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Genetic Rare Variants Affecting Multiple Pathways in Japanese Patients with Palindromic Rheumatism

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Palindromic rheumatism (PR) is a type of cryptogenic paroxysmal arthritis. Several genes may be involved in PR pathogenesis; however, conducting comprehensive case-control genetic studies for PR poses challenges owing to its rarity as a disease. Moreover, case-control studies may overlook rare variants that occur infrequently but play a significant role in pathogenesis. This study aimed to identify disease-related genes in Japanese patients with PR using whole-genome sequencing (WGS) and rare-variant analysis. Genomic DNA was obtained from two familial cases and one sporadic case, and it was subjected to WGS. WGS data of 104 healthy individuals obtained from a public database were used as controls. We performed data analysis for rare variants on detected variants using SKAT-O, KBAC, and SKAT, and subsequently defined significant genes. Significant genes combined with variants shared between the cases were defined as disease-related genes. We also performed pathway analysis for disease-related genes using Reactome. We identified 2,695,244 variants shared between cases; after excluding polymorphisms and noise, 74,640 variants were detected. We identified 540 disease-related genes, including 1,893 variants. Furthermore, we identified 32 significant pathways. Our results indicate that the detected genes and pathways in this study may be involved in PR pathogenesis.

INTRODUCTION

Palindromic rheumatism (PR) is a cryptogenic paroxysmal arthritis characterized by the periodic repetition of paroxysmal arthritis (1). It has also been suggested to represent a risk factor of developing rheumatoid arthritis (RA) and Sjögren's syndrome (2). Although the pathogenesis remains unknown, it has been suggested that PR not only exhibits the aspects of autoinflammatory diseases caused by abnormalities in the innate immune system, but also the aspects of autoimmune diseases caused by abnormalities in the acquired immune system (3). We have previously identified the splicing variant of the ASC/PYCARD gene encoding an inflammasome signaling protein complex adapter in patients with PR (4). The human leukocyte antigen (HLA) gene has been additionally suggested as a genetic factor of PR (5, 6). Therefore, several genes may be involved in the pathogenesis of PR.

In recent years, next-generation sequencing (NGS) breakthroughs have made it possible to obtain human genome sequences at a relatively lower cost and shorter time. Consequently, whole-exome sequencing (WES) and whole-genome sequencing (WGS) are being widely used, and the progression of various medical studies has accelerated (7). In particular, the identification of disease-related genes is increasing every year, and the number of genes associated with rare diseases in the Online Mendelian Inheritance in Man (OMIM) database is also increasing (8). Although genetic profiling by WES in Chinese patients with PR has been reported, no WGS analysis of PR has been reported (9).

Japanese patients with PR are even rarer, making it difficult to perform case-control analysis with a large sample size. Moreover, case-control studies may overlook rare variants that occur infrequently but play a significant role in pathogenesis. Therefore, in this study, we performed a comprehensive genetic analysis of Japanese patients with PR by combining multiple variant-analysis methods, such as variant analysis, burden test,

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and variance component test, in the DNA region of interest. Here, we present the first report identifying disease-related genes in Japanese patients with PR based on rare variant and pathway analyses.

MATERIALS AND METHODS

Samples

Three cases of PR were analyzed in the study: two were family cases involving mothers and children, whereas the third case was sporadic. These patients were examined at the Shichikawa Arthritis Center at Osaka Rehabilitation Hospital, Osaka, Japan. This study was approved by the Institutional Ethical Committee of Kobe University Graduate School of Health Sciences. The study was conducted in accordance with the principles of the Declaration of Helsinki (approval no. 140-3). Case 1: the mother was a 60-year-old Japanese woman who developed PR at the age of 57 years. Blood tests revealed the following data: C-reactive protein (CRP), 2.35 mg/dL; erythrocyte sedimentation rate (ESR), 53 mm/h; matrix metalloproteinase 3 (MMP-3), 19.0 ng/mL; rheumatoid arthritis hemagglutination (RAHA), 40×; anti-cyclic citrullinated peptide (CCP) antibody, 0.6 U/mL (negative); anti-nuclear antibody, 40×. Case 2: a 27-year-old adult who developed PR at the age of 22 years, the daughter of Case 1. Blood tests revealed the following data: CRP, 2.96 mg/dL; ESR, 21 mm/h; MMP-3, 19.5 ng/mL; uric acid (UA), 2.9 mg/dL; RAHA, 40×; anti-CCP antibody, 2.4 U/mL (negative); anti-nuclear antibody, 40×. Case 3: a 42-year-old Japanese woman whose age at onset of PR was unknown. Blood tests revealed the following data: CRP, 0.34 mg/dL; ESR, 11 mm/h; MMP-3, 43.9 ng/mL; UA, 3.2 mg/dL; RAHA, <40×; anti-CCP antibody, 1.0 U/mL (negative); anti-nuclear antibody, 40×; lupus erythematosus test, negative. Written informed consent was obtained from the patient for publication of this report.

We also obtained WGS data of 104 healthy samples from a public database (1000 Genomes Project, Phase 3, JPT samples; https://www.ncbi.nlm.nih.gov/sra) as controls.

WGS

Genomic DNA fractions of patients with PR were isolated using a PAXgene blood DNA kit (QIAGEN; Hilden, Germany). WGS was outsourced to Eurofin Genomics, Inc (Ota-ku, Tokyo, Japan). The assigned index sequences for Cases 1, 2, and 3 were ACAGTG, GTGAAA, and GCCAATAT, respectively. The NGS platform used was HiSeq X (Illumina; San Diego, CA, US), the library insert size was 300 bp, and sequencing was performed using a 150 bp paired-end sequence. The DNA Databank of Japan DRA accession number is DRA015459.

Data analysis

Variant calling was performed using the Genome Analysis Tool Kit (GATK; https://software.broadinstitute.org/gatk/) for every sample, and hg38 was used as the reference sequence. The data from the merged control samples obtained using Bcftools (http://samtools.github.io/bcftools/bcftools.html) and from the case samples were subjected to disease-related variant detection and associated rare variant analysis.

