The importance of the Wnt/ β -catenin pathway and LRP5 protein in bone metabolism of postmenopausal women

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Advances in Clinical and Experimental Medicine, ISSN 1899–5276 (print), ISSN 2451–2680 (online)

Adv Clin Exp Med. 2019;28(2):179–184

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Funding sources

None declared

Conflict of interest

None declared

Received on January 16, 2017

Reviewed on February 12, 2017

Accepted on November 8, 2017

Published online on July 2, 2018

Cite as

Kamiński A, Karasiewicz M, Bogacz A, Dziekan K, Seremak-Mrozikiewicz A, Czerny B. The importance of the Wnt/ β -catenin pathway and LRP5 protein in bone metabolism of postmenopausal women. *Adv Clin Exp Med.* 2019;28(2):179–184. doi:10.17219/acem/79969

DOI

10.17219/acem/79969

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Abstract

Background. Postmenopausal osteoporosis is the most common metabolic bone disease among women. The Wnt signaling pathway has been known to be the critical regulator of osteoblastogenesis. Alterations in this mechanism may have consequences for bone remodeling in humans.

Objectives. The aim of the study was to evaluate the frequency of genotypes and alleles of single nucleotide polymorphism (SNP) rs4988321 and rs312009 of *LRP5* in Polish postmenopausal women with osteopenia ($n = 109$) and osteoporosis ($n = 333$). Potential correlations between genetic polymorphisms, bone mineral density (BMD), risk for bone fractures, and other clinical parameters were analyzed.

Material and methods. Genomic DNA was extracted from the blood samples and the sequence polymorphisms of *LRP5* gene were detected using real-time polymerase chain reaction (RT-PCR) methods with melting curve analysis. We also calculated the odds ratio (OR) for the *LRP5* genotypes and the alleles. Then, we evaluated the effect of the *LRP5* polymorphism on T-score, Z-score, L2L4AM, L2L4YA, L2L4BMD, body mass index (BMI), and other clinical parameters.

Results. No statistically significant differences in the distribution of *LRP5* rs312009 genotypes between the groups were observed. Furthermore, our findings indicate that there is no correlation between *LRP5* genotypes and the clinical characteristics of women with osteopenia/osteoporosis. In contrast, there was an increased value of OR in heterozygotes for rs4988321, both in patients with osteopenia ($OR = 1.47$) and in those with osteoporosis ($OR = 1.33$). In our study, we were not able to calculate the OR parameter for the AA genotype due to its low prevalence in the population.

Conclusions. Our results suggest that the Val667Met *LRP5* (rs312009) polymorphism may contribute to an elevated risk for fractures in postmenopausal Polish women.

Key words: bone mineral density, LRP5, postmenopausal women, osteoporosis

Introduction

Osteoporosis is typically manifested by disturbances in the microstructure of bone tissue and increased susceptibility to bone fracture due to reduced bone mass. Due to its high incidence among patients over 50 years of age and the considerable costs of treatment, osteoporosis continues to be one of the greatest health challenges of an aging society. Osteoporotic fractures, which constitute the main consequences of the asymptomatic course of the disease, usually lead to long-term disability and even death. Over the last decade, studies on candidate genes, family analysis and genome-wide association studies (GWAS) resulted in the identification of numerous genetic loci related to osteoporosis. Genetic factors are believed to constitute 50–80% of individual differences in bone mineral density (BMD).¹ Studies on the background of skeletal dysplasias with decreased bone mass, e.g., brittle bone disease and the osteoporosis-pseudoglioma syndrome, pointed to the role of the Wnt/ β -catenin pathway – including low-density lipoprotein receptor-related protein 5 (LRP5) and WNT1 – in the regulation of bone tissue homeostasis.^{2–4}

