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Hereditary Pancreatitis Model WBN/Kob Rat Strain Has a Unique Haplotype in the *Pdwk1* Region on Chromosome 7

Masayuki MORI, Xiaoying FU, Lei CHEN, Guohong ZHANG, and Keiichi HIGUCHI

Department of Aging Biology, Institute on Aging and Adaptation, Shinshu University Graduate School of Medicine, 3–1–1 Asahi, Matsumoto 390-8621, Japan

Abstract: The WBN/Kob rat strain is a hereditary animal model of chronic pancreatitis and diabetes mellitus. The major WBN/Kob loci for pancreatitis (*Pdwk1* and *Pdwk2*) are located on chromosomes 7 and X, respectively. In this study, polymorphisms were sought for candidate genes in the *Pdwk1* and *Pdwk2* regions. Nucleotide polymorphisms were found in 14 candidate genes examined in the *Pdwk1* region. These polymorphisms were not associated with functional changes, and hence were unlikely to be a cause of pancreatitis. Seven nucleotide polymorphisms in three candidate genes, *Rac2*, *Grap2*, and *Xpnpep3*, located within a 3.3-Mb region were not found in 14 other inbred rat strains. These results suggest that WBN/Kob has a unique haplotype block in the chromosomal region contatining *Pdwk1*.
Key words: gene polymorphism, pancreatitis, WBN/Kob

Chronic pancreatitis (CP) is a continuing or recurring inflammatory disease of the pancreas that typically causes pain and leads to irreversible morphological and functional damage [12]. The etiology of most CP cases is still unclear. Male WBN/Kob rats represent an animal model of CP and diabetes mellitus [3, 6, 10]. The abnormality initiates at approximately 12 weeks of age with marked fibrosis around the pancreatic ducts and blood vessels. Then fibrous tissue gradually and extensively invades the pancreas. The islets are also affected by fibrotic degeneration, leading to an obvious decrease in islet number and size. In addition, inflammatory cells infiltrate and damage tissue around the islets and among adjacent acinar cells. This endocrine-exocrine dysfunction eventually leads to diabetes mellitus at 60–90 weeks of age. The processes leading to pancreatitis in WBN/Kob have been extensively studied. These studies have revealed the involvement of sex hormones [4, 7], imbalances of prolyl hydroxylase and collagenase [2], and autoimmunity [8] in pancreatitis. However, the etiology of pancreatitis in WBN/Kob rats is still unknown. Genetic factors are strongly suspected. Thus, a genetic approach should help to elucidate the fundamental cause of WBN/Kob pancreatitis and diabetes mellitus. Chromosomal mapping of WBN/Kob rats for pancreatitis and diabetes mellitus genes was performed by breeding F_2 hybrid progeny between WBN/Kob and BN [11], and peaks of likely ratio statistical scores for linkage to pancreatitis were observed on two chromosomes. One was a broad peak between *D7Rat97* (70.3 cM) and *D7Rat4* (80.5 cM) on chromosome 7, and the other was at *DXRat103* (37.6 cM) on chromosome X. These loci were designated *Pdwk1* and *Pdwk2* (pancreatitis and diabetes mellitus in WBN/Kob locus 1 and 2), respectively. Identification of *Pdwk1* and *Pdwk2* genes should shed new light on pancreatitis mechanisms, and to this

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Address corresponding: M. Mori, Department of Aging Biology, Institute on Aging and Adaptation, Shinshu University Graduate School of Medicine, 3–1–1 Asahi, Matsumoto 390-8621, Japan