Data analysis for single sample

We performed a quality check for each sample using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), and low-quality reads were trimmed using Trimmomatic (http://www.usadellab.org/cms/?page=trimmomatic; trim primer sequence, read length was 50 bp or less, the bases of Q value was less than 20 from the start or end of the read). After trimming, a quality check was performed to confirm that the low-quality reads were removed. Trimmed samples were mapped using BWA (http://bio-bwa.sourceforge.net/), followed by variant calling using GATK software.

Detection of disease-related variants

Sequence data of the three case samples were compared using Bcftools to detect hereditary variants. First, variants with a minor allele frequency (MAF) of less than 5% were removed from the merged control samples using Snpsift (http://snpeff.sourceforge.net/SnpSift.html) to obtain a Japanese-specific variant group. Next, the shared variants between the case samples were compared with Japanese-specific variant groups using Bcftools. Variant groups detected only in the shared variants between the case samples were extracted. Japanese-specific variants with a MAF ≥5% were excluded. We also excluded unreliable variants with a quality of less than 30 and a depth of five or fewer, using Snpsift. Furthermore, the variants were annotated using SnpEff (http://snpeff.sourceforge.net/), variants with an MAF ≥5% in all populations of the 1000 Genomes Project were removed, and disease-related variants were detected.

Data analysis for associated rare variants

Case and merged control samples were further merged and used for the analysis of associated rare variants. Three methods were applied for the associated rare-variant analysis: Kernel-Based Adaptive Cluster (KBAC) (10) as weighted burden test, Sequence Kernel Association Test (SKAT) (11) as variance component test, and Optimized SKAT (SKAT-O) (12) as variance component test combined with burden test using the analysis tool Rvtests (https://zhanxw.github.io/rvtests/). Each method has its assumptions. For example, KBAC assumes that rare variants affect proteins in the same direction. Samples are divided into cases and controls for each variant. During the analysis, the variant is weighted depending on the group that it is more frequently present in. In contrast, SKAT assumes that rare variants include those that have no effect and those that have different directions of influence. In addition, the variants are weighted according to the frequency at which they present within the population. SKAT-O combines the burden test and variance component test to provide robust detection power for various scenarios. However, if the scenario assumed in each test is strongly relevant, it has less power than these tests. Therefore, in this study, to avoid the detection of genes as false positives, genes that can be detected robustly and found to be significant by SKAT-O, and found to be significant by either KBAC or SKAT, which have different assumed scenarios, were defined as significant genes. Finally, the case samples were individually analyzed, and significant genes in which only unique variants were detected in each case were removed and defined as disease-related genes (Figure 1).

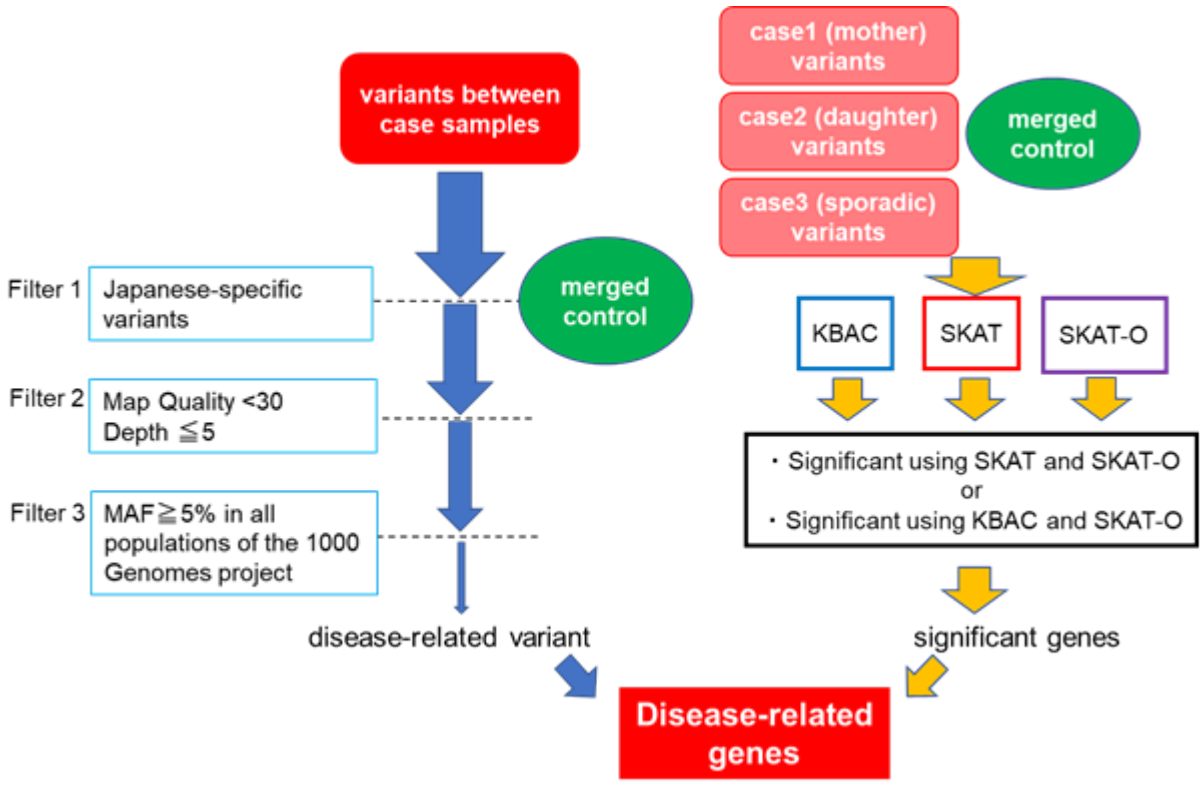

Figure 1. Strategy for the detection of disease-related genes
Japanese-specific variants were obtained from shared variants in case samples (Filter 1: MAF ≥5% in controls), noise was removed (Filter 2: Map Quality <30, Depth ≤5), and polymorphisms were removed (Filter 3: MAF ≥5% in all populations of the 1000 Genomes project), and remaining variants were identified as disease-related variants. Rare variant association analysis (SKAT-O, KBAC, and SKAT) was performed for cases and controls, and genes significant in SKAT-O and either KBAC or SKAT were defined as significant genes. Disease-related variants and significant genes were combined as disease-related genes. KBAC, Kernel-Based Adaptive Cluster; SKAT, Sequence Kernel Association Test; SKAT-O, Optimized SKAT; MAF, minor allele frequency.

