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ORIGINAL ARTICLE

Genetic Relatedness of WNIN and WNIN/Ob with Major Rat Strains in Biomedical Research

Kiran Kumar Battula[1] · Giridharan Nappanveettil[1] · Satoshi Nakanishi[2] · Takashi Kuramoto[2] · Jeffry M. Friedman[3] · Rajender Rao Kalashikam[1]

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Abstract WNIN (Wistar/NIN) is an inbred rat strain maintained at National Institute of Nutrition (NIN) for more than 90 years, and WNIN/Ob is an obese mutant originated from it. To determine their genetic relatedness with major rat strains in biomedical research, they were genotyped at various marker loci. The recently identified markers for albino and hooded mutations which clustered all the known albino rats into a single lineage also included WNIN and WNIN/Ob rats. Genotyping using microsatellite DNA markers and phylogenetic analysis with 49 different rat strains suggested that WNIN shares a common ancestor with many Wistar originated strains. Fst estimates and Fischer's exact test suggest that WNIN rats differed significantly from all other strains tested. WNIN/Ob though shows hyperleptinemia, like Zucker fatty rat, did not share the Zucker fatty rat mutation. The above analyses suggest WNIN as a highly differentiated rat strain and WNIN/Ob a novel obese mutant evolved from it.

Keywords Disease models · Obesity · Microsatellite markers · Phylogeny · Rat

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✉ Rajender Rao Kalashikam
rkrajender@yahoo.com

[1] Molecular Genetics Laboratory, National Centre for Laboratory Animal Science (NCLAS), National Institute of Nutrition (NIN), Tarnaka, Hyderabad 500007, India

[2] Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University, Kyoto, Japan

[3] Molecular Genetics Laboratory, Howard Hughes Medical Institute, The Rockefeller University, New York, NY 10065, USA

Introduction

Rat has facilitated many studies on physiology, pharmacology and metabolism, and over the last 100 years, more than 200 inbred rat strains representing various human diseases like diabetes, hypertension, cardiovascular disorders, immunological disorders, neurological disorders and behavioural disorders have been identified (Aitman et al. 2008; Mashimo and Serikawa 2009; Dwinell et al. 2011; Szpirer and Levan 2012). All these strains are known to have their origin from less than a dozen original outbred stocks, among which Wistar stock (maintained at Wistar Institute, Philadelphia) was known to be widely distributed and contributed to major animal models in biomedical research (Lindesy and Baker 2006). Wistar rats were introduced to the animal facility of the National Institute of Nutrition in 1920, and they were maintained in an inbred status ever since. It is named as WNIN, and it also finds a place in the International Index of Laboratory Animals (Festing 1993). An obese mutant has originated from this inbred rat in early 1990s, and this was maintained as a separate strain designated as WNIN/Ob. The mutant strain, WNIN/Ob, shows autosomal incomplete dominant inheritance of obese trait with three phenotypes viz., lean, heterozygote (carrier) and obese (WNIN/$Ob^{+/+}$, WNIN/$Ob^{+/-}$ and WNIN/$Ob^{-/-}$). The rats of carrier (+/−) and obese (−/−) phenotype have kinky tails which make them unique among the known rodent models of obesity. The obese rats of WNIN/Ob are hyper-phagic, hyper-lipedemic, hyper-insulinemic, hyper-leptinemic and hyper-cholesterolemic, and they exhibit degenerative diseases like cataract and retinal degeneration, infertility, polycystic ovaries, impaired immunity and tumours and show accelerated ageing as well (Giridharan 1998; Giridharan et al. 1996; Reddy et al. 2009; Harishankar et al. 2011a, b; Bandaru et al. 2011; Kalashikam et al. 2014).

Since WNIN-inbred rats are maintained at NIN for more than 90 years, it is felt essential to characterize them genetically and establish their genetic relatedness with known rat strains. Such knowledge will be highly useful for further genetic studies/biomedical research using these rats.

Majority of rat strains used in biomedical research are albinos which have taken their origin from different parental stocks and were not known to have a shared ancestry. Tyrosinase is reported as the causative gene for albino phenotype in rat and mouse strains. Multiple natural mutations at this albino locus were reported in different albino mouse strains (Beermann et al. 2004). Unlike mice, different strains of known albino rats were reported to have a single mutation (*Arg299His*) at this locus. Further, it was reported that this albino mutation has occurred in a hooded rat thus suggesting a single ancestor for all the known albino rats of the present day world (Kuramoto et al. 2012). To determine whether WNIN rats share the same lineage, they were genotyped for the albino and hooded mutations.

