Molecular Biology: Open Access
ISSN: 2168-9547

Valles-Ayoub, et al., Mol Biol 2012, 1:1
DOI: 10.4172/2168-9547.1000104

Research Article Open Access

Mixed Inbred FVB;B6 Background Strain Attenuates Kidney Disease and Improves Survival of Gne$^{M712T/M712T}$ Mice

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Abstract

Recessive form of Hereditary Inclusion Body Myopathy (HIBM, IBM2, MIM:600737) is an adult onset muscle wasting disorders caused by hypomorphic GNE, the rate-limiting enzyme of sialic acid (Sia) biosynthesis. Unlike human patients, mice bearing the Gne$^{M712T/M712T}$ genotype in C57BL/6 background strain suffer severe glomerular hematuria, incomplete podocyte development, and do not survive beyond the first few days of life. We crossed heterozygous mice (Gne$^{M712T/+}$) of B6 strain with FVB strain mice. In mixed inbred FVB;B6 background, the homozygous mice show attenuated glomerular disease and survive longer (mean survival 22±13 weeks, n=26). Paradoxically, the homozygous mice showed increased total Sia levels in serum (2x control), and Neu5Ac:Neu5Gc ratios are slightly shifted towards Neu5Ac in serum and towards Neu5Gc in muscle tissue. Increase in serum Sia levels may be caused by altered glomerular filtration. This paradoxical increase in serum Sia may contribute to Sia pools of muscle, and exert a potential beneficial effect. In summary, the background strain of mouse model can significantly affect the disease phenotype.

Keywords: Muscular dystrophy; Nonaka; Distal myopathy with rimmed vacuoles (DMRV); Sialylation; Glycosylation

Introduction

Inclusion Body Myositis & Myopathies (IBMs) are a group of adult-onset slowly progressive muscle disorders, often leading to severe disability [1]. The most common of the hereditary forms of IBMs is the autosomal recessive Hereditary Inclusion Body Myopathy (HIBM, IBM2, MIM:600737, DMRV, MIM:605820) or GNE myopathy. At the cellular level, histological phenotype of affected myofibers in HIBM/DMRV reveals presence of rimmed autophagic-like vacuoles and inclusion bodies with filamentous components containing β-amyloid, lysosomal proteins, tau and presenilin [2]. HIBM/DMRV is caused by mutations in the GNE gene (MIM:603824) [3-5]. GNE encodes for the bifunctional enzyme UDP-N-Acetylglucosamine 2-Epimerase/N-Acetylmannosamine Kinase (GNE/MNK), which is the rate-limiting enzyme for sialic acid (Sia) biosynthesis [6]. HIBM/DMRV mutations lead to hypomorphic GNE/MNK enzyme [7,8]. GNE expression in mammalian cells is ubiquitous, with higher levels in placenta, liver, secretory organs, mucosa, and some cancer cells [9]. It remains unknown why clinical symptoms and pathology in HIBM/DMRV patients are limited to skeletal muscle tissue. To date, two mouse models have been produced for HIBM/DMRV:

1) Gne^{M712T} knock-in mouse model bearing the most common founder genotype of HIBM/DMRV patients, GNE p.M712T. This model mouse was produced by homologous recombination, and Gne is driven by the endogenous promoter. In C57BL/6 mouse strain, the Gne$^{M712T/M712T}$ mice do not survive beyond early postnatal days due to severe kidney disease, glomerular hematuria, hyposialylation of podocalyxin, and seemingly incomplete development of podocyte foot processes. Additionally, N-Acetylmannosamine (ManNAc) is salutary to the kidney disease and significantly improves survival [10].

2) Transgenic mice expressing human GNE with p.D176V mutation, in Gne-null background. The GNE transgene is driven by the strong mammalian promoter CAG, which is a hybrid of chicken beta-actin and CMV promoters [11,12]. After the age of 40 weeks, this mouse exhibits myopathy similar to the HIBM/DMRV patients. Prophylactic administration of N-Acetylmannosamine, N-acetylneuraminate, and Sialyllactose precludes the development of myopathic phenotype in this model [13].

