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Article

Genetic Association of the Functional *WDR4* Gene in Male Fertility

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Abstract: Infertility is one of the important problems in the modern world. Male infertility is characterized by several clinical manifestations, including low sperm production (oligozoospermia), reduced sperm motility (asthenozoospermia), and abnormal sperm morphology (teratozoospermia). *WDR4*, known as Wuho, controls fertility in Drosophila. However, it is unclear whether *WDR4* is associated with clinical manifestations of male fertility in human. Here, we attempted to determine the physiological functions of *WDR4* gene. Two cohorts were applied to address this question. The first cohort was the general population from Taiwan Biobank. Genomic profiles from 68,948 individuals and 87 common physiological traits were applied for phenome-wide association studies (PheWAS). The second cohort comprised patients with male infertility from Wan Fang Hospital, Taipei Medical University. In total, 81 male participants were recruited for the genetic association study. Clinical records including gender, age, total testosterone, follicle-stimulating hormone (FSH), luteinizing hormone (LH), total sperm number, sperm motility, and sperm morphology were collected. In the first cohort, results from PheWAS exhibited no associations between *WDR4* genetic variants and 87 common physiological traits. In the second cohort, a total of four tagging single-nucleotide polymorphisms (tSNPs) from *WDR4* gene (rs2298666, rs465663, rs2248490, and rs3746939) were selected for genotyping. We found that SNP rs465663 solely associated with asthenozoospermia. Functional annotations through the GTEx portal revealed the correlation between TT or TC genotype and low expression of *WDR4*. Furthermore, we used mouse embryonic fibroblasts cells from mwdr4 heterozygous (+/–) mice for functional validation by western blotting. Indeed, low expression of WDR4 contributed to ROS-induced DNA fragmentation. In conclusion, our results suggest a critical role of *WDR4* gene variant as well as protein expression in asthenozoospermia.

Keywords: *WDR4*; genetic variants; male fertility; sperm quality

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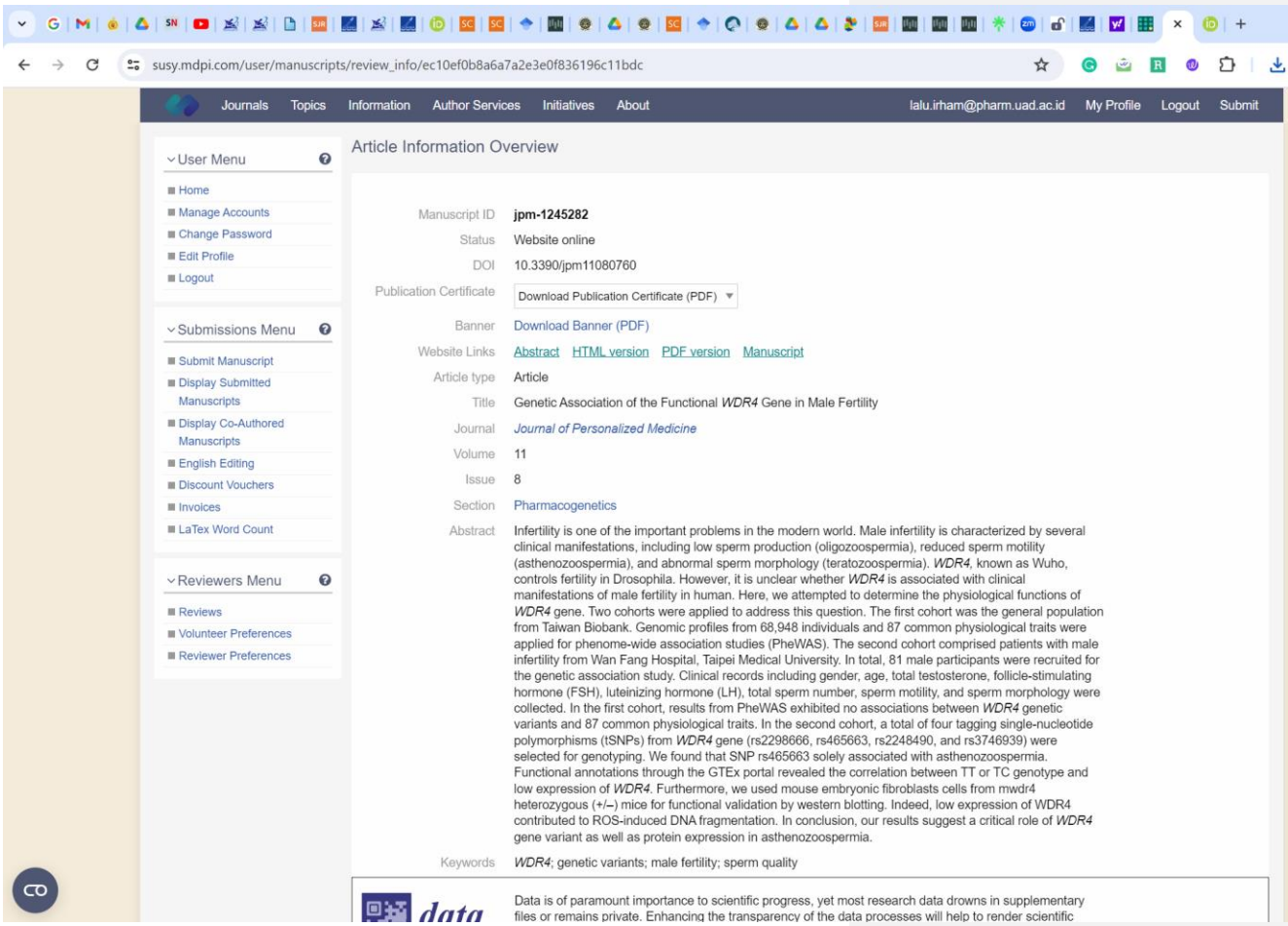
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Abstract
Infertility is one of the important problems in the modern world. Male infertility is characterized by several clinical manifestations, including low sperm production (oligozoospermia), reduced sperm motility (asthenozoospermia), and abnormal sperm morphology (teratozoospermia). WDR4, known as Wuho, controls fertility in Drosophila. However, it is unclear whether WDR4 is associated with clinical manifestations of male fertility in human. Here, we attempted to determine the physiological functions of WDR4 gene. Two cohorts were applied to address this question. The first cohort was the general population from Taiwan Biobank. Genomic profiles from 68,948 individuals and 87 common physiological traits were applied for phenome-wide association studies (PheWAS). The second cohort comprised patients with male infertility from Wan Fang Hospital, Taipei Medical University. In total, 81 male participants were recruited for the genetic association study. Clinical records including gender, age, total testosterone, follicle-stimulating hormone (FSH), luteinizing hormone (LH), total sperm number, sperm motility, and sperm morphology were collected. In the first cohort, results from PheWAS exhibited no associations between WDR4 genetic variants and 87 common physiological traits. In the second cohort, a total of four tagging single-nucleotide polymorphisms (tSNPs) from WDR4 gene (rs2298666, rs465663, rs2248490, and rs3746939) were selected for genotyping. We found that SNP rs465663 solely associated with asthenozoospermia. Functional annotations through the GTEx portal revealed the correlation between TT or TC genotype and low expression of WDR4. Furthermore, we used mouse embryonic fibroblasts cells from mwdr4 heterozygous (+/–) mice for functional validation by western blotting. Indeed, low expression of WDR4 contributed to ROS-induced DNA fragmentation. In conclusion, our results suggest a critical role of WDR4 gene variant as well as protein expression in asthenozoospermia.
Keywords
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data
Data is of paramount importance to scientific progress, yet most research data drowns in supplementary files or remains private. Enhancing the transparency of the data processes will help to render scientific

Keywords *WDR4*; genetic variants; male fertility; sperm quality

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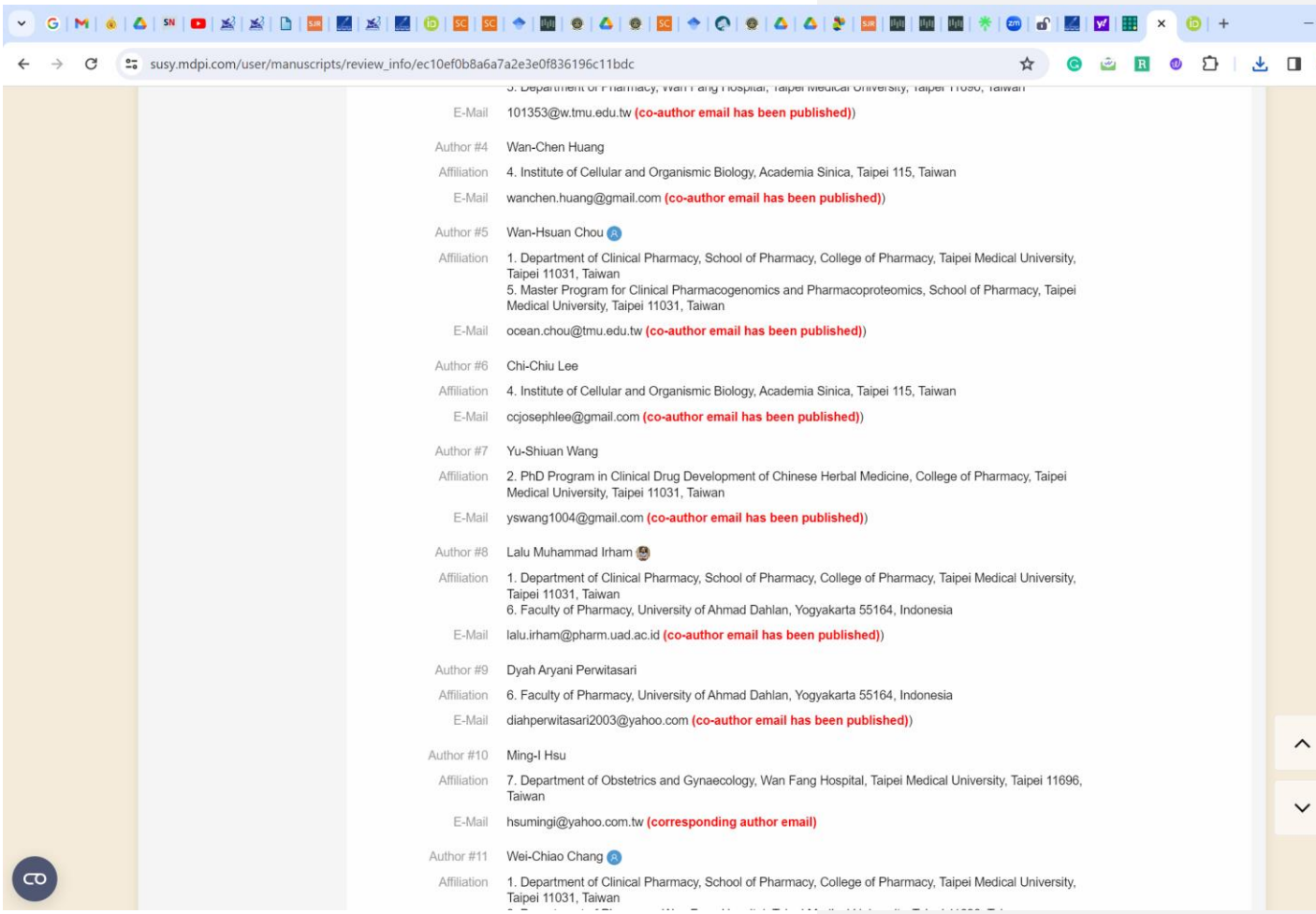
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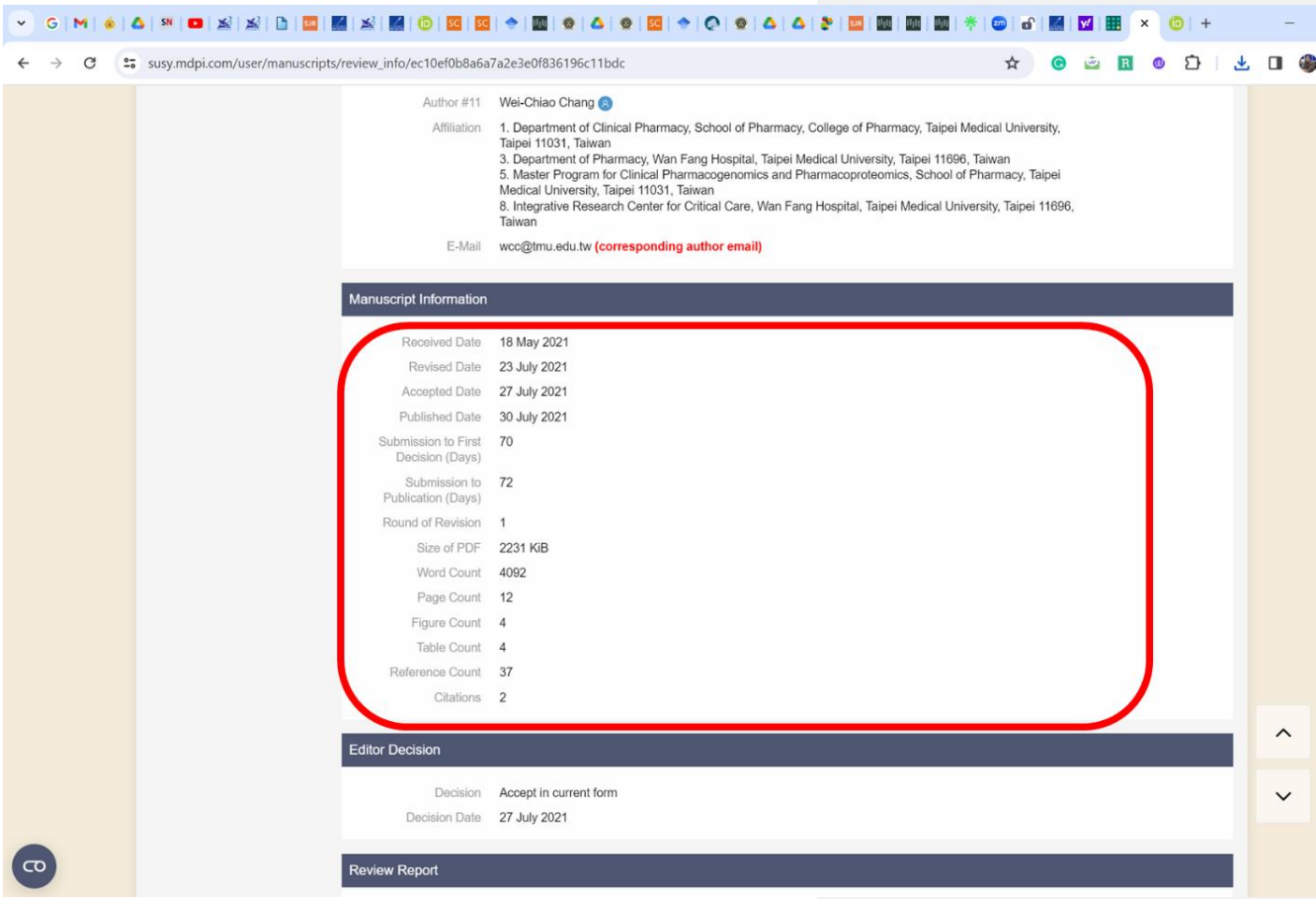
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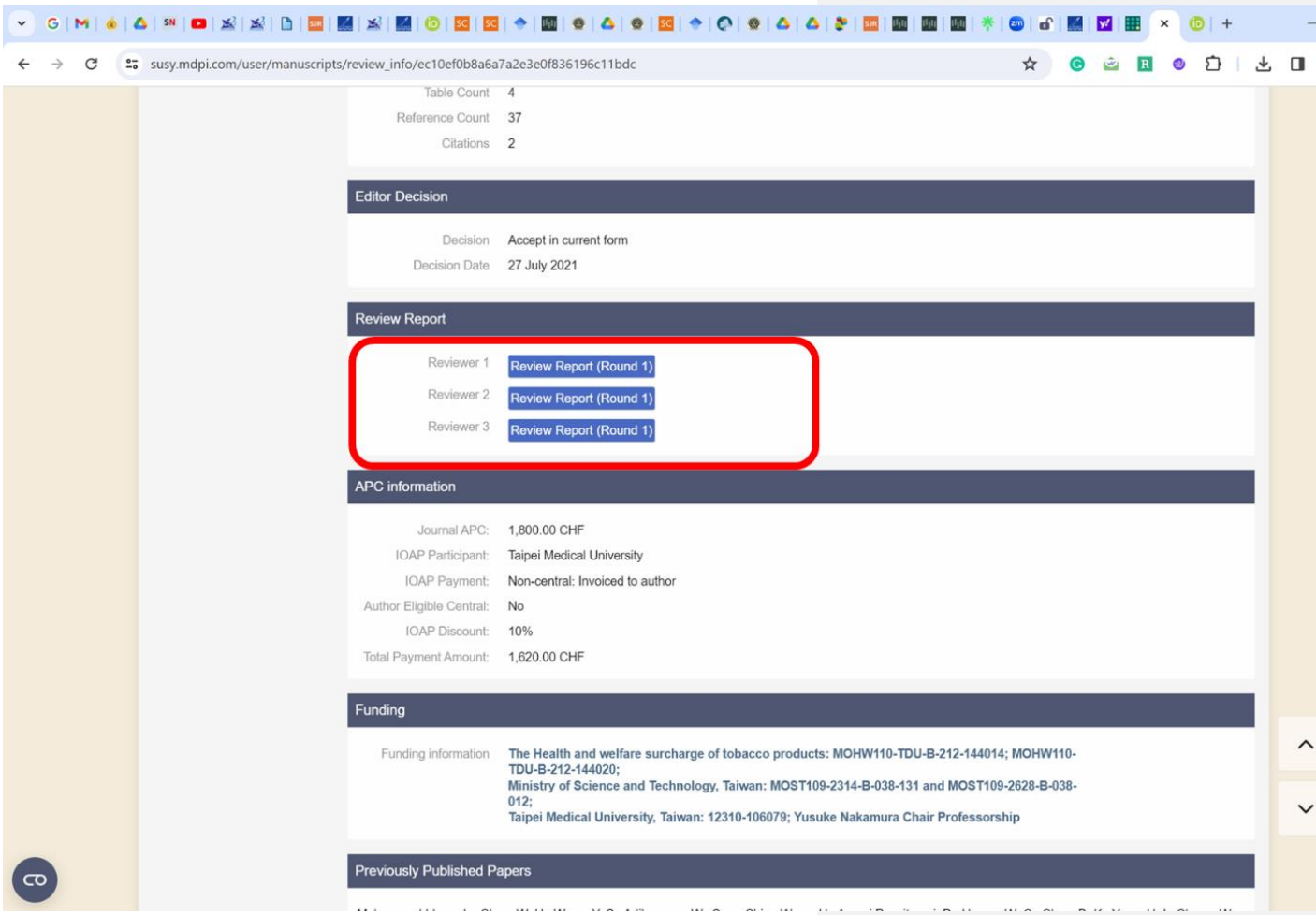
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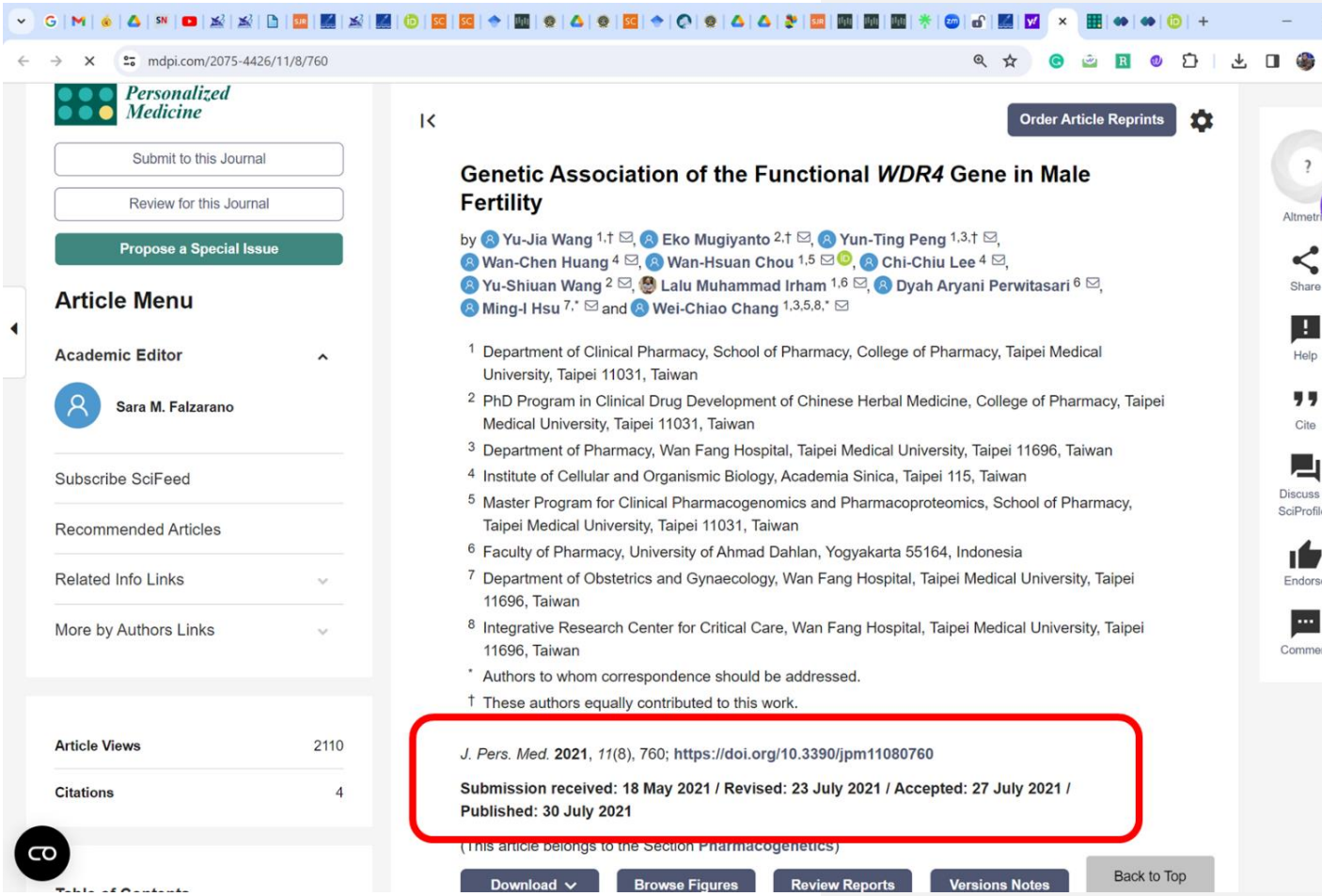