The Wnt signaling pathway increases the activity and differentiation of bone marrow stem cells and stimulates the osteoblast lines, with a simultaneous decrease toward chondrogenic and adipogenic cell lines. Activation of that pathway inhibits the apoptosis of mature osteoblasts, thus extending their resilience.⁵ Additionally, the Wnt/ β -catenin pathway decreases osteoclast differentiation by stimulating the synthesis and secretion of osteoprotegerin, which regulates bone resorption by inhibiting osteoclast differentiation into mature forms.⁶ The canonical Wnt signaling pathway plays an essential role in the regulatory processes of bone formation, so its disturbed activity may result in grave consequences for bone metabolism.^{7–10} In short, LRP-5 and LRP-6 proteins, which belong to the low-density lipoprotein-receptor-related proteins (LDL) receptor family, function in this pathway as 1-domain co-receptors which, together with Fzd 7-domain endothelial receptor, facilitate the binding of the Wnt glycoproteins and the activation of cell response through β -catenin and Tcf/Lef factor-dependent transcription. The details of the Wnt signaling pathway have already been described.^{11,12} The Wnt/ β -catenin pathway is subject to antagonist regulation, sclerostin, Dkk1 and Dkk2, and Wnt modulator of surface ectoderm (Wise), for example, which – by binding to PRP5 – block access for the Wnt proteins and inhibit the classic signaling pathway, thus inhibiting osteoblastogenesis, induced via the Wnt/ β -catenin pathway. The *LRP5* gene is expressed in the osteoblasts, and in vitro and in vivo studies have confirmed that its deletion inhibited osteoblast function, causing serious anomalies in bone phenotype.^{7–9} This protein, via the Wnt pathway, influences the bone mass. The connection between the sequence polymorphism of that gene and bone mineral density (BMD) has been extensively studied.¹³

The review of the latest literature reports revealed that single nucleotide polymorphism (SNP) changes in genes encoding the LRP family proteins may also affect bone phenotype and, at the same time, present a risk for osteoporosis.¹⁴ Studies on the phenotype–genotype relationship regarding osteoporosis seem to be crucial for early diagnosis and the development of treatment for postmenopausal osteoporosis, osteoarthritis, rheumatoid arthritis, and rare bone diseases.

Objectives

The aim of the study was to evaluate the frequency of the genotypes and alleles of SNP rs4988321 and rs312009 of *LRP5* in Polish postmenopausal women with osteopenia ($n = 109$) and osteoporosis ($n = 333$). The potential correlations between genetic polymorphisms, BMD, risk for bone fractures, and other clinical parameters were analyzed.

Material and methods

Patients

The study included 109 unrelated postmenopausal Caucasian women with osteopenia (mean age: 53.24 ± 8.12 years) and 333 patients with osteoporosis (mean age: 56.06 ± 8.83 years). The control group consisted of 233 postmenopausal women with normal T-scores (mean age: 53.38 ± 8.2 years) recruited from the patients of the same hospital. Blood samples were collected at the Department of Orthopedics and Traumatology, Pomeranian Medical University in Szczecin, Poland. Bone densitometry was performed at the Laboratory of Densitometry, Clinical Hospital No. 1, Pomeranian Medical University in Szczecin, Poland, to determine the BMD values, T-scores and Z-scores. Bone mineral density was detected in the lumbar spine from L2 to L4 vertebrae using the dual energy X-ray absorptiometry (DEXA) method. Densitometry was performed using a Lunar DPX 100 camera (Lunar Corp., Madison, USA). A normal BMD value by DEXA is between one standard deviation (SD) from the mean with respect to the age of peak bone mass ($-1 < \text{T-score} < 1$). Based on these measurements, the women were classified into the following groups: osteopenia ($-2.5 < \text{T-score} \leq 1$), osteoporosis ($\text{T-score} \leq -2.5$) and normal T-score ($\text{T-score} \geq 1$). The ratio of the average BMD in relation to the mean value for young adults (YA) and in comparison to age (age-matched – AM) was also evaluated. Furthermore, height and weight were measured and the body mass index (BMI) was calculated. During an interview with each patient, data on disease manifestation, drug use, age at 1st and last menstruation, gravidity, and birth weight were collected. The inclusion criteria for the genetic research were as follows: menopause at least 1 year before participation in the study, and no

hormone replacement therapy (HRT) or drugs taken affecting bone mass (selective estrogen receptor modulators (SERMs), calcitonin, bisphosphonates, heparin, steroids, thyroid hormones, antiepileptic drugs, GnRH analogues, or tibolone). The study excluded patients with endocrine and metabolic disorders, hematological diseases, kidney disease, cancer, and autoimmune and connective tissue disease, as well as who had undergone bilateral ovariectomy. Written informed consent was obtained from each patient. The Pomeranian Medical University Ethics Committee approved the study (approval No. KB-0012/100/15).