Pathway analysis

Pathway analysis was performed on disease-related genes using the web analysis tool Reactome, which is weighted according to the genes and roles in the pathway and analyzed for significance (https://reactome.org/) (13, 14).

RESULTS

WGS

The WGS results were as follows: In Case 1 (the mother), the number of reads was 508,069,320, the call bases were 76,718 Mbp, and the Q30 was 94.28%. In Case 2 (the daughter), the number of reads was 494,944,820; the call bases were 74,737 Mbp; and Q30 was 94.28%. In Case 3 (sporadic), the number of reads was 661,385,994, the call bases were 99,869 Mbp, and the Q30 was 92.48% (Table I).

Table I. WGS result

Sample	Index	number of reads	Called Bases (Mbp)	%Q30
Case1 (the mother)	ACAGTG	508,069,320	76,718	94.28
Case2 (the daughter)	GTGAAA	494,944,820	74,737	94.28
Case3 (sporadic)	GCCAATAT	661,385,994	99,869	92.48

Number of reads and called bases obtained for each case, percentage of Q30.

Disease-related variants

We detected 4,798,618, 4,780,461, and 4,740,764 variants in Cases 1, 2, and 3, respectively. The number of shared variants between cases was 2,695,244. After the removal of Japanese-specific variants, 128,023 variants were identified as candidates. After removing the unreliable variants, 127,554 variants were detected. Furthermore, after removing variants with an MAF ≥5% and referencing the 1000 Genomes Project, 74,640 variants were detected (Table II). The classification of detected disease-related variants by region and effect is shown in Table III.

Table II. Disease-related variants

	number of variants
Case1 (the mother)	4,780,461
Case2 (the daughter)	4,740,764
Case3 (sporadic)	4,798,618
Shared variant	2,695,244
after filter1	128,023
after filter2	127,554
after filter3	74,640

The number of shared variants in each case was 2,695,244, and 74,640 Disease-related variants were finally detected after removing polymorphisms and noise.

Table III. Types of disease-related genes

Region	Variant effect	Count
Exon	conservative_in-frame_deletion	4
	conservative_in-frame_insertion	10
	disruptive_in-frame_deletion	6
	disruptive_in-frame_insertion	14
	frameshift_variant	10
	non_coding_transcript_exon_variant	264
	missense_variant	55
	stop_gained	7
	synonymous_variant	86
Intron	3_prime_UTR_variant	992
	5_prime_UTR_premature_start_codon_gain_variant	25
	5_prime_UTR_variant	169
	downstream_gene_variant	7,661
	intron_variant	11,418
	intergenic_region	45,126
	intragenic_variant	1
	sequence_feature	724
	splice_region_variant	134
	structural_interaction_variant	4
	upstream_gene_variant	7,930

Classification of disease-related variants by effect in exon and intron regions.

Disease-related genes

We detected 1,884, 875, and 1,036 significant genes using SKAT-O, KBAC, and SKAT, respectively ($P < 0.05$). In total, 424 genes were significant based on both SKAT-O and KBAC. In total, 958 genes were significant based on both SKAT-O and SKAT. In total, 1,073 genes were significant based on SKAT-O and based on KBAC or SKAT. These 1,073 genes were defined as significant genes ($P < 0.05$) (Table IV, Figure 2). Disease-related variants and the significant genes were combined, and 540 genes were identified as disease-related genes (Table V). Furthermore, Table VI presents the variants in which putative annotation on their impact using snpEff was MODERATE or HIGH among the disease-related genes.

Table IV. Significant genes

Collapsing Methods	number of detected genes
KBAC	875
SKAT	1,036
SKAT-O	1,884
Significant genes (Significant using SKAT and SKAT-O or Significant using KBAC and SKAT-O)	1,073

Genes that were significant in SKAT-O and significant in KBAC or SKAT were defined as significant genes, and 1,073 significant genes were detected.

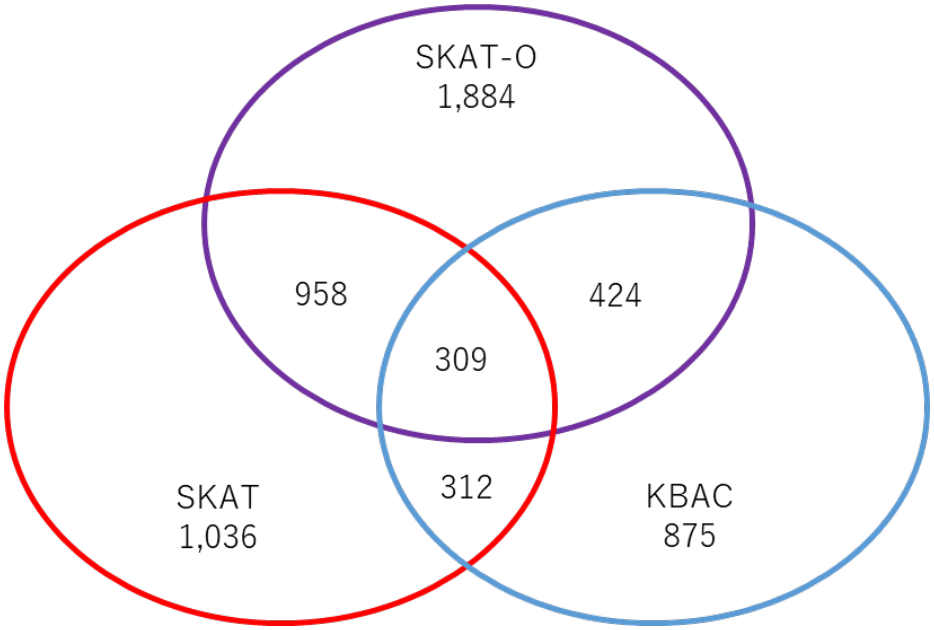

Figure 2. Genes detected by each test
A total of 958 genes were commonly detected in SKAT-O and SKAT, and 424 genes commonly detected in SKAT-O and KBAC. After removing duplicates, 1073 significant genes were finally detected.