Authors' Responses to Reviewer's Comments (Reviewer 1)

Author's Notes

Respond to Reviewer 1 comment:

Q1: In the introduction, the authors reference CF mutations and their association with impaired spermatogenesis and impaired sperm quality. This is a relatively poor choice of an example, as the relationship of CF mutations and male infertility is weak (beyond structural anomalies in the Wolffian tract.)

Ans: We sincerely thank the reviewer's comment. As suggested by the reviewer, we have removed the references of CF mutations and have modified the introduction part as stated by the following sentences (**page 2, lines 59-65**): "In addition, HSFY, which is related to the heat-shock transcription factor (HSF) family that found on chromosome Yq, has a long open reading frame carrying an HSF-type DNA binding domain involved in azoospermia. Some point mutations in the testis-specific HSFY gene family, such as microdeletions, may be a cause of unexplained cases of idiopathic male infertility [1]. Furthermore, a study evaluated the role of genetic variants in male infertility; There were three SNPs (rs4920566, rs11763979, and rs3741843) were associated with taste receptor genes (*TAS*R) and male infertility [2]. Besides the genomic variants, the epigenetic modifications also play important roles in spermatogenesis and male infertility."

Reference:

1. Ozdemir O, Gul E, Kilicarslan H, Gokce G, Beyaztas FY, Ayan S, Sezgin I: SRY and AZF gene variation in male infertility: a cytogenetic and molecular approach. *Int Urol Nephrol* 2007, 39(4):1183-1189.
2. Gentiluomo M, Crifasi L, Luddi A, Locci D, Barale R, Piomboni P, Campa D: Taste receptor polymorphisms and male infertility. *Human Reproduction* 2017, 32(11):2324-2331.

Author's Notes File: Report Notes

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	Yes	Can be improved	Must be improved	Not applicable
Does the introduction provide sufficient background and include all relevant references?	( )	(x)	( )	( )
Is the research design appropriate?	(x)	( )	( )	( )
Are the methods adequately described?	(x)	( )	( )	( )
Are the results clearly presented?	(x)	( )	( )	( )
Are the conclusions supported by the results?	( )	(x)	( )	( )

Comments and Suggestions for Authors
In the introduction, the authors reference CF mutations and their association with impaired spermatogenesis and impaired sperm quality. This is a relatively poor choice of an example, as the relationship of CF mutations and male infertility is weak (beyond structural anomalies in the Wolffian tract.)

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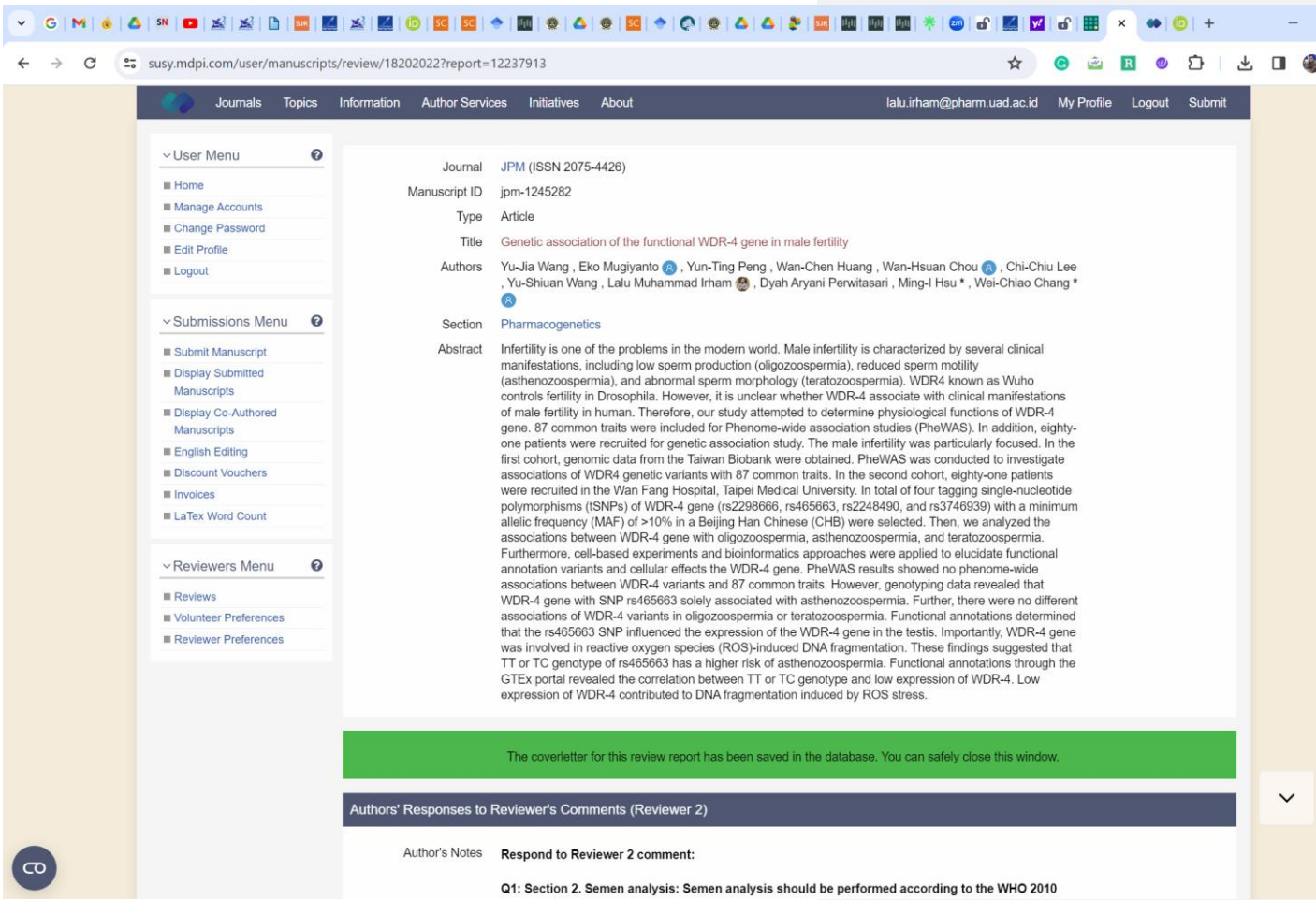
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Title Genetic association of the functional WDR-4 gene in male fertility
Authors Yu-Jia Wang , Eko Mugiyanto , Yun-Ting Peng , Wan-Chen Huang , Wan-Hsuan Chou , Chi-Chiu Lee , Yu-Shiuan Wang , Lalu Muhammad Irham , Dyah Aryani Perwitasari , Ming-I Hsu * , Wei-Chiao Chang *
Section Pharmacogenetics
Abstract Infertility is one of the problems in the modern world. Male infertility is characterized by several clinical manifestations, including low sperm production (oligozoospermia), reduced sperm motility (asthenozoospermia), and abnormal sperm morphology (teratozoospermia). WDR4 known as Wuho controls fertility in Drosophila. However, it is unclear whether WDR-4 associate with clinical manifestations of male fertility in human. Therefore, our study attempted to determine physiological functions of WDR-4 gene. 87 common traits were included for Phenome-wide association studies (PheWAS). In addition, eighty-one patients were recruited for genetic association study. The male infertility was particularly focused. In the first cohort, genomic data from the Taiwan Biobank were obtained. PheWAS was conducted to investigate associations of WDR4 genetic variants with 87 common traits. In the second cohort, eighty-one patients were recruited in the Wan Fang Hospital, Taipei Medical University. In total of four tagging single-nucleotide polymorphisms (tSNPs) of WDR-4 gene (rs2298666, rs465663, rs2248490, and rs3746939) with a minimum allelic frequency (MAF) of >10% in a Beijing Han Chinese (CHB) were selected. Then, we analyzed the associations between WDR-4 gene with oligozoospermia, asthenozoospermia, and teratozoospermia. Furthermore, cell-based experiments and bioinformatics approaches were applied to elucidate functional annotation variants and cellular effects the WDR-4 gene. PheWAS results showed no phenome-wide associations between WDR-4 variants and 87 common traits. However, genotyping data revealed that WDR-4 gene with SNP rs465663 solely associated with asthenozoospermia. Further, there were no different associations of WDR-4 variants in oligozoospermia or teratozoospermia. Functional annotations determined that the rs465663 SNP influenced the expression of the WDR-4 gene in the testis. Importantly, WDR-4 gene was involved in reactive oxygen species (ROS)-induced DNA fragmentation. These findings suggested that TT or TC genotype of rs465663 has a higher risk of asthenozoospermia. Functional annotations through the GTEx portal revealed the correlation between TT or TC genotype and low expression of WDR-4. Low expression of WDR-4 contributed to DNA fragmentation induced by ROS stress.
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Authors' Responses to Reviewer's Comments (Reviewer 2)
Author's Notes Respond to Reviewer 2 comment:
Q1: Section 2. Semen analysis: Semen analysis should be performed according to the WHO 2010

Authors' Responses to Reviewer's Comments (Reviewer 2)

Author's Notes

Respond to Reviewer 2 comment:

Q1: Section 2. Semen analysis: Semen analysis should be performed according to the WHO 2010 guideline. Please provide this information. Most important, WHO define asthenozoospermia as <32% progressives sperm motility, this parameter is more important than total sperm motility (WHO 2010: page 226 Table A1.3: 'percentage of progressively motile (PR) spermatozoa below the lower reference limit'), similarly in case of oligozoospermia WHO give different definition, more important is total sperm count than concentration (WHO 2010: total number (or concentration, depending on outcome reported) of spermatozoa below the lower reference limit; Preference should always be given to total number, as this parameter takes precedence over concentration). Because the authors divide the group according to asthenozoospermia, oligozoospermia or teratozoospermia it is extremely important to assign patients the appropriate category of semen disorders.