In this study, we crossed the B6 heterozygous Gne^{M712T} mice with FVB mice to produce a mixed inbred B6;FVB strain, and to evaluate the effect of the Gne^{M712T} mutation in different inbred mouse strains. Crossing with FVB background strain was chosen because FVB mice have vigorous reproductive performance with large litters [14], and often show modified genetic disease phenotype compared to B6 [15-17].

Methods

Animals

FVB/NJ mice were obtained from the Jackson Laboratories, and Gne^{M712T} mouse was provided by HIBM Research Group. Breeding were performed at Greater Los Angeles VA Healthcare System, Veterminary medical unit. Animals had access ad libitum to water and Mouse Diet 5015 chow (PMI Nutrition International, LLC, Brentwood, MO) or Teklad Rodent Diet (Harlan Laboratories, Indianapolis, IN). The B6-Gne$^{M712T/+}$ mice were crossed with FVB/NJ strain mice, and the N1 generation were further crossed to produce mixed inbred homozygous

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Received March 03, 2012; **Accepted** March 26, 2012; **Published** April 01, 2012

Citation: Valles-Ayoub Y, Haghighatgoo A, Saechao C, Khokher Z, Creencia C, et al. (2012) Mixed Inbred FVB;B6 Background Strain Attenuates Kidney Disease and Improves Survival of Gne$^{M712T/M712T}$ Mice. Mol Biol 1:104. doi:10.4172/2168-9547.1000104

mice (FVB;B6-Gne$^{M712T/M712T}$). Studies were conducted in accordance with the guidelines set forth by the Greater Los Angeles VA Healthcare System Institutional Animal Care and Use Committee and by NIH's Guidelines for the Care and Use of Laboratory Animals.

Molecular analysis

Mouse genotyping was performed on either or both tail genomic DNA and cDNA isolated from skeletal muscle, liver, kidneys, and brain using standard protocols. Total RNA was isolated from murine tissues using the TRIZOL reagent (Invitrogen, Carlsbad, California, USA), and cDNA prepared using the SuperScript III system (Invitrogen). Multiplex PCR based genotyping was performed across Gne exon 12 on genomic DNA as template, using three custom designed primer sets (Figure 1). For amplification of cDNA template, we used the custom designed primer set mGne-E11F and mGne-E12R, using PuReTaq Ready-To-Go PCR beads (GE HealthCare, Waukesha, Wisconsin, USA) or REDTaq and appropriate buffer (Sigma-Aldrich, St. Louis, Missouri, USA), by standard PCR conditions. Sequencing was performed using DYEnamic ET kit (GE Healthcare) on ABI 377-96 Prism (Applied Biosystems, Foster City, CA, USA).

Sialic acid analysis

Serum and tissue sialic acid analysis was performed at UCSD Glycotechnology Core Resource using HPLC-DMB method [18]. Sample is dissolved in a final concentration of 2 M HOAc and heated to 80°C for 3 hours to release sialic acids. The released sialic acids are collected by ultra-filtration through a 3,000 NMWCO filter and derivatized with 1,2-diamino-4,5-methylenedioxybenzene dihydrochloride (DMB). The fluorescent sialic acid derivatives are analyzed by reverse-phase HPLC with on-line fluorescence detection.

Serum chemistry

Creatine Kinase (CK) and Blood Urea Nitrogen (BUN) was analyzed by UCLA Division of Laboratory Animal Medicine (DLAM).