Table V. Disease-related genes

ABCG5	*BNIPL*	*CLMP*	*FBXW8*	*ING1*	*LOC101927588*	*MTNR1A*	*POLR2J3*	*SERPING1*	*SYT1*	*USP10*
ACAT1	*BORCS8*	*CLUH*	*FERMT2*	*INTS6*	*LOC101928177*	*MTNR1B*	*PPP1R14A*	*SFTPA2*	*SYT10*	*VCPKMT*
ACTN1	*BORCS8-MEF2B*	*CLVS1*	*FGF12-AS1*	*INTS6L*	*LOC101928389*	*MYHAS*	*PPP1R7*	*SGCG*	*SYT11*	*VGLL3*
ADAMTS14	*BSND*	*CNGB3*	*FGFR2*	*IPO5*	*LOC101928535*	*MYOM1*	*PPP2CA*	*SGTA*	*SYT12*	*VLDLR*
ADAP1	*BST1*	*CNOT6L*	*FHL1*	*ISX*	*LOC101929208*	*MYRF*	*PPP4R3A*	*SH2D3C*	*SYT14*	*VLDLR-AS1*
ADARB1	*C11orf65*	*COL16A1*	*FLJ43315*	*ITGA1*	*LOC101929563*	*MYRFL*	*PPRC1*	*SH3GL1*	*SYT15.2*	*VPS28*
ADGRE3	*C1orf146*	*COPS4*	*FMN1*	*ITGBL1*	*LOC101929583*	*NAA38*	*PRDM16*	*SH3PXD2A*	*SYT16*	*VPS45*
ADGRF2	*C1orf56*	*COX4I1*	*FOXN3*	*JAKMIP1*	*LOC102031319*	*NANOS1*	*PRICKLE1*	*SHMT1*	*SYT17*	*VRK2*
ADNP	*C1QTNF7*	*COX7B2*	*FOXO1*	*KATNAL1*	*LOC102724404*	*NAPB*	*PRKAR2A*	*SHOX2*	*TFAP4*	*WASL*
ADNP2	*C6orf223*	*CRMP1*	*FRG1DP*	*KBTBD6*	*LOC102724421*	*NAV2*	*PRKCD*	*SIRPA*	*THEM4*	*WDR72*
AFDN	*CA5A*	*CRTAM*	*FTX*	*KCNC2*	*LOC102724580*	*NCOA4*	*PRKCDBP*	*SKA3*	*THOP1*	*WDR86-AS1*
AFF2	*CAMKK2*	*CRTAP*	*GAB2*	*KCNIP4*	*LOC102724708*	*NDST3*	*PRPS2*	*SLC15A4*	*THY1*	*WDR93*
AGRN	*CAPN8*	*CTH*	*GABPB2*	*KCNMA1-AS1*	*LOC105379514*	*NEIL2*	*PRR16*	*SLC15A5*	*TIAM2*	*WHRN*
AKAP13	*CARMN*	*CTTNBP2*	*GALC*	*KDSR*	*LOC202181*	*NES*	*PRRG3*	*SLC1A3*	*TLE1*	*WNK2*
AKAP8L	*CARS2*	*CYB5D1*	*GALNT16*	*KLHL23*	*LOC284395*	*NHS*	*PRUNE1*	*SLC22A12*	*TMC6*	*WWOX*
AKIRIN2	*CAST*	*CYP4F12*	*GDAP2*	*KLK14*	*LOC285000*	*NHSL1*	*PTGER4P2-CDK2AP2P2*	*SLC30A9*	*TMC8*	*WWP2*
ALKBH8	*CATSPER2P1*	*CYP7B1*	*GGA2*	*KPRP*	*LOC440910*	*NHSL2*	*PTGIS*	*SLC35F6*	*TMCO5B*	*YEATS2*
ANKDD1A	*CATSPERD*	*DAGLA*	*GIMAP8*	*KSR1*	*LOC643072*	*NME9*	*PTPRB*	*SLC36A1*	*TMEM114*	*YPEL5*
ANKRD20A5P	*CATSPERG*	*DAP*	*GKN1*	*L2HGDH*	*LRRC72*	*NNT-AS1*	*PVT1*	*SLC39A4*	*TMEM132E*	*YWHAG*
ANKUB1	*CBLN2*	*DAP3*	*GNAI1*	*LAMA5*	*LRRC74A*	*NOS1*	*PWWP2A*	*SLC45A1*	*TMEM221*	*ZBTB26*
ANOS1	*CC2D2A*	*DAPK1*	*GNG10*	*LAMB4*	*LRRTM1*	*NOS1AP*	*PXYLP1*	*SLC45A4*	*TMEM235*	*ZC3HC1*
ANTXR1	*CCDC14*	*DAPK2*	*GPC6*	*LDHC*	*LSM12*	*NSMAF*	*PYROXD1*	*SLC52A3*	*TMEM267*	*ZCCHC4*
ANXA2	*CCDC140*	*DNAH2*	*GPR65*	*LINC00032*	*LY75*	*NUDCD2*	*QSER1*	*SLFN14*	*TMEM35A*	*ZFAND2A*
AQP1	*CCDC141*	*DNAJC25-GNG10*	*GRID2IP*	*LINC00343*	*LY75-CD302*	*NXPE4*	*RAB30*	*SLIT3*	*TMEM52B*	*ZFP1*
AQP12B	*CCDC142*	*DNM1*	*GRK7*	*LINC00364*	*MACF1*	*OLR1*	*RAB30-AS1*	*SMARCA5*	*TMTC3*	*ZFP14*
ARHGAP17	*CCDC144B*	*DNM1P41*	*GSG1*	*LINC00365*	*MAF*	*OTOGL*	*RABGAP1*	*SMARCA5-AS1*	*TNR*	*ZMYM2*