Whether the men had an isolated asthenozoospermia, oligozoospermia or teratozoospermia? If yes, it should be clearly written, if not authors should use term: non-asthenozoospermia group (men with other disorders than asthenozoospermia: oligoteratozoospermia, teratozoospermia, oligozoospermia..); non-oligozoospermia group and non-teratozoospermia group. Unfortunately, if the subjects didn't have isolated seminological disorders, these changes should be implemented consistently throughout the rest of the manuscript (tables and statistics).

Ans: Thanks for your valuable comments and suggestions. According to your suggestions, we have modified the definition of asthenozoospermia (PR<32%) and oligozoospermia (total sperm number <$39x10^6$). The data are shown in table 2 and 3. The paragraphs have been amended in the methods and results sections. For clarification, we showed sample numbers for both isolated and combined defects among groups in Table 1. In addition, we changed the term "controls" to "non-oligozoospermia", "non-asthenozoospermia"; and "non-teratozoospermia" throughout the revised manuscript. We revised accordingly in the method section part 2.2 (**page 2-3, lines 92-108**). The following paragraph is the revised version. "Semen samples were examined after liquefaction. Semen analysis was conducted separately by two experienced technicians in the reproductive laboratory. Semen parameters, including sperm count, sperm motility, and sperm morphology were evaluated. The World Health Organization (WHO) 2010 recommendations for semen analysis were followed. A semen sample (10 µL) was first transferred to a MAKLER counting chamber (Irvine Scientific, Santa Ana, CA, USA) to measure the sperm count. Oligozoospermia was defined as a number of sperms <$39x10^6$ spermatozoa. The motility of each spermatozoon was graded into three categories: progressive motility (PR), non-progressive motility (NP), and immotile spermatozoa. Asthenozoospermia was defined as PR<32%. For sperm morphological evaluation, each spermatozoon was classified into eight groups as follows: normal sperm (NS), amorphous head (AH), duplicated head (DH), large head (LH), small head (SH), normal head with other defect (NH),

Ans: Thanks for your valuable comments and suggestions. According to your suggestions, we have modified the definition of asthenozoospermia (PR<32%) and oligozoospermia (total sperm number <39x10^6). The data are shown in table 2 and 3. The paragraphs have been amended in the methods and results sections. For clarification, we showed sample numbers for both isolated and combined defects among groups in Table 1. In addition, we changed the term "controls" to "non-oligozoospermia", "non-asthenozoospermia"; and "non-teratozoospermia" throughout the revised manuscript. We revised accordingly in the method section part 2.2 (**page 2-3, lines 92-108**). The following paragraph is the revised version. "Semen samples were examined after liquefaction. Semen analysis was conducted separately by two experienced technicians in the reproductive laboratory. Semen parameters, including sperm count, sperm motility, and sperm morphology were evaluated. The World Health Organization (WHO) 2010 recommendations for semen analysis were followed. A semen sample (10 μL) was first transferred to a MAKLER counting chamber (Irvine Scientific, Santa Ana, CA, USA) to measure the sperm count. Oligozoospermia was defined as a number of sperms <39x10^6 spermatozoa. The motility of each spermatozoon was graded into three categories: progressive motility (PR), non-progressive motility (NP), and immotile spermatozoa. Asthenozoospermia was defined as PR<32%. For sperm morphological evaluation, each spermatozoon was classified into eight groups as follows: normal sperm (NS), amorphous head (AH), duplicated head (DH), large head (LH), small head (SH), normal head with other defect (NH), tapering head (TH), and other defect (OD). Teratozoospermia was defined as <4% of morphologically normal spermatozoa. In addition, combined oligozoospermia defined as participants with sperm number less than 39x10^6 spermatozoa and other defects (astheno- and/or teratozoospermia), which rationale applies to other two sperm characteristics (combined asthenozoospermia and combined teratozoospermia)".

Furthermore, we also adjusted the sentence in the result section **page 6 (lines 207-209)**. "The effects of four SNPs on total sperm number were first evaluated. There were no statistically significant differences in genotypic distributions between oligozoospermia and non-oligozoospermia groups (Table 2)". And **page 7, lines 214-217.** "Next, the effects of four SNPs on total sperm motility were analyzed. As shown in Table 3, the genotypic distribution of rs465663 differed between asthenozoospermia and non-asthenozoospermia groups. The proportions of TT and TC genotypes were higher in asthenozoospermia compared to non-asthenozoospermia (p=0.006)".

Q2: Section 3.7. Effects of *WDR4* in DNA fragmentation: The authors did not investigate directly DNA fragmentation but the protein expression. In my opinion, this section requires.

Ans: We sincerely thank the reviewer's comments. Yes. We did not investigate directly DNA fragmentation but the protein expression. Therefore, we improve the sentences as follows: "Previous studies showed that the sperm DNA fragmentation level was increased by ROS stress, and was correlated with sperm motility [5, 6]. The higher expression level of γH2AX (biomarker of DNA fragmentation level) has been revealed to correlate with male infertility [20]. To investigate the role of *WDR4* in male infertility, the MEF (mouse embryonic fibroblasts) cells were isolated from mwdr4 heterozygous (+/-) mice, and the H_2O_2 was used to induce DNA fragmentation for mimicking oxidative stress. The mwdr4 and γH2AX protein expression level were confirmed with western blotting (**Figure 4**). The results showed that the mWdr4 protein expression

Furthermore, we also adjusted the sentence in the result section **page 6 (lines 207-209)**. "The effects of four SNPs on total sperm number were first evaluated. There were no statistically significant differences in genotypic distributions between oligozoospermia and non-oligozoospermia groups (Table 2)". And **page 7, lines 214-217.** "Next, the effects of four SNPs on total sperm motility were analyzed. As shown in Table 3, the genotypic distribution of rs465663 differed between asthenozoospermia and non-asthenozoospermia groups. The proportions of TT and TC genotypes were higher in asthenozoospermia compared to non-asthenozoospermia (p=0.006)".

Q2: Section 3.7. Effects of *WDR4* in DNA fragmentation: The authors did not investigate directly DNA fragmentation but the protein expression. In my opinion, this section requires.

Ans: We sincerely thank the reviewer's comments. Yes. We did not investigate directly DNA fragmentation but the protein expression. Therefore, we improve the sentences as follows: "Previous studies showed that the sperm DNA fragmentation level was increased by ROS stress, and was correlated with sperm motility [5, 6]. The higher expression level of γH2AX (biomarker of DNA fragmentation level) has been revealed to correlate with male infertility [20]. To investigate the role of *WDR4* in male infertility, the MEF (mouse embryonic fibroblasts) cells were isolated from mwdr4 heterozygous (+/-) mice, and the H_2O_2 was used to induce DNA fragmentation for mimicking oxidative stress. The mwdr4 and γH2AX protein expression level were confirmed with western blotting (**Figure 4**). The results showed that the mWdr4 protein expression level in mWdr4 heterozygous MEF group is low. Importantly, the H_2O_2-induced γH2AX protein level was highly increased in mwdr4 heterozygous MEF group compare to wild type MEF group. These results highlighted an important role of *WDR4* in male infertility."

Q3: Section 4. Discussion: sentences from lines 256 to 264 about epidemiology of infertility should be compiled with sentences in introduction. This background does not fit in with the discussion. The authors should focus on discussing their own results, comparing them with published data and explaining the role of the studied gene in male fertility.

Ans: Thank you very much for reviewer's suggestions. We already adjusted and compile the epidemiology of infertility in the Introduction part (**Page 1-2, lines 46-49**). "Infertility is a global health issue affecting 8%~12% of couples worldwide, and male infertility factors are responsible for about 20%~30% of infertility cases. Approximately 7% of the male population is affected by infertility".

Q4: As mentioned above, in discussion authors should describe in detail the role of the *WDR-4* in spermatogenesis. I suggest creating a separate subsection in which the role of *WDR-4* in spermatogenesis and the pathomechanism associated with mutations of this gene will be described.

Ans: We sincerely thank the reviewer for the time taken to review our work and the important suggestion given. References of WDR4 are very limited. The function of WDR4 has been mentioned in the separate subsection of discussion part (**Page 9, line 260-269**). "Wuho is a member of the evolutionarily conserved WD repeat protein family that is expressed by the genes wuho in Drosophila, *TRM82* in yeast, and *WDR4* in humans [1]. WD40 domains usually contain four to eight repeating sequences, which are separated by approximately 40 amino acids. Each repeat consists of two sites, poorly conserved site and well-conserved

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Reference:

1. Cheng IC, Chen BC, Shuai H-H, Chien F-C, Chen P, Hsieh T-s: Wuho Is a New Member in Maintaining Genome Stability through its Interaction with Flap Endonuclease 1. PLoS biology 2016, 14(1):e1002349.
2. Riedl SJ, Salvesen GS: The apoptosome: signalling platform of cell death. Nature Reviews Molecular Cell Biology 2007, 8(5):405-413.
3. Wu J, Hou JH, Hsieh TS: A new Drosophila gene wh (wuho) with WD40 repeats is essential for spermatogenesis and has maximal expression in hub cells. Developmental biology 2006, 296(1):219-230.
4. Tahmasebi S, Khoutorsky A, Mathews MB, Sonenberg N: Translation deregulation in human disease. Nature Reviews Molecular Cell Biology 2018, 19(12):791-807.

Q5: Conclusions: It is worth mentioning that in the case of infertile men, when intracytoplasmic sperm injection is used as a method of infertility treatment, there is a risk of passing these changes on to the next generation, which patients should be informed about.

Ans: Thank you very much for the reviewer's suggestions. This study indicated that genetic variants and expression level of the *WDR4* gene is associated with infertility in males in cases of asthenozoospermia. It is ideal to translate medical research into clinical usage. However, the sample size is still small in this study. Larger sample size in different populations are required to confirm the polymorphism. Then, the clinical advices should be provided in the future.

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( ) Extensive editing of English language and style required
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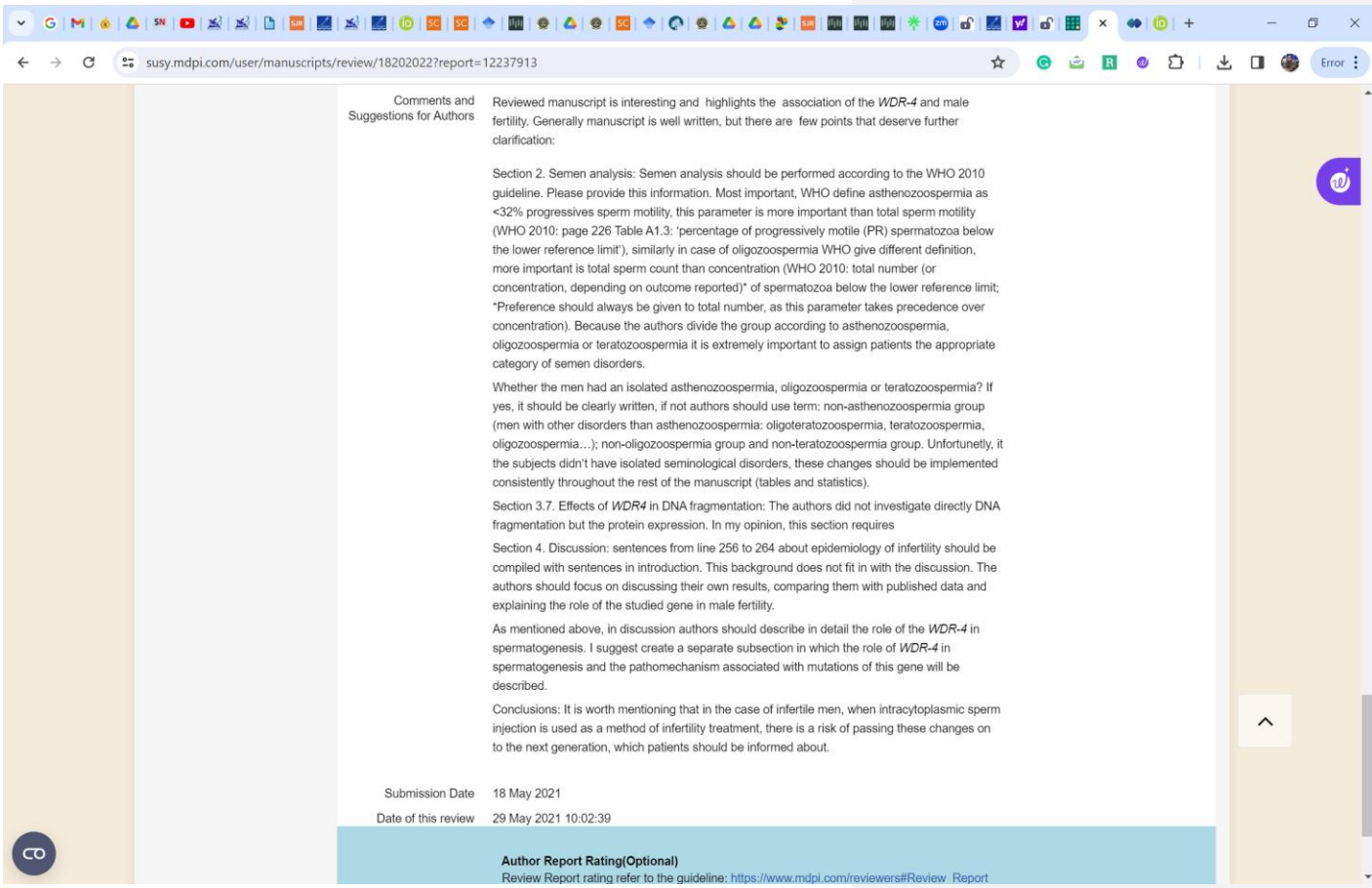
	Yes	Can be improved	Must be improved	Not applicable
Does the introduction provide sufficient background and include all relevant references?	(x)	( )	( )	( )
Is the research design appropriate?	( )	(x)	( )	( )
Are the methods adequately described?	( )	(x)	( )	( )
Are the results clearly presented?	( )	(x)	( )	( )
Are the conclusions supported by the results?	( )	(x)	( )	( )

Comments and Suggestions for Authors

Reviewed manuscript is interesting and highlights the association of the *WDR-4* and male fertility. Generally manuscript is well written, but there are few points that deserve further clarification:

Section 2. Semen analysis: Semen analysis should be performed according to the WHO 2010 guideline. Please provide this information. Most important, WHO define asthenozoospermia as <32% progressives sperm motility, this parameter is more important than total sperm motility (WHO 2010: page 226 Table A1.3: 'percentage of progressively motile (PR) spermatozoa below the lower reference limit'), similarly in case of oligozoospermia WHO give different definition, more important is total sperm count than concentration (WHO 2010: total number (or concentration, depending on outcome reported)* of spermatozoa below the lower reference limit; *Preference should always be given to total number, as this parameter takes precedence over concentration). Because the authors divide the group according to asthenozoospermia, oligozoospermia or teratozoospermia it is extremely important to assign patients the appropriate category of semen disorders.

