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ORIGINAL ARTICLE—ALIMENTARY TRACT

Thiopurines exert harmful effects on spermatogenesis in *Nudt15*^{R138C} knock-in mice

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Abstract

Background The association between thiopurine use and testicular reproductive functions remains unclear. In this study, we investigated whether thiopurines affect testicular functions based on the *NUDT15* genotypes using *Nudt15*^{R138C} knock-in mice.

Methods The male *Nudt15*^{R138C} knock-in mice (9–12 weeks) were treated with mercaptopurine (MP: 0.5 mg/kg/day) for 4 or 12 weeks. To examine reversibility, some mice were maintained for a further 12 weeks under MP-free condition.

Results After MP treatment for 4 weeks, *Nudt15*$^{R138C/R138C}$ mice exhibited a significant reduction of testis weight compared to *Nudt15*$^{+/+}$ mice and *Nudt15*$^{+/R138C}$ mice. The epithelial height and diameter of seminiferous tubules were significantly reduced in *Nudt15*$^{R138C/R138C}$ mice compared to *Nudt15*$^{+/+}$ and *Nudt15*$^{+/R138C}$ mice. Apoptotic cells were significantly increased in *Nudt15*$^{R138C/R138C}$ mice, and most of apoptotic cells were spermatogonia. There were no significant changes in sperm counts and sperm morphology in MP-treated *Nudt15*$^{R138C/R138C}$ mice after 4-week MP treatment. On the other hand, after MP treatment for 12 weeks, the *Nudt15*$^{+/R138C}$ mice, but not *Nudt15*$^{+/+}$ mice, exhibited a significant reduction in the testis weight and atrophic changes of seminiferous tubules, but these changes disappeared after 12-week rearing under MP-free condition. Despite a significant increase in abnormal sperm rate, there were no changes in the ability to conceive. No differences in serum levels of follicle-stimulating hormone or testosterone were observed between MP-treated *Nudt15*$^{+/R138C}$ and *Nudt15*$^{+/+}$ mice after 12-week MP treatment.

Conclusions Thiopurines exert harmful effects on testicular reproductive function according to host *NUDT15* genotypes.

Keywords Inflammatory bowel disease · Mercaptopurine · NUDT15 · Spermatogenesis

Introduction

The thiopurine drugs azathioprine (AZA) and 6-mercaptopurine (MP) are widely used for maintenance of clinical remission in steroid-dependent inflammatory bowel disease (IBD) [1–6]. Although thiopurines are key drugs in the treatment of IBD, adverse events such as myelosuppression, hepatotoxicity, hair loss, pancreatitis and gastrointestinal intolerance have been reported [4, 7–9]. Myelosuppression is most common, and its incidence is higher in East Asian populations (20 ~ 40%) than in Caucasians (5%) [10].

Yang et al. identified a strong association between a single nucleotide polymorphism (SNP) in *NUDT15* and thiopurine-induced myelosuppression [11]. This variant leads to an amino acid substitution at position 139 [NUDT15 p.Arg139Cys (R139C)] and induces a loss of enzyme activity [12]. NUDT15 converts cytotoxic 6-thioguanosine triphosphate (TGTP) and 6-thio-deoxyguanosine triphosphate (TdGTP) to non-cytotoxic 6-thioguanosine monophosphate (TGMP) and 6-thio-deoxyguanosine monophosphate (TdGMP) [12, 13]. Loss of function of NUDT15 induces an accumulation of cytotoxic 6-TGTP and 6-TdGTP, causing myelosuppression [12]. A previous study of thiopurine-treated IBD patients in Japan ($n = 1291$) revealed that

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more than 90% of patients carrying homozygotes for the *NUDT15*^{R139C} variant ($n=49$) developed leukocytopenia and alopecia, and many of them were serious and early onset [14]. The *NUDT15*^{R139C} risk variant is most common in East Asians (9.8%) and Hispanics (3.9%), rare in Europeans (0.2%) and not observed in Africans [15], supporting ethnic-related differences in thiopurine tolerance [16]. In Japanese, the homozygotes frequency for the *NUDT15*^{R139C} variant is about 1%, and the heterozygotes frequency is about 20% [17, 18]. Based on these findings, *NUDT15* genotyping is recommended prior to thiopurine introduction to mitigate drug toxicity [14, 19, 20].

We have previously established *Nudt15*^{R138C} knock-in mice which corresponds to the human *NUDT15*^{R139C} polymorphism [21, 22]. In this model, thiopurine causes hematopoietic stem cell (HSC) toxicity in *Nudt15*$^{+/R138C}$ and *Nudt15*$^{R138C/R138C}$ mice. Hematopoietic tissue was promptly injured by MP treatment in *Nudt15*$^{+/R138C}$ and *Nudt15*$^{R138C/R138C}$ mice, and HSCs were damaged according to *Nudt15*^{R138C} allele number [22].

IBD occurs in women and men of young reproductive age, and many of them are treated with thiopurines. Thiopurines are approved for administration to pregnant women [20], but their safety is under debate. Therefore, we previously investigated how thiopurine use during pregnancy affects offspring carrying the *NUDT15* risk allele using *Nudt15*^{R138C} knock-in mice. Thiopurine use during pregnancy caused serious damage to the fetus, depending on the *NUDT15* genotype of the offspring. In particular, *Nudt15*$^{R138C/R138C}$ offspring in *Nudt15*$^{+/R138C}$ pregnant mice suffered from serious damage when the pregnant mice were exposed to a therapeutic thiopurine dose [21]. Since the *NUDT15* genotype of offspring is dependent on the genotype of the parents, we recommended to check the *NUDT15* genotype of not only an IBD mother but also their partner to avoid possible adverse outcomes in their offspring prior to thiopurine initiation.

Thiopurines inhibit purine synthesis and possibly affect spermatogenesis, but the association between thiopurine use and testicular function has not been extensively studied. The aim of this study was to investigate whether thiopurine use affects male fertility based on their *NUDT15* genotypes using *Nudt15* ^{R138C} knock-in mice.