ARHGAP19-SLIT1	*CCDC144CP*	*DNM1P46*	*GSG1L*	*LINC00486*	*MAF1*	*P2RX7*	*RABGAP1L*	*SMCHD1*	*TNRC18*	*ZMYM6*
ARHGAP21	*CCDC146*	*DSG2*	*GSG1L2*	*LINC00598*	*MAFA*	*P3H1*	*RAD17.2*	*SMCO4*	*TNRC18P1*	*ZNF292*
ARHGAP30	*CCDC148*	*DTNA*	*HDAC9*	*LINC00649*	*MAFF*	*P3H2*	*RAD52*	*SMCR8*	*TNRC6B*	*ZNF329*
ARHGAP42	*CCDC148-AS1*	*DUOX1*	*HEATR5A*	*LINC01091*	*MAFG*	*P3H2-AS1*	*RAMP3*	*SNAP29*	*TNRC6C*	*ZNF333*
ARHGEF28	*CCDC149*	*DUSP10*	*HECTD1*	*LINC01136*	*MAFG-AS1*	*PARP6*	*RASAL2*	*SNCAIP*	*TRIM42*	*ZNF398*
ARHGEF38	*CD302*	*DUT*	*HERC2*	*LINC01169*	*MAFIP*	*PCYT1B-AS1*	*RASEF*	*SNTG1*	*TRIM69.2*	*ZNF580*
ARHGEF38-IT1	*CDK8*	*DUXA*	*HERC2P2*	*LINC01221*	*MAFTRR*	*PDE4D*	*RCBTB1*	*SNUPN*	*TRIO*	*ZNF584*
ARSA	*CECR2*	*EEF1AKMT1*	*HERC2P3*	*LINC01266*	*MELK*	*PDE4DIP*	*REC114*	*SNX27*	*TRIOBP*	*ZNF600*
ASB18	*CENPI*	*EFCAB9*	*HERC2P9*	*LINC01312*	*METTL22*	*PHACTR1*	*RECQL*	*SNX33*	*TSPO*	*ZNF610*
ASTN2-AS1	*CEP128*	*EFNB3*	*HLA-DQA1*	*LINC01324*	*MINOS1*	*PI4KA*	*RECQL5*	*SORBS1*	*TTC21B*	*ZNF749*
ASXL1	*CEP152*	*EGFL7*	*HLA-DQB1*	*LINC01349*	*MINOS1-NBL1*	*PIK3CD-AS1*	*RHEB*	*SPAM1*	*TTC23*	*ZNF845*
ATP11B	*CEP170P1*	*ELP4*	*HLA-DQB1-AS1*	*LINC01378*	*MINOS1P1*	*PIK3CD-AS2*	*RHPN2*	*SPANXA2.2*	*TTC23L*	*ZSCAN18*
ATP2B2	*CEP41*	*EPDR1*	*HNF1A*	*LINC01410*	*MIPOL1*	*PIK3R6*	*RNF175*	*SPANXA2-OT1*	*TTC39A*	*ZSCAN21*
AVEN	*CEP89*	*EPHA3*	*HOMER2*	*LINC01599*	*MIR1-1HG*	*PKM*	*RPE65*	*SPARC*	*TTLL13P*	*ZSCAN5A*
B3GLCT	*CERS3*	*ERC2*	*HPN*	*LINGO4*	*MIR31HG*	*PLEKHG1*	*RPS10*	*SPARCL1*	*TUBB8*	
B3GNT6	*CERS3-AS1*	*ERC2-IT1*	*HPN-AS1*	*LIPE-AS1*	*MIR3689A*	*PLEKHO1*	*RPS10-NUDT3*	*SPTSSA*	*UBASH3B*	
BCAS4	*CFTR*	*ESYT1*	*HTR3C*	*LOC100128317*	*MIR4487*	*PLG*	*RSU1*	*SRGAP3*	*UBE2H*	
BCL2L14	*CHD7*	*EYA2*	*HTT*	*LOC100129603*	*MIR548AQ*	*PLK5*	*RUNX2*	*SRL*	*UBE3C*	
BCL3	*CHDH*	*FAAP24*	*IFFO2*	*LOC100132249*	*MLIP-IT1*	*PMS2P3*	*SAA1*	*SRSF10*	*UBL4A*	
BIN2	*CHODL*	*FAM174B*	*IFT88*	*LOC100506207*	*MLLT10*	*PNOC*	*SAP18*	*ST6GALNAC5*	*UNC5B*	
BMP2K	*CHODL-AS1*	*FAM90A1*	*IKZF4*	*LOC100507002*	*MLLT10P1*	*POLG2*	*SARAF*	*SULF2*	*UNC5C*	
BMP8A	*CHRM5*	*FANCD2*	*IL17D*	*LOC101927050*	*MRPL52*	*POLQ*	*SCAP*	*SUPT3H*	*UNC5D*	
BMPR2	*CIAPIN1*	*FAR1*	*IL1R1*	*LOC101927124*	*MRPS10*	*POLR1A*	*SCAPER*	*SYN2*	*UPB1*	
BMT2	*CIB1*	*FARSA*	*IL20RA*	*LOC101927189*	*MSC-AS1*	*POLR1B*	*SEMA3D*	*SYNM*	*USF3*	

The 540 disease-related genes were defined as genes that were significant in SKAT-O and significant in either KBAC or SKAT analysis, for which shared variants were detected between cases.