To determine phylogenetic relationship of WNIN and WNIN/Ob with major rat strains in biomedical research, a genome scan was performed using microsatellite DNA markers. Microsatellites are 2–4 base pairs tandem repeats, dispersed randomly throughout the genome. Polymorphisms at these sites are usually due to variation in the number of repeats and hence are multi-allelic. Unique sequences

flanking microsatellites make them locus-specific markers. The relatively low mutation rate and existence of a high amount of polymorphism at microsatellite sites have made them attractive markers for strain identification/genotyping, construction of genetic maps and in many experiments for mapping genetic traits. Microsatellite DNA markers have also proven efficient in reconstruction of rat phylogeny. (Serikawa et al. 1992; Canzian 1997; Watanabe et al. 1999; Thomas et al. 2003; Mashimo et al. 2006). Rat genome database has now enriched the genotype information of many microsatellite markers (SSLP) for many rat strains (Shimoyama et al. 2015). WNIN rats were genotyped using such microsatellite DNA markers for which genotype information of many different rat strains is available. This analysis was intended to derive the phylogenetic relationship of WNIN rats using minimum number of markers. Distance and UPGMA methods were considered for this analysis over latest methods like parsimony and maximum likelihood as the latter could not recover the correct topology of the phylogenetic tree. 'Nei's unbiased minimum distance' was shown to be an efficient estimator to recover a correct topology of a phylogenetic tree, when sampled loci are 50 and above and the sampled individuals are less than 10 numbers. Even a single individual seemed sufficient as long as the obtained genetic distance is 0.168 and above (Nei 1978). Hence, the above distance estimator was used in the present analysis. To determine strain differentiation, Fst (a measure of population differentiation, Wright's F-statistic) and Fischer's exact probability tests were conducted.

Zucker fatty rat, a well-known obese rat, shows hyper-leptinemia like WNIN/Ob rat strain. The mutation causing obesity in Zucker fatty rat was reported on leptin receptor mapped on Chr.5 (Leibel et al. 1997). Since WNIN/Ob rats are hyper-leptinemic and the locus of mutation was mapped in the vicinity of Leptin receptor (Kalashikam et al. 2013), they were also genotyped for the Zucker fatty mutation to determine whether they share the same mutation.

Materials and Methods

Ethical Approval

The study was approved by Institutional Animal Ethical Committee, NIN.

Animals

Male rats representing 20% of the breeders were randomly selected from each of the inbred strains, WNIN ($n = 6$), WNIN/Ob ($n = 5$) and Fischer-344/NIN ($n = 12$) maintained at National Centre for Laboratory Animal Sciences (NCLAS), NIN, India.

Genomic DNA Isolation

Tail clips were obtained from the above rats, and genomic DNA was isolated from them using Qiagen genomic DNA isolation kit (Netherlands).

Genotyping

Screening for Albino, Hooded and Zucker fatty mutation: WNIN and WNIN/Ob rats were screened for the above mutations at Kyoto University, Japan. Albino mutation was determined by PCR–RFLP analysis. The PCR products amplified by the primer pair 5′ TTTCATTCATATGTAAGTCCCTTG 3′ and 5′ GCTTAGCATTG-CAAAACTCACA 3′ were digested with SnaB1 restriction endonuclease (New England Biolabs) and electrophoresed on agarose gel. To define hooded mutation, rats were genotyped using the primer sets, ERV-positive (GGCCTGTGAGTGT-GAATTTG and GGACGAGCCCCCATAAATA) and ERV-negative (ACTTAAA-GACCACTGAGGACA and AATGCGGAACATCTTTCAA). WNIN/Ob rats were also genotyped for Zucker fatty mutation Q269P (CAG to CCG) on Leptin receptor.

Genotyping at microsatellite markers: WNIN, WNIN/Ob and Fischer-344/NIN (F-344/NIN), were genotyped at 76 unlinked microsatellite DNA markers/SSLPs (Supplementary Table S1) spread on 20 autosomes and 'X' chromosome at the genotyping facility, Rockefeller University, USA. Primer sequences for the SSLP markers were obtained from Rat Genome Database (Shimoyama et al. 2015).