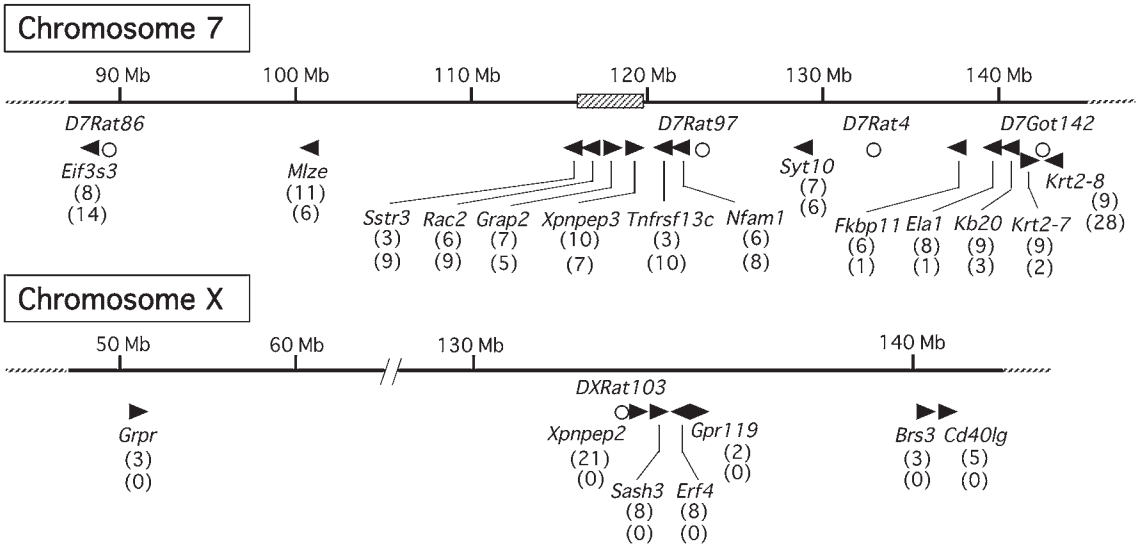

Fig. 1. Maps of candidate genes on chromosomes 7 and X. Arrowheads and circles represent candidate genes and marker loci, respectively. Numbers in parenthesis are exon counts for the genes (up) and for the identified SNPs. A hatched box indicates the 3.3-Mb region of WBN/Kob-specific haplotype.

end, we examined polymorphisms in candidate genes located in the WBN/Kob *Pdwk1* and *Pdwk2* regions.

Specific pathogen-free 21-week-old male WBN/Kob and BN/SsN rats were purchased from Japan SLC (Hamamatsu, Japan). After euthanasia with ether inhalation, the pancreas and spleen were removed. WBN/Kob rats were confirmed to have pancreatitis, while BN rats had normal pancreata (data not shown). Genomic DNA and mRNA were isolated by standard procedures. All experimental procedures were carried out in accordance with the Regulations for Animal Experimentation of Shinshu University.

Candidates with the following criteria were selected from genes located around *D7Rat97*, *D7Rat4*, and *DXRat103* marker loci: (i) genes reported to be associated with pancreatitis in humans or mice; (ii) genes associated with pancreatic development or function; (iii) genes associated with leukocyte development or function. Fourteen candidate genes on chromosome 7, eukaryotic transcription initiation factor 3 (*Eif3s3*), melanoma-derived leucine zipper, extra-nuclear factor (*Mlze*), somatostatin receptor 3 (*Sstr3*), RAS-related C3 botulinum substrate 2 (*Rac2*), GRB2-related adaptor protein 2 (*Grap2*), X-prolyl aminopeptidase 3 (*Xpnpep3*), tumor necrosis factor receptor superfamily, member 13c (*Tnfrsf13c*), NFAT activating protein with ITAM motif 1 (*Nfam1*), synaptotagmin X (*Syt10*), FK506 binding protein 11 (*Fkbp11*), elastase 1 (*Ela1*), type II keratin Kb20 (*Kb20*), keratin complex 2, basic, gene 7 (*Krt2-7*), and keratin complex 2, basic, gene 8 (*Krt2-8*), were examined (Fig. 1). Seven candidate genes on chromosome X, gastrin releasing peptide receptor (*Grpr*), X-prolyl aminopeptidase 2 (*Xpnpep2*), SAM and SH3 domain containing 3 (*Sash3*), E74-like factor 4 (*Elf4*), G protein-coupled receptor 119 (*Gpr119*), bombesin-like receptor 3 (*Brs3*), and CD40 ligand (*Cd40lg*) were also examined. Primers for PCR amplification and sequencing were designed based on the rat genome sequence (rat genome assembly version 3.4 (RGSCv3.4)), so that approximately 500–1,000 bp of DNA fragments containing exon(s) and partial intron sequences could be amplified from genomic DNA. PCR products were purified with an UltraClean PCR Clean-up DNA Purification Kit (MO BIO Laboratories, Carlsbad, USA). An aliquot was sequenced by using a BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, USA), according to the manufacturer's instructions, and analyzed on an ABI PRISM Genetic analyzer 3130 (Applied Biosystems).

