FSSG scored did not differ in NAFL and NASH patients, with the prevalence of GERD being 25 % in each group.

## Factors positively correlating with AIS and FSSG scores

AIS score was positively correlated with FSSG score ($r = 0.4108$, $P < 0.001$) (Fig. 2), but not with any other parameter (Table 3). FSSG score was positively correlated with platelet count ($r = 0.2197$, $P = 0.0159$), and negatively correlated with age ($r = -0.2363$, $P = 0.0085$), FPG concentration ($r = -0.2010$, $P = 0.0277$), HOMA-IR score ($r = -0.1948$, $P = 0.0330$) and hyaluronic acid concentration ($r = -0.2246$, $P = 0.0125$). BMI, transaminase activities, lipid profiles, and iron parameters were not correlated with AIS or FSSG score.

## Correlation between histological findings and AIS scores

Assessment of histological findings in the 123 patients with NAFLD showed that 53 (43 %), 40 (33 %), and 30 (24 %) had steatosis grades 1, 2, and 3, respectively; 30 (24 %), 70 (57 %), 18 (15 %), and 5 (4 %) had inflammation grades 0, 1, 2, and 3, respectively; and 44 (36 %), 38 (31 %), 21 (17 %), 13 (11 %), and 7 (6 %) had fibrosis grades 0, 1, 2, 3, and 4, respectively. Evaluation of correlations between AIS scores and histological findings showed that AIS score was not correlated with steatosis, inflammation, or fibrosis grade (Fig. 3).

## Clinical findings in patients with and without insomnia

Comparisons of clinical and laboratory findings in patients with and without insomnia showed that γGT concentrations and FSSG scores were higher, and the prevalence of hypertension and IRI and HOMA-IR scores were lower, in patients with insomnia (Table 4). Moreover, GERD symptoms were significantly more prevalent in patients with than without insomnia (56 vs. 13 %, $P < 0.0001$).

## Drug usage

Drug usage in patients involved in this study was shown in Table 5. Beta-blockers users were more prevalent in patients with GERD compared to those without. The prevalence of other drug users was not different between patients with GERD/insomnia and those without.

**Table 4** The comparison between insomniacs and non-insomniacs

| Clinical parameter | Insomniacs ($n$ = 34 [28 %]) | Non-insomniacs ($n$ = 89 [72 %]) | $P$ value |
|---|---|---|---|
| Age (years) | 56 (35–74) | 60 (14–82) | 0.8100 |
| Gender (female) | 22 (65 %) | 54 (61 %) | 0.8359 |
| BMI (kg/m$^2$) | 26.4 (21.9–38.6) | 26.6 (16.6–43.4) | 0.5028 |
| Obesity (BMI > 25) | 25 (74 %) | 62 (70 %) | 0.8252 |
| Dyslipidemia | 21 (62 %) | 56 (63 %) | 1.0000 |
| Hypertension (yes) | 8 (24 %) | 40 (45 %) | 0.0385 |
| Type 2 diabetes (yes) | 16 (47 %) | 39 (44 %) | 0.8400 |
| Hemoglobin (g/dl) | 14.4 (11.0–18.3) | 14.0 (10.5–17.1) | 0.9072 |
| Platelet count (× 10$^4$/μl) | 22.3 (8.7–33.5) | 21.1 (4.6–78.5) | 0.7137 |
| AST (IU/l) | 47 (20–182) | 44 (17–186) | 0.4682 |
| ALT (IU/l) | 71 (12–358) | 69 (15–218) | 0.6233 |
| GGT (IU/l) | 77 (24–391) | 59 (20–268) | 0.0063 |
| Cholinesterase (IU/l) | 373 (208–547) | 371 (167–545) | 0.7992 |
| Total cholesterol (mg/dl) | 206 (125–314) | 214 (87–335) | 0.3670 |
| Triglyceride (mg/dl) | 146 (68–424) | 164 (61–659) | 0.3864 |
| Ferritin (ng/ml) | 128 (11–1100) | 170 (5–923) | 0.7315 |
| FPG (mg/dl) | 94 (70–452) | 97 (60–171) | 0.7708 |
| IRI (μU/ml) | 9.8 (2.8–11.2) | 12.0 (1.6–49.5) | 0.0326 |
| HOMA-IR | 2.10 (0.65–33.04) | 2.96 (0.38–16.34) | 0.0335 |
| Hyaluronic acid (ng/ml) | 32 (9–392) | 37 (9–3480) | 0.9774 |
| Type IV collagen 7S (ng/ml) | 4.7 (2.7–13.0) | 4.4 (2.8–10.0) | 0.9054 |
| FSSG | 8 (0–38) | 3 (0–17) | <0.0001 |
| FSSG ≥ 8 ($n$ [%]) | 19 [56 %] | 12 [13 %] | <0.0001 |
| NASH ($n$ [%]) | 24 [71 %] | 59 [66 %] | 0.8299 |