Rat Strains Used for Comparison and Their SSLP Data Source

For comparative genetic analysis, 48 strain's SSLP genotype data for the above 76 markers were mined from Rat Genome Database (Shimoyama et al. 2015).

Phylogenetic Analysis

The genotype data in base pairs format of the 51 strains (WNIN, WNIN/Ob, F-344/NIN, and 48 rat strains mined above) were converted to allele numbers format (Supplementary Table S1) using web version of 'GENEPOP' computational program (Rousset, 2008). The above data were used to derive genetic distances between strains by 'Nei's unbiased minimum distance' (Nei 1978) estimator implemented in 'TFPGA' computational program (Miller 1997). The distance matrix obtained above was subjected to hierarchical clustering by 'UPGMA' method implemented in 'NEIGHBOR' computational program of Phylip 3.69 version (Felsenstein 2009). The tree file obtained from 'NEIGHBOUR' program was provided as input file to 'Tree view' computational program (Page 1996) to generate dendrogram/phylogenetic tree in graphical form. Permitted branch swaps were performed on the phylogenetic tree using the 'Tree view' program.

Differentiation Between Rat Strains

Fst (Wright's F-statistic) analysis and Fisher's exact Probability tests were conducted on rat strain pairs. Web version of 'GENEPOP' computational program (Rousset 2008) was used for the above analyses.

Results and Discussion

WNIN rats like all other albino rats showed *Arg299His* mutation in Tyrosinase gene (Fig. 1). Further, as observed in all other albino rats, WNIN rats also showed the endogenous retrovirus element insertion in the first intron of the 'kit' gene, known for hooded phenotype (Fig. 2). These results suggest that WNIN rats share the same ancestral lineage as like other albino rats.

To determine genetic relatedness of WNIN rats with known rat strains, they were compared with albinos originated from different parental stocks (Wistar, WKY, SD, F-344), hooded rats and brown Norway rat. Many of these strains were disease models of diabetes, hypertension, immunological, neurological and developmental disorders. Phylogenetic analysis was performed to determine the clustering of WNIN rats with other rat strains. The topology of the phylogenetic tree obtained in the present analysis is in well agreement with the phylogenetic trees published by other researchers and also with the known phylogenetic histories of rat strains (Canzian 1997; Thomas et al. 2003; Atanur et al. 2013). Brown Norway rat formed the root of the phylogenetic tree. The sub-strains of Wistar, WKY and SD essentially grouped into individual clusters. F344/NIN genotyped in the present analysis clustered with F-344/Pit. As expected the rat strains, WNIN and WNIN/Ob were clustered together. It was observed that WNIN and WNIN/Ob share a common ancestor with many albino rats of Wistar origin (NP9, P5C, BBDP/Rhw, BDR/Rhw, MHS/Gib, MNS/Gib, WN/N, WF/Pit, MNR/N, MR/Pit, MNRA/N, WIST/Nhg, LEW/Pit, LOU/CHan) (Fig. 3).

We have previously reported that WNIN rats are genetically distinct from WKY and F-344 rat strains (Kiran et al. 2007). The proportion of genetic differences between WNIN rats and other rat strains estimated by Fst (Wright's F-statistic) analysis was 70 % and above (Supplementary Table S2). Fischer's exact test suggests that WNIN rats are significantly different from all other rat strains tested

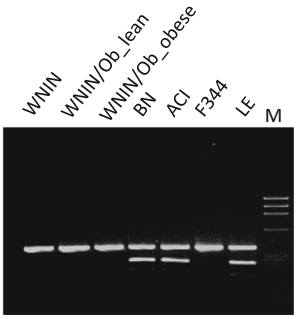
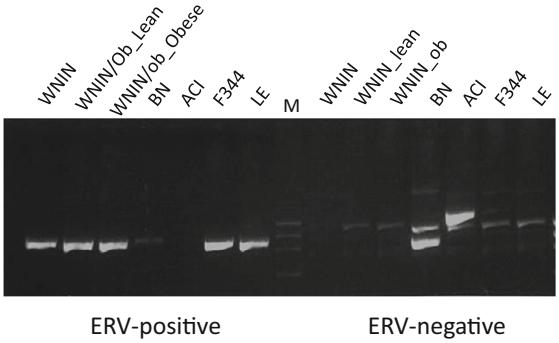


Fig. 1 Arg299His mutation in Rat Tyrosinase gene causing albino phenotype

PCR products of wild type rats (BN, ACI, LE) were digested whereas those from albino phenotype (F-344, WNIN, WNIN/Ob: lean and obese)were not. M is 100bp ladder



PCR products(997bp) on the left side of the marker(M) indicate ERV insertion (WNIN, WNIN/Ob_lean, WNIN/Ob_Obese). PCR products (1006bp & 1590bp observed in BN and ACI respectively) on the right side of the marker indicate no ERV insertion.

