Association of microRNA-related gene polymorphisms and idiopathic azoospermia in a south-east Turkey population

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ABSTRACT

MicroRNAs (miRNAs) are small conserved non-coding RNA molecules that post-transcriptionally regulate gene expression. Although it is reported in many studies that there are associations between alterations of miRNA homeostasis and pathological conditions such as cancer, psychiatric and neurological diseases, cardiovascular disease and autoimmune disease, the effects of common genetic variants of these genes on male infertility are unclear. To better understand this effect, we performed a case-control study including a total of 108 infertile men with idiopathic azoospermia and 125 fertile control subjects. Real-time polymerase chain reaction was used to genotype six single-nucleotide polymorphisms (SNPs) of miRNA biogenesis pathway genes and the associations between individual and combined genotypes and idiopathic azoospermia were analysed. The results showed significant difference between the individual AA genotype frequency of the GEMIN3 (rs197388) gene in the patient and control groups, indicating that the AA genotype may be considered as indicative of a higher predisposition to idiopathic azoospermia. The combined genotype analysis, including six SNPs, revealed statistically significant differences between the patients and control subjects for some combinations. For example, the frequency of genotype distributions of the AA/CA-CC-TT-AT genotype combination for the XPO5-RAN-DICER1-GEMIN3 combined loci was significantly different, and it may be considered a predisposition to idiopathic azoospermia. According to the obtained results, both individual and combined genotypes of SNPs from miRNA genes may be used to predict the risk of male infertility with idiopathic azoospermia.

Introduction

Infertility is one of the most frequently diagnosed diseases in reproductive health [1–4]. Infertility affects approximately 8%–12% of couples worldwide. Of all infertility cases, approximately 40%–50% is due to ‘male factor’ infertility and a significant proportion of male infertility is accompanied by idiopathic azoospermia [5]. Azoospermia, described as the absence of spermatozoa in semen, affects approximately 1% of all men. Azoospermia is classified as obstructive or non-obstructive; while obstructive azoospermia is the consequence of physical blockage in the genital tract, non-obstructive azoospermia is caused by spermatogenic failure [4]. In approximately 40% of all cases, the aetiology is still unknown; therefore, those cases are considered idiopathic azoospermia. Although several risk factors for idiopathic azoospermia have been identified, such as chromosomal abnormalities and Y-chromosome microdeletions, the genetic mechanisms underlying spermatogenic failure and sperm dysfunction still remain unclear [3].

One of the important mechanisms regulating gene expression is performed transcriptionally and post-transcriptionally by small RNA molecules such as miRNAs and piwi-interacting RNAs [6]. Most miRNAs are 19–25 nucleotides in length and they are typically encoded within introns, which are first transcribed as a long RNA transcript [3]. They are called primary miRNAs (pri-miRNAs), ranging between hundreds of nucleotides and tens of kilobases in size [7]. Drosha protein, the nuclear RNase encoded by the DROSHA gene, performs the processing of primary pri-miRNAs within the microprocessor complex, including DGCR8, which produces the 70–100-nt pre-miRNAs. Then, the Exportin-5/Ran-GTP complex exports the pre-miRNAs from the nucleus into the
cytoplasm and is cleaved by Dicer, Gemin3 and Gemin4 as parts of the RNA-induced silencing complex (RISC). The RISC not only contributes to miRNA processing, but also targets gene silencing [8].

In the human genome, the most frequent form of DNA variation is single-nucleotide polymorphisms (SNPs) [9]. The SNPs of the silencing machinery, miRNA precursors and their target sites interfere with miRNA function and may contribute to phenotypic variation, as disease susceptibility [10]. There have been many studies on the relationship between SNP variants in the miRNA biogenesis pathway genes and diseases, including cancers [3,11]. However, there have only been a few reports regarding the relationship between male infertility and these variants [3,8,10,12,13]. Considering the essential role of miRNA regulatory genes in a large variety of basic cellular functions via post-transcriptional regulations on their target genes, we hypothesize that genetic variation in miRNA-related genes has the potential to affect normal spermatogenesis. In order to examine this hypothesis, we genotyped DROSHA, DGCR8, XPO5, RAN, DICER1 and GEMIN3 polymorphisms in a case-control study on infertile male subjects with idiopathic azoospermia in a Turkish population. To the best of our knowledge, this is the first study to investigate the associations between DGCR8, XPO5, RAN and GEMIN3 gene polymorphisms and susceptibility to azoospermia.

Materials and methods

Patients and controls

In this study, we selected 108 azoospermic men as the patient group; the mean age was 31.3 ± 5.5 years. All of the azoospermic cases (n = 108) were non-obstructive and idiopathic, with at least one year of infertility history. We performed at least two semen analyses on each patient, in accordance with the guidance of the World Health Organization [14]. Azoospermic men with known causes of infertility, such as genetic factors (chromosome anomalies, azoospermia factor microdeletions), lifestyle factors (e.g. alcoholism and occupation), or clinical factors (varicocele, cryptorchidism, orchitis, obstruction of the vas deferens) and those whose wives were infertile were excluded from the study. The control group was composed of 125 fertile men (mean age 37.8 ± 7.6 years) chosen from among men who had at least one child. The control subjects also lacked any history of assisted reproductive technology. Both the patient and control groups were selected from the same geographical region, and they were referred by the Urology Department of Dicle University Hospital to the Medical Biology and Genetics Department. The study was certified by the Ethics Committee of Dicle University’s Faculty of Medicine (134/25.03.2013) and written informed consent was obtained from all participants.