Genetic analysis

Genetic polymorphisms were determined at the Department of Stem Cell and Regenerative Medicine at the Institute of Natural Fibers and Medicinal Plants in Poznań, Poland. Genomic DNA was obtained from 5 mL of whole blood using a Qiagen DNA isolation kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. DNA concentration was measured using a DeNovix DS-11 Spectrophotometer (DeNovix Inc., Wilmington, USA). A LightCycler FastStart DNA Master HybProbe (Roche Diagnostics, Risch-Rotkreuz, Switzerland) assay and a LightCycler® 480 instrument for *LRP5* gene genotyping were used. The *LRP5* gene polymorphisms were performed using a LightSNiP *LRP5* (TIBMolbiol, Berlin, Germany), which contained the primers and probes specific for the amplified fragment. Polymerase chain reaction (PCR) was

performed in 10 µL of reaction mixture, according to the manufacturer's protocol, under the following conditions: initial denaturation at 95°C for 10 min, 35 cycles (denaturation at 95°C for 10 s, annealing at 60°C for 10 s, elongation for 15 s at 72°C, and melting for 30 s at 95°C and 40°C for 120 s). Polymorphism *LRP5* gene sequences were observed as different melting curves of PCR products. SPSS Statistics v. 17.0 for Windows (IBM Corp., Armonk, USA) was used for statistical analysis. We used the Hardy-Weinberg equation to calculate the expected genotype frequencies for each polymorphism, which were compared with the observed values using χ^2 test. The expected results are presented with 95% confidence intervals (CI). We also calculated the odds ratio (OR) for the genotypes and the alleles. Then, we evaluated the effect of the *LRP5* polymorphism on T-score, Z-score, L2L4AM, L2L4YA, L2L4BMD, BMI, and other clinical parameters. A correlation analysis between genotypes and clinical parameters using one-way analysis of variance (ANOVA) test was performed. A p-value <0.05 was considered statistically significant.

Results

The distribution of genotype frequency is consistent with the Hardy-Weinberg equation, both for *LRP5* rs312009 (Tables 1–3) and rs4988321 (Table 3, 4). The χ^2 test found no statistically significant differences between the values obtained for the rs312009 ($p = 0.334$) and rs4988321

Table 1. Frequency of the genotype and allele of the *LRP5* rs312009 polymorphism in women with osteopenia and the controls

Genotype/ allele	Osteopenia		Controls		OR	95% CI	p-value
	observed value n [%]	expected value [%]	observed value n [%]	expected value [%]			
CC	76 (69.7)	68.9	141 (60.5)	61.6	1.50	0.90–2.53	0.06
CT	29 (26.6)	28.2	84 (36.1)	33.8	0.64	0.37–1.09	0.05
TT	4 (3.7)	2.9	8 (3.4)	4.6	1.09	0.23–4.11	0.57
Total	109 (100)	100	233 (100)	100	–	–	–
C	181 (83)	–	366 (78.5)	–	1.34	0.87–2.09	0.10
T	37 (17)	–	100 (21.5)	–	0.75	0.48–1.15	0.10
Total	218 (100)	–	466 (100)	–	–	–	–

OR – odds ratio; CI – confidence interval.

Table 2. Frequency of the genotype and allele of the *LRP5* rs312009 polymorphism in women with osteoporosis and the controls

Genotype/ allele	Osteoporosis		Controls		OR	95% CI	p-value
	observed value n [%]	expected value [%]	observed value n [%]	expected value [%]			
CC	196 (58.9)	59.6	141 (60.5)	61.6	0.93	0.65–1.33	0.38
CT	122 (36.6)	35.2	84 (36.1)	33.8	1.02	0.71–1.48	0.48
TT	15 (4.5)	5.2	8 (3.4)	4.6	1.33	0.52–3.68	0.34
Total	333 (100)	100	233 (100)	100	–	–	–
C	514 (77.2)	–	366 (78.5)	–	0.92	0.69–1.24	0.32
T	152 (22.8)	–	100 (21.5)	–	1.08	0.81–1.46	0.32
Total	666 (100)	–	466 (100)	–	–	–	–

OR – odds ratio; CI – confidence interval.