Fig. 2 ERV insertion in the intron 1 of Rat Kit gene

(Supplementary Table S3). All the above results thus suggest that WNIN is a highly differentiated rat strain of Wistar origin. Fischer exact test and Fst analysis also suggested that WNIN/Ob has differentiated significantly from WNIN.

WNIN/Ob is hyper-leptinemic, and the mutation causing obesity in this rat was mapped on chromosome 5, in the vicinity of Leptin receptor, a candidate gene for obesity (Kalashikam et al. 2013). Several alleles were reported for this gene causing obesity in rat and mouse strains, and all of them have altered coding sequences of Leptin receptor (Leibel et al. 1997; Giridharan 1998). Unpublished data from our lab suggest that the coding sequence of Leptin receptor is unaltered in WNIN/Ob (NIN annual report 2003–2004, p101). To complement the above, WNIN/Ob rats were genotyped for the well-known leptin receptor mutation, *Q269P* observed in Zucker fatty rat. As expected, WNIN/Ob rats showed a normal allele for the above suggesting that WNIN/Ob is a novel obese-mutant rat strain.

Obesity leading to many degenerative disorders like diabetes, hypertension, cardiovascular disorders, ageing, infertility, etc. is a major public health problem in the present day world (Swinburn et al. 2011; Ginter and Simko 2014; Lifshitz and Lifshitz 2014). Mainly, an imbalance in energy homeostasis is poorly understood due to the complex metabolic system (Schutz et al. 2014; Albuquerque et al. 2015). Single-gene mutants (Lutz and Woods 2012) have played a major role in addressing many questions, yet the metabolic syndrome was not completely understood. Hyper-leptinemia is commonly observed in human obesity, and the causes seem to be multiple (Wauman and Tavernier 2011; Sáinz et al. 2015). WNIN/Ob, a novel mutant with hyper-leptinemia, is expected to help us solve some of the unanswered questions on this debilitating syndrome.

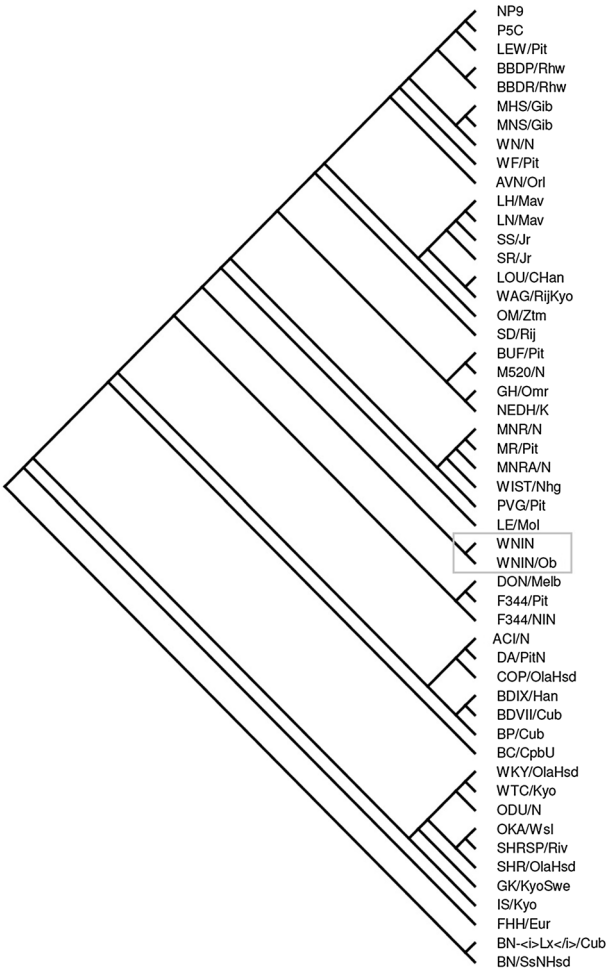


Fig. 3 Phylogenetic tree showing genetic relationship of WNIN and WNIN/Ob with 49 rat strains

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Conflict of interest The authors declare that they have no conflict of interest.

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