First, nucleotide sequences of candidate genes were compared between the WBN/Kob and BN strains. No polymorphisms were found for seven genes on chromosome X. The reason for the absence of polymorphisms

is unclear. One possibility is that WBN/Kob and BN share an identical haplotype in this chromosomal region. In contrast, one hundred and nine nucleotide alterations were found in fourteen genes within an approximately 53-Mb segment of the *Pdwk1* region on chromosome 7. Sixteen of these were found in Ensembl (http://www.ensembl.org/). Others were considered to be newly identified polymorphisms, and registered in the NCBI SNP database (http://www.ncbi.nlm.nih.gov/SNP/) with a handle name of DABSUGSM. Other rat strains were then genotyped for the polymorphisms to examine if the polymorphisms were unique to WBN/Kob. The WBN/Kob strain originated from a Wistar rat colony of the Institute of Experimental Gerontology in Basel and has been inbred at the Institute of Pathology, University of Bonn since 1961 [3]. To our knowledge, both the origin and current state of the Wistar colony are uncertain. According to phylogenetic cluster analysis of rat strains with single nucleotide polymorphisms (SNPs), WBN/Kob has a close phylogenetic relationship to the clusters of BD and ACI, but not to the cluster of WKY (Wistar) [supplemental information to 9]. Thus, we examined BDIX (from the BD cluster), ACI, DA, DONRYU, and SHR/Izm rats. When the polymorphic nucleotides of the WBN/Kob allele were not found in these strains, IS, TM, NIG-III, LEW, WM, PVG/c, LOU/M, KHR, and KMI strains were also examined. Genomic DNA samples of BDIX, ACI, WM/Ms, PVG/c, LOU/M, IS, DONRYU, DA, TM, LEW, and NIG-III were kindly donated by Dr. T Nishikawa of the National Institute of Radiological Sciences, Japan. Genomic DNA samples of KHR and KMI strains were provided by the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University, through the National BioResource Project (NBRP) of the MEXT, Japan. Most polymorphic nucleotides were found in one or more of rat strains. However, five nucleotide changes in three genes, *Rac2*, *Grap2*, and *Xpnpep3* were not found in any of these strains, suggesting that these changes are specific to the WBN/Kob strain. G to A nucleotide substitutions in exon 2 of the *Rac2* (RGSCv3.4 position 116,530,532) and in intron 4 of *Grap2* (position 118,928,554) were unique to WBN/Kob rats. Also, WBN/Kob rats had unique repeat numbers of (TG)n and (AG)n microsatellites in introns 1 and 2 of *Grap2* (positions 118,906,980–118,907,009 and 118,920,663–118,920,706). Three polymorphisms in *Xpnpep3* on WBN/Kob rats were not found in the other rat strains examined. One polymorphism was a microsatellite in intron 4 (position 119,756,405–119,756,470) in which WBN/Kob uniquely had five repeats of GCCTCT, while the other strains had ten or eleven repeats. The second polymorphism was a G to A nucleotide substitution at 259 bases upstream of the initiating methionine codon (position 119,729,247). The third polymorphism was a G to T nucleotide substitution in exon 2 (position 119,743,296), leading to a substitution of a histidine for a glutamine at codon 28 (Q28H; Fig. 2). In addition to this missense nucleotide substitution, there was an A to G substitution in exon 1 between BN and WBN/Kob strains, leading to a substitution of a threonine for an alanine at codon 15 (A15T). Screening of the other rat strains revealed that 15A is unique to the BN strain. Combining the two amino acid substitutions, there were three XPNPEP3 isoforms in the rat strains (Fig. 2).