Whether the men had an isolated asthenozoospermia, oligozoospermia or teratozoospermia? If yes, it should be clearly written, if not authors should use term: non-asthenozoospermia group (men with other disorders than asthenozoospermia: oligoteratozoospermia, teratozoospermia, oligozoospermia ...); non-oligozoospermia group and non-teratozoospermia group. Unfortunetly, it



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Submission Date	18 May 2021
Date of this review	29 May 2021 10:02:39

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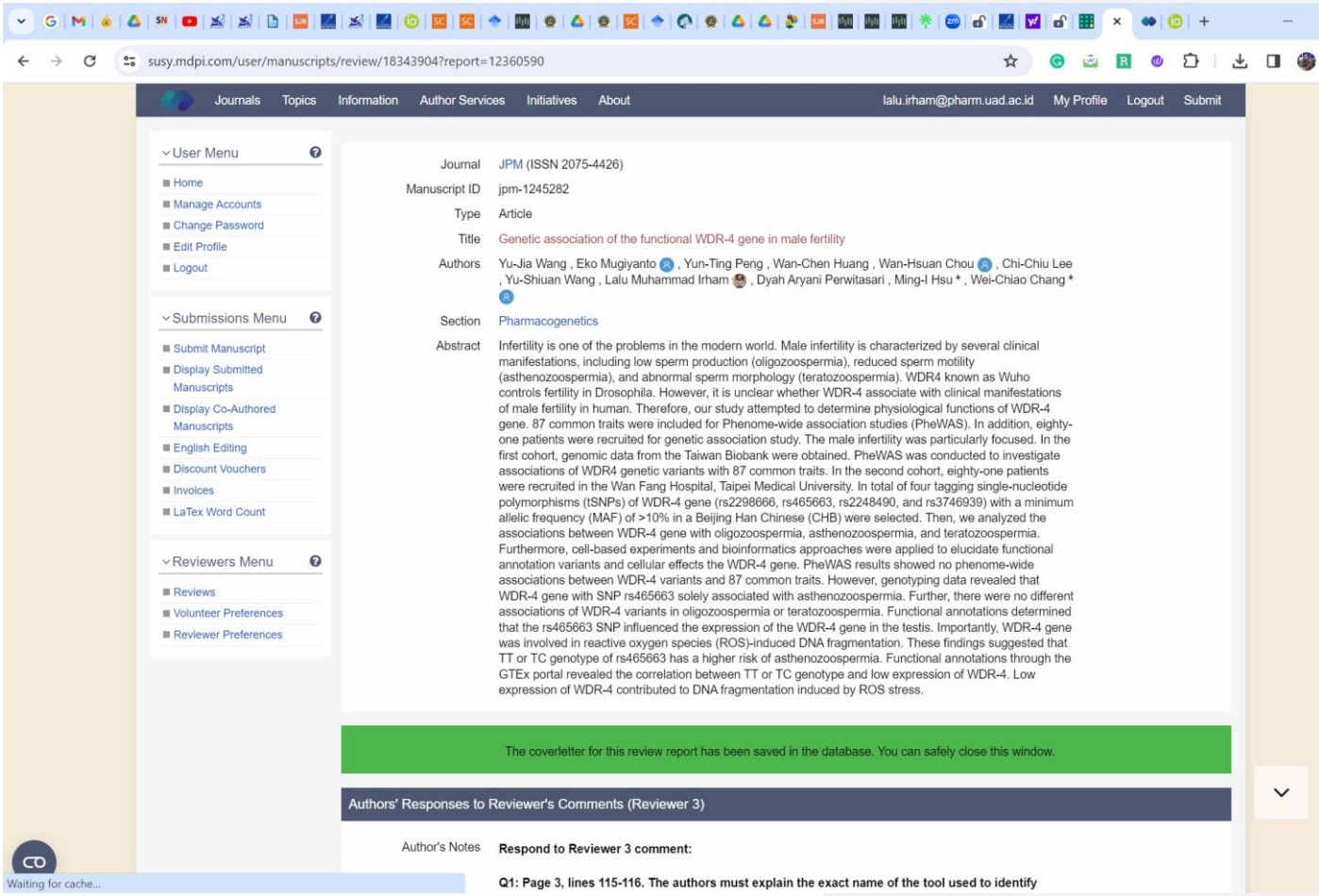
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Journal JPM (ISSN 2075-4426)
Manuscript ID jpm-1245282
Type Article
Title Genetic association of the functional WDR-4 gene in male fertility
Authors Yu-Jia Wang , Eko Mugiyanto , Yun-Ting Peng , Wan-Chen Huang , Wan-Hsuan Chou , Chi-Chiu Lee , Yu-Shiuan Wang , Lalu Muhammad Irham , Dyah Aryani Perwitasari , Ming-I Hsu * , Wei-Chiao Chang *
Section Pharmacogenetics
Abstract Infertility is one of the problems in the modern world. Male infertility is characterized by several clinical manifestations, including low sperm production (oligozoospermia), reduced sperm motility (asthenozoospermia), and abnormal sperm morphology (teratozoospermia). WDR4 known as Wuho controls fertility in Drosophila. However, it is unclear whether WDR-4 associate with clinical manifestations of male fertility in human. Therefore, our study attempted to determine physiological functions of WDR-4 gene. 87 common traits were included for Phenome-wide association studies (PheWAS). In addition, eighty-one patients were recruited for genetic association study. The male infertility was particularly focused. In the first cohort, genomic data from the Taiwan Biobank were obtained. PheWAS was conducted to investigate associations of WDR4 genetic variants with 87 common traits. In the second cohort, eighty-one patients were recruited in the Wan Fang Hospital, Taipei Medical University. In total of four tagging single-nucleotide polymorphisms (tSNPs) of WDR-4 gene (rs2298666, rs465663, rs2248490, and rs3746939) with a minimum allelic frequency (MAF) of >10% in a Beijing Han Chinese (CHB) were selected. Then, we analyzed the associations between WDR-4 gene with oligozoospermia, asthenozoospermia, and teratozoospermia. Furthermore, cell-based experiments and bioinformatics approaches were applied to elucidate functional annotation variants and cellular effects the WDR-4 gene. PheWAS results showed no phenome-wide associations between WDR-4 variants and 87 common traits. However, genotyping data revealed that WDR-4 gene with SNP rs465663 solely associated with asthenozoospermia. Further, there were no different associations of WDR-4 variants in oligozoospermia or teratozoospermia. Functional annotations determined that the rs465663 SNP influenced the expression of the WDR-4 gene in the testis. Importantly, WDR-4 gene was involved in reactive oxygen species (ROS)-induced DNA fragmentation. These findings suggested that TT or TC genotype of rs465663 has a higher risk of asthenozoospermia. Functional annotations through the GTEx portal revealed the correlation between TT or TC genotype and low expression of WDR-4. Low expression of WDR-4 contributed to DNA fragmentation induced by ROS stress.
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Authors' Responses to Reviewer's Comments (Reviewer 3)
Author's Notes
Respond to Reviewer 3 comment:
Q1: Page 3, lines 115-116. The authors must explain the exact name of the tool used to identify
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Authors' Responses to Reviewer's Comments (Reviewer 3)

Author's Notes

Respond to Reviewer 3 comment:

Q1: Page 3, lines 115-116. The authors must explain the exact name of the tool used to identify tagging SNPs and the selection parameters.

Ans: Thanks for the reviewer's comments. Following your suggestions, the paragraph have been added in the method section (2.4. Genotyping of SNPs in the *WDR4* gene) as below: "Tagging (t)SNPs with a minimum allele frequency (MAF) of >10% in a Beijing Han Chinese (CHB) population were selected through UCSC (http://genome.ucsc.edu) and HapMap vers. 2010-08_phase II + III (http://hapmap.ncbi.nlm.hig.gov/). Haploview 4.2 was applied for tSNP selection".

Q2: Page3, line 130. Add the date of access to GTEx and the version number consulted. Just the access date is present in Sup.table2 footnote; this information is needed in the main text.

Ans: Thanks for your kind reminder. We added the accession date of the GTEx as mentioned in **page 3, line 136-137**: "Data were obtained from the GTEx (V8) Portal on May 4, 2020".

Q3: Page 3, line 141. To increase the author's analysis' reproducibility, I recommended adding a reference or the specific PLINK commands used for PheWAS.

Ans: We sincerely thank the reviewer's comment. Regarding to PLINK commands or reference, we followed the guidance from PLINK website (https://www.cog-genomics.org/plink/). The reference of PLINK was added in the revised manuscript.

Q4: Page 4, lines 172-174. I can suppose that the authors adopted just the recessive model of inheritance and excluded the codominant and dominant model in the statistical analyses due to the very few cases. In this study, the selected tagging SNPs have a MAF >10%, and the largest number of cases was 30 for Asthenozoospermia. I would suggest that the authors perform a power calculation and mention the power in the main text. As also admitted by the authors, the number of subjects in the study is the major limitation. I agree with this statement; these numbers make any conclusion speculative regarding studying the association between genetic variants and any outcome. The authors could re-perform the analyses changing the setting of outcomes from a dichotomous variable to a continuous variable and from a logistic to a generalised linear model (GLM) analyses of the association, like suggested in this manuscript PMID: 29040583 DOI: 10.1093/humrep/dex305.

Ans: Thanks for your valuable comments and suggestions. Indeed, number of the subjects is the major limitation of our study, which may lead to false negative findings. In this study, the nonsignificant findings in

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Ans: Thanks for your valuable comments and suggestions. Indeed, number of the subjects is the major limitation of our study, which may lead to false negative findings. In this study, the nonsignificant findings in the association between *WDR4* variations and oligozoospermia as well as teratozoospermia may be a victim of this. Furthermore, we have performed the analysis on continuous outcome variable in this revision. Results demonstrated no statistically significant association of *WDR4* variants with sperm counts and morphology. The variants of *WDR4* associated with the sperm motility, which is consistent with our findings with dichotomous outcomes. All results have been added in Supplementary Table S3-S5., and we also added sentences in the revised manuscript. "Here we performed the analysis by using dichotomous model. However, we also analyzed the data using continuous model. The results are consistent with our findings by utilizing dichotomous model (**Table S3-5**)".

Table S3. Associations between genetic variants of the *WDR4* gene and total sperm number

SNP	Genotype	No	Value		*p*-value		
			Mean	S.E.	Dominant	Recessive	Additive
rs2298666	GG	58	221.50	25.85	0.924	0.801	0.995
	GA	20	217.81	47.97			
	AA	2	254.00	76.00			

Figure S1. Spearman's rank correlation coefficient between age, serum hormones, and semen parameters

Reference:

1. Pasqualotto FF, Sobreiro BP, Hallak J, Pasqualotto EB, Lucon AM: Sperm concentration and normal sperm morphology decrease and follicle-stimulating hormone level increases with age. BJU Int 2005, 96(7):1087-1091.

Q6: Page 9, lines 278-290. Reword this sentence. The phrase formulated in this way misunderstands the meaning of eQTL. "in the presence of a specific genotype, an altered expression of a gene X is observed". We can speak of the presence or non-presence of a genotype, but a genotype is not expressed, i.e. a gene is expressed. Finally, the authors should emphasise that their results are less than suggestive, given the small number of samples that makes this study the victim of numerous errors due to statistical fluctuations. So the results presented in this current form may be a mild suggestion for future studies done on larger populations.

Furthermore, the authors throughout the manuscript, from introduction to conclusion, cite and compare the WDR-4 gene of Homo sapiens, Drosophila and Mouse. Personally, it is little functional to support this study the comparison with orthologous genes beyond the introduction. However, if the authors maintain this structure of the manuscript, I believe a section is necessary where the WDR-4 gene is compared in the three species, indicating the degree of conservation and identity, a comparison that can be done with the BLAST tool https://blast.ncbi.nlm.nih.gov/Blast.cgi.

Ans: Thanks so much for reviewer's suggestions. We already added your concern regarding to limitation this study in the part of discussion (**Page 10, 328-333**). "Our research contributes to the understanding of the WDR-4 gene's variations. However, some limitations still exist in this study; first, although the subject number (68,948 individuals) for PheWAS is good, none of significant physiological traits was found. Regarding the second cohort (male infertility) from hospital, small sample size limits the statistical power. Thus, a larger sample sizes with different populations are necessary to confirm these findings".

Regarding to *WDR4*-degree conservation, we add some related information as below:

"Wuho is a member of the evolutionarily conserved WD repeat protein family that is expressed by the genes wuho in Drosophila, *TRM82* in yeast, and *WDR4* in humans [1]. The *WDR4* domains usually contain four to eight repeating sequences, which are separated by approximately 40 amino acids. Each repeat consists of two sites, poorly conserved site and well-conserved site [2]"

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1. Cheng IC, Chen BC, Shuai H-H, Chien F-C, Chen P, Hsieh T-s: Wuho Is a New Member in Maintaining Genome Stability through its Interaction with Flap Endonuclease 1. PLoS biology 2016, 14(1):e1002349.
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(x) I am not qualified to assess the quality of English in this paper

	Yes	Can be improved	Must be improved	Not applicable
Does the introduction provide sufficient background and include all relevant references?	(x)	( )	( )	( )
Is the research design appropriate?	( )	(x)	( )	( )
Are the methods adequately described?	( )	(x)	( )	( )
Are the results clearly presented?	(x)	( )	( )	( )
Are the conclusions supported by the results?	( )	( )	(x)	( )

Comments and Suggestions for Authors

The manuscript entitled "Genetic association of the functional *WDR-4* gene in male fertility" is well written and structured. The authors have explored the possible association between *WDR-4* gene variants and several male infertility outcomes.

Although I appreciate the quality of this work, in my opinion, some clarifications and changes are still needed.

Page 3, lines 115-116. The authors must explain the exact name of the tool used to identify tagging SNPs and the selection parameters.

Page3, line 130. Add the date of access to GTEx and the version number consulted. Just the access date is present in Sup.table2 footnote; this information is needed in the main text.

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Page 9, lines 278-290. Reword this sentence. The phrase formulated in this way misunderstands the meaning of eQTL. "in the presence of a specific genotype, an altered expression of a gene X is observed". We can speak of the presence or non-presence of a genotype, but a genotype is not expressed, i.e. a gene is expressed.

Finally, the authors should emphasise that their results are less than suggestive, given the small number of samples that makes this study the victim of numerous errors due to statistical fluctuations.

So the results presented in this current form may be a mild suggestion for future studies done on larger populations.

Furthermore, the authors throughout the manuscript, from introduction to conclusion, cite and compare the WDR-4 gene of Homo sapiens, Drosophila and Mouse. Personally, it is little

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Poor			Normal			Good		Excellent	

Article

Genetic Association of the Functional *WDR4* Gene in Male Fertility

Yu-Jia Wang [1,†], Eko Mugiyanto [2,†], Yun-Ting Peng [1,3,†], Wan-Chen Huang [4], Wan-Hsuan Chou [1,5], Chi-Chiu Lee [4], Yu-Shiuan Wang [2], Lalu Muhammad Irham [1,6], Dyah Aryani Perwitasari [6], Ming-I Hsu [7,*] and Wei-Chiao Chang [1,3,5,8,*]

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2 PhD Program in Clinical Drug Development of Chinese Herbal Medicine, College of Pharmacy, Taipei Medical University, Taipei 11031, Taiwan
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5 Master Program for Clinical Pharmacogenomics and Pharmacoproteomics, School of Pharmacy, Taipei Medical University, Taipei 11031, Taiwan
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† These authors equally contributed to this work.