Results are presented as numbers with percentages in parenthesis for qualitative data or as mean ± SD for quantitative data

*BMI* body mass index, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *GGT* gamma glutamyl transpeptidase, *FPG* fasting plasma glucose, *IRI* immuno-reactive insulin

$P$ values were calculated by $t$ test or $\chi^2$ analysis

**Table 5** Drug usage

| Drug usage | FSSG < 8 ($n = 92$) | FSSG ≥ 8 ($n = 31$) | *P* value | Insomniacs ($n = 34$) | Non-insomniacs ($n = 89$) | *P* value |
|---|---|---|---|---|---|---|
| Use of antihypertensive drugs | | | | | | |
| Calcium antagonists | 26 [28 %] | 10 [32 %] | 0.6560 | 7 [21 %] | 29 [33 %] | 0.2680 |
| ARBs | 4 [4 %] | 2 [6 %] | 0.6412 | 2 [6 %] | 4 [4 %] | 0.6679 |
| Beta-blockers | 2 [2 %] | 4 [13 %] | 0.0348 | 2 [6 %] | 4 [4 %] | 0.6679 |
| Use of NSAIDs | 10 [11 %] | 2 [6 %] | 0.7285 | 1 [3 %] | 11 [12 %] | 0.1762 |
| Use of anticoagulants | 2 [2 %] | 0 [0 %] | 1.0000 | 0 [0 %] | 2 [2 %] | 1.0000 |
| Use of digestive drugs | 2 [2 %] | 0 [0 %] | 1.0000 | 0 [0 %] | 2 [2 %] | 1.0000 |

*ARBs* angiotensin receptor blockers, *NSAIDs* non-steroidal anti-inflammatory drugs

**Table 6** Results of multivariate analysis: independent predictors of insomniacs

| Variables | Adjusted (multivariate) | | |
|---|---|---|---|
| | OR | 95 % CI | *P* value |
| FSSG | 1.2315 | 1.1221–1.3516 | <0.0001 |
| GGT | 1.0109 | 1.0031–1.0189 | 0.0063 |
| IRI | 0.9515 | 0.8908–1.0164 | 0.1396 |
| Hypertension | 0.5503 | 0.1823–1.6610 | 0.2893 |

*OR* odds ratio, *CI* confidence interval, *FSSG* frequency scale for the symptoms of GERD, *GGT* gamma glutamyl transpeptidase, *IRI* immuno-reactive insulin

### Factors independently predictive of insomnia in patients with NAFLD

Multivariate logistic regression analysis showed that plasma GGT concentration [odds ratio (OR) 1.2315, 95 % confidence interval (CI) 1.1221–1.3516, $P < 0.0001$] and FSSG score (OR 1.0109, 95 % CI 1.0031–1.0189, $P = 0.0063$) were significant independent predictors of insomnia (Table 6). In contrast, IRI and HOMA-IR scores and hypertension were not predictive.

### Effects of RPZ on insomnia

RPZ treatment of patients with GERD significantly reduced FSSG and AIS scores (Fig. 4). Of the 9 patients with insomnia treated with RPZ, 4 (44 %) showed resolution of insomnia after treatment.

## Discussion

This study of Japanese NAFLD patients demonstrated that (1) 28 % had AIS scores ≥ 6, indicative of insomnia; (2) 25 % had FSSG scores ≥ 8, indicative of GERD; (3) AIS and FSSG scores did not differ significantly in patients with NASH and NAFL; (4) FSSG score was independently associated with AIS score; and (5) insomnia could be relieved after treatment with RPZ.