X-prolyl aminopeptidase specifically catalyzes the removal of any unsubstituted N-terminal amino acid adjacent to a penultimate proline residue (N-terminal imido bond). The fibrotic area in the WBN/Kob rat pancreas is mainly composed of type-III collagen [2]. N-terminal imido bonds are common to several collagen degradation products. Thus, it is possible that a mutation in the *Xpnpep3* gene disturbs collagen metabolism, eventually leading to fibrosis. Both of the amino acid substitutions in WBN/Kob rats are located in the putative mitochondrial localization signal sequence of XPNPEP3 [1]. The Q28H substitution in particular is located at three amino acids upstream of the predicted cleavage site of the signal sequence. The influence of the substitutions on mitochondrial localization signal function was then examined. DNA fragments containing the coding sequence of rat *Xpnpep3* were PCR-amplified from 1st-strand pancreas cDNA of SCR, BN, and WBN/Kob rats, and were inserted into the plasmid vector pEGFP-N3 (Clontech, Palo Alto, USA) so that the XPNPEP3 protein was expressed as a green fluorescent protein (GFP) fusion protein. Cultured COS cells were transfected with expression plasmids. Two days after transfection, cells were fixed with 4% formalin, and observed by fluorescence microscopy. Mitochondria were stained with

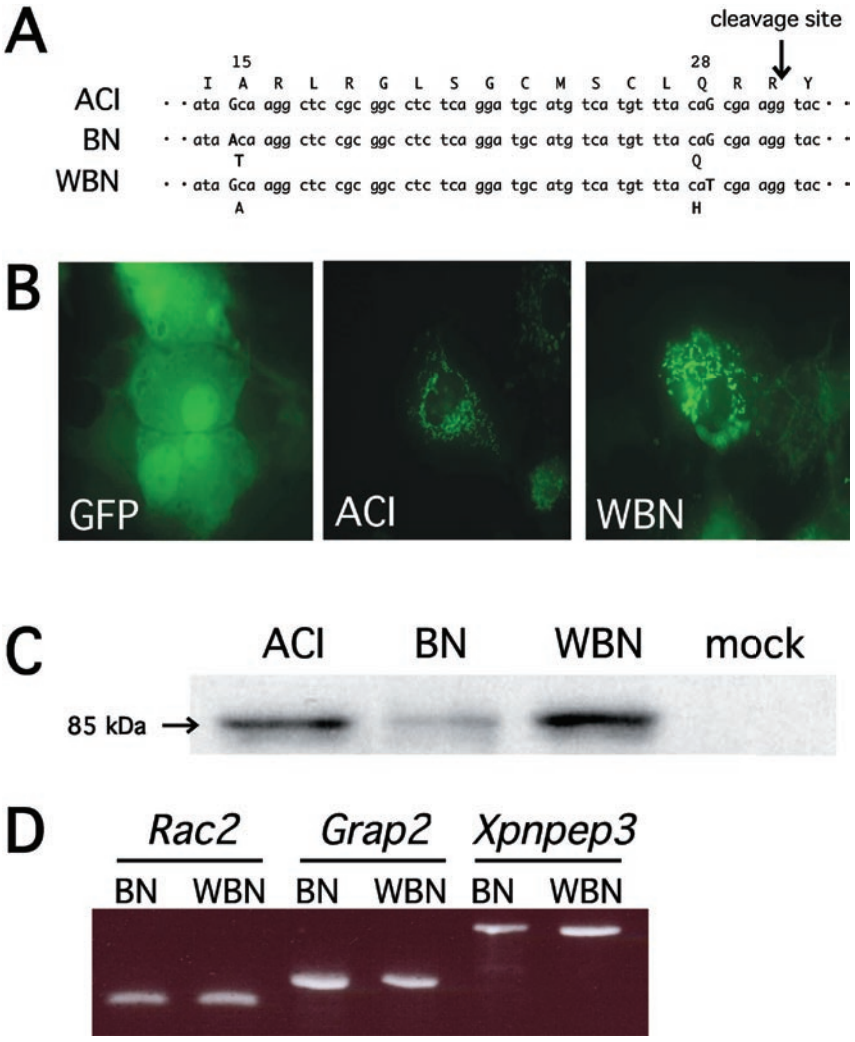

Fig. 2. Mitochondrial localization of three XPNPEP3 isoforms. (A) Comparison of nucleotide and deduced amino acid sequences for three *Xpnpep3* alleles derived from ACI, BN, and WBN/Kob rat strains. Nucleotide and amino acid substitutions are indicated in bold. Position of a cleavage site predicted by the TargetP program [http://www.cbs.dtu.dk/services/TargetP/] is indicated by an arrow. (B) COS cells expressing XPNPEP3 isoforms derived from ACI and WBN/Kob rat strains. (C) Western blot analysis of three XPNPEP3 isoforms expressed in COS cells. (D) RT-PCR analysis of *Rac2*, *Grap2*, and *Xpnpep3* transcripts for WBN/Kob and BN.

MitoTracker (Molecular Probes, Eugene, USA). All three isoforms transiently expressed in COS cells were localized in mitochondria (Fig. 2). Furthermore, all isoforms had identical molecular weight by western blot analysis with anti-GFP antibody (Rockland, Gilbertsville, USA). These results indicate that these amino acid substitutions do not influence mitochondrial localization of XPNPEP3 and subsequent removal of the signal peptide. Also, RT-PCR analysis of the spleen revealed the same transcript level with the expected size for *Xpnpep3*, as well as for *Rac2*, and *Grap2* for WBN/Kob and BN rats (Fig. 2). Thus, the alterations in the three genes were indicated to be functionally neutral.

In general, two conditions must be fulfilled in order to establish an etiological link between nucleotide alteration in the candidate gene to the disease. One is to demonstrate that the alteration is found only in the strain with the disease (strain-specificity). The other is to dem-

