliance and good health.

We, therefore, hypothesize that any variants in the ER gene could have an effect on the development of peak bone mass and thus on the development of osteoporosis; these variants could also account for the lack of response to HRT in nonresponders. We examined three established RFLPs represented by *Bst*UI (or B variant), *Pvu*II, and *Xba*I at the ER gene locus (5–7) and analyzed the association of each genotype with lumbar spine BMD in healthy postmenopausal Korean women. We also analyzed differences between genotypes concerning the changes in BMD and bone markers after 1 yr of HRT.

Subjects and Methods

Subjects

Two hundred and forty-eight healthy postmenopausal women of ethnic Korean background were studied. Their mean age was 52 yr (range, 41–68 yr). All subjects took HRT with conjugated equine estrogen (Premarin) alone or combined with medroxyprogesterone acetate (Provera) in a cyclic or continuous regimen. Women with good drug compliance who were not taking any drugs that would affect bone turnover rate and did not switch to other drugs during 1 yr of HRT were eligible. Women with early menopause (before 40 yr of age) and those who had had an ovariectomy were excluded. None had a history of bone disease, illness, or drug use that might affect bone turnover. Women were excluded if they had a spine density less than 3 SD below an

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age-matched reference mean or a spinal degenerative disease detected by conventional spine radiographs.

Measurements of BMD and bone markers

BMD and bone markers were measured before and after 1 yr of HRT. BMD at the lumbar spine was measured by dual energy x-ray absorptiometry using a QDR-2000 (Hologic, Waltham, MA) or a DPX-L (Lunar Co., Madison, WI). The precision errors (coefficient of variation of repeated measurements on individuals) were 0.65% and 1.2%, respectively. At the time of follow-up, we used the same densitometer as that used at baseline. The scores measured by the DPX-L were converted to those of the QDR-2000 by the conversion equation: QDR-2000 = (0.847 × DPX-L) + 0.019 (8). The z score (the value of the SD obtained when the average of the data was adjusted to 0) was calculated by using the data of BMD obtained from up to 2000 Korean women. Plasma osteocalcin (OC) was determined by RIA using an Incstar Osteocalcin ^{125}I RIA kit (Stillwater, MN). The intra- and interassay variations were 4.8% and 9.8%, respectively. Carboxy-terminal propeptide of type I collagen (PICP) was measured by the enzyme-linked immunosorbent assay method using a prolagen-C kit from Metra Biosystems (Winooski, VE). The intra- and interassay variations were 2.8% and 7.2%, respectively. Urine deoxypyridinoline (DPD) was assayed by the enzyme-linked immunosorbent assay method using a Pyrilinks-D kit and corrected for creatinine. The intra- and interassay variations were 5.7% and 3.5%, respectively. Spot urine specimens were collected between 0800–1000 h. Serum alkaline phosphatase (ALP) and urinary creatinine were measured by automatized routine procedures. Data for PICP and DPD were obtained from only 67 and 87 women, respectively, because of a delayed test start.

DNA analysis

DNA was isolated from peripheral blood leukocytes using conventional methods. The 143-bp fragment of genomic DNA containing the polymorphic portion of exon 1 (GCG to GCC; codon 87) described by Garcia *et al.* as the ER B variant was amplified by PCR using previously described oligonucleotide primers (5). The PCR fragments were digested with the *Bst*UI restriction endonuclease and separated on a 7.5% polyacrylamide gel. In every case of enzyme digestion, the PCR product with the restriction site (87, 54, and 2 base pair), provided by Dr. Schatchter, was used as a control (Fig. 1). To analyze the *Pvu*II and *Xba*I RFLPs in intron 1, approximately 1.3-kilobase (kb) fragments were amplified by PCR using the same oligonucleotide primers and PCR reaction steps originally described by Yaich *et al.* (2, 6, 7). The PCR products were digested by the *Pvu*II or *Xba*I restriction endonuclease and separated on a 4% agarose gel. PP and XX, signifying the absence of restriction sites, gave one 1.3-kb fragment, and pp, signifying the presence of restriction sites on both alleles, was digested into two fragments (~0.85 and 0.45 kb). The cutting fragment of the *Xba*I polymorphism near the *Pvu*II RFLP site (~50 bp apart) revealed two fragments (~0.9 and 0.4 kb) and was labeled as xx (Fig. 1) (2). To identify the *Xba*I polymorphic site, the PCR product was cloned by TA cloning kit (Invitrogen, San Diego, CA) and sequenced using a U.S. Biochemical Corp. sequencing kit (Cleveland, OH).