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Abstract: Infertility is one of the important problems in the modern world. Male infertility is characterized by several clinical manifestations, including low sperm production (oligozoospermia), reduced sperm motility (asthenozoospermia), and abnormal sperm morphology (teratozoospermia). *WDR4*, known as Wuho, controls fertility in Drosophila. However, it is unclear whether *WDR4* is associated with clinical manifestations of male fertility in human. Here, we attempted to determine the physiological functions of *WDR4* gene. Two cohorts were applied to address this question. The first cohort was the general population from Taiwan Biobank. Genomic profiles from 68,948 individuals and 87 common physiological traits were applied for phenome-wide association studies (PheWAS). The second cohort comprised patients with male infertility from Wan Fang Hospital, Taipei Medical University. In total, 81 male participants were recruited for the genetic association study. Clinical records including gender, age, total testosterone, follicle-stimulating hormone (FSH), luteinizing hormone (LH), total sperm number, sperm motility, and sperm morphology were collected. In the first cohort, results from PheWAS exhibited no associations between *WDR4* genetic variants and 87 common physiological traits. In the second cohort, a total of four tagging single-nucleotide polymorphisms (tSNPs) from *WDR4* gene (rs2298666, rs465663, rs2248490, and rs3746939) were selected for genotyping. We

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found that SNP rs465663 solely associated with asthenozoospermia. Functional annotations through the GTEx portal revealed the correlation between TT or TC genotype and low expression of *WDR4*. Furthermore, we used mouse embryonic fibroblasts cells from mwdr4 heterozygous (+/–) mice for functional validation by western blotting. Indeed, low expression of WDR4 contributed to ROS-induced DNA fragmentation. In conclusion, our results suggest a critical role of *WDR4* gene variant as well as protein expression in asthenozoospermia.

Keywords: *WDR4;* genetic variants; male fertility; sperm quality

1. Introduction

Infertility is defined as a disease characterized by the inability of couples to conceive a pregnancy after 12 months of regular unprotected intercourse [1]. Infertility is a global health issue affecting 8%~12% of couples worldwide, and male infertility factors are responsible for about 20%~30% of infertility cases. Approximately 7% of the male population is affected by infertility [2]. Evaluating male infertility is mainly based on semen analyses. The most significant abnormalities of semen quality include a low sperm number (oligozoospermia), poor sperm motility (asthenozoospermia), and abnormal sperm morphology (teratozoospermia) [3,4]. Oxidative stress has been considered as a critical factor to infertility that possibly associated with the DNA fragmentation [5]. Moreover, the DNA fragmentation levels were reported to correlate with sperm motility [6].

Genetic variations are considered as one of the important factors in male infertility. For example, the azoospermia factor (AZF) region in the long arm of the Y chromosome contains three sub-regions referred to as AZFa, AZFb, and AZFc. Microdeletions in these sub-regions cause impaired sperm production [7]. In addition, HSFY, which is related to the heat-shock transcription factor (HSF) family, has a long open reading frame carrying an HSF-type DNA binding domain involved in azoospermia. Some point mutations in the testis-specific HSFY gene family, such as microdeletions, may be a cause of unexplained cases of idiopathic male infertility [1]. Furthermore, Ozdemir et al. evaluated the genetic variants of SRY and AZF in male infertility [8]. Moreover, three SNPs (rs4920566, rs11763979, and rs3741843) on taste receptor genes (TASR) are associated with male infertility [9]. Several genes were further identified to correlate with infertility-related azoospermia in humans, such as *RNU7-6P*, *ZFP64* [10], *SOX5*, *PLCH2* [11], and *ART3* genes [12].

Wu et al. reported that the *wh* gene (*Wuho* gene with WD40 repeats, also named *WDR4* in homo sapiens) is important in *Drosophila* spermatogenesis [13]. However, the physiological functions as well as the clinical manifestation for genetic variations of *WDR4* gene are still unclear. Here, two cohorts were applied to address this question. The first cohort was the general population from Taiwan Biobank used for phenome-wide association study (PheWAS). The association between *WDR4*

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variants and 87 common traits was comprehensively screened. The second cohort was male participants recruited from Wan Fang Hospital. The effects of *WDR4* variants on human male infertility (oligozoospermia, asthenozoospermia, teratozoospermia) were investigated.

2. Materials and Methods

2.1. Participant Recruitment and Sample Collection

Men aged ≥ 20 years old were recruited from the infertility clinic of Wan Fang Hospital, Taipei Medical University between November 2013 and June 2015. Participants with a history of vasectomy or cancer were excluded from the study. Semen samples were collected from every participant by masturbation after 3~5 days of sexual abstinence. These samples were kept at room temperature and delivered to the reproductive laboratory for semen analysis within 1 h. Peripheral blood samples were collected for serum hormone analysis and genomic DNA extraction. The study protocol was approved by the Taipei Medical University-Joint Institutional Review Board (TMU-JIRB) (No: 201302040), and written informed consent was received from all participants.

2.2. Semen Analysis

Semen samples were examined after liquefaction. Semen analysis was conducted separately by two experienced technicians in the reproductive laboratory. Semen parameters including total sperm number, sperm motility, and sperm morphology were evaluated. The World Health Organization (WHO) 2010 recommendations for semen analysis were followed [14]. A semen sample (10 μL) was first transferred to a MAKLER counting chamber (Irvine Scientific, Santa Ana, CA, USA) to measure total number of spermatozoa. Oligozoospermia was defined as a number of sperm < 39×10^6 spermatozoa. The motility of each spermatozoon was graded into three categories: progressive motility (PR), non-progressive motility (NP), and immotile spermatozoa. Asthenozoospermia was defined as PR < 32%. For sperm morphological evaluation, each spermatozoon was classified into eight groups as follows: normal sperm (NS), amorphous head (AH), duplicated head (DH), large head (LH), small head (SH), normal head with other defect (NH), tapering head (TH), and other defect (OD). Teratozoospermia was defined as <4% of morphologically normal spermatozoa. In addition, combined oligozoospermia was defined as participants with sperm number less than 39×10^6 spermatozoa and other defects (astheno- and/or teratozoospermia), a rationale also applied to two other sperm characteristics (combined asthenozoospermia and combined teratozoospermia).

2.3. Genomic DNA Extraction

Peripheral blood samples were first centrifuged at 3000 rpm and 4 °C for 10 min to separate serum and blood cells. The buffy coat layer was extracted and washed with red blood cell (RBC) lysis buffer to isolate peripheral blood mononuclear cells

(PBMCs). PBMCs were then lysed using a cell lysis buffer. Proteins were precipitated using a protein precipitation solution followed by 95% isopropanol and 80% alcohol to isolate total genomic DNA. Finally, the DNA purity and concentration were measured using NanoDrop (Thermo Fisher Scientific, Wilmington, DE, USA).

2.4. Genotyping of SNPs in the WDR4 Gene

Tagging (t)SNPs with a minimum allele frequency (MAF) of >10% in a Beijing Han Chinese (CHB) population were selected through UCSC (http://genome.ucsc.edu) and HapMap vers. 2010-08_phase II + III (http://hapmap.ncbi.nlm.hig.gov/). Haploview 4.2 was applied for tSNP selection. A total of four SNPs of the *WDR4* gene were selected and genotyped (Figure 1). Characteristics of these SNPs are shown in Table S1. Genotyping was performed using the TaqMan Allelic Discrimination Assay (Applied Biosystems, Foster City, CA, USA). A polymerase chain reaction (PCR) was carried out with an ABI StepOnePlus Thermal Cycler (Applied Biosystems). The fluorescence from different probes was measured and analyzed by System SDS software vers. 2.2.2 (Applied Biosystems).

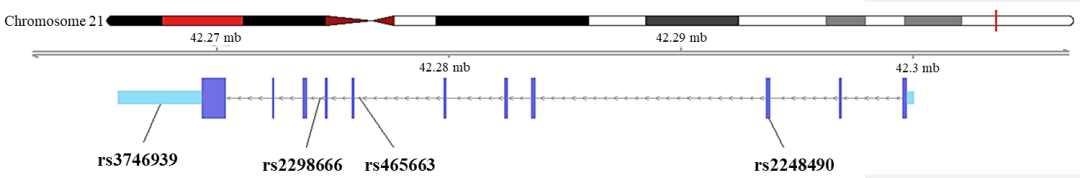

Figure 1. Graphic view of the genotyped *WDR4* gene.

2.5. Functional Annotation Data Query

Tissue-specific *cis*-expression quantitative trait loci (*cis*-eQTL) were queried from the GTEx Portal (http://www.gtexportal.org/home/) to evaluate the effects of the SNPs on gene expressions in different human tissues. Data were obtained from the Genotype-Tissue Expression (GTEx) Portal on 4 May 2020.

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2.6. Phenome-Wide Association Study (PheWAS)

A phenome-wide association study (PheWAS) was conducted to screen the association between WDR4 genetic variation and common traits. Imputed Axiom Genome-Wide TWB 2.0 Array data from 68,948 individuals were obtained from the Taiwan Biobank. The data were subjected to quality control based on a variant call rate (>98%), sex check, sample call rate (>98%), Hardy–Weinberg equilibrium (HWE; $p > 10^{-6}$), heterozygosity (within the mean ± 3 standard deviations (SDs)), and identical by descent (IBD) check (<0.1875). After quality control and extracting common variants (with minor allele frequencies of >0.05), 3,982,815 variants and 59,448 individuals remained. Furthermore, 105 variants of the *WDR4* gene were extracted to conduct the PheWAS with 87 phenotypes using Plink v1.9 (www.cog-genomics.org/plink/1.9/) [15,16].

2.7. Cell Culture

The mouse embryonic fibroblasts (MEF) used in this study were purchased from mWh heterozygous mice (+/−) [17] and maintained in DMEM (GIBCO, 11995) contained with 10% FBS, 1% Pen/Strep (GIBCO, 15140122). The 3×10^5 cells were seeded in 6 cm culture dish for 24 h before treated with H_2O_2.

2.8. Western Blotting

To detect the reactive oxygen species (ROS)-caused DNA damage, cells were treated with 100 μM H_2O_2 for 24 h. Cells were then lysed, and the total proteins were analyzed by 10% SDS–polyacrylamide gel. After running the gel, proteins were transferred to a PVDF membrane and blocked with 5% non-fat dry milk for 1 h at RT. Membranes were then washed with PBST (PBS contained with 0.1% Tween 20) and incubated with primary antibodies in 1:1000 dilutions overnight at 4 °C; second antibodies were incubated in 1:5000 dilutions for 1 h at RT. The peptides LKKKRQRSPFPGSPEQTK were synthesized to detect mouse Wdr4 protein (mWdr4). The antibody was purified by affinity chromatography with peptide antigens before use [17]. The protein band intensities were detected by an ECL-plus detection system (GE Healthcare, RPN2235).

2.9. Statistical Analysis

For the PheWAS, plink v1.9 was used for analysis (www.cog-genomics.org/plink/1.9/) [15,16]. Furthermore, the simpleM method was used for variants (18 independent variants after correction), and Pearson's correlation was used for phenotypes (76 independent phenotypes after correction with $r^2 > 0.7$) to adjust the number of independent tests for multiple testing correction in PheWAS [18]. In this study, tests with $p < 3.65 \times 10^{-5}$ ($0.05/18 \times 76$) were regarded as significant for phenome-wide associations. For the *WDR4* genetic association study with male infertility, R 3.2.0 (http://www.r-project.org) was used for the statistical analysis. HWE of SNPs was evaluated using a Chi-squared test. Magnitudes of associations between SNPs and semen quality parameters were examined through a

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linear regression analysis under a recessive model. Age, total testosterone, follicle-stimulating hormone (FSH), and luteinizing hormone (LH) were included as covariates in the regression model. $p < 0.05$ was considered statistically significant.

3. Results

3.1. PheWAS for WDR4 Variants in A Taiwanese Population

To further explore the roles of *WDR4* in human traits, a PheWAS was conducted to investigate associations between *WDR4* variants and common traits in a Taiwanese population. The associations between 105 variants of*WDR4* gene and 87 phenotypes were tested through the PheWAS. A variety of associations between the *WDR4* genetic variants and the common phenotypes were identified at the significance level of $p < 0.05$. However, none of them remained significant after multiple testing correction (Figure 2).

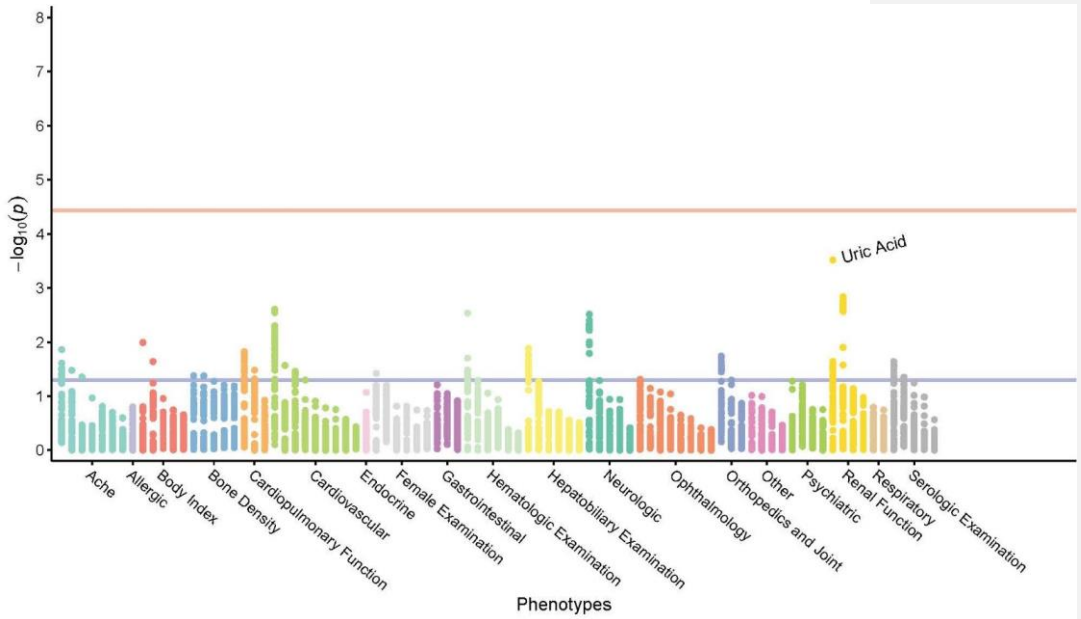

Figure 2. Phenome-wide scan for associations with *WDR4* variants. The blue line represents $p = 0.05$, and the red line represents the significance level for phenome-wide associations after multiple testing corrections ($p = 3.65 \times 10^{-5}$).

3.2. Participant Characteristics

Eighty-one males were recruited from the infertility clinic. Participants were aged 28~51 years, with a mean age of 36 years. Men with oligozoospermia, asthenozoospermia, and teratozoospermia accounted for 15%, 27%, and 7%, respectively. Their clinical characteristics are summarized in Table 1. Furthermore, correlation coefficient of age, serum hormones, and semen parameters were shown by Spearman's rank correlation. In agreement with the previous study, our data demonstrated

morphology decline with age, whereas follicle-stimulating hormone (FSH) levels rose (Figure S1) [19].

Table 1. Characteristics of the 81 male participants.