Methods

Animal experiments

We previously established two independent *Nudt15*^{R138C} knock-in mouse strains using two different sgRNAs [22], and both strains were used in this study. The human and mouse NUDT15 are 89% identical at the protein level. The arginine at amino acid sequence 139 in human NUDT15 is preserved at position 138 in mouse NUDT15. We have previously reported that overexpression of the murine *Nudt15*^{R138C} mutant enhances sensitivity to MP in a murine blood cell line [22]. Thus, by using CRISPR–CAS9 genome editing and homology-directed repair to convert c.412C to T, we established knock-in mice carrying the *Nudt15*^{R138C} allele in the C57BL/6 background. Genotypes were determined by sequencing with the forward primer, GGCATC TAGCCTGTAATATAGACAT, the reverse primer, CAG AGGTAGGTAGGCAGATCTGAG, and the sequencing primer, CCCGGCCTGCAGGTCTATGCCACCAGGACA ATTCAG. *Nudt15*^{R138C} knock-in mice were reared under specific pathogen-free conditions. The animal research committee of Shiga University of Medical Science approved this project (permission number 2019–2-5).

Mercaptopurine (MP) administration

MP (Sigma-Aldrich Japan, Tokyo, Japan) was dissolved in water and orally administered to the *Nudt15*^{R138C} knock-in mice [22]. Since mouse was reported to drink about 5 ml of water per day [23], MP was dissolved in water adjusted to 0.2 μg × mouse body weight (g) × ml^{-1} (1 mg/kg MP). Drinking water containing the same amount of dimethyl sulfoxide (DMSO) was given to the control mice. As described in our previous report [22], the daily dose of MP that allows long-term survival of more than 2 months is 1.0 mg/kg for *Nudt15*$^{+/+}$, 0.5 mg/kg for *Nudt15*$^{+/R138C}$, and 0.2 mg/kg for *Nudt15*$^{R138C/R138C}$ mice. In this study, we used an MP dose of 0.5 mg/kg/day for all experiments.

MP treatment and histological analysis

For evaluation of the effect of MP on the histopathological parameters of spermatogenesis, the male mice (9–12 weeks) were treated with MP for 4 or 12 weeks and sacrificed, and the right testis and cauda epididymis were immediately removed and weighed. The testis was fixed in Bouin's solution and embedded in paraffin using a routine method. Sections perpendicular to the long axis of the testis were made and stained with hematoxylin and eosin. The epithelial height and diameter of the seminiferous tubules were measured using an ocular micrometer. At least 30 round tubules were measured in each mouse. The mean tubular diameter and the epithelial height of 30 tubules were calculated for each mouse. These mean values were then used to determine the tubular diameter and the epithelial height.

To evaluate whether or not the effects of MP on spermatogenesis are reversible, after 12-week treatment with MP some mice were maintained for a further 12 weeks in the absence of MP. Then, histological evaluation was performed.

Evaluation of male fertility

After MP treatment for 12 weeks, male *Nudt15*^{R138C} knock-in mice were placed with wild-type females (male: female, 1:2) for 3 days. The females were checked for pregnancy, and pregnant were moved to a holding cage and number of offspring birth was counted.

Sperm analysis

For sperm sampling, the cauda epididymis was minced with small scissors and the sperm suspensions were allowed to disperse in 1 mL of modified HTF medium (Nippon Medical & Chemical Instruments Ltd., Osaka, Japan) for 30 min at 37 °C. The sperm suspensions were filtered through a 40 µm nylon filter to remove any debris, and the sperm count and sperm morphology were evaluated.

The number of epididymal sperm was determined by hemocytometer counts. All counts were made in duplicate and averaged. For evaluation of sperm morphology, sperm cells on glass slides were fixed with methanol for 5 min. After air-drying, they were incubated with Giemsa's stain solution (Nacalai Tesque, Kyoto, Japan) for 20 min. The slides were observed under an inverted phase contrast microscope (BX51, Olympus Co., Tokyo, Japan). The sperm cells were classified according to the site of abnormality, and at least 200 sperm cells were analyzed per mouse. Morphological classification of sperm cells was evaluated according to previous reports [24, 25].

Serum hormone levels

Serum levels of mouse testosterone, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) were measured using enzyme-linked immune-sorbent assays (ELISAs), purchased from Enzo Life Sciences, Inc. (Tokyo, Japan) and CUSABIO (Houston, TX).

TUNEL (terminal deoxynucleotidyl transferase dUTP-biotin nick end labeling) assay

Apoptosis was determined using the DeadEnd™ Fluorometric TUNEL assay (Promega, Madison, WI) according to the manufacturer's instructions. For quantification, 20 seminiferous tubules per sample were analyzed and the average number of TUNEL positive cells per one seminiferous tubule was determined.

Statistical analysis

Data are presented as the mean ± SD. ANOVA followed by Tukey's tests was adopted for multiple comparison analyses using GraphPad Prism version 9 (GraphPad software, La Jolla, CA, USA). All tests were two-tailed, and a p value < 0.05 was considered significant.

Results

Short-term (4 weeks) effects of MP on testicular function in *Nudt15*^{R138C} knock-in mice

To evaluate the association of thiopurine toxicity with testicular function in *Nudt15*^{R138C} knock-in mice, male *Nudt15*$^{R138C/R138C}$ mice were orally administered MP at 0.5 mg/kg/day for 4 weeks. This is the therapeutic dose used for IBD patients carrying the *NUDT15*^{R139C} heterozygote. We have previously reported that MP at 0.5 mg/kg/day induces severe myelosuppression and serious damage to the fetus in *Nudt15*$^{R138C/R138C}$ mice [21, 22]. At this dose, *Nudt15*$^{+/+}$ and *Nudt15*$^{+/R138C}$ mice survive long-term (> 2 months), but *Nudt15*$^{R138C/R138C}$ mice die earlier [22].