Table 3. Frequency of the genotype and allele of the *LRP5* rs4988321 polymorphism in women with osteopenia and the controls

Genotype/ allele	Osteopenia		Controls		OR	95% CI	p-value
	observed value n [%]	expected value [%]	observed value n [%]	expected value [%]			
GG	98 (89.9)	89.3	1,218 (93.6)	93.7	0.61	0.25–1.54	0.17
GA	10 (9.2)	10.4	15 (6.4)	6.2	1.47	0.57–3.63	0.24
AA	1 (0.9)	0.3	0	0.1	–	–	–
Total	109 (100)	100	233 (100)	100	–	–	–
G	206 (94.5)	–	451 (96.8)	–	0.57	0.24–1.36	0.11
A	12 (5.5)	–	15 (3.2)	–	1.75	0.73–4.09	0.11
Total	218 (100)	–	466 (100)	–	–	–	–

OR – odds ratio; CI – confidence interval.

Table 4. Frequency of the genotype and allele of the *LRP5* rs4988321 polymorphism in women with osteoporosis and the controls

Genotype/ allele	Osteoporosis		Controls		OR	95% CI	p-value
	observed value n [%]	expected value [%]	observed value n [%]	expected value [%]			
GG	305 (91.6)	91.8	218 (93.6)	93.7	0.75	0.36–1.49	0.24
GA	28 (8.4)	8.0	15 (6.4)	6.2	1.33	0.67–2.75	0.24
AA	0	0.2	0	0.1	–	–	–
Total	333 (100)	100	233 (100)	100	–	–	–
G	638 (95.8)	–	451 (96.8)	–	0.76	0.37–1.49	0.25
A	28 (4.2)	–	15 (3.2)	–	1.32	0.67–2.69	0.25
Total	666 (100)	–	466 (100)	–	–	–	–

OR – odds ratio; CI – confidence interval.

($p = 0.179$) study groups. An analysis of genotype frequency for the *LRP5* rs312009 polymorphism in women with osteopenia and osteoporosis and the controls revealed no statistically significant differences. No statistically significant differences were noted for allele frequency either (Tables 1, 2).

Increased OR values in heterozygotes for rs4988321 were noted in women with both osteopenia (OR = 1.47, 95% CI = 0.57–3.63, $p = 0.24$) and osteoporosis (OR = 1.33, 95% CI = 0.67–2.75, $p = 0.24$). In light of the sample size of our study, the OR for the AA genotype could not be established due to its relatively low incidence in the population. Increased OR values for the A allele were found in women with osteopenia (OR = 1.75, 95% CI = 0.73–4.09, $p = 0.11$) and osteoporosis (OR = 1.32, 95% CI = 0.67–2.69, $p = 0.25$) (Tables 3, 4). The genotype analysis for both *LRP5* polymorphisms revealed no statistically significant correlations in clinical features between the groups of women. Furthermore, characteristics of the study population (perimenopausal women with osteopenia, osteoporosis and normal T-scores) are presented in Table 5.

Discussion

The analyses of the genetic background of osteoporosis have indicated a relationship between a mutation in the 11q12-13 locus, the same one where the *LRP5* gene is located, and the incidence of osteoporosis-pseudoglioma syndrome (OPPG), autosomal dominant phenotype of high

bone mass (HBM) and autosomal dominant osteoporosis type I.¹⁵ Polymorphic variants, including Val667Met, have been suggested as being associated with BMD and the risk for fracture of various intensity and effect in different populations, which was confirmed by numerous multicenter studies.¹⁴ The aim of our study was to evaluate the frequency of the polymorphisms in the *LRP5* gene in Polish postmenopausal women with osteopenia, osteoporosis, and normal T-scores, as well as to assess a possible relationship between genotypes and BMD values and other clinical features.