Table VI. Classification of variants with putative annotation on their impact in Disease-related Genes

gene	Putative Impact	variant_effect	Nucleotide_change	Amino_acid_change	rs_ID
ACTN1	MODERATE	sequence_feature modified-residue: phosphoserine	c.515+14T>G		rs743128
ARHGAP21	MODERATE	missense_variant	c.5849G>C	p.Ser1950Thr	rs1127893
C6orf223	MODERATE	disruptive_in-frame_insertion	c.392_397dupCGGCGG	p.Ala131_Ala132dup	rs778896183
CAMKK2	MODERATE	conservative_in-frame_insertion	c.1612_1614dupAAA	p.Lys538dup	rs398021385
COPS4	MODERATE	sequence_feature modified-residue: N6-acetyllysine	c.75-4865_75-4864delTT		rs146126553
DAP	MODERATE	sequence_feature modified-residue: Phosphoserine	c.152+13002dupT		rs57849320
LY75	MODERATE	sequence_feature glycosylation-site: N-linked (GlcNAc...)	c.3959-15dupA		rs36120198
SLC22A12	HIGH	stop_gained	c.774G>A	p.Trp258*	rs121907892

Variants of disease-related genes with HIGH or MODERATE impact using snpEff. Variants classified as high are assumed to influence disruptive impact in the protein, probably causing protein truncation, loss of function, or triggering nonsense-mediated decay (e.g., stop gained, frameshift). Variants classified as MODERATE are assumed non-disruptive variants that may change protein function (e.g., missense, in-frame).

Pathway analysis

We detected 32 significant pathways associated with disease-related genes based on Reactome analysis ($P < 0.05$). Among the significant pathways, four were involved in the regulation of gene expression (four genes), 13 were involved in cell growth and proliferation (16 genes), two were involved in development (six genes), two were involved in the neuronal system (eight genes), three were involved in cell–cell communication (five genes), one was involved in programmed cell death (three genes), one was involved in vesicle-mediated transport (five genes), and six were involved in other functions (four genes) (Table VII). The variants detected in the genes associated with each pathway are shown in Table VIII.

Table VII. Function of each pathway affected by disease-related genes

Function	classification of pathway	Pathway name	Entities *P* Value	Entities FDR	Submitted entities found
regulation of gene expression	Transcriptional regulation by RUNX2	RUNX2 regulates genes involved in cell migration	0.002554321	0.729436064	*ITGBL1, RUNX2*
		RUNX2 regulates genes involved in differentiation of myeloid cells	0.02427843	0.729436064	*RUNX2*
		RUNX2 regulates chondrocyte maturation	0.032202418	0.729436064	*RUNX2*
	Gene Silencing by RNA	Post-transcriptional silencing by small RNAs	0.032202418	0.729436064	*TNRC6C, TNRC6B*
cell growth, proliferation	Signaling by FGFR	Phospholipase C-mediated cascade; FGFR2	0.018517558	0.729436064	*FGFR2*
		FGFR2 ligand binding and activation	0.021012613	0.729436064	*FGFR2*
		Negative regulation of FGFR2 signaling	0.025190644	0.729436064	*PPP2CA, FGFR2*
		PI-3K cascade:FGFR2	0.036489082	0.729436064	*FGFR2*
		SHC-mediated cascade:FGFR2	0.044113664	0.729436064	*FGFR2*
		FRS-mediated FGFR2 signaling	0.048235436	0.729436064	*FGFR2*
	RHO GTPase cycle	RAC3 GTPase cycle	0.020262898	0.729436064	*ARHGAP21, ARHGAP42, TRIO, NHS, DSG2, ARHGAP17, VRK2, ESYT1, FERMT2*
		RAC2 GTPase cycle	0.032849692	0.729436064	*ARHGAP21, ARHGAP42, TRIO, NHS, DSG2, ARHGAP17, VRK2, ESYT1*
		RAC1 GTPase cycle	0.045108319	0.729436064	*PLEKHG1, TRIO, ARHGAP17, WASL, VRK2, TIAM2, ARHGAP21, ARHGAP42, ARHGAP30, NHS, SRGAP3, ESYT1, FERMT2*
	IRS-mediated signaling	PI3K Cascade	0.030014691	0.729436064	*THEM4, GAB2, FGFR2*
		IRS-mediated signalling	0.047507509	0.729436064	*THEM4, GAB2, FGFR2*
	PTEN Regulation	Competing endogenous RNAs (ceRNAs) regulate PTEN translation	0.041118945	0.729436064	*TNRC6C, CNOT6L, TNRC6B*
	Signaling by ERBB4	Nuclear signaling by ERBB4	0.041313975	0.729436064	*WWOX, SPARC, ADAP1*
Developmental Biology	Netrin-1 signaling	Netrin mediated repulsion signals	0.007768538	0.729436064	*UNC5B, UNC5C, UNC5D*
		Netrin-1 signaling	0.032203918	0.729436064	*TRIO, UNC5B, UNC5C, SLIT3, WASL, UNC5D*
Neuronal System	Protein-protein interaction at synapses	Neurexins and neuroligins	0.011078363	0.729436064	*NHSL1, SYT1, SYT12, HOMER2, LRRTM1, SYT10, DAP3*
		Protein-protein interactions at synapses	0.034647971	0.729436064	*LINGO4, NHSL1, SYT1, SYT12, HOMER2, LRRTM1, SYT10, DAP3*
Cell-Cell communication	Signal regulatory protein family interactions	Signal regulatory protein family interactions	0.035974514	0.729436064	*SFTPA2, SIRPA*
	Cell junction organization	Regulation of cytoskeletal remodeling and cell spreading by IPP complex components	0.040990706	0.729436064	*ACTN1, RSU1*
		Cell-extracellular matrix interactions	0.041118945	0.729436064	*ACTN1, RSU1, FERMT2*
Programed Cell Death	Caspase activation via extrinsic apoptotic singnaling pathway	Caspase activation via Dependence Receptors in the absence of ligand	0.012667173	0.729436064	*DAPK1, UNC5B, DAPK2*
Vesicle-mediated transport	Intra-Golgi and retrograde Golgi-to-ER traffic	Intra-Golgi traffic	0.047806738	0.729436064	*NAPB, RAB30, SYT1, VPS45, SNAP29*
other	Disease of signal transduction by growth factor receptors and second messengers	Signaling by FGFR2 IIIa TM	0.002972328	0.729436064	*FGFR2*
		FGFR2 mutant receptor activation	0.008105055	0.729436064	*FGFR2*
		Signaling by FGFR2 amplification mutants	0.017303037	0.729436064	*FGFR2*
		Signaling by FGFR in disease	0.018257814	0.729436064	*ZMYM2, GAB2, FGFR2*
		Signaling by FGFR2 in disease	0.030014691	0.729436064	*FGFR2*
	Diseases associated with surfactant metabolism	Defective SFTPA2 causes IPF	0.038978291	0.729436064	*SFTPA2*