As shown in the representative pictures (Fig. 1A), the testis of *Nudt15*$^{R138C/R138C}$ mice was morphologically small. *Nudt15*$^{R138C/R138C}$ mice exhibited a significant reduction of the ratio of testis/body weight compared to *Nudt15*$^{+/+}$ mice and *Nudt15*$^{+/R138C}$ mice [*Nudt15*$^{R138C/R138C}$ 2.8 ± 0.2 mg/g vs. *Nudt15*$^{+/+}$ 3.7 ± 0.2 ($p < 0.01$) and vs. *Nudt15*$^{+/R138C}$ 3.8 ± 0.2 ($p < 0.01$)] (Fig. 1B). No morphological alterations of the testis were observed in either *Nudt15*$^{+/+}$ or *Nudt15*$^{+/R138C}$ mice compared to control mice.

Histological examination of the testis revealed that the epithelial height and the diameter of seminiferous tubules were significantly reduced in *Nudt15*$^{R138C/R138C}$ mice compared to *Nudt15*$^{+/+}$ mice and *Nudt15*$^{+/R138C}$ mice [epithelial height: *Nudt15*$^{R138C/R138C}$ 29.0 ± 1.2 µm vs. *Nudt15*$^{+/+}$ 37.2 ± 1.5 ($p < 0.01$) and vs. *Nudt15*$^{+/R138C}$ 36.5 ± 1.3 ($p < 0.01$); diameter: 154.0 ± 4.3 µm vs. 178.9 ± 7.9 ($p < 0.01$) and vs 178.1 ± 7.0 ($p < 0.01$)] (Fig. 2A–C).

Apoptotic cells in the seminiferous tubules, detected by TUNEL staining, were significantly increased in *Nudt15*$^{R138C/R138C}$ mice compared to *Nudt15*$^{+/+}$ mice and *Nudt15*$^{+/R138C}$ mice [*Nudt15*$^{R138C/R138C}$ 5.9 ± 2.7/seminiferous tubule vs. *Nudt15*$^{+/+}$ 1.3 ± 0.3 ($p < 0.01$) and vs. *Nudt15*$^{+/R138C}$ 2.0 ± 0.6 ($p < 0.01$)] (Fig. 3A, B). Localization of TUNEL-positive cells indicates that MP treatment induced apoptosis of spermatogonia (the mitotic germ cells including the stem cells and differentiation-destinated amplifying cells) in *Nudt15*$^{R138C/R138C}$ mice (Fig. 3A) [26].

At this time point, there were no significant changes in spermatozoa (sperm counts and morphological abnormality) in the cauda epididymis of *Nudt15*$^{R138C/R138C}$ mice compare to *Nudt15*$^{+/+}$ and *Nudt15*$^{+/R138C}$ mice [sperm counts:

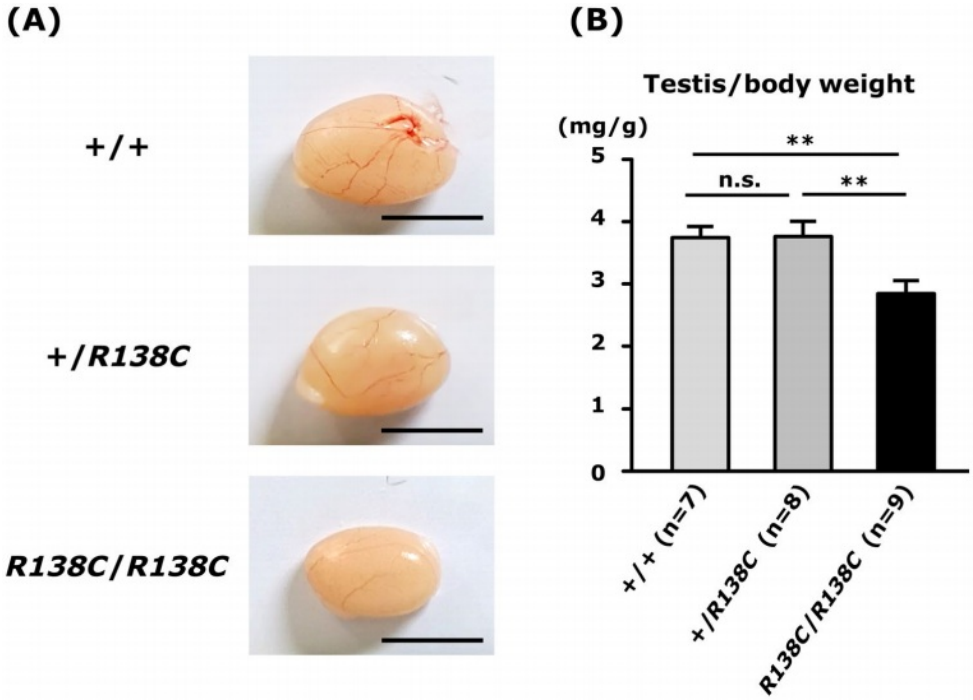

Fig. 1 Effects of short-term mercaptopurine (MP) treatment (4 weeks) on the testes of *Nudt15*^{R138C} knock-in mice. **A** Morphology of testes after treatment with MP (0.5 mg/kg/day) for 4 weeks. Scale bar: 5 mm. **B** Statistics of the testis/body weight ratio. Data are presented as the mean ± SD. $^{**}p < 0.01$. *n.s.* not significant, +/+: *Nudt15*$^{+/+}$ mice (wild type), +/*R138C*: *Nudt15*$^{+/R138C}$ mice, *R138C/R138C*: *Nudt15*$^{R138C/R138C}$ mice