Characteristic	Participants	Normal Range
Age (years) [a]	36.21 ± 4.58	
Range (years)	27~51	
Semen analysis, no. (%)		
Oligozoospermia (total sperm number < 39 × 10^6)		
Oligozoospermia	12 (15)	
Isolated	9	
Combined	3	
Non-oligozoospermia	69 (85)	
Asthenozoospermia (PR < 32%)		
Asthenozoospermia	22 (27)	
Isolated	10	
Combined	12	
Non-asthenozoospermia	59 (73)	
Teratozoospermia (sperm with normal morphology <4%)		
Teratozoospermia	6 (7)	
Isolated	0	
Combined	6	
Non-teratozoospermia	75 (93)	
Serum hormone analysis [a]		
Total testosterone (ng/mL)	4.87 ± 1.59	2.51~10.60
FSH (mIU/mL)	5.42 ± 4.24	1~14
LH (mIU/mL)	2.40 ± 1.59	1.5~9.2
SHBG (nmol/L)	28.88 ± 14.82	14.5~48.4
Inhibin B (pg/mL)	252.40 ± 162.44	
TSH (μIU/mL)	1.63 ± 0.82	
T3 (ng/dL)	112.20 ± 18.06	
T4 (μg/dL)	8.15 ± 1.28	
Free T4 (ng/dL)	0.90 ± 0.10	0.61~1.12
AMH (ng/mL)	12.51 ± 7.84	–
Zinc (μg/L)	1076.10 ± 217.26	800~1200

[a] Mean ± standard deviation. PR, progressive motility; FSH, follicle-stimulating hormone; LH, luteinizing hormone; SHBG, sex hormone-binding globulin; TSH, thyroid-stimulating hormone; T3, triiodothyronine; T4, thyroxine; AMH, anti-müllerian hormone. – No optimal recommendation available for male

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3.3. Associations between WDR4 Variants and Oligozoospermia

The effects of four SNPs on total sperm number were first evaluated. There were no statistically significant differences in genotypic distributions between oligozoospermia cases and the non-oligozoospermia group (Table 2).

Table 2. Associations between genetic variants of the *WDR4* gene and oligozoospermia.

SNP	Genotype	Cases		Non-Oligozoospermia		Recessive Model
		No.	%	No.	%	*p*-Value
rs2298666	GG	10	83.3	48	70.6	0.665

	GA	2	16.7	18	26.5	
	AA	0	0	2	2.9	
rs465663	TT	9	75.0	33	49.3	0.157
	TC	3	25.0	24	35.8	
	CC	0	0	10	14.9	
rs2248490	CC	8	66.7	30	43.5	0.095
	CG	4	33.3	29	42.0	
	GG	0	0	10	14.5	
rs3746939	AA	8	66.7	37	53.6	0.165
	AC	4	33.3	27	39.1	
	CC	0	0	5	7.2	

The *p*-value was adjusted for age, total testosterone, follicle-stimulating hormone, and luteinizing hormone. SNP, single-nucleotide polymorphism.

3.4. Associations between WDR4 Variants and Asthenozoospermia

Next, the effects of four SNPs on total sperm motility were analyzed. As shown in Table 3, the genotypic distribution of rs465663 differed between asthenozoospermia and non-asthenozoospermia groups. The proportions of TT and TC genotypes were higher in asthenozoospermia compared to non-asthenozoospermia ($p = 0.025$).

Table 3. Associations between genetic variants of the *WDR4* gene and asthenozoospermia.

SNP	Genotype	Cases		Non-Asthenozoospermia		Recessive Model
		No.	%	No.	%	*p*-Value
rs2298666	GG	17	81	41	69.5	0.399
	GA	4	19	16	27.1	
	AA	0	0	2	3.4	
rs465663	TT	15	71.4	27	46.6	
	TC	6	28.6	21	36.2	
	CC	0	0	10	17.2	
rs2248490	CC	15	68.2	23	39	
	CG	5	22.7	28	47.5	
	GG	2	9.1	8	13.6	
rs3746939	AA	15	68.2	30	50.8	0.513
	AC	6	27.3	25	42.4	
	CC	1	4.5	4	6.8	

The *p*-value was adjusted for age, total testosterone, follicle-stimulating hormone, and luteinizing hormone. *$p < 0.05$. SNP, single-nucleotide polymorphism.

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3.5. Associations between WDR4 Variants and Teratozoospermia

The effects of four SNPs on normal sperm morphology were also examined. As shown in Table 4, no statistically significant differences in genotypic distributions were observed between teratozoospermia cases and the non-teratozoospermia group.

Table 4. Associations between genetic variants of the *WDR4* gene and teratozoospermia.

SNP	Genotype	Cases		Non-Teratozoospermia		Recessive Model
		No.	%	No.	%	*p*-Value
rs2298666	GG	5	83.3	53	71.6	0.759
	GA	1	16.7	19	25.7	
	AA	0	0	2	2.7	
rs465663	TT	5	83.3	37	50.7	0.395
	TC	1	16.7	26	35.6	
	CC	0	0	10	13.7	
rs2248490	CC	5	83.3	33	44	0.154

	CG	1	16.7	32	42.7	
	GG	0	0	10	13.3	
rs3746939	AA	4	66.7	41	54.7	0.186
	AC	2	33.3	29	38.7	
	CC	0	0	5	6.7	

The *p*-value was adjusted for age, total testosterone, follicle-stimulating hormone, and luteinizing hormone. SNP, single-nucleotide polymorphism.

3.6. Functional Annotation from GTEx Portal for rs465663

The profile of *WDR4* gene expression in various tissues was determined by the GTEx portal database (Figure 3). In the genetic association analysis, rs465663, an intronic variant of *WDR4*, was associated with asthenozoospermia. To further elucidate the possible functions of rs465663, *cis*-eQTL results were retrieved from the GTEx portal. rs465663 could affect the expression level of several genes in different tissue types. Subjects carrying the T allele showed a lower expression level of *WDR4* in testes, whole blood, and esophageal mucosal tissue (Table S2).

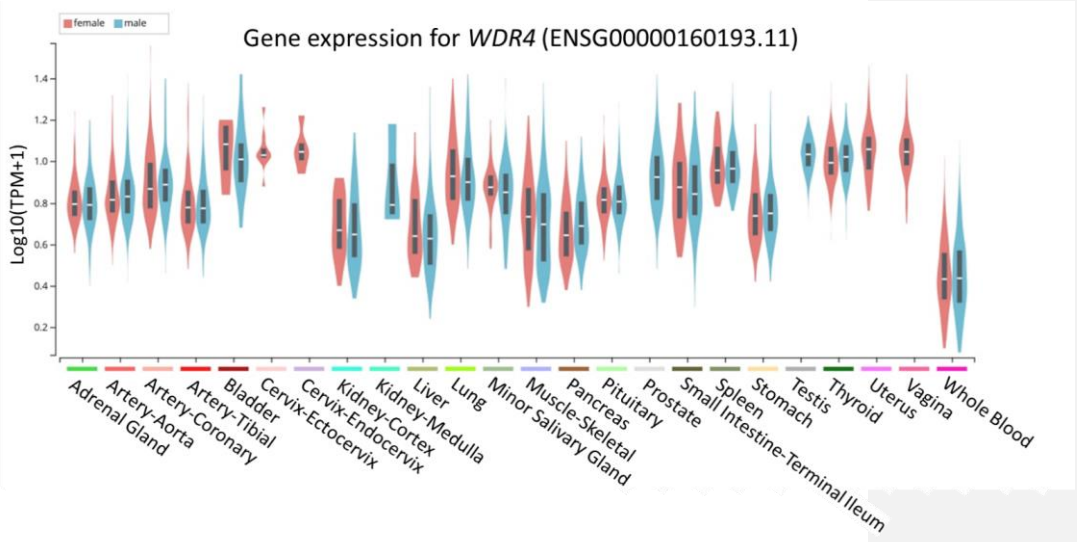

Figure 3. Profile of *WDR4* gene expression in various tissues. Blue represents the male gender and red the female gender.

3.7. Effects of WDR4 in DNA Fragmentation through γH2AX Expression

Previous studies showed that the sperm DNA fragmentation level was increased by ROS stress and was correlated with sperm motility [5,6]. The higher expression level of γH2AX (biomarker of DNA fragmentation level) was revealed to correlate with male infertility [20]. To investigate the role of

WDR4 in male infertility, the MEF (mouse embryonic fibroblasts) cells were isolated from mwdr4 heterozygous (+/−) mice, and the H_2O_2 was used to induce DNA fragmentation for mimicking oxidative stress. The mwdr4 and the γH2AX protein expression levels were confirmed with western blotting (Figure 4). The results showed that the mWdr4 protein expression level in the mWdr4 heterozygous MEF group was low. Importantly, the H_2O_2-induced γH2AX protein level was highly increased in the mwdr4 heterozygous MEF group compared to the wild type MEF group. These results highlighted an important role of *WDR4* in male infertility.

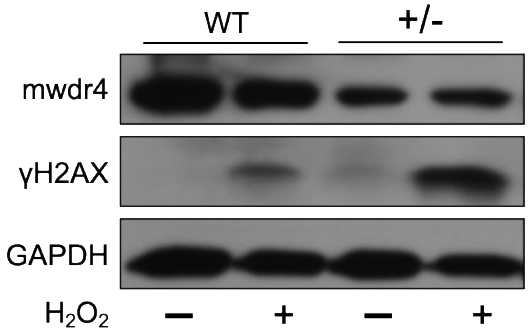

Figure 4. H_2O_2-induced γH2AX expression increased in wdr4 heterozygous (+/−) MEF cells. The MEFs cells were subcultured into 6 cm culture dish 24 h before being treated with 100 μM H_2O_2. The mwdr4, the γH2AX, and the GAPDH protein expression levels were determined by western blot after H_2O_2 treatment for 24 h.

4. Discussion

Wuho is a member of the evolutionarily conserved WD repeat protein family that is expressed by the genes *wuho* in Drosophila, *TRM82* in yeast, and *WDR4* in humans [17]. The *WDR4* domains usually contain four to eight repeating sequences, which are separated by approximately 40 amino acids. Each repeat consists of two sites, a poorly conserved site and a well-conserved site [21]. Wu at al. reported that the lack of *WDR4* function is associated with dramatic germline-specific phenotypes by arresting the spermatogenesis at the spermatid elongating stage [13]. The study indicated that approximately 20% of the ovarioles in WDR4 mutant female have apparent defects in oogenesis with an over-proliferation of cystocytes. Additionally, *WDR4* is associated with germline cell development through cytosolic tRNA modifications [22].

Male infertility is characterized (manifested) by low sperm production (oligozoospermia), reduced sperm motility (asthenozoospermia), and abnormal sperm morphology (teratozoospermia). In this study, we wanted to seek out whether any genetic variants associate with the clinical outcomes. Importantly, the analysis using continuous traits showed

consistent results with dichotomous models (Tables S3–S5). Asthenozoospermia and oligozoospermia are the most common factors responsible for male infertility [23]. In line with this, our study indicated that asthenozoospermia comprised the most cases followed consecutively by oligozoospermia and teratozoospermia. Here, we identified variants of the *WDR4* gene that related to male infertility. rs465663 significantly associated with the susceptibility to asthenozoospermia. The variation in rs465663 was located in the intronic region of chromosome 21, while proportions of the TT and the TC genotypes of rs465663 were higher than the CC genotype in cases with asthenozoospermia. Interestingly, the C allele is the minor allele of rs465663. The distribution of the minor allele of rs465663 in current study was lower than those reported in other populations, including Asians, Europeans, and Americans (Table S1). Trends of infertility globally showed that two populations of Europeans, represented by Central and Eastern Europe (8%~12%), and Americans, represented by North America (4.5%~6%), were higher compared to Sub-Saharan Africans (2.5~4.8%); unfortunately, those studies did not provide the percentage of Asians, possibly due to underreporting [24].

Our findings also emphasized that functional annotations through a bioinformatic approach using the GTEx portal revealed that subjects had lower expression of the TT genotype of *WDR4* than the CC and the TC genotypes in several human tissues, including testes and whole blood. Meanwhile, the major allelic frequency of rs465663 of *WDR4* had lower expression in testes. This result implied that rs465663 might influence expression of the *WDR4* gene, especially in testes. Disease-related male infertility can be caused by testicular deficiencies and spermatogenesis [4]. This evidence provided additional clues that variations of the *WDR4* gene may affect the testes, including their production of sperm (Figure 3). Furthermore, we noticed that ROS-induced DNA fragmentation level was significantly increased in the low *WDR4* group (Figure 4). The results further support the possibility that lower expression of *WDR4* in males resulted in infertility.

Several phenotypes are associated with *WDR4* variants, such as rs370189685, which was correlated with the fasting plasma glucose level [25]. A study from *Drosophila* germ cells suggested that *WDR4* is a regulator of Mei-p26, and it interacts with *TRIM32* to control tissue homeostasis in other stem cell systems [26]. Other genes related to male infertility were identified to associate with azoospermia (e.g., *SOHLH1* [27], *SYCP3* [28], and *TEX11* [29]). *WDR4* gene with a missense mutation was reported to be related to primordial dwarfism through m^7G_{46} methylation, which impaired transfer (t)RNAs [30]. Sperm carries thousands of different RNAs [31]. Interestingly, according to the REACTOME database (R-HSA-6782315) [32], variations in rs465663 of the *WDR4* gene might involve in RNA metabolism and tRNA modifications in nuclei and cytosol. tRNAs play pivotal roles in protein synthetases. Mutations in tRNAs which modify enzymes are associated with human diseases, including cancer, type 2 diabetes (T2D),

neurological disorders, and mitochondrion-linked disorders [33]. Furthermore, as reported by a previous study, tRNA modifications can affect proteostasis in humans [34]. A similar study supported that tRNA modifications in mice resulted in increased apoptosis in male germ cells and male infertility [35]. In addition, epigenomic assays are also essential for relating noncoding genetic variations to regulatory mechanisms underlying phenotypic changes, including genomic variations of the *WDR4* gene. The Encyclopedia of DNA Elements (ENCODE) database revealed that rs465663 is located in an intronic region with known histone modifications [32]. Subsequent evidence revealed that an epigenomic histone modification was involved in male fertility [36]. Some genes, such as *FAM50B* and *GNAS*, were reported to involve in the quality of sperm in asthenozoospermia through histone modification-type methylation [37]. Taken together, previous studies provided comprehensive integration between the identified genetic variants and male infertility.

Our research is focusing on the genetic variants of *WDR4* in male fertility s. However, some limitations still exist in this study. First, although the subject number (68,948 individuals) for PheWAS was good, no significant physiological traits were found. Regarding the second cohort (male participants) from the hospital, the small sample size limits the statistical power. Thus, larger sample sizes with different populations are necessary to confirm our findings. Second, as the candidate gene approach was performed, the influence of variants in other unexamined genes cannot be ruled out. Since the pathophysiology of infertility in humans is complexly regulated by many signaling pathways, using whole genome sequencing technology may yield further insights into the genomic variations in infertility.

5. Conclusions

This study offers important information related to genetic variants and expression level of the *WDR4* which might affect infertility in the cases of asthenozoospermia. However, further functional studies and larger sample sizes are required to validate the variants.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: Spearman's rank correlation coefficient between age, serum hormones, and semen parameters, Table S1: Characteristics of tagging single-nucleotide polymorphisms (tSNPs) of the WDR4 gene, Table S2: Cis-expression quantitative trait loci (cis-eQTL) of rs465663, Table S3: Associations between genetic variants of the WDR4 gene and total sperm number, Table S4: Associations between genetic variants of the WDR4 gene and sperm progressive motility, Table S5: Associations between genetic variants of the WDR4 gene and sperm morphology

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writing review & editing: Y.-J.W., Y.-T.P., E.M., W.-C.H., W.-H.C., C.-C.L., Y.-S.W., L.M.I., D.A.P., M.-I.H., and W.-C.C.; supervision: W.-C.H., D.A.P., M.-I.H., and W.-C.C. All authors have made significant contributions to this study. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study

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Conflicts of Interest: The authors declare no conflict of interest.