SNP selection and genotyping

There were three main criteria for selection. First, all SNPs were reported with a minor allele frequency > 0.01, and second, they needed to reside within a 3′-untranslated region (UTR) or promoter region. Third, all SNPs were selected from the HapMap project and PubMed (http://www.ncbi.nlm.nih.gov/pubmed). As a result, we identified six potentially functional SNPs from six miRNA biogenesis pathway genes for male infertility.

Venous blood samples were drawn from all of the individuals after receiving written informed consent, and the samples were kept in tubes containing ethylenediaminetetraacetic acid. The salting-out procedure was used to extract DNA from whole blood [15]. Through collaboration between the Medical Biology and Genetics Department of Dicle University and Mersin University, genotypes were determined by real-time polymerase chain reaction (PCR) using a TaqMan™ fluorogenic 5′-nuclease assay with TaqMan™ probes (Applied Biosystems, Inc., Foster City, CA, USA). The specific primers and fluorogenic probes for the miRNA biogenesis pathway gene SNPs were designed with Primer Express 3.0 software (Applied Biosystems, Foster City, CA, USA) (Table 1). The primers and probes were purchased from Metabion International AG (Martinsried, Germany). SNP amplification assays were performed according to the manufacturer’s instructions. Briefly, the samples were assayed in a 25 mL reaction mixture containing 30 ng of DNA and mixed with 12.5 µL of 2X TaqMan Universal PCR Master Mix (Applied Biosystems, Inc., Foster City, CA, USA) and 900 nmol/L of forward and reverse primers and 200 nmol/L of each of the probes. Reaction conditions consisted of preincubation at 60 °C for 1 min and at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and at 60 °C for 1 min. Amplifications and analyses were performed in an ABI Prism 7500 Real-Time PCR System (Applied Biosystems, Inc., Foster City, CA, USA), using SDS 2.0.6 software for allelic discrimination (Applied Biosystems).

Statistical analysis

Arlequin Software 3.1.1 [16] was used for the statistical analysis of allele frequencies, Hardy–Weinberg equilibrium (HWE), linkage disequilibrium between SNP pairs and overall comparison between patients and controls. The distribution of gene polymorphisms between the patients and the control subjects, as well as their
deviations from the HWE, was compared using Fisher’s exact χ² test; p-values < 0.05 were considered significant. Associations between gene polymorphisms and idiopathic azoospermia were estimated as odds ratios (OR) and 95% confidence intervals (95% CI) using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). The combined results suggested that FAS-670A/G SNP (rs13078) and FAS1 670 and RAN611X in NOA in the Turkish population.

In the present study, 108 idiopathic azoospermic patients and 125 fertile men were evaluated for the existence of SNPs at the RNASEN (DROSHA, rs10719), DGCGR8 (rs1640299), XPOS (rs11077), RAN (rs14035), DICER1 (rs13078) and GEMIN3 (DDX20, rs197388) loci. The genotype frequencies of these SNPs within the patient and control groups are shown in Table 2. The individual polymorphism analysis revealed that the genotype distributions of the RNASEN, DGCGR8, XPOS, RAN and DICER1 gene polymorphisms were not statistically different between the patients and controls (p > 0.05), suggesting no associations between the polymorphisms at these five individual genes and male infertility in the studied cohort. However, the genotype frequencies of SNP at GEMIN3 (rs197388) were found to differ significantly between the patient and control groups.
frequency of the GEMIN3 AA genotype was determined to be significantly higher in the patient group ($p = 0.034$). The risk confidence intervals were found to be within significance limits (OR, 3.15; 95% CI, 1.04–9.54), which might be regarded as a higher predisposition for idiopathic azoospermia.

Although the association between miRNA biogenesis pathway gene polymorphisms and cancer has been reported in earlier studies [20], several studies have recently explored the underlying mechanism of male infertility in gene polymorphisms of the miRNA biogenesis pathway [3,10,12,21,22]. The studies have shown that some miRNAs that downregulate gene expression via base pairing in the 3’-UTR of the mRNA of target genes, which, in turn, could regulate the expression levels and may result in the upregulation of DICER1 gene may cause global alteration of miRNA production, implying that SNP in DICER1 reported that polymorphisms in DROSHA could affect miRNA production, implying that SNP in DICER1 and DROSHA could affect susceptibility to male infertility when considering their function in miRNA biogenesis and spermatogenesis. It was also reported that conditional knockout of DICER1 in primary germ cells caused spermatogenesis defects in mice [22]. The presence of many apoptotic spermatocytes seen in defective seminiferous tubules implies that DICER1 might be a guard of cell survival in spermatogenesis. In early embryonic development, miRNA belonging to the miRNA 17–92 cluster is highly expressed in the primary germ cells until gender is determined. It is possible that the miRNA 17–92 cluster is crucial for the survival and proliferation of spermatogonia because it is related to apoptosis [23]. It was reported in another study that a mutation in the DICER1 gene may cause global alteration of miRNA expression levels and may result in the upregulation of their target genes, which, in turn, could regulate the other interacting factors, such as the miRNA 17–92 cluster, and then cause apoptosis of the spermatozoa and abnormal semen quality [3]. The results from the present study showed that the genotype frequencies of SNP at GEMIN3 and DROSHA had no associations with male infertility in this Turkish population. Other different specific mutations of these genes could better account for this condition.