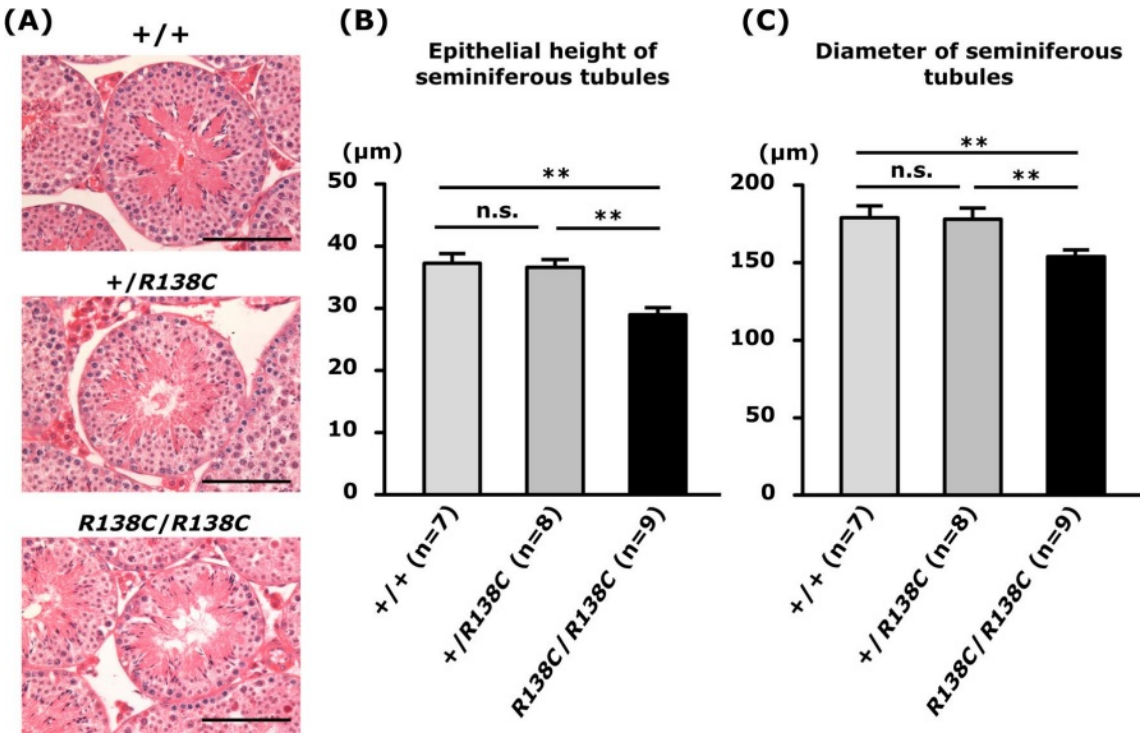

Fig. 2 Histology of the testes from *Nudt15*^{R138C} knock-in mice after treatment with MP (0.5 mg/kg/day) for 4 weeks. **A** Representative images (×400) of HE-stained sections of the testes. Scale bar: 100 μm. **B** Statistics of the epithelial height of seminiferous tubules. **C** Statistics of the diameter of seminiferous tubules. Data are presented as the mean ± SD. $^{**}p < 0.01$. n.s., not significant, +/+: *Nudt15*$^{+/+}$ mice (wild type), +/*R138C*: *Nudt15*$^{+/R138C}$ mice, *R138C/R138C*: *Nudt15*$^{R138C/R138C}$ mice

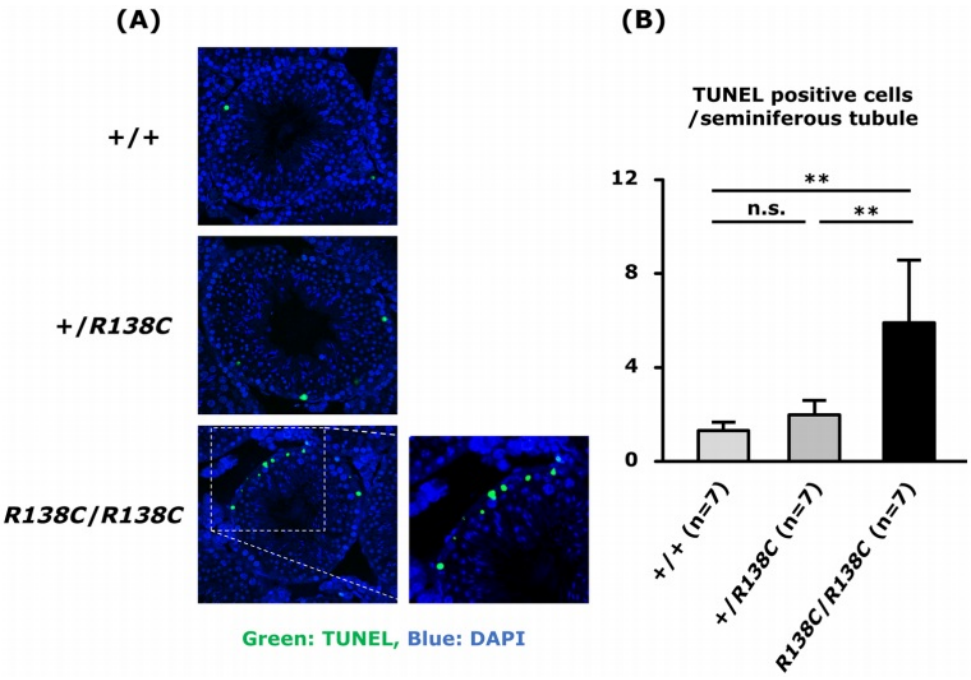

Fig. 3 Evaluation of apoptosis in the testes from *Nudt15*^{R138C} knock-in mice after treatment with MP (0.5 mg/kg/day) for 4 weeks. **A** Representative images showing apoptotic cells stained with the TUNEL method. **B** Statistics of the number of TUNEL-positive apoptotic cells per seminiferous tubule. Data are presented as the mean ± SD. $^{**}p < 0.01$. *n.s.* not significant, +/+ : *Nudt15*$^{+/+}$ mice (wild type), +/*R138C*: *Nudt15*$^{+/R138C}$ mice, *R138C/R138C*: *Nudt15*$^{R138C/R138C}$ mice