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Article

Genetic Association of the Functional *WDR4* Gene in Male Fertility

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Abstract: Infertility is one of the important problems in the modern world. Male infertility is characterized by several clinical manifestations, including low sperm production (oligozoospermia), reduced sperm motility (asthenozoospermia), and abnormal sperm morphology (teratozoospermia). *WDR4*, known as Wuho, controls fertility in Drosophila. However, it is unclear whether *WDR4* is associated with clinical manifestations of male fertility in human. Here, we attempted to determine the physiological functions of *WDR4* gene. Two cohorts were applied to address this question. The first cohort was the general population from Taiwan Biobank. Genomic profiles from 68,948 individuals and 87 common physiological traits were applied for phenome-wide association studies (PheWAS). The second cohort comprised patients with male infertility from Wan Fang Hospital, Taipei Medical University. In total, 81 male participants were recruited for the genetic association study. Clinical records including gender, age, total testosterone, follicle-stimulating hormone (FSH), luteinizing hormone (LH), total sperm number, sperm motility, and sperm morphology were collected. In the first cohort, results from PheWAS exhibited no associations between *WDR4* genetic variants and 87 common physiological traits. In the second cohort, a total of four tagging single-nucleotide polymorphisms (tSNPs) from *WDR4* gene (rs2298666, rs465663, rs2248490, and rs3746939) were selected for genotyping. We found that SNP rs465663 solely associated with asthenozoospermia. Functional annotations through the GTEx portal revealed the correlation between TT or TC genotype and low expression of *WDR4*. Furthermore, we used mouse embryonic fibroblasts cells from mwdr4 heterozygous (+/–) mice for functional validation by western blotting. Indeed, low expression of WDR4 contributed to ROS-induced DNA fragmentation. In conclusion, our results suggest a critical role of *WDR4* gene variant as well as protein expression in asthenozoospermia.

Keywords: *WDR4*; genetic variants; male fertility; sperm quality

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1. Introduction

Infertility is defined as a disease characterized by the inability of couples to conceive a pregnancy after 12 months of regular unprotected intercourse [1]. Infertility is a global health issue affecting 8%~12% of couples worldwide, and male infertility factors are responsible for about 20%~30% of infertility cases. Approximately 7% of the male population is affected by infertility [2]. Evaluating male infertility is mainly based on semen analyses. The most significant abnormalities of semen quality include a low sperm number (oligozoospermia), poor

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sperm motility (asthenozoospermia), and abnormal sperm morphology (teratozoospermia) [3,4]. Oxidative stress has been considered as a critical factor to infertility that possibly associated with the DNA fragmentation [5]. Moreover, the DNA fragmentation levels were reported to correlate with sperm motility [6].

Genetic variations are considered as one of the important factors in male infertility. For example, the azoospermia factor (AZF) region in the long arm of the Y chromosome contains three sub-regions referred to as AZFa, AZFb, and AZFc. Microdeletions in these sub-regions cause impaired sperm production [7]. In addition, HSFY, which is related to the heat-shock transcription factor (HSF) family, has a long open reading frame carrying an HSF-type DNA binding domain involved in azoospermia. Some point mutations in the testis-specific HSFY gene family, such as microdeletions, may be a cause of unexplained cases of idiopathic male infertility [1]. Furthermore, Ozdemir et al. evaluated the genetic variants of SRY and AZF in male infertility [8]. Moreover, three SNPs (rs4920566, rs11763979, and rs3741843) on taste receptor genes (TASR) are associated with male infertility [9]. Several genes were further identified to correlate with infertility-related azoospermia in humans, such as *RNU7-6P*, *ZFP64* [10], *SOX5*, *PLCH2* [11], and *ART3* genes [12].

Wu et al. reported that the *wh* gene (*Wuho* gene with WD40 repeats, also named *WDR4* in homo sapiens) is important in *Drosophila* spermatogenesis [13]. However, the physiological functions as well as the clinical manifestation for genetic variations of *WDR4* gene are still unclear. Here, two cohorts were applied to address this question. The first cohort was the general population from Taiwan Biobank used for phenome-wide association study (PheWAS). The association between *WDR4* variants and 87 common traits was comprehensively screened. The second cohort was male participants recruited from Wan Fang Hospital. The effects of *WDR4* variants on human male infertility (oligozoospermia, asthenozoospermia, teratozoospermia) were investigated.

2. Materials and Methods

2.1. Participant Recruitment and Sample Collection

Men aged ≥ 20 years old were recruited from the infertility clinic of Wan Fang Hospital, Taipei Medical University between November 2013 and June 2015. Participants with a history of vasectomy or cancer were excluded from the study. Semen samples were collected from every participant by masturbation after 3~5 days of sexual abstinence. These samples were kept at room temperature and delivered to the reproductive laboratory for semen analysis within 1 h. Peripheral blood samples were collected for serum hormone analysis and genomic DNA extraction. The study protocol was approved by the Taipei Medical University-Joint Institutional Review Board (TMU-JIRB) (No: 201302040), and written informed consent was received from all participants.

2.2. Semen Analysis

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Semen samples were examined after liquefaction. Semen analysis was conducted separately by two experienced technicians in the reproductive laboratory. Semen parameters including total sperm number, sperm motility, and sperm morphology were evaluated. The World Health Organization (WHO) 2010 recommendations for semen analysis were followed [14]. A semen sample (10 μL) was first transferred to a MAKLER counting chamber (Irvine Scientific, Santa Ana, CA, USA) to measure total number of spermatozoa. Oligozoospermia was defined as a number of sperm < 39×10^6 spermatozoa. The motility of each spermatozoon was graded into three categories: progressive motility (PR), non-progressive motility (NP), and immotile spermatozoa. Asthenozoospermia was defined as PR < 32%. For sperm morphological evaluation, each spermatozoon was classified into eight groups as follows: normal sperm (NS), amorphous head (AH), duplicated head (DH), large head (LH), small head (SH), normal head with other defect (NH), tapering head (TH), and other defect (OD). Teratozoospermia was defined as <4% of morphologically normal spermatozoa. In addition, combined oligozoospermia was defined as participants with sperm number less than 39×10^6 spermatozoa and other defects (astheno- and/or teratozoospermia), a rationale also applied to two other sperm characteristics (combined asthenozoospermia and combined teratozoospermia).

2.3. Genomic DNA Extraction

Peripheral blood samples were first centrifuged at 3000 rpm and 4 °C for 10 min to separate serum and blood cells. The buffy coat layer was extracted and washed with red blood cell (RBC) lysis buffer to isolate peripheral blood mononuclear cells (PBMCs). PBMCs were then lysed using a cell lysis buffer. Proteins were precipitated using a protein precipitation solution followed by 95% isopropanol and 80% alcohol to isolate total genomic DNA. Finally, the DNA purity and concentration were measured using NanoDrop (Thermo Fisher Scientific, Wilmington, DE, USA).

2.4. Genotyping of SNPs in the WDR4 Gene

Tagging (t)SNPs with a minimum allele frequency (MAF) of >10% in a Beijing Han Chinese (CHB) population were selected through UCSC (http://genome.ucsc.edu) and HapMap vers. 2010-08_phase II + III (http://hapmap.ncbi.nlm.hig.gov/). Haploview 4.2 was applied for tSNP selection. A total of four SNPs of the *WDR4* gene were selected and genotyped (Figure 1). Characteristics of these SNPs are shown in Table S1. Genotyping was performed using the TaqMan Allelic Discrimination Assay (Applied Biosystems, Foster City, CA, USA). A polymerase chain reaction (PCR) was carried out with an ABI StepOnePlus Thermal Cycler (Applied Biosystems). The fluorescence from different probes was measured and analyzed by System SDS software vers. 2.2.2 (Applied Biosystems).

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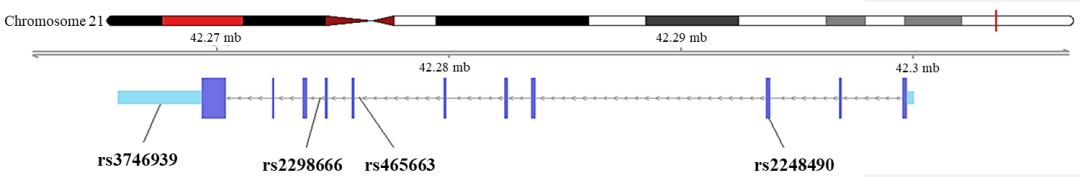

Figure 1. Graphic view of the genotyped *WDR4* gene.

2.5. Functional Annotation Data Query

Tissue-specific *cis*-expression quantitative trait loci (*cis*-eQTL) were queried from the GTEx Portal (http://www.gtexportal.org/home/) to evaluate the effects of the SNPs on gene expressions in different human tissues. Data were obtained from the Genotype-Tissue Expression (GTEx) Portal on 4 May 2020.

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2.6. Phenome-Wide Association Study (PheWAS)

A phenome-wide association study (PheWAS) was conducted to screen the association between WDR4 genetic variation and common traits. Imputed Axiom Genome-Wide TWB 2.0 Array data from 68,948 individuals were obtained from the Taiwan Biobank. The data were subjected to quality control based on a variant call rate (>98%), sex check, sample call rate (>98%), Hardy–Weinberg equilibrium (HWE; $p > 10^{-6}$), heterozygosity (within the mean ± 3 standard deviations (SDs)), and identical by descent (IBD) check (<0.1875). After quality control and extracting common variants (with minor allele frequencies of >0.05), 3,982,815 variants and 59,448 individuals remained. Furthermore, 105 variants of the *WDR4* gene were extracted to conduct the PheWAS with 87 phenotypes using Plink v1.9 (www.cog-genomics.org/plink/1.9/) [15,16].

2.7. Cell Culture

The mouse embryonic fibroblasts (MEF) used in this study were purchased from mWh heterozygous mice (+/−) [17] and maintained in DMEM (GIBCO, 11995) contained with 10% FBS, 1% Pen/Strep (GIBCO, 15140122). The 3×10^5 cells were seeded in 6 cm culture dish for 24 h before treated with H_2O_2.

2.8. Western Blotting

To detect the reactive oxygen species (ROS)-caused DNA damage, cells were treated with 100 μM H_2O_2 for 24 h. Cells were then lysed, and the total proteins were analyzed by 10% SDS–polyacrylamide gel. After running the gel, proteins were transferred to a PVDF membrane and blocked with 5% non-fat dry milk for 1 h at RT. Membranes were then washed with PBST (PBS contained with 0.1% Tween 20) and incubated with primary antibodies in 1:1000 dilutions overnight at 4 °C; second antibodies were incubated in 1:5000 dilutions for 1 h at RT. The peptides LKKKRQRSPFPGSPEQTK were synthesized to detect mouse Wdr4 protein (mWdr4). The antibody was purified by affinity chromatography with peptide antigens before use [17]. The protein band intensities were detected by an ECL-plus detection system (GE Healthcare, RPN2235).

2.9. Statistical Analysis

For the PheWAS, plink v1.9 was used for analysis (www.cog-genomics.org/plink/1.9/) [15,16]. Furthermore, the simpleM method was used for variants (18 independent variants after correction), and Pearson's correlation was used for phenotypes (76 independent phenotypes after correction with $r^2 > 0.7$) to adjust the number of independent tests for multiple testing correction in PheWAS [18]. In this study, tests with $p < 3.65 \times 10^{-5}$ ($0.05/18 \times 76$) were regarded as significant for phenome-wide associations. For the *WDR4* genetic association study with male infertility, R 3.2.0 (http://www.r-project.org) was used for the statistical analysis. HWE of SNPs was evaluated using a Chi-squared test. Magnitudes of associations between SNPs and semen quality parameters were examined through a

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linear regression analysis under a recessive model. Age, total testosterone, follicle-stimulating hormone (FSH), and luteinizing hormone (LH) were included as covariates in the regression model. $p < 0.05$ was considered statistically significant.

3. Results

3.1. PheWAS for WDR4 Variants in A Taiwanese Population

To further explore the roles of *WDR4* in human traits, a PheWAS was conducted to investigate associations between *WDR4* variants and common traits in a Taiwanese population. The associations between 105 variants of*WDR4* gene and 87 phenotypes were tested through the PheWAS. A variety of associations between the *WDR4* genetic variants and the common phenotypes were identified at the significance level of $p < 0.05$. However, none of them remained significant after multiple testing correction (Figure 2).

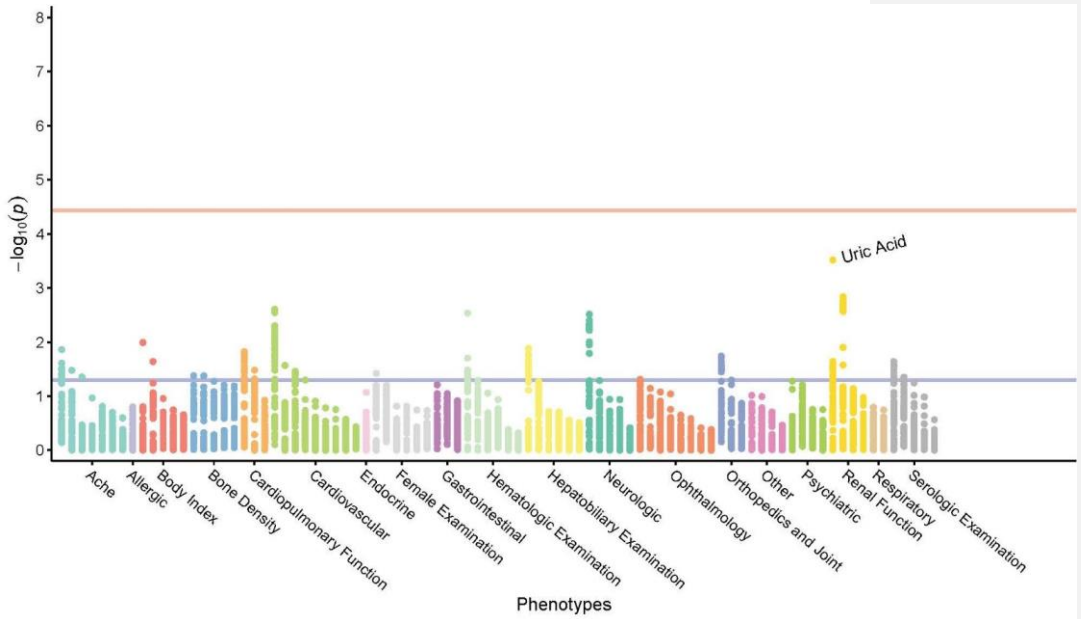

Figure 2. Phenome-wide scan for associations with *WDR4* variants. The blue line represents $p = 0.05$, and the red line represents the significance level for phenome-wide associations after multiple testing corrections ($p = 3.65 \times 10^{-5}$).

3.2. Participant Characteristics

Eighty-one males were recruited from the infertility clinic. Participants were aged 28~51 years, with a mean age of 36 years. Men with oligozoospermia, asthenozoospermia, and teratozoospermia accounted for 15%, 27%, and 7%, respectively. Their clinical characteristics are summarized in Table 1. Furthermore, correlation coefficient of age, serum hormones, and semen parameters were shown by Spearman's rank correlation. In agreement with the previous study, our data demonstrated

morphology decline with age, whereas follicle-stimulating hormone (FSH) levels rose (Figure S1) [19].

Table 1. Characteristics of the 81 male participants.