Statistical analysis

To determine whether the proportions observed in our data were those to be expected in a random mating equilibrium population, they were explored using the χ^2 method under the Hardy-Weinberg law. Distribution of characteristics among each genotype was evaluated with one-way ANOVA or the Kruskal-Wallis H test. Comparisons of z score values of BMD, percent change in BMD, and percent change in bone markers in each genotype were examined using the Kruskal-Wallis H test. Rates of change in BMD and those in each bone marker were expressed as the percent change from initial levels. Pearson's χ^2 test was used for evaluation of the association between genotypes and responsiveness to HRT (*i.e.* responder or nonresponder). Independent Student's t test and logistic regression were used to evaluate the independency between genotypes and responsiveness to HRT after adjusting possible confounding biases. Responder or nonresponder was set as a dependent variable in logistic regression.

Results

The B variant could not be found in our samples even though we used the control DNA containing the *Bst*UI restriction site. The distribution of the *Pvu*II and *Xba*I RFLPs was as follows: PP, 35 (14.1%); Pp, 136 (54.8%); pp, 77 (31.1%); and XX, 18 (7.3%); Xx, 72 (29.0%); and xx, 158 (63.7%), respectively. The genotype distribution of these RFLPs was compatible with the populations in the Hardy-Weinberg equilibrium. Sequencing of the *Xba*I PCR product revealed the putative site, approximately 0.35 kb upstream from exon 2, which differed by only 1 base from the *Xba*I recognition sequence (TCTAGA to TCTGGA; Fig. 2).

The clinical characteristics of the study groups are provided in Table 1. The groups were all matched for age, height, weight, age at menarche, and years since menopause (YSM). At baseline, there were no statistically significant differences in bone markers such as ALP, OC, PICP, and DPD among the genotypes. Figure 3 shows that no significant relationship

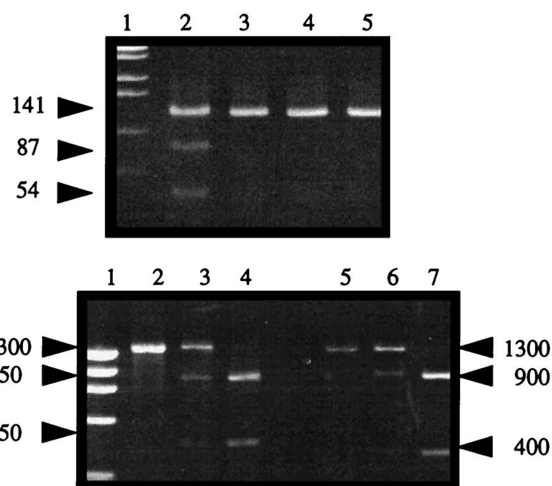

FIG. 1. Genotype analysis of the B variant in exon 1 of the ER gene. Genomic leukocyte DNA from the control with the *Bst*UI restriction site (lane 2) and from three study subjects (lanes 3–5) was PCR amplified, digested with *Bst*UI, and separated on a 7.5% polyacrylamide gel. The restriction site of *Bst*UI was not found in our samples (*top*). Genotype analysis of the *Pvu*II (lane 2, PP; lane 3, Pp; lane 4, pp) and *Xba*I (lane 5, XX; lane 6, Xx; lane 7, xx) RFLP sites in intron 1 of the ER gene was performed. Genomic leukocyte DNA was PCR amplified using the same primers, digested with *Pvu*II or *Xba*I, and separated on a 4% agarose gel (*bottom*). The fragment sizes are indicated in base pairs.

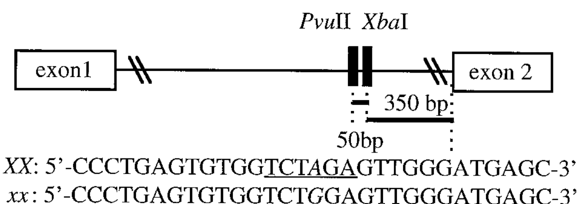

FIG. 2. Sequence analysis of the *Xba*I RFLP site in intron 1 of the ER gene. The *Xba*I RFLP is located approximately 50 bp apart from the *Pvu*II RFLP site and is approximately 400 bp upstream from exon 2 (*top*), with a point mutation (A→G) that differed by one base pair from the *Xba* I recognition site (TCT**A**GA) to TCT***G***GA for the *Xba*I allele (*bottom*). The fragment sizes are indicated in base pairs.