An analysis of genotype and allele frequency for the *LRP5* rs312009 polymorphism in women with osteopenia and osteoporosis and healthy controls revealed no statistically significant differences. An earlier study by Agueda et al. postulated a link between BMD of the lumbar region of the spine and SNP *LRP5* rs312009.¹⁶ A nucleotide change (SNP rs312009) was proved to be located in the *LRP5* 5' region at the RUNX2 binding site. The rs312009-dependent variability was experimentally confirmed in 2 osteoblastic cell lines, with higher transcription activity in the T allele as compared to the C allele. In vitro studies found the mRNA *LRP5* value to be RUNX2-dependent, thus proving a link between 2 bone-regulating systems – the RUNX2 transcription-factor cascade and the Wnt signaling pathway.¹⁷ Panach et al. found significantly lower L2–L4 BMD values in women with the CC genotype, and elevated risk for osteoporosis as compared to TT and CT.¹⁸

Table 5. Characteristics of the study population (perimenopausal women with osteopenia, osteoporosis and normal T-scores)

Parameter		p-value	Mean	SEM	95% CI	
					lower limit	upper limit
T-score	osteopenia	<0.001 ^b	–3.1640	0.05627	–1.9072	–1.7530
	osteoporosis		0.0779	0.11321	–3.2757	–3.0522
	controls		–1.8138	0.08072	–0.1482	0.3040
	total				–1.9727	–1.6549
Z-score	osteopenia	0.117 ^a	–0.8448	0.08470	–1.0143	–0.6754
	osteoporosis	<0.001 ^b	–3.5691	1.94626	–7.4572	0.3190
	controls		0.6425	0.19620	0.2423	1.0427
	total		–1.6696	0.81499	–3.2794	–0.0597
Weight [kg]	osteopenia	0.026 ^a	65.1721	0.99502	63.2022	67.1420
	osteoporosis	0.001 ^b	61.2088	0.93755	59.3462	63.0714
	controls		68.7273	1.49288	65.7458	71.7088
	total		64.7204	0.65801	63.4251	66.0157
Height [cm]	osteopenia	0.08 ^a	162.6311	0.45083	161.7386	163.5237
	osteoporosis	0.01 ^b	160.2527	0.52869	159.2024	161.3031
	controls		163.0758	0.73612	161.6056	164.5459
	total		161.9606	0.32149	161.3277	162.5934
BMI	osteopenia	0.04 ^a	24.6445	0.35747	23.9368	25.3522
	osteoporosis	0.04 ^b	23.7879	0.31784	23.1564	24.4193
	controls		25.8802	0.55665	24.7685	26.9919
	total		24.6574	0.23299	24.1988	25.1161
Age [years]	osteopenia	0.54 ^a	53.2377	0.73506	51.7825	54.6930
	osteoporosis	0.014 ^b	56.0643	0.74650	54.5883	57.5403
	controls		53.3788	1.01176	51.3582	55.3994
	total		54.4726	0.47138	53.5452	55.3999
Birth weight [g]	osteopenia	0.026 ^a	3,226.7857	77.68484	3,067.3896	3,386.1818
	osteoporosis	0.005 ^b	3,141.2500	134.07981	2,855.4656	3,427.0344
	controls		3,628.9474	110.29173	3,397.2330	3,860.6617
	total		3,326.3492	63.20235	3,200.0095	3,452.6889
Years of reproduction	osteopenia	0.724 ^a	36.2000	0.63682	34.9257	37.4743
	osteoporosis	0.528 ^b	35.6154	0.62160	34.3736	36.8572
	controls		36.3750	0.94586	34.4459	38.3041
	total		35.9936	0.40144	35.2007	36.7866
Age of first menstruation	osteopenia	0.636 ^a	13.1167	0.30908	12.4982	13.7351
	osteoporosis	0.754 ^b	12.9385	0.26843	12.4022	13.4747
	controls		13.3750	0.33224	12.6974	14.0526
	total		13.0955	0.17517	12.7495	13.4416
Age of last menstruation	osteopenia	0.069 ^a	49.2099	0.49588	48.2230	50.1967
	osteoporosis	0.058 ^b	48.1585	0.54804	47.0681	49.2490
	controls		50.1707	0.68512	48.7860	51.5554
	total		48.9804	0.32876	48.3322	49.6286
Number of pregnancies	osteopenia	0.869 ^a	1.8852	0.09821	1.6908	2.0797
	osteoporosis	0.902 ^b	1.9560	0.13525	1.6873	2.2247
	controls		1.9394	0.14940	1.6410	2.2378
	total		1.9211	0.07075	1.7819	2.0604
Years after menopause	osteopenia	0.854 ^a	7.1833	0.77769	5.6272	8.7395
	osteoporosis	0.001 ^b	10.6308	0.71305	9.2063	12.0553
	controls		7.0313	0.98832	5.0156	9.0469
	total		8.5796	0.48209	7.6273	9.5319
BMD L2–L4 [g/cm ²]	osteopenia	0.986 ^a	0.9674	0.02003	0.9276	1.0072
	osteoporosis	0.944 ^b	0.9752	0.01495	0.9456	1.0048
	controls		0.9694	0.02186	0.9254	1.0133
	total		0.9713	0.01066	0.9503	0.9923
BMD L2–L4 YA [%]	osteopenia	0.965 ^a	80.9022	1.71939	77.4868	84.3175
	osteoporosis	0.982 ^b	81.2783	1.24077	78.8203	83.7362
	controls		81.0204	1.77293	77.4557	84.5851
	total		81.0938	0.89537	79.3305	82.8570
BMD L2–L4 AM [%]	osteopenia	0.989 ^a	89.1304	1.82717	85.5010	92.7599
	osteoporosis	0.968 ^b	89.5043	1.23064	87.0665	91.9422
	controls		89.7755	1.93765	85.8796	93.6714
	total		89.4219	0.93156	87.5873	91.2564