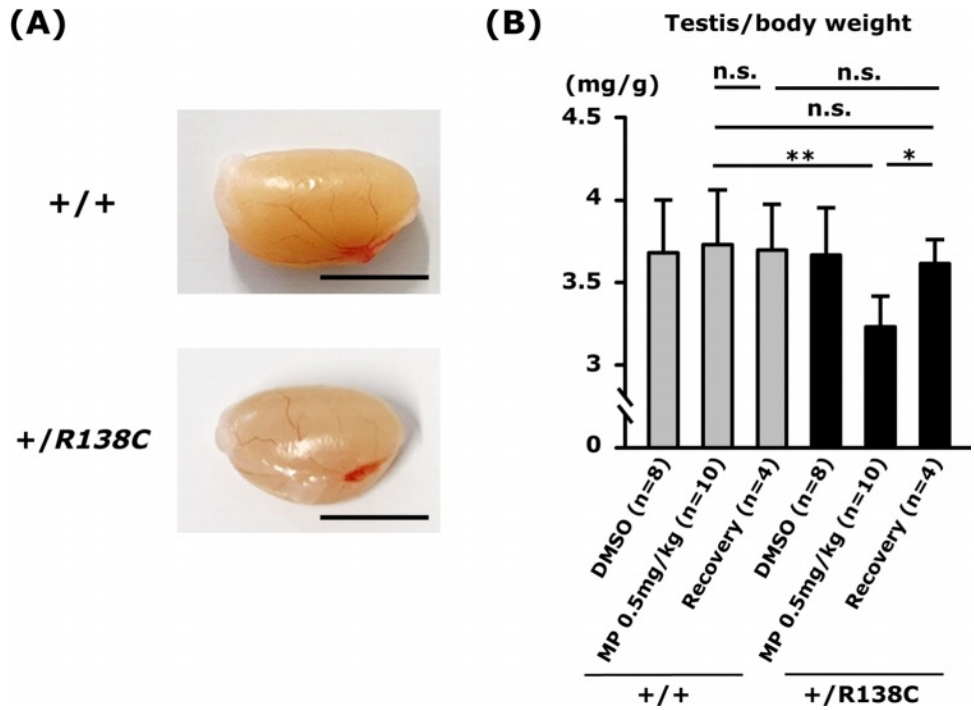

Fig. 4 Effects of long-term MP treatment (12 weeks) on the testes of *Nudt15*^{R138C} knock-in mice. **A** Morphology of testes after treatment with MP (0.5 mg/kg/day) for 12 weeks. Scale bar: 5 mm. **B** Statistics of the testis/body weight ratio. After MP treatment for 12 weeks, some mice were maintained for a further 12 weeks under MP-free condition (Recovery). Data are presented as the mean ± SD. $^{**}p < 0.01$, $^{*}p < 0.05$. n.s., not significant. +/+ : *Nudt15*$^{+/+}$ mice (wild type), +/*R138C*: *Nudt15*$^{+/R138C}$ mice

Nudt15$^{R138C/R138C}$ $71.0 \pm 16.9 \times 10^5$/cauda epididymis vs. *Nudt15*$^{+/+}$ $86.6 \pm 18.4 \times 10^5$ ($p = 0.19$) and vs. *Nudt15*$^{+/R138C}$ 86.7 ± 15.3 ($p = 0.18$); abnormal sperm rate: *Nudt15*$^{R138C/R138C}$ $32.9 \pm 4.7\%$ vs. *Nudt15*$^{+/+}$ $30.4 \pm 7.2\%$ ($p = 0.73$) and vs. *Nudt15*$^{+/R138C}$ $26.8 \pm 7.3\%$ ($p = 0.15$)].

Long-term (12 weeks) effects of MP on testicular function of *NUDT15*$^{+/R138C}$ mice

Since MP (0.5 mg/kg/day) was extremely toxic for *Nudt15*$^{R138C/R138C}$ mice, long-term effects could be evaluated in *Nudt15*$^{+/+}$ mice and *Nudt15*$^{+/R138C}$ mice. *Nudt15*$^{+/+}$ mice and *Nudt15*$^{+/R138C}$ mice were treated with MP (0.5 mg/

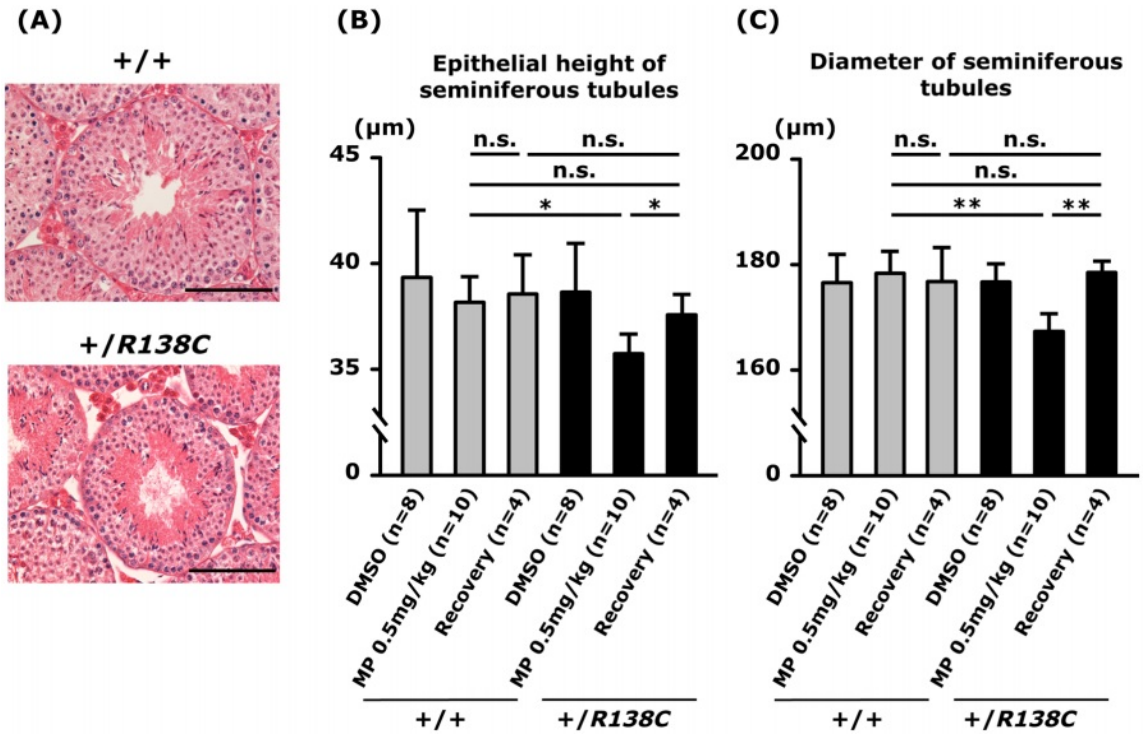

Fig. 5 Histology of the testes from *Nudt15*^{R138C} knock-in mice after treatment with MP (0.5 mg/kg/day) for 12 weeks. **A** Representative images (×400) of HE-stained sections of the testes. Scale bar: 100 μm. **B** Statistics of the epithelial height of seminiferous tubules. After MP treatment for 12 weeks, some mice were maintained for a further 12 weeks under MP-free condition (Recovery). **C** Statistics of the diameter of seminiferous tubules. Data are presented as the mean ± SD. $^{**}p < 0.01$, $^{*}p < 0.05$. n.s., not significant. +/+: *Nudt15*$^{+/+}$ mice (wild type), +/*R138C*: *Nudt15*$^{+/R138C}$ mice