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TABLE 1. The profile for the *Pvu*II and *Xba*I RFLP genotypes

Characteristic	*Pvu*II Genotype			*Xba*I Genotype		
	PP	Pp	pp	XX	Xx	xx
Number of patients	35	136	77	18	72	158
Age (yr)	51.7 ± 4.4	51.9 ± 4.7	52.2 ± 4.2	52.1 ± 3.8	52.0 ± 4.7	52.0 ± 4.7
Height (cm)	157.7 ± 4.3	157.1 ± 4.3	157.1 ± 4.2	159.0 ± 3.1	157.1 ± 4.9	157.0 ± 4.1
Weight (kg)	56.7 ± 6.4	58.4 ± 7.5	59.1 ± 6.7	60.8 ± 7.0	57.5 ± 7.4	58.4 ± 7.0
Age at menarche (yr)	15.8 ± 1.9	16.5 ± 1.6	16.3 ± 1.7	15.9 ± 1.7	16.7 ± 1.9	16.2 ± 1.5
Years since menopause (yr)	2.0 [1.0–4.0]	2.0 [1.0–5.8]	2.0 [1.0–4.0]	2.0 [0.8–4.0]	2.0 [1.0–5.0]	2.0 [1.0–4.3]
ALP (IU/L)	70.0 [63.5–90.0]	66.5 [58.3–78.0]	75.5 [59.0–94.0]	70.0 [61.0–91.0]	74.0 [64.5–97.0]	68.5 [58.0–84.3]
BGP (ng/mL)	5.2 [4.0–6.3]	4.9 [3.7–6.8]	5.0 [2.5–6.0]	4.4 [3.6–6.5]	5.3 [3.7–7.1]	4.9 [3.5–6.2]
PICP (ng/mL)	80.0 [77.5–88.0]	76.5 [61.8–96.8]	109.0 [67.0–133.5]	80.0 [79.0–85.0]	94.5 [66.5–115.3]	80.0 [64.8–108.5]
n	(9)	(39)	(19)	(6)	(17)	(44)
DPD (pmol/uL Cr)	8.4 [5.9–11.3]	7.1 [5.5–8.2]	8.0 [6.6–9.4]	8.4 [4.9–11.8]	6.9 [4.8–8.1]	7.1 [6.2–8.6]
n	(11)	(52)	(24)	(8)	(26)	(53)

All data shown were obtained at the study baseline. Age, height, weight, and age at menarche are presented as the mean ± SD by one-way ANOVA test and year since menopause, bone markers are presented as the median [IQR] by the Kruskal-Wallis H test. Sample sizes are shown in parentheses if different from the total in each genotype.

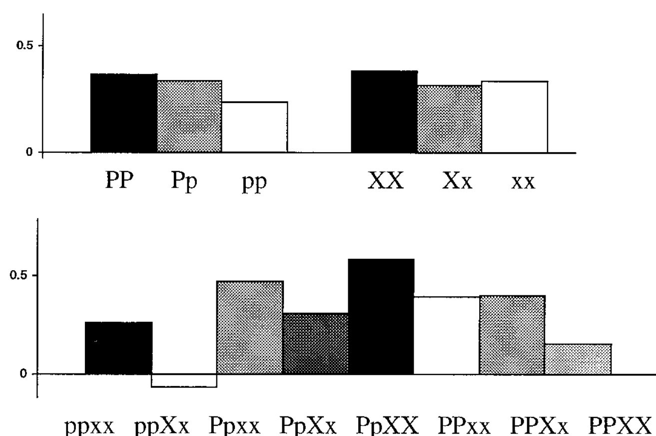

FIG. 3. The z score values of the lumbar spine BMD of the *Pvu*II (*top left*) and *Xba*I (*top right*) RFLP genotypes and the combined genotypes of the *Pvu*II and *Xba*I RFLPs (*bottom*). Values are expressed as medians. No significant difference between the groups was found by the Kruskal-Wallis H test.