Significant pathways ($P < 0.05$) in the results of pathway analysis using Reactome were classified based on function.

Table VIII. List of variants detected in genes via pathway analysis

gene	variant_effect	number of variants
ACTN1	intron_variant	1
ADAP1	intron_variant	3
ARHGAP17	downstream_gene_variant	1
	intron_variant	1
	upstream_gene_variant	1
ARHGAP21	missense_variant	1
	intron_variant	2
ARHGAP30	intron_variant	5
ARHGAP42	intron_variant	8
	downstream_gene_variant	2
CNOT6L	3_prime_UTR_variant	2
	intron_variant	3
	splice_region_variant&intron_variant	1
DAP3	intron_variant	5
	downstream_gene_variant	1
DAPK1	intron_variant	2
DAPK2	intron_variant	4
DSG2	downstream_gene_variant	1
ESYT1	downstream_gene_variant	1
FERMT2	intron_variant	2
FGFR2	downstream_gene_variant	2
	intron_variant	3
	5_prime_UTR_variant	1
GAB2	intron_variant	2
HOMER2	intron_variant	5
ITGBL1	intron_variant	10
	3_prime_UTR_variant	1
	downstream_gene_variant	1
LINGO4	downstream_gene_variant	1
LRRTM1	downstream_gene_variant	1
NAPB	intron_variant	1
NHS	intron_variant	6
NHSL1	intron_variant	10
PLEKHG1	intron_variant	9
PPP2CA	intron_variant	2
RAB30	intron_variant	1
	upstream_gene_variant	1
RSU1	intron_variant	14
RUNX2	intron_variant	2
SFTPA2	downstream_gene_variant	1
SIRPA	intron_variant	1
SLIT3	intron_variant	10
SNAP29	intron_variant	2
	3_prime_UTR_variant	1
SPARC	intron_variant	1
SRGAP3	intron_variant	6
SYT1	intron_variant	15
SYT10	intron_variant	3
SYT12	intron_variant	1
THEM4	intron_variant	1
	upstream_gene_variant	1
TIAM2	intron_variant	9
TNRC6B	intron_variant	9
TNRC6C	intron_variant	2
TRIO	intron_variant	8
UNC5B	downstream_gene_variant	1
UNC5C	intron_variant	8
UNC5D	intron_variant	9
	downstream_gene_variant	1
VPS45	intron_variant	8
	downstream_gene_variant	1
VRK2	intron_variant	5
WASL	intron_variant	1
WWOX	upstream_gene_variant	1
	intron_variant	41
	downstream_gene_variant	1
ZMYM2	intron_variant	5

The number and effects of variants were detected in genes related to 32 significant pathways.

DISCUSSION

Several HLA class II genes (HLA-DRA, HLA-DR1, and HLA-DR4) are associated with PR (5, 6, 9). Our study detected HLA-DQB1 and HLA-DQB1-AS1 as disease-related HLA genes. The WES report by Zheng et al. (2023) also suggested an association with HLA-DQB1. These findings suggest that several HLA genes are involved in PR.

Interestingly, a nonsense mutation p.W258X in Solute Carrier Family 22 Member 12 (SLC22A12) was detected as the high putative impact variant. SLC22A12 functions as a uric acid transporter that regulates blood uric acid levels, and this gene mutation causes renal hypouricemia (15–17). Uric acid is a powerful antioxidant, and oxidative stress increases when blood uric acid decreases (18). In addition, interleukin 6 is produced by active oxygen via MAP 3 kinase 1 and SAPK kinase kinase and induces inflammation (19). Our finding suggests that SLC22A12 dysfunction may contribute to pathogenesis in PR. The risk of cardiovascular diseases is 4.7-fold higher in patients with RA with hypouricemia than in patients with normouricemia (20). Our result suggests that hypouricemia in patients with PR may be a potential risk of cardiovascular diseases when transitioning to RA.

Pathway analyses revealed multiple gene regulatory pathways involving Runt-related transcription factor 2 (RUNX2), integrin subunit beta-like 1, trinucleotide repeat-containing adaptor 6B (TNRC6B), and TNRC6C. RUNX2 is a well-known transcription factor that induces the differentiation of mesenchymal stem cells into osteoblasts (21). In addition, Homer Scaffolding Protein 2 (HOMER2) and Calcium/Calmodulin Dependent Protein Kinase Kinase 2 (CAMKK2) were detected as disease-related genes. HOMER2 has been suggested as a key regulator of receptor activator of nuclear factor-kappa B ligand-mediated osteoclastogenesis along with Homer Scaffold Protein 3 (22), which was also pointed out in the WES report by Zheng et al. (2023). CaMKK2 has been suggested to regulate osteoblast formation via the Protein Kinase A pathway and osteoclast differentiation via the regulation of Nuclear Factor of Activated T Cells 1 (23). It has been found that some patients with PR show transition into RA (2). Further, abnormal osteoclasts are involved in the pathogenesis of bone destruction in RA (24, 25). Our results suggest that the pathogenesis of bone destruction in RA transitioning from PR is caused not only by abnormal osteoclasts but also by abnormal bone formation via RUNX2-related pathways.