The precise prevalence of insomnia in NAFLD patients has been unclear. Using the AIS, we found that 28 % of patients with biopsy-proven NAFLD had insomnia. Although sleep dysfunction has been defined as a Pittsburgh Sleep Quality Index (PSQI) score > 5.5 in other studies [17, 31], the AIS is satisfactorily validated, simple to perform, and well accepted based on ICD-10 criteria. The AIS has been used to assess insomnia in the general population in Japan. For example, a study of approximately 3000 individuals found that 21.4 % had experienced insomnia during the previous month [32]. The prevalence of insomnia in our NAFLD patients does not seem to be markedly different from that in the general population. Though this precise reason is unknown, one plausible explanation is that about 20–30 % of the general population is estimated to have NAFLD. We should obtain data from sex- and age-matched non-NAFLD population to clarify whether the prevalence of insomnia in NAFLD is really higher compared to that in the general population. Assessments of employees of two local governments in Japan found that 1382 of 5951 males (23.2 %) and 465 of 1500 females (31.0 %), aged 34–59 years, had insomnia [33, 34]. Assessments of middle-aged women found that 27.5–43.6 % had AIS scores ≥ 6 [35, 36]. Taken together, these findings indicate that the prevalence of insomnia is higher in women than in men, across countries and cultures. In contrast, we observed no differences in AIS scores between men and women with NAFLD. Since the discrepancy between our results and previous studies can be explained by a small number of patients involved in this study, a larger number of patients should be examined in the future. Since Yoshioka et al. [34] showed that the gender difference disappeared after adjustment for paid work and family responsibilities, detailed characteristics of patients should be considered to clarify the gender differences. In Japanese studies in which insomnia was

diagnosed using AIS, factors associated with insomnia included work at visual display terminals for ≥ 6 h per day [37], job stress [38, 39], and reduced illumination in the workplace [40]. In this study, however, we did not evaluate these work environmental factors.

The mechanisms by which insomnia arises in patients with NAFLD have never been clarified. We found that AIS scores and the incidence of insomnia were similar in patients with NAFL and NASH. AIS scores did not correlate with any histological findings, such as steatosis, inflammation, and fibrosis scores, indicating that histological severity is not important in the pathogenesis of insomnia in patients with NAFLD. In contrast, many studies have explored the associations between life-style related disorders/obesity and sleep disturbance. Changes in secretion of the hormones cortisol, leptin, and ghrelin, and increased insulin resistance due to short sleep duration were found to increase the risks of obesity and diabetes [41–43]. In contrast, insomniacs were more likely to have insulin resistance than non-insomniacs. This study demonstrated that FSSG score was significantly correlated only with AIS score. Multivariate analysis showed that FSSG was an independent risk factor associated with insomnia, suggesting that GERD symptoms are responsible for insomnia in NAFLD patients, findings consistent with previously reported results [9, 17]. For example, sleep disorders, such as inability to sleep, difficult falling asleep, and awakening during the night, were observed in 56.3 % of patients with heartburn [9]. Similarly, we found that 61.3 % (19/31) of NAFLD patients with GERD had insomnia. A study in 134 Japanese patients with GERD found that FSSG score was significantly positively correlated with PSQI score [11]. GERD can affect sleep through two primary mechanisms. First, nighttime reflux, which occurs in 47–79 % of patients with GERD, can cause awakening during the night. Second, GERD can cause short, amnestic arousals (approximately 30 s), resulting in sleep fragmentation. However, recent studies also suggested that the link between GERD and sleep problems may be bidirectional. Sleep stage may influence the esophago-upper esophageal sphincter contractile reflex [44]. Sleep disturbance may reinforce the perception of intra-esophageal acid [45]. The association of NAFLD with GERD has been assessed in only two studies, which reported that 37 and 51 % of patients with NAFLD had GERD symptoms [7, 8], percentages higher than observed in the present study. Plausible explanations of a lower prevalence (25 %) of GERD in our NAFLD patients were the difference of ethics, sex/age distribution, and the diagnostic method of GERD or NAFLD between previous studies [7, 8] and ours. Another explanation is the possibility that our NAFLD patients receiving dietary educations might avoid irregular diet habits, which are known to be the most significant risk factors for GERD symptoms [15]. In the future, sex- and age-matched controlled studies using a larger population is essential to draw conclusions. A recent study of Japanese patients with NAFLD found that GERD symptoms were significantly more severe in the group with higher than lower total cholesterol (T-CHO) and triglyceride (TG) levels [7]. In contrast, we observed no correlation between GERD symptoms and either T-CHO or TG. These conflicting results may be due to our inclusion of only patients with biopsy-proven NAFLD, who are not representative of the general population of patients diagnosed with NAFLD. In contrast, the patients included in the previous study were diagnosed with NAFLD by ultrasound [7]. Moreover, that study did not assess the association of insomnia with GERD symptoms. Thus, to our knowledge, our study is the first to clarify the relationship between insomnia and GERD symptoms in patients with NAFLD.