Behavioral analysis

Motor function testing using Rotarod treadmill, Grip strength analysis, and open field was performed using standard methods. The Rotarod treadmill (Ugo-Basile, Italy) instrument consists of a rotating rod, elevated so as to discourage the animals from stepping

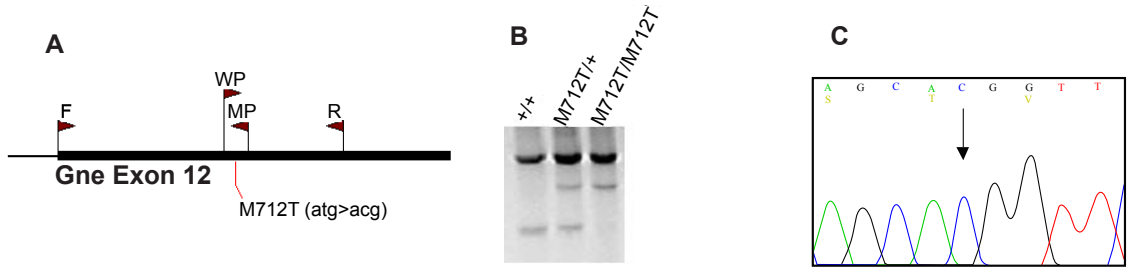

Figure 1: Genomic and cDNA analysis. A) Genotyping design showing Gne exon 12. B) Genotyping of Gne locus. Bilateral PCR amplification of specific alleles (Bi-PASA) was used for Gne locus using primers F (5'ctggaactgctttgggactt), R (5'atttgccttcgcagaaacactga), inner primers requiring gc rich tails (underlined), wildtype allele WP (5'gggcggggcggcgcagccagcat), and mutant allele primer MP (5'ggggcgggcgtgtagtccagaaccg). C) Muscle cDNA sequencing of FVB;B6-Gne$^{M712T/M712T}$ mouse showing homozygous M712T (atg>acg).

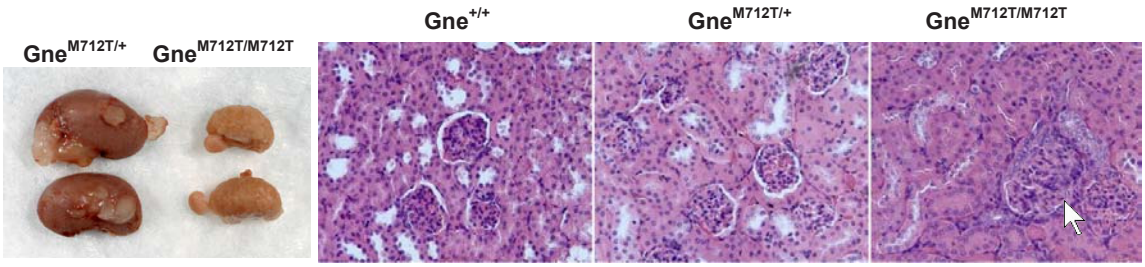

Figure 2: Kidneys of homozygous mouse at age of 40 weeks and age matched heterozygous and wildtype control littermates. Kidneys of homozygous M712T mice are grossly smaller and granular, and histologically show severe membranous glomerulonephritis and loss of Bowman's space (White Arrow).

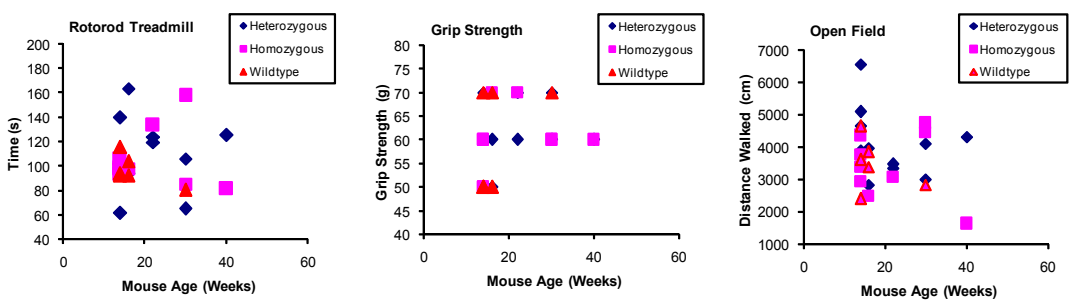

Figure 3: Motor function analysis did not result in statistically significant difference between homozygous, heterozygous, or wildtype mice at various age groups.