Multiple mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) signaling pathways related to cell growth and proliferation via fibroblast growth factor receptor 2 (FGFR2), protein phosphatase 2 catalytic subunit alpha, thioesterase superfamily member 4, GRB2-associated-binding protein 2, TNRC6B, TNRC6C, and CCR4-NOT transcription complex subunit 6-like were identified. The MAPK and PI3K signaling pathways activated by FGFR2 have been suggested to be associated with RA (26), and our results suggest the involvement of similar signaling pathways in PR. The MAPK and PI3K signaling pathways activated by FGFR2 may be involved in the pathogenesis of various diseases, including PR.

The involvement of the RHO GTPase cycle including the Rac family small GTPase 1 (RAC1), RAC2, and RAC3 GTPase cycles have shuttled between GDP-bound inactive and GTP-bound active forms via GTP hydrolysis regulated by Rho GTPase-activating protein 17 (ARHGAP17), ARHGAP21, ARHGAP30, ARHGAP42, extended synaptotagmin 1, fermitin family homolog 2 (FERMT2), NHS actin remodeling regulator (NHS), pleckstrin homology and RhoGEF domain-containing G1, SLIT-ROBO Rho GTPase-activating protein 3, TIAM Rac1-associated GEF 2, Trio Rho guanine nucleotide exchange factor (TRIO), VRK serine/threonine kinase 2, and WASP-like actin nucleation-promoting factor (WASL) was suggested. The RHO GTPase cycle has been reported to be involved in various autoimmune diseases as a cytokine regulatory factor (27–29), and involvement of the RHO GTPase cycle in PR has also been suggested.

A netrin-1 signaling pathway, which is regulated by slit guidance ligand 3, TRIO, Unc-5 netrin receptor B (UNC5B), UNC5C, UNC5D, and WASL, and is relevant to development, has been identified. The netrin family of axon-inducing factors is involved in the regulation of macrophages and these proteins represent important molecules in inflammation and immune responses (30–32). Our results suggest that the pathology of PR may be affected by abnormalities in inflammation and immune responses mediated by the netrin-1 signaling pathway.

Multiple pathways involved in cell–cell communication mediated by alpha-actinin-1, FERMT2, Ras suppressor protein 1, surfactant protein A2, and signal regulatory protein alpha were identified. Cell–cell communication is important for mediating the immune response, and these genes have been suggested to be associated with autoimmune diseases (33, 34). Our results suggest that the pathology of PR may be mediated by an abnormal immune response via cell–cell communication.

The pathways involved in intra-Golgi trafficking mediated by NSF attachment protein beta, Ras-related protein Rab-30, synaptotagmin-1, vacuolar protein sorting 45 homolog, and synaptosome-associated protein 29 were identified. Intra-Golgi trafficking is important for facilitating transport of proteins involved in the immune response. The accumulation of mutant proteins in the Golgi promotes the formation of pyrin inflammasomes, which results in the overproduction of inflammation-inducing substances interleukin 1 beta (IL-1β) and IL-18 (35). Our results suggested that the pathology of PR may be affected by the transport of immune-related proteins.

This WGS study is the second comprehensive NGS analysis of patients with PR, following Zheng et al.'s (2023) study using WES (9). In the present study, we identified many genetic variants not reported by Zheng et al. (2023); however, we found similarities in their association with HLA-DQB1 and functional deviations in osteogenesis. Possible reasons for the different variants found include racial differences, sample size differences, and our report being based on WGS rather than WES, and mainly due to the predominant analysis method we used being focused on rare variants. Genetic variants affecting pathogenesis can be broadly classified into variants that are significant in frequency and variants that are rare in frequency but have a significant effect on pathogenesis. Therefore, while it is difficult to compare the previous study with ours, we believe that the combined results of both studies will cover the genetic variants involved in the pathogenesis of palindromic rheumatism.

We previously identified a splice variant form of ASC, which is the common adaptor of inflammasomes, in patients with PR (4). It was not detected as one of the disease-related genes in the present study, which is attributed to the fact that the association between ASC and PR is not related to rare variants and it is also suggested that ASC is secondarily associated with other regulatory genes; the affected inflammasome pathways related to IL-1β and IL-18 secretion also represent candidates related to the pathogenesis of PR.

It has been assumed that large sample sizes are required for the analysis of disease-associated rare variants. Zuk et al. reported that a well-powered analysis of associated rare variants in a multifactorial disease would require a sample size of 25,000 cases (36). Conversely, it has been suggested that even with 5,000 cases, it may be possible to detect genes with a 10–20 times higher risk; however, it is difficult to this number of cases for rare diseases. In fact, although future work to confirm our results should aim for larger sample sizes, it is not realistic to collect cases in the thousands, even taking into account the number of all patients with PR in Japan. Thus, considering genetic racial differences, an international framework to collect cases also seems difficult. Therefore, for the analysis of associated rare variants, verification experiments using a combination of multiple analysis methods, multi-omics including proteome and metabolome, and wet experiments are recommended. Our study aimed to detect more related gene groups by combining multiple analyses of associated rare variants, focusing on variants that are shared among cases, and performing pathway analysis, using a small sample size. Further validation is required to advance genetic analysis using a small sample size.

In conclusion, we report the first WGS analysis of PR and identified 540 disease-related genes, including 32 pathways which are significantly associated with PR. This suggests that several rare variants of multiple genes and multiple pathway abnormalities affected by them are involved in the pathogenesis of PR. Our analysis was limited to rare variants shared in all cases owing to the small number of cases in this study; however, we propose that previously overlooked genes represent candidates for disease-related genes.

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