onstrate that the alteration functionally changes the gene. In this context, the nucleotide changes reported above are unlikely to be a cause of pancreatitis of WBN/Kob rats. However, if a threshold exists for the pathogenesis in WBN/Kob pancreatitis, one gene may not be enough to cause a functional difference, but two or more genes may have a coordinated action, and result in an altered function. Indeed, it was revealed in a previous study that the two loci on chromosomes 7 (*Pdwk1*) and X (*Pdwk2*) were genetically associated with WBN/Kob pancreatitis [11]. If complex interactions of the genes underlie WBN/Kob pancreatitis, a certain type of polymorphism which is specific to the WBN/Kob strain would not have been found by the molecular genetic strategy employed in this study. One standard way to confirm an effect of each locus and precisely define the chromosomal region is to breed congenic strains in which the *Pdwk1* or *Pdwk2* regions are separately introduced into the background of a normal rat strain. Also, there remains the possibility that a nucleotide alteration for pancreatitis exists in introns of the candidate genes. In this study, only exons and portions of introns were examined. A large part of introns awaits further study. In fact, recent genome-wide association studies have revealed many intronic SNPs associated with common diseases in humans.

Further continuation of the candidate gene approach will hopefully lead to the identification of *Pdwk1* and *Pdwk2*. Notably, five WBN/Kob-specific nucleotide alterations were found in three genes located within a 3.3-Mb region. The results obtained in this study suggest that a distinct haplotype is preserved in this relatively small chromosomal segment of WBN/Kob. It is unlikely that these nucleotide changes occurred during or after the establishment of the WBN/Kob strain as a spontaneous pancreatitis model. Rather, it would be reasonable to assume that the chromosomal segment with a unique haplotype was introduced to the WBN/Kob strain from its ancestry. It is premature to conclude, but tempting to speculate that *Pdwk1* might be located within the unique haplotype block. Perhaps, *Pdwk1* itself has a peculiar nucleotide alteration(s). This may be why the WBN/Kob strain uniquely develops hereditary pancreatitis. It was impossible to precisely define the chromosomal region of the particular haplotype because only portions of selected genes were examined in this study. According to Ensembl, approximately 100 genes are in the region between the *Rac2* and *Xpnpep3* loci. In this study, candidate genes with a reported association with pancreatitis in humans or mice, or genes associated with pancreas development or function, or genes associated with leukocyte development or function, were examined. It is possible that *Pdwk1* does not have any of the functional categories of the candidate genes that were examined. If this is the case, identification of the gene(s) should shed new light on the mechanisms of pancreatitis.

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