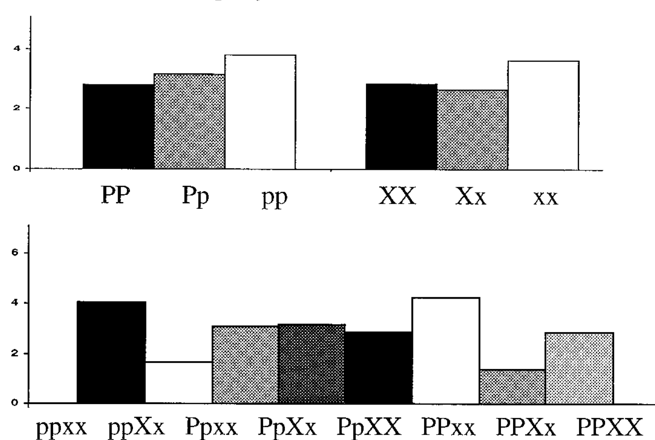

FIG. 4. Adjusted rates of bone loss by the *Pvu*II (*top left*) and *Xba*I (*top right*) RFLP genotypes, and the combined genotypes of the *Pvu*II and *Xba*I RFLPs (*bottom*) before and after 1 yr of HRT. Values are expressed as medians. No significant difference between the groups was found by the Kruskal-Wallis H test.

between genotypes and the z score of BMD could be found. With HRT, the median increment in BMD was 3.1% [0.7–5.7%, interquartile range (IQR)] for 1 yr. The decrement in bone markers for 1 yr was as follows: ALP, −24.3% (−34.0% to 9.8%); OC, −38.0% (−53.3% to 13.6%); PICP, −21.6% (−41.3% to 7.5%); and DPD, −39.7% (−50.0% to 14.1%; median, IQR). No significant genotypic differences were found between percent change in BMD and that in bone markers after 1 yr of treatment (Figs. 4 and 5). In the combination of two RFLPs, the distribution was as follows: PPXX, 13 (5.3%); PPXx, 12 (4.8%); PPxx, 10 (4.0%); PpXX, 5 (2.0%); PpXx, 56 (22.6%); Ppxx, 75 (30.3%); ppXx, 4 (1.6%); and ppxx, 73 (29.4%). ppXX was not detected. Mean age, height, weight, age at menarche, YSM, and biochemical markers were not statistically different among these groups (data not shown). There was no significant relationship between the combination of genotypes and the z scores of BMD (Fig. 3). After HRT, we also did not find a significant relationship between genotypes and percent change in BMD before and after therapy (Fig. 4).

If we defined the nonresponder group as women who had lost more than 1% BMD/yr, 10.5% of the women studied were included in the nonresponder group. Weight, height, age at menarche, YSM, and biochemical markers were not different between the two groups, but in the nonresponder group, mean age was significantly younger (50.4 ± 3.3 *vs.* 52.2 ± 4.8 yr in the nonresponder and responder groups, respectively; mean ± SD), and initial BMD was statistically higher (1.0087 ± 0.08 *vs.* 0.9451 ± 0.11 g/cm^2 in the nonresponder and responder groups, respectively; mean ± SD). After adjusting age and initial BMD, no genotype was significantly associated with responsiveness.

Discussion

The ER gene on chromosome 6q25.1 is comprised of more than 140 kb and has eight exons and five functional domains, designated A/B–F (9). All three RFLPs we examined are located in the A/B domain, which is called transactivating factor 1. It is an important site for stimulating transcription from certain estrogen-responsive promoters. The B variant in the exon 1 site is a silent mutation that changes codon 87 from

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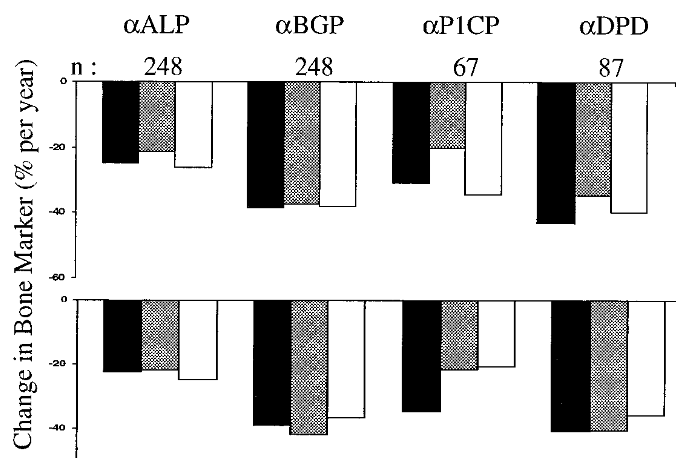

FIG. 5. Percent change in each bone marker before and after HRT in the *Pvu*II (*top*) and *Xba*I (*bottom*) RFLP genotypes. The *black bar* represents the PP or XX genotype, the *gray bar* the Pp or Xx genotype, and the *white bar* the pp or xx genotype. Values are expressed as medians. n, Number of subjects. No significant difference between groups was found by Kruskal-Wallis H test.