down. Mice were first conditioned to the device by running them at progressive speeds up to 40 rpm during 10 minutes for three sessions, separated by 30 minutes intervals during the first day of exposure to the instrument. During the second day the speed was incremented from 40 rpm at 5 rpm increments every 5 minutes until they fall from the treadmill. Three sessions was performed for each animal, separated by 30 min intervals. The average running time was recorded for each animal. For Grip strength analysis, a digital force gauge DFIS 10 (John Chatillon & Sons, Inc, Greensboro, NC) instrument is fitted with a bar that the animals grasp. As the mice are moved away from the bar by manually pulling them from the body, the force exerted on the instrument is continuously measured and the maximal force developed at the time when the animal releases the grip on the bar is recorded as the grip strength. The animals were a flat surface and their bodies will be at zero angle with the direction of the pulling force. The average of three attempts, separated by three minutes, was recorded for each animal. For open-field activity analysis, each mouse was placed in circular open field chambers of 25 cm diameter under low level illumination for a duration of 20 minutes. This is done to maximize exploratory activity, which is normally inhibited in rodents by daylight or bright illumination, and to eliminate unwanted visual clues from the surrounding environment. The animal movements was recorded with a video tracking and motion analysis system, which consists of a Sony CCD video camera (sensitive to the wavelength of light used), Targa M16 Plus video digitizing board on a Microcomputer, and Ethovision software (Noldus, Inc, The Netherlands). Tracking was performed at a rate of 1 Hz during the entire 20 min session and stored in memory. Movement analysis included total distance traveled, habituation (decay slopes of distance traveled as a function of time), average speed, and maximum speed over 5, and 20 sec intervals, average heading, turn angle and angular velocity, turn bias, and sinuosity.

Statistical methods

Means were compared using the parametric one way analysis of variance model and post hoc t tests were computed under this model. Medians were compared using the corresponding non-parametric Kruskal-Wallis method. P values <0.10 were considered significant.

Results

We produced the mixed inbred B6;FVB background mice bearing the most common mutation seen in HIBM patients, the Middle East founder Gne:p.M712T (atg>acg) [3]. In B6;FVB strain, the number of homozygous pups surviving to weaning age is improved, but remains below Mendelian expected rate of 25% from heterozygous-heterozygous mating. Of 59 litters, totaling 409 mice, pups surviving to weaning age included 56% wildtype, 38% heterozygous, and 6% homozygous. The surviving homozygous mice (mean survival 22±13 weeks, n=26) invariably show moderate to severe kidney disease (Figure 2).

As previously reported, the homozygous pups in B6 background are physically much smaller than their heterozygous and wildtype littermates [10]. However, in mixed inbred FVB;B6 strain, the homozygous are similar is size (length and weight) compared to their littermates. Motor strength/endurance testing (i.e. Rotarod treadmill and grip strength), exercise induced creatine kinase elevation, and open-field for gross locomotor activity level, did not show statistically significant difference in homozygous and littermate controls (Figure 3 and Figure 4).

Serum was analyzed for Blood Urea Nitrogen (BUN), which showed 2X higher in homozygous mice (45.5±18.6mg/dL, n=9, reference/control range 12-28). Unlike humans, who only produce N-Acetylneuraminic acid (Neu5Ac), rodents produce both Neu5Ac N-glycolylneuraminic acid (Neu5Gc) [19]. The homozygous mice show altered Sia profile, with reduced Neu5Ac fraction in muscle, and higher Neu5Ac fraction in serum (Figure 5). Higher total serum Sia levels may be the result of chronic renal failure and reduced total serum protein [20].