Characteristic	Participants	Normal Range
Age (years) [a]	36.21 ± 4.58	
Range (years)	27~51	
Semen analysis, no. (%)		
Oligozoospermia (total sperm number < 39 × 10^6)		
Oligozoospermia	12 (15)	
Isolated	9	
Combined	3	
Non-oligozoospermia	69 (85)	
Asthenozoospermia (PR < 32%)		
Asthenozoospermia	22 (27)	
Isolated	10	
Combined	12	
Non-asthenozoospermia	59 (73)	
Teratozoospermia (sperm with normal morphology <4%)		
Teratozoospermia	6 (7)	
Isolated	0	
Combined	6	
Non-teratozoospermia	75 (93)	
Serum hormone analysis [a]		
Total testosterone (ng/mL)	4.87 ± 1.59	2.51~10.60
FSH (mIU/mL)	5.42 ± 4.24	1~14
LH (mIU/mL)	2.40 ± 1.59	1.5~9.2
SHBG (nmol/L)	28.88 ± 14.82	14.5~48.4
Inhibin B (pg/mL)	252.40 ± 162.44	
TSH (μIU/mL)	1.63 ± 0.82	
T3 (ng/dL)	112.20 ± 18.06	
T4 (μg/dL)	8.15 ± 1.28	
Free T4 (ng/dL)	0.90 ± 0.10	0.61~1.12
AMH (ng/mL)	12.51 ± 7.84	—
Zinc (μg/L)	1076.10 ± 217.26	800~1200

[a] Mean ± standard deviation. PR, progressive motility; FSH, follicle-stimulating hormone; LH, luteinizing hormone; SHBG, sex hormone-binding globulin; TSH, thyroid-stimulating hormone; T3, triiodothyronine; T4, thyroxine; AMH, anti-müllerian hormone. – No optimal recommendation available for male

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3.3. Associations between WDR4 Variants and Oligozoospermia

The effects of four SNPs on total sperm number were first evaluated. There were no statistically significant differences in genotypic distributions between oligozoospermia cases and the non-oligozoospermia group (Table 2).

Table 2. Associations between genetic variants of the *WDR4* gene and oligozoospermia.

SNP	Genotype	Cases		Non-Oligozoospermia		Recessive Model
		No.	%	No.	%	*p*-Value
rs2298666	GG	10	83.3	48	70.6	0.665

	GA	2	16.7	18	26.5	
	AA	0	0	2	2.9	
rs465663	TT	9	75.0	33	49.3	0.157
	TC	3	25.0	24	35.8	
	CC	0	0	10	14.9	
rs2248490	CC	8	66.7	30	43.5	0.095
	CG	4	33.3	29	42.0	
	GG	0	0	10	14.5	
rs3746939	AA	8	66.7	37	53.6	0.165
	AC	4	33.3	27	39.1	
	CC	0	0	5	7.2	

The *p*-value was adjusted for age, total testosterone, follicle-stimulating hormone, and luteinizing hormone. SNP, single-nucleotide polymorphism.

3.4. Associations between WDR4 Variants and Asthenozoospermia

Next, the effects of four SNPs on total sperm motility were analyzed. As shown in Table 3, the genotypic distribution of rs465663 differed between asthenozoospermia and non-asthenozoospermia groups. The proportions of TT and TC genotypes were higher in asthenozoospermia compared to non-asthenozoospermia ($p = 0.025$).

Table 3. Associations between genetic variants of the *WDR4* gene and asthenozoospermia.

SNP	Genotype	Cases		Non-Asthenozoospermia		Recessive Model
		No.	%	No.	%	*p*-Value
rs2298666	GG	17	81	41	69.5	0.399
	GA	4	19	16	27.1	
	AA	0	0	2	3.4	
rs465663	TT	15	71.4	27	46.6	
	TC	6	28.6	21	36.2	
	CC	0	0	10	17.2	
rs2248490	CC	15	68.2	23	39	
	CG	5	22.7	28	47.5	
	GG	2	9.1	8	13.6	
rs3746939	AA	15	68.2	30	50.8	0.513
	AC	6	27.3	25	42.4	
	CC	1	4.5	4	6.8	

The *p*-value was adjusted for age, total testosterone, follicle-stimulating hormone, and luteinizing hormone. $*p < 0.05$. SNP, single-nucleotide polymorphism.

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3.5. Associations between WDR4 Variants and Teratozoospermia

The effects of four SNPs on normal sperm morphology were also examined. As shown in Table 4, no statistically significant differences in genotypic distributions were observed between teratozoospermia cases and the non-teratozoospermia group.

Table 4. Associations between genetic variants of the *WDR4* gene and teratozoospermia.

SNP	Genotype	Cases		Non-Teratozoospermia		Recessive Model
		No.	%	No.	%	*p*-Value
rs2298666	GG	5	83.3	53	71.6	0.759
	GA	1	16.7	19	25.7	
	AA	0	0	2	2.7	
rs465663	TT	5	83.3	37	50.7	0.395
	TC	1	16.7	26	35.6	
	CC	0	0	10	13.7	
rs2248490	CC	5	83.3	33	44	0.154

	CG	1	16.7	32	42.7	
	GG	0	0	10	13.3	
rs3746939	AA	4	66.7	41	54.7	0.186
	AC	2	33.3	29	38.7	
	CC	0	0	5	6.7	

The *p*-value was adjusted for age, total testosterone, follicle-stimulating hormone, and luteinizing hormone. SNP, single-nucleotide polymorphism.

3.6. Functional Annotation from GTEx Portal for rs465663

The profile of *WDR4* gene expression in various tissues was determined by the GTEx portal database (Figure 3). In the genetic association analysis, rs465663, an intronic variant of *WDR4*, was associated with asthenozoospermia. To further elucidate the possible functions of rs465663, *cis*-eQTL results were retrieved from the GTEx portal. rs465663 could affect the expression level of several genes in different tissue types. Subjects carrying the T allele showed a lower expression level of *WDR4* in testes, whole blood, and esophageal mucosal tissue (Table S2).

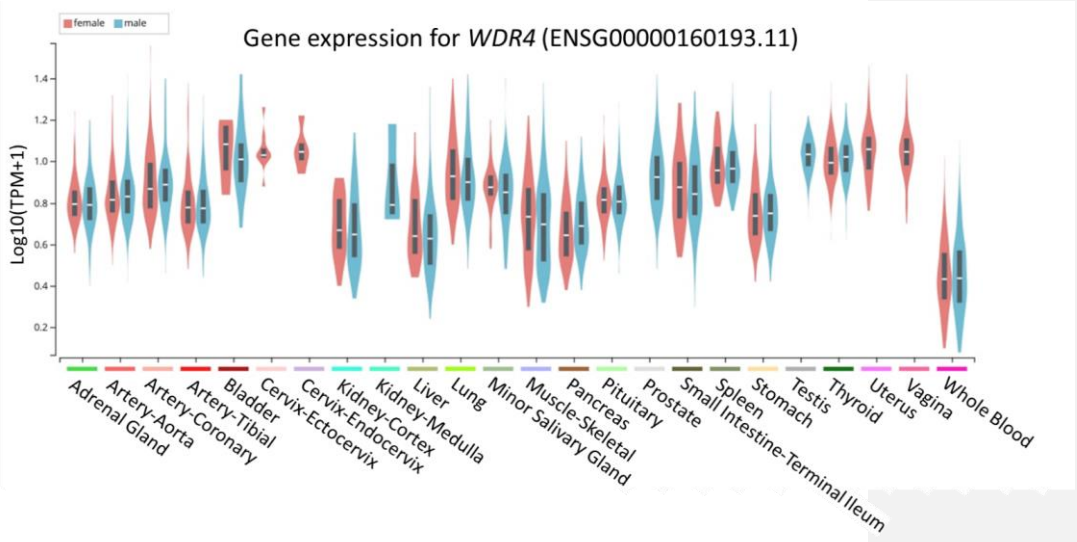

Figure 3. Profile of *WDR4* gene expression in various tissues. Blue represents the male gender and red the female gender.

3.7. Effects of WDR4 in DNA Fragmentation through γH2AX Expression

Previous studies showed that the sperm DNA fragmentation level was increased by ROS stress and was correlated with sperm motility [5,6]. The higher expression level of γH2AX (biomarker of DNA fragmentation level) was revealed to correlate with male infertility [20]. To investigate the role of

WDR4 in male infertility, the MEF (mouse embryonic fibroblasts) cells were isolated from mwdr4 heterozygous (+/−) mice, and the H_2O_2 was used to induce DNA fragmentation for mimicking oxidative stress. The mwdr4 and the γH2AX protein expression levels were confirmed with western blotting (Figure 4). The results showed that the mWdr4 protein expression level in the mWdr4 heterozygous MEF group was low. Importantly, the H_2O_2-induced γH2AX protein level was highly increased in the mwdr4 heterozygous MEF group compared to the wild type MEF group. These results highlighted an important role of *WDR4* in male infertility.

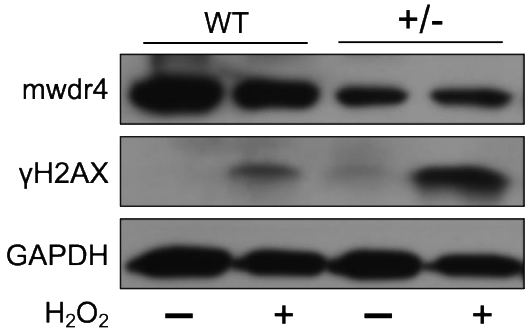

Figure 4. H_2O_2-induced γH2AX expression increased in wdr4 heterozygous (+/−) MEF cells. The MEFs cells were subcultured into 6 cm culture dish 24 h before being treated with 100 μM H_2O_2. The mwdr4, the γH2AX, and the GAPDH protein expression levels were determined by western blot after H_2O_2 treatment for 24 h.

4. Discussion

Wuho is a member of the evolutionarily conserved WD repeat protein family that is expressed by the genes *wuho* in Drosophila, *TRM82* in yeast, and *WDR4* in humans [17]. The *WDR4* domains usually contain four to eight repeating sequences, which are separated by approximately 40 amino acids. Each repeat consists of two sites, a poorly conserved site and a well-conserved site [21]. Wu at al. reported that the lack of *WDR4* function is associated with dramatic germline-specific phenotypes by arresting the spermatogenesis at the spermatid elongating stage [13]. The study indicated that approximately 20% of the ovarioles in WDR4 mutant female have apparent defects in oogenesis with an over-proliferation of cystocytes. Additionally, *WDR4* is associated with germline cell development through cytosolic tRNA modifications [22].

Male infertility is characterized (manifested) by low sperm production (oligozoospermia), reduced sperm motility (asthenozoospermia), and abnormal sperm morphology (teratozoospermia). In this study, we wanted to seek out whether any genetic variants associate with the clinical outcomes. Importantly, the analysis using continuous traits showed

consistent results with dichotomous models (Tables S3–S5). Asthenozoospermia and oligozoospermia are the most common factors responsible for male infertility [23]. In line with this, our study indicated that asthenozoospermia comprised the most cases followed consecutively by oligozoospermia and teratozoospermia. Here, we identified variants of the *WDR4* gene that related to male infertility. rs465663 significantly associated with the susceptibility to asthenozoospermia. The variation in rs465663 was located in the intronic region of chromosome 21, while proportions of the TT and the TC genotypes of rs465663 were higher than the CC genotype in cases with asthenozoospermia. Interestingly, the C allele is the minor allele of rs465663. The distribution of the minor allele of rs465663 in current study was lower than those reported in other populations, including Asians, Europeans, and Americans (Table S1). Trends of infertility globally showed that two populations of Europeans, represented by Central and Eastern Europe (8%~12%), and Americans, represented by North America (4.5%~6%), were higher compared to Sub-Saharan Africans (2.5~4.8%); unfortunately, those studies did not provide the percentage of Asians, possibly due to underreporting [24].

Our findings also emphasized that functional annotations through a bioinformatic approach using the GTEx portal revealed that subjects had lower expression of the TT genotype of *WDR4* than the CC and the TC genotypes in several human tissues, including testes and whole blood. Meanwhile, the major allelic frequency of rs465663 of *WDR4* had lower expression in testes. This result implied that rs465663 might influence expression of the *WDR4* gene, especially in testes. Disease-related male infertility can be caused by testicular deficiencies and spermatogenesis [4]. This evidence provided additional clues that variations of the *WDR4* gene may affect the testes, including their production of sperm (Figure 3). Furthermore, we noticed that ROS-induced DNA fragmentation level was significantly increased in the low *WDR4* group (Figure 4). The results further support the possibility that lower expression of *WDR4* in males resulted in infertility.

Several phenotypes are associated with *WDR4* variants, such as rs370189685, which was correlated with the fasting plasma glucose level [25]. A study from *Drosophila* germ cells suggested that *WDR4* is a regulator of Mei-p26, and it interacts with *TRIM32* to control tissue homeostasis in other stem cell systems [26]. Other genes related to male infertility were identified to associate with azoospermia (e.g., *SOHLH1* [27], *SYCP3* [28], and *TEX11* [29]). *WDR4* gene with a missense mutation was reported to be related to primordial dwarfism through m^7G_{46} methylation, which impaired transfer (t)RNAs [30]. Sperm carries thousands of different RNAs [31]. Interestingly, according to the REACTOME database (R-HSA-6782315) [32], variations in rs465663 of the *WDR4* gene might involve in RNA metabolism and tRNA modifications in nuclei and cytosol. tRNAs play pivotal roles in protein synthetases. Mutations in tRNAs which modify enzymes are associated with human diseases, including cancer, type 2 diabetes (T2D),

neurological disorders, and mitochondrion-linked disorders [33]. Furthermore, as reported by a previous study, tRNA modifications can affect proteostasis in humans [34]. A similar study supported that tRNA modifications in mice resulted in increased apoptosis in male germ cells and male infertility [35]. In addition, epigenomic assays are also essential for relating noncoding genetic variations to regulatory mechanisms underlying phenotypic changes, including genomic variations of the *WDR4* gene. The Encyclopedia of DNA Elements (ENCODE) database revealed that rs465663 is located in an intronic region with known histone modifications [32]. Subsequent evidence revealed that an epigenomic histone modification was involved in male fertility [36]. Some genes, such as *FAM50B* and *GNAS*, were reported to involve in the quality of sperm in asthenozoospermia through histone modification-type methylation [37]. Taken together, previous studies provided comprehensive integration between the identified genetic variants and male infertility.

Our research is focusing on the genetic variants of *WDR4* in male fertility s. However, some limitations still exist in this study. First, although the subject number (68,948 individuals) for PheWAS was good, no significant physiological traits were found. Regarding the second cohort (male participants) from the hospital, the small sample size limits the statistical power. Thus, larger sample sizes with different populations are necessary to confirm our findings. Second, as the candidate gene approach was performed, the influence of variants in other unexamined genes cannot be ruled out. Since the pathophysiology of infertility in humans is complexly regulated by many signaling pathways, using whole genome sequencing technology may yield further insights into the genomic variations in infertility.

5. Conclusions

This study offers important information related to genetic variants and expression level of the *WDR4* which might affect infertility in the cases of asthenozoospermia. However, further functional studies and larger sample sizes are required to validate the variants.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: Spearman's rank correlation coefficient between age, serum hormones, and semen parameters, Table S1: Characteristics of tagging single-nucleotide polymorphisms (tSNPs) of the WDR4 gene, Table S2: Cis-expression quantitative trait loci (cis-eQTL) of rs465663, Table S3: Associations between genetic variants of the WDR4 gene and total sperm number, Table S4: Associations between genetic variants of the WDR4 gene and sperm progressive motility, Table S5: Associations between genetic variants of the WDR4 gene and sperm morphology

Author Contributions: Conceptualization: Y.-J.W., Y.-T.P., W.-H.C., M.-I.H., and W.-C.C.; sample collection: Y.-J.W., and Y.-T.P.; data curation: Y.-J.W., Y.-T.P., M.-I.H., and W.-C.C.; formal analyses: Y.-J.W., Y.-T.P., W.-H.C., M.-I.H., and W.-C.C.; experimental preparation: C.-C.L., Y.-S.W., W.-H.C.; data interpretation and discussion: Y.-J.W., Y.-T.P., E.M., W.-C.H., W.-H.C., C.-C.L., Y.-S.W., L.M.I., D.A.P., M.-I.H., and W.-C.C.;

writing review & editing: Y.-J.W., Y.-T.P., E.M., W.-C.H., W.-H.C., C.-C.L., Y.-S.W., L.M.I., D.A.P., M.-I.H., and W.-C.C.; supervision: W.-C.H., D.A.P., M.-I.H., and W.-C.C. All authors have made significant contributions to this study. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study

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Conflicts of Interest: The authors declare no conflict of interest.

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