kg/day) for 12 weeks, and some of them were followed for a further 12 weeks under MP-free condition.

As shown in Fig. 4, MP administration induced a significant reduction of testis/body weight ratio in *Nudt15*$^{+/R138C}$ mice (*Nudt15*$^{+/R138C}$ 3.2 ± 0.2 mg/g vs. *Nudt15*$^{+/+}$ 3.7 ± 0.3, $p < 0.01$), but this change disappeared after 12 weeks of MP-free condition (3.6 ± 0.2 mg/g, $p = 0.73$ vs *Nudt15*$^{+/+}$ mice). Histological examination revealed that MP administration induced a significant reduction of the height and diameter of the seminiferous tubules in *Nudt15*$^{+/R138C}$ mice compared to *Nudt15*$^{+/+}$ mice [epithelial height: *Nudt15*$^{+/R138C}$ 35.7 ± 0.9 μm vs. *Nudt15*$^{+/+}$ 38.2 ± 1.2 ($p < 0.05$); diameter: 167.4 ± 3.4 μm vs. 178.4 ± 4.0 ($p < 0.01$)] (Fig. 5A–C), and these changes disappeared after 12 weeks of MP-free condition (epithelial height: 37.6 ± 1.0 μm, $p = 0.61$; diameter, 178.5 ± 2.1 μm, $p = 1.00$ vs. *Nudt15*$^{+/+}$ mice) (Fig. 5B, C).

As shown in Fig. 6, there was no significant change in epididymal sperm number, but the abnormal sperm rate was significantly increased in *Nudt15*$^{+/R138C}$ mice as compared to *Nudt15*$^{+/+}$ mice [*Nudt15*$^{+/R138C}$ 35.6 ± 4.1% vs. *Nudt15*$^{+/+}$ 31.1 ± 3.6% ($p < 0.05$)]. This increase disappeared after 12-week MP-free condition (29.1 ± 3.7%, $p = 0.67$ compared to *Nudt15*$^{+/+}$ mice). Despite a significant increase of abnormal sperm rate, mating experiments with females showed that pregnancy rate and number of offspring birth were not affected after MP treatment for 12 weeks [pregnancy rate: *Nudt15*$^{+/R138C}$ 31.8% vs. *Nudt15*$^{+/+}$ 40.9% ($p = 0.53$); average number of offspring birth/pregnant: 7.6 ± 2.0 vs. 5.9 ± 1.5 ($p = 0.07$)] (Fig. 7A). No teratogenicity associated with MP treatment was evident in MP-treated *Nudt15*$^{+/R138C}$ and *Nudt15*$^{+/+}$ mice.

LH has been reported to stimulate spermatogenesis indirectly via testosterone, whereas FSH acts directly on the seminiferous tubules [27–29]. Therefore, we evaluated serum levels of LH, FSH and testosterone in *Nudt15*$^{+/R138C}$ mice treated with MP (0.5 mg/kg/day) for 12 weeks (Fig. 7B). LH was not detected in *Nudt15*$^{+/R138C}$ mice and *Nudt15*$^{+/+}$ mice, and there were no significant differences in serum FSH and testosterone levels between *Nudt15*$^{+/R138C}$ mice and *Nudt15*$^{+/+}$ mice [FSH: *Nudt15*$^{+/R138C}$ 3.2 ± 1.7 mIU/mL vs. *Nudt15*$^{+/+}$ 4.0 ± 3.5 ($p = 0.58$); testosterone: 2.8 ± 0.8 ng/mL vs. 2.7 ± 0.4 ($p = 0.74$)].

Discussion

Thiopurines play an important role in maintaining remission in the treatment of patients with IBD, which often affects