Discussion

This study describes follow up data pertinent to the model #1 described above [10]. This work demonstrates the variable phenotype severity seen in two commonly available inbred mouse strains. Because the background mouse strain influences the severity of renal disease caused by Gne$^{M712T/M712T}$ genotype, and since human HIBM patients with GNE$^{M712T/M712T}$ do not suffer from kidney disease, we believe the efficiency of the sialic biosynthetic pathway in different organs is dependent on other background genes. Additionally, the paradoxical high serum Sia levels in this mouse model may contribute to delayed onset of muscle disease because the myofibers may benefit from higher serum Sia levels [13]. The reason for altered Neu5Ac:Neu5Gc ratio seen in skeletal muscles of this mouse model can be attributed to higher levels of Neu5Gc in muscle and higher levels of Neu5Ac in serum, however significance of the altered Neu5Ac:Neu5Gc ratios to human disease remains unclear.

We did not examine the muscle histologically in this inbred strain because motor function and activity (rotarod, grip strength, open field activity) showed signifcant variability even within the same age matched genotype group, and thus did not show statistically significance difference between the genotype groups at any age.

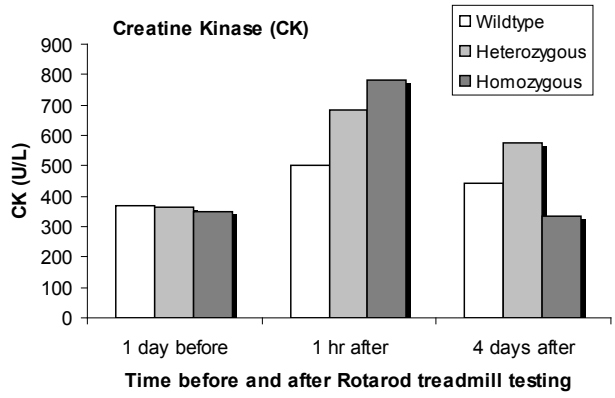

Figure 4: Exercise induced CK elevation did not result in statistically significant difference between homozygous, heterozygous, or wildtype mice.

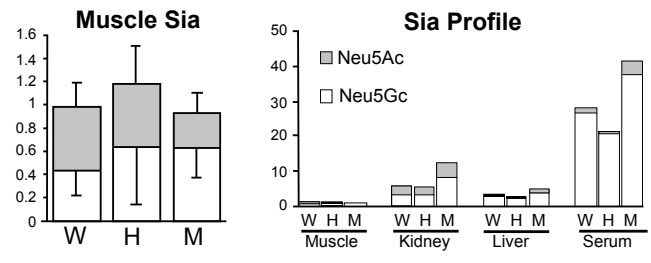

Figure 5: Sia profile. W=Wildtype, H=Heterozygous, M=Homozygous Gne:p.M712T. Homozygous mice show altered Sia profile. In muscle, ratio of Neu5Gc/Neu5Ac is increased. Serum Sia is 1.5 – 2X control littermates (n=4 each genotype group, p<0.05).

Since skeletal muscle is the only organ clinically affected in HIBM/DMRV human patients [21-25], our future direction for this project includes production of a hypomorphic Gne mouse model that shows significant pathology only in skeletal muscles. Our future strategies to produce such model include development of congenic FVB strain, and skeletal muscle specific Gne knock-out mouse.

In Summary, the background strain of mouse model can significantly affect the disease phenotype, and producing mouse models of human disease in several inbred background strains may prove useful in developing an optimal model that most closely resembles the human disorder.

Acknowledgement

We thank Dr. Jeffrey Gornbein (UCLA) for assistance with statistical analysis, Juan Orozco and Jennifer Kay for assistance with mouse colony management, rotorod treadmill, open-field, and grip-strength evaluation. Funding was provided by Advancement of Research for Myopathies (A.R.M.) and David and Laura Merage Foundation.

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