CI – confidence interval; ANOVA – analysis of variance; BMD – bone mineral density; BMI – body mass index; SEM – standard error of the mean;

^a comparison between the groups with osteopenia and normal T-scores (one-way ANOVA); ^b comparison between the groups with osteoporosis and normal T-scores (one-way ANOVA).

In our study, we detected higher OR values in heterozygotes for rs4988321 SNP in women with both osteopenia (OR = 1.47, 95% CI = 0.57–3.63, $p = 0.24$) and osteoporosis (OR = 1.33, 95% CI = 0.67–2.75, $p = 0.24$). We were not able to determine the OR for the AA genotype in our groups due to its low incidence in the population. Elevated OR values for the A allele were found in women with osteopenia (OR = 1.75, 95% CI = 0.73–4.09, $p = 0.11$) and osteoporosis (OR = 1.32, 95% CI = 0.67–2.69, $p = 0.25$), which is consistent with the results of the study by van Meurs et al., who demonstrated the rs4988321 *LRP5* polymorphism (Val667Met) to be associated with vertebral fractures and lowered BMD values, with the OR for fracture estimated at 1.26 (95% CI = 1.08–1.47).¹⁴ The rs4988321(A) allele was associated with a reduced BMD of the lumbar vertebrae ($p = 3.3 \times 10^{-8}$) and of the femoral neck ($p = 3.8 \times 10^{-5}$). Similarly, Stathopoulou et al. observed an association between the rs4988330 polymorphism with the corrected BMD value, and the presence of the A allele was connected with significantly lower BMD as compared to the GG genotype, although a higher calcium intake (>680 mg/day) diminished the unfavorable influence of that polymorphism on BMD.¹⁹ In our study, all BMD values were higher only in the heterozygotes from the osteopenia group. Significant differences in the genotype distribution of the Val667Met (rs4988321) polymorphism were also reported for a population of Mexican women.²⁰ Analyses of the *LRP5* sequence polymorphism have visibly demonstrated ethnicity-related variations, as Sassi et al. found no correlation between the Val667Met genotype and the development of osteoporosis in Tunisian women.¹ Previously, SNP Val667Met had been suggested to determine BMD in men as well.²¹ This might be explained by the fact that the polymorphic locus is located in the extracellular domain of the receptor, responsible for Dkk-1 binding, which – being the inhibitor of the pathway – might influence Wnt signaling activity.¹ The genetic factor continues to play an important role in BMD determination and in the variability of its values between individuals. Based on the International HapMap Project and GWAS, candidate genes which might modify BMD values have been selected. Regardless, despite numerous candidate gene association studies, identification of genetic predispositions for osteoporosis continues.¹³ The literature offers countless examples that the *Lrp5*/Wnt/ β -catenin pathway seems to be the main direction to establish effective therapies for diseases associated with lowered bone mass and increased risk for fractures. At present, although the influence of *LRP5* and its homologues on BMD seems to be indisputable, the exact details of this association continue to be investigated.^{22,23}

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