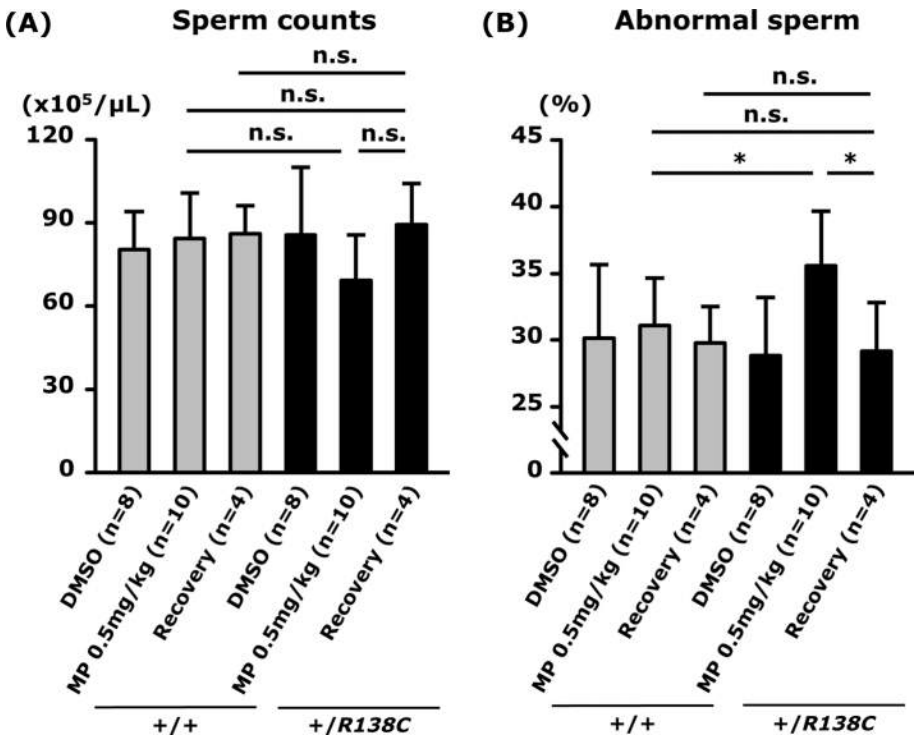

Fig. 6 Sperm evaluation. (A) Statistics of the number of sperm per cauda epididymis after treatment with MP (0.5 mg/kg/day) for 12 weeks. (B) Statistics of the percentage of abnormal sperm. Morphological classification of spermatozoa was evaluated according to the previous reports [24, 25]. Data are presented as the mean ± SD. $^{**}p < 0.01$, $^{*}p < 0.05$. n.s., not significant. +/+ : *Nudt15*$^{+/+}$ mice (wild type), +/*R138C*: *Nudt15*$^{+/R138C}$ mice

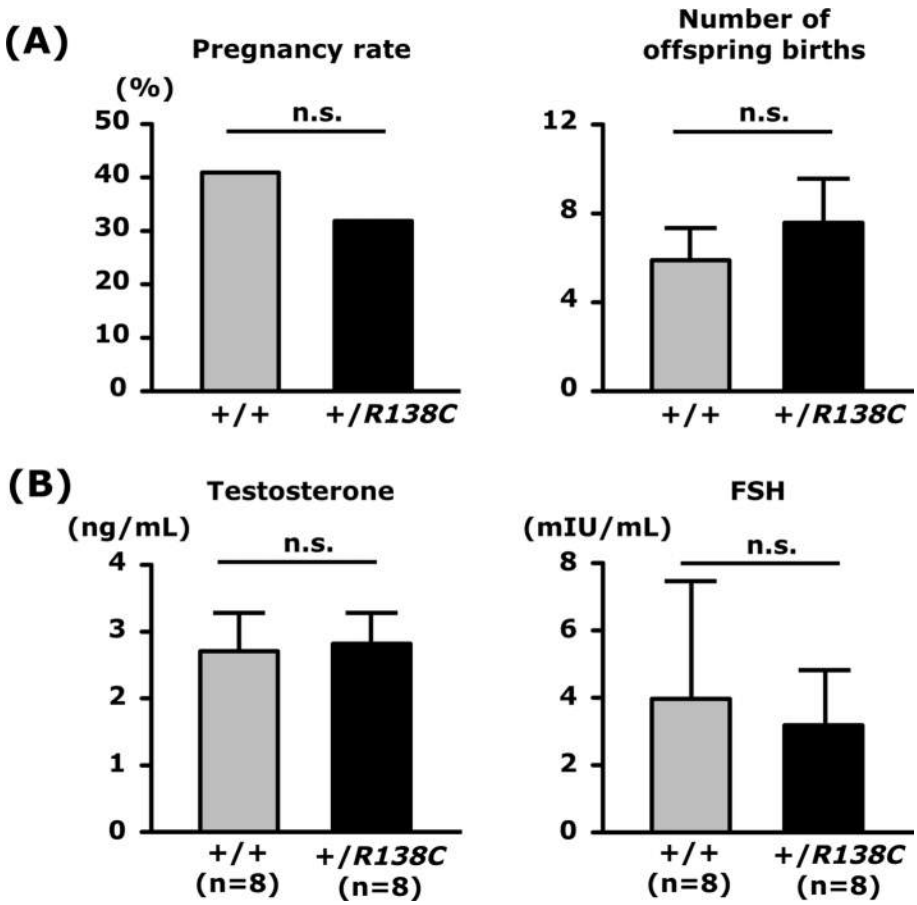

Fig. 7 Evaluation of fertility and serum hormones. **A** Statistics of pregnancy rate and the number of offspring births per female mouse. After treatment with MP (0.5 mg/kg/day) for 12 weeks, male *Nudt15*^{R138C} knock-in mice ($n = 10$) were placed with wild-type females (n = 20) for 3 days, and the pregnancy rate and number of offspring births per pregnant female were evaluated. **B** Statistics of serum testosterone and FSH (follicle-stimulating hormone) levels after treatment with MP (0.5 mg/kg/day) for 12 weeks. Data are presented as the mean ± SD. n.s., not significant. +/+ : *Nudt15*$^{+/+}$ mice (wild type), +/*R138C*: *Nudt15*$^{+/R138C}$ mice

young men and women. It is estimated that there are more than 1 million IBD patients in the USA and 2.5 million in Europe, about one-third of whom receive thiopurines during the course of their disease [30, 31]. On the other hand, since thiopurines exert their effects by inhibiting nucleic acid synthesis, there is concern about their toxicity on actively-dividing somatic cells and germ cells, such as spermatozoa. In this context, we have recently reported using *NUDT15*^{R138C} knock-in mice that thiopurines at a clinical dose to the pregnant mother led to serious consequences for the fetus based on fetal *NUDT15* genotypes [21]. Like female reproductive function, spermatogenesis is a highly organized and complex process [32]. This study represents the first report on the impact of thiopurines on male reproductive function, taking into account the *NUDT15* genotypes, using *Nudt15*^{R138C} knock-in mice.