Previous studies suggested that acid suppression can improve sleep problems in GERD patients. For example, a prospective randomized clinical trial found that a significantly higher percentage of patients treated with esomeprazole than placebo showed resolution of GERD-related sleep disturbances [46]. RPZ treatment also significantly improved subjective indices of sleep quality over placebo [16]. Moreover, an 8-week course of RPZ treatment significantly decreased both FSSG and PSQI scores in Japanese patients [11]. Consistent with these findings, we found that treatment with RPZ significantly decreased both AIS and FSSG scores. These results also indicate that GERD symptoms are at least partly responsible for the occurrence of insomnia in NAFLD patients.

In addition to AIS score, GGT concentration was found to be an independent predictor of insomnia in patients with NAFLD. Serum GGT activity is a marker of oxidative stress [47]. The primary function of GGT is to maintain intracellular concentrations of glutathione, a critical anti-oxidant molecule. Thus, increased GGT activity can be regarded as a response to oxidative stress, aimed at increasing the intracellular concentration of glutathione. Although little is known about the relationship between insomnia and oxidative stress, a preliminary study showed that anti-oxidant activity was significantly lower and lipid peroxidation levels significantly higher in patients with primary insomnia than in controls [48]. Serum concentrations of GGT and 8-hydroxydeoxyguanosine are correlated in patients with NAFLD, indicating the occurrence of oxidative stress [49]. GGT has also been associated with the occurrence of metabolic syndrome, early atherosclerosis, and cardiovascular events [50]. Moreover, GGT concentrations are higher in patients with obstructive sleep

apnea syndrome (OSAS) than in controls [51] and have been associated with nocturnal arterial oxygen desaturation. Furthermore, continuous positive airway pressure treatment has been shown to decrease GGT, further suggesting that the increase in GGT is directly associated with OSAS. OSAS has also been associated with the presence and severity of NAFLD [52], suggesting that insomnia in patients with NAFLD is at least partly associated with the occurrence of OSAS. Our finding, that elevated GGT is an independent predictor of insomnia, suggests that these patients also have OSAS, although this was not directly evaluated in our patient population.

This study had several limitations. First, its design was cross-sectional, making it difficult to establish a cause–effect relationship, and suggesting the need for prospective studies. Second, the AIS is a self-administered questionnaire and subjective measure of insomnia, suggesting the need for assessment of objective sleep variables such as those obtained during polysomnography. Third, GERD was diagnosed based on FSSG scores alone, without endoscopic examination or 24 h pH monitoring. Because all participants were Japanese, there is a possibility that our results may not be applicable for NAFLD patients of other races or ethnic groups. The current study also did not assess the effects of smoking habits, mental status, dietary habit, and work environments. Additional studies, in larger populations, are needed to assess these variables.

In conclusion, we found that about 30 % of Japanese patients with biopsy-proven NAFLD have insomnia. GERD symptoms may be important in the development of insomnia. PPIs may be clinically useful for treating insomnia in NAFLD patients.

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**Determination of the site preference of G to T mutation in di- and trinucleotide sequences.** To analyze site preference of G to T mutation caused by *Mth1/Ogg1/Mutyh* deficiency, the 239 data of G to T mutation detected in G2–G8 were subjected (C to A mutations were converted to G to T mutation). The reference exon sequences and the 101 nucleotides those containing each mutation site (shown in Supplementary Data S1 online) were used to determine the site preference of mutation. The ratio shown in Fig. 5b, c were calculated as follows (data were summarized in Supplementary Table S2 online).

(A) The number of each di- or tri-nucleotides sequences in the reference exon sequence were counted by 1 nucleotide sliding.
(B) The number of each di- or tri-nucleotides sequences that include mutated guanine site were counted.
(C) The frequency of each di- or tri-nucleotides sequences was calculated as follows: (A) /number of total nucleotide in reference exon sequence.
(D) Total number of di- or tri-nucleotides sequences that include mutated guanine site were 478 and 717, respectively.
(E) The expected value for a random mutation for each di- or tri-nucleotides sequences were calculated as (C) × (D).
(F) The ratio (observed mutation for the expected value for a random mutation) was calculated as (B)/(E).


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## Author contributions

M.O. and K.S. designed the study, analysed data, and wrote the paper. R.F. and Y.G. designed and performed exome and sequencing analyses. Y.I. and T.I. performed bioinformatic analysis. T.T. gave conceptual advice. M.F. quantitated 8-oxodG. M.H. analysed hydrocephalus mice. Y.N. was involved in the study design and preparation of the paper. All authors discussed the results and commented on the manuscript.

## Additional information

**Supplementary information** accompanies this paper at http://www.nature.com/scientificreports

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