GCG to GCC of alanine (5, 10). Several reports have shown that the frequency of BB′ heterozygotes is about 12% in Caucasians (5, 7, 11) and approximately the same in African-Americans (Schachter, B., personal communication), but there have been no reported data about the distribution of this RFLP among Asians. In our study, the B variant could not be found even though we used the control DNA containing the *Bst*UI restriction site. This finding might be explained by ethnic differences in the B variant; the *Bst*UI RFLP may be rare or absent in ethnic Koreans. The *Pvu*II RFLP site was sequenced in the first intron, 0.4 kb upstream from exon 2, with a point mutation (T→C) in the recognition sequence CAGCTG responsible for the P allele (6). The *Xba*I RFLP site has not been sequenced yet, but it has been studied by Southern blot analysis using an M72 probe that is a 1.6-kb *Eco*RI fragment containing exon 2 with flanking intron sequences (7). We sequenced the putative *Xba*I site in intron 1, approximately 50 bp apart from the known *Pvu*II site. The distribution of these two RFLPs was similar to that of RFLPs demonstrated in other studies of Caucasians and Japanese women (2–3, 7), and it did not deviate significantly from the Hardy-Weinberg equilibrium.

The genetic influence on bone density has been confirmed by a number of family and twin studies. An estimated 46–80% of the total variance in adult bone mass is attributed to genetic determinants (12–15). Recently, great interest has been generated by a report from Morrison *et al.* (16), who investigated this genetic mechanism at the molecular basis. They claimed that a natural polymorphism within the VDR gene was responsible for as much as 75% of the total genetic effect on bone density (16). However, the consistency of this effect has not been established, and controversy over the reported relationship between the *Bsm*I genotype and BMD demands further investigation (17). There are fewer studies about the relationship between the genotypes of the ER gene and BMD. Kobayashi *et al.* (2) showed that the PPxx genotype of combined *Pvu*II and *Xba*I RFLPs was associated with low lumbar spine BMD in 238 Japanese healthy volunteer postmenopausal women, but in a population-based study by Qi *et al.* (3), the pp genotype of the *Pvu*II RFLP was significantly related to lower lumbar spine BMD in women and lower femoral neck BMD in men. However, we could not find any significant associations between the ER genotypes and lumbar spine BMD in postmenopausal women. As in the case of VDR polymorphisms, the effect of ER polymorphisms on bone mass may need further study and evaluation.

After 12 months, about 11% of the treated women had lost more than 1% of their bone density in our study. In some studies in which the raw data are provided, spinal BMD diminished in 3–30% of the women who took accepted bone-sparing doses of estrogen (18–21). The Christiansen group reported that if a nonresponder to HRT was defined as a woman who had lost more than 1% bone/yr, then about 0–5% of healthy early postmenopausal women were classified as nonresponders. However, no studies on the mechanism of occurrence of estrogen resistance have been reported. In other steroid receptors, such as the glucocorticoid, androgen, and vitamin D_3 receptors, hormone resistance has clearly been linked to deletions and point mutations of the respective genes (22–24). As the steroid hormone receptors are closely related in their domain structure and function as ligand-inducible transcriptional regulators, one would expect the type of ER defects associated with estrogen resistance to be analogous to those described for other steroid receptors. In the ER, it has been thought that mutation would be lethal in the embryo stage (25). However, recently, a case report of a man with complete estrogen deficiency was revealed to be caused by a cystine to thymine transition at codon 157 of both alleles, resulting in a premature stop codon and a severely truncated nonfunctioning protein (1). From this case, one would predict the possibility that any other variants at the ER gene locus could cause estrogen resistance. We examined three ER variants, but there were no significant genotypic differences concerning the changes in BMD and bone markers after 1 yr of HRT. Because no other data about the effect of ER variants on estrogen resistance have been reported, further studies must be conducted to find other sites of the ER gene.

In conclusion, BMD in Koreans could not be associated with three ER genotypes: the B variant, *Pvu*II, and *Xba*I RFLPs. After 1 yr of HRT, the changes in bone density are not associated with any of these ER genotypes.

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