Short-term (4 weeks) administration of 0.5 mg/kg/day MP resulted in a decrease in testicular size and a significant decrease in testicular weight in *Nudt15*$^{R138C/R138C}$ mice, while *Nudt15*$^{+/R138C}$ mice and *Nudt15*$^{+/+}$ mice showed no change. In addition, histology showed an atrophy of seminiferous tubules in *Nudt15*$^{R138C/R138C}$ mice, and TUNEL assay indicated that these changes were accompanied by a significant increase in apoptotic cells. Most apoptotic cells were located in contact with the basement membrane of the seminiferous tubules, suggesting that spermatogonia were mainly injured. Spermatogonia are the mitotic germ cells including the stem cells and differentiation-destined amplifying cells and are located in the basal compartment [26]. A previous study reported that thiopurine-sensitive cells in the testes are spermatocytes in mice [33], but the localization of apoptotic cells in this study is not consistent with the anatomical localization of spermatocytes. In *Nudt15*$^{R138C/R138C}$ mice, it is likely that loss of NUDT15 activity may have induced a systemic accumulation of the cytotoxic 6-TGTP and 6-TdGTP and injured spermatogonia (stem cells and amplifying cells), leading to testicular atrophy. These results suggest that in the IBD patients with homozygous *NUDT15* risk allele, thiopurine cytotoxicity might extend to actively-dividing cells throughout the body, not only to the stem cells in the bone marrow and hair follicle. Atrophic changes in seminiferous tubules were evident in MP-treated *Nudt15*$^{R138C/R138C}$ mice, but there were no changes in sperm count and morphology. The germ cells have been reported to require 34.4 days or longer for seminiferous epithelial cycles (one cycle, 8.6 days or longer)] to complete spermatogenesis in mice [26]. This suggests that 4 weeks may have been too short to reflect histological changes of the testes in epididymal sperm counts. Thus, short-term administration of 0.5 mg/kg/day MP was harmful to the testicular function in *Nudt15*$^{R138C/R138C}$ mice, although it did not affect that of *Nudt15*$^{+/R138C}$ mice.

Long-term (12 weeks) administration of 0.5 mg/kg/day MP to *Nudt15*$^{+/R138C}$ mice caused an atrophy of the seminiferous tubules and an increase in abnormal sperms, although these changes were reversible with drug withdrawal. It was possible that changes in MP-treated *Nudt15*$^{+/R138C}$ mice could be due to hormonal changes affecting spermatogenesis such as testosterone or FSH [27–29], but there were no differences in serum levels of testosterone and FSH. Mating experiments with females showed no changes in male fertility or offspring malformations. This is supported by a similar observation in the *act* (activator of cAMP-responsive element modulator in testis) gene knockout (KO) mice which exhibit more severe morphological abnormalities and reduced number of spermatozoa than our models. The changes in the sperms of the act KO mice were more serious compared to the findings in this study, but they showed normal fertility [34]. These suggest that fertility in male mice may be preserved even in the presence of considerable sperm abnormalities. Based on the findings so far in MP-treated *Nudt15*$^{+/R138C}$ mice, MP administration at a therapeutic dose has the potential to induce a reversible toxicity on spermatogenesis while preserving fertility in patients with heterozygotes for the *NUDT15* risk allele. Since we clinically prescribe thiopurines for heterozygous male carrying the *NUDT15* risk allele for a long period, a longer-term study (more than 12 weeks) using this mouse model will be necessary in the future.

Although the relationship between thiopurine use and male fertility has been reported in several mouse studies, conclusions about the safety of thiopurines remain confusing [35]. Ligumsky et al. have previously reported a slightly different observation in mice [36]. Administration of relatively higher doses of MP (2, 5 or 8 mg/kg/day) for 51 days did not affect sperm morphology or sperm production. However, a significant increase in embryonic resorption in all exposed mice, suggested a potential sperm damage. In another study, there were no changes in testicular function or spermatogenesis in male rats treated with 3 or 5 mg/kg/day MP for 75 days [37]. A number of animal model studies have revealed that the toxic effects of MP were dose dependent, and testicular function and fertility remained unaffected at low dosages [35]. However, our findings in this study suggested that such experiments are preferable to be performed with consideration with thiopurine-susceptible genotypes when experimental results are applied to actual clinical practice.

The safety of fetal exposure to thiopurines and the importance of controlling the disease activity for pregnancy using thiopurines has been extensively evaluated [38–41], but there are few studies of the association between paternal exposure to thiopurines with male reproductive activity and related pregnancy outcomes. In a systematic review by Simsek et al. [35], it was reported that none of the included

studies revealed an association between thiopurine exposure and poor sperm quality or testicular function, and impaired fertility was often related to underlying disorders. The estimated odds ratio (OR) for congenital anomalies in paternally thiopurine exposed offspring was 1.32 (95% CI, 0.75 and 2.34). They concluded that the slightly increased risk of birth abnormality could be a consequence of paternal chronic disease rather than thiopurine exposure. A recent systematic review by Gubatan et al. reported that paternal thiopurine use was not associated with preterm birth compared with no thiopurine exposure [42], and paternal thiopurine use was not associated with congenital malformations [42]. They also reported that thiopurine use in male patients with IBD was not associated with increased risk of early pregnancy loss, preterm birth, or congenital malformations. Friedman et al. used Danish registries to examine long-term outcomes and no negative impact of paternal preconception use of thiopurines was reported [43]. Thus, there is an increasing body of evidence suggesting that thiopurine use is safe for IBD fathers wishing to promote pregnancy, but conclusive studies are insufficient. Furthermore, previous reports have not taken into account any genetic background related to thiopurine metabolism. Since the importance of the *NUDT15* genotype in thiopurine use has been recognized, the genetic background of patients should also be analyzed as a contributory factor in the future.

In conclusion, previous studies have shown that thiopurines do not impair spermatogenesis or testicular function and do not injure paternally exposed offspring. However, these did not consider the genetic background involved in thiopurine metabolism, such as NUDT15. In actual clinical settings, it is necessary to set doses based on the genetic background of patients such as *NUDT15* genotypes and to remember the potential effects of thiopurines on male fertility associating with genetic susceptibility. To clarify whether thiopurines affect spermatogenesis and paternally exposed offspring, large epidemiological studies evaluating the safety of thiopurines in men and their offspring should be performed in the future.

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Declarations

Conflict of interest AA receiving lecture fee from Takeda Pharmaceutical Co. Ltd., AbbVie GK, and Miyarisan Pharmaceutical Co. Ltd. YK received patent royalties from Medical & Biological Laboratories Co., Ltd, and lecture fee from Takeda Pharmaceutical Co. Ltd., AbbVie GK, and Janssen Pharmaceutical K.K. All other authors declare that they have no conflict of interest in this study.

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