GEMIN3 is a miRNA biogenesis factor [24]; in addition, it has recently been found to bind PUMILIO2 and NANOS1 proteins within the chromatoid body [25]. It has been suggested that the NANOS1–PUMILIO2

| Locus (P/C) | Patients | Controls | Genotype | Patients | Controls |
|------------|----------|----------|----------|----------|----------|
| RNASEN rs10719 (108/125) | C 145 (67.13) 171 (68.40) | CC 49 | $\chi^2 = 0.02$ | p = 0.8852 | 56 | $\chi^2 = 1.55$ | NS | NS |
| DGCRR8 rs1640299 (105/125) | T 71 (32.87) 79 (31.60) | CT/TT 59 | $\chi^2 = 3.13$ | p = 0.0764 | 69 | $\chi^2 = 2.76$ | NS | NS |
| XPO5 rs11077 (106/125) | T 86 (40.95) 117 (46.80) | GT/TT 66 | $\chi^2 = 2.40$ | p = 0.1214 | 85 | $\chi^2 = 1.25$ | NS | NS |
| RAN rs14035 (105/125) | C 159 (75.71) 179 (71.60) | CC 62 | $\chi^2 = 0.92$ | p = 0.3375 | 72 | $\chi^2 = 1.13$ | NS | NS |
| DICER1 rs13078 (85/119) | T 51 (24.29) 71 (28.40) | CT/TT 43 | $\chi^2 = 0.09$ | p = 0.7660 | 53 | $\chi^2 = 0.82$ | NS | NS |
| GEMIN3 rs197388 (83/120) | A 122 (57.55) 150 (60.00) | CA/AA 83 | $\chi^2 = 0.92$ | p = 0.3375 | 108 | $\chi^2 = 0.005$ | NS | NS |
| | C 159 (75.71) 179 (71.60) | CC 62 | $\chi^2 = 0.09$ | p = 0.7660 | 72 | $\chi^2 = 0.82$ | NS | NS |
| | T 51 (24.29) 71 (28.40) | CT/TT 43 | $\chi^2 = 0.09$ | p = 0.7660 | 53 | $\chi^2 = 0.82$ | NS | NS |
| | A 21 (12.35) 32 (13.45) | AA 10 | $\chi^2 = 23.80$ | p = 0.0000 | 108 | $\chi^2 = 1.20$ | 0.034 | 3.15, 1.04–9.54 |
| | C 159 (75.71) 179 (71.60) | CC 62 | $\chi^2 = 0.09$ | p = 0.7660 | 72 | $\chi^2 = 0.82$ | NS | NS |
| | T 51 (24.29) 71 (28.40) | CT/TT 43 | $\chi^2 = 0.09$ | p = 0.7660 | 53 | $\chi^2 = 0.82$ | NS | NS |
| | A 32 (19.28) 40 (16.67) | AA 10 | $\chi^2 = 23.80$ | p = 0.0000 | 108 | $\chi^2 = 1.20$ | 0.034 | 3.15, 1.04–9.54 |
| | T 134 (80.72) 200 (83.33) | AT/TT 73 | $\chi^2 = 0.09$ | p = 0.7660 | 115 | $\chi^2 = 0.2733$ | NS | NS |

Note: NS (not significant), $p < 0.05$. 

$\chi^2$, number of alleles; $N$, number of subjects; HWE: Hardy–Weinberg equilibrium.
P/C, total number of patients; C, total number of control subjects) are given in brackets.

**Bold value** is significant at $p < 0.05$. 

**Note:** $N$ (number of subjects); $N$, number of alleles; $N$, number of subjects; HWE: Hardy–Weinberg equilibrium.
complex, together with GEMIN3 and small non-coding RNAs, regulate mRNA translation within the chromatoid body of human germ cells. In our study, the genotype frequencies of SNP at GEMIN3 (rs197388) were observed to be significantly different between the patient and control groups, and rs197388 was significantly associated with the risk of male infertility in this Turkish population, implying that SNP from GEMIN3 (rs197388) could modify the risk of abnormal semen parameters and result in azoospermia in male infertility. Because this polymorphism in GEMIN3 could affect miRNA production, it could affect susceptibility to male infertility with azoospermia when considering their function in miRNA biogenesis and spermatogenesis.

In the test for deviation from HWE, deviation was shown for the genotype distribution at RAN (rs14035) in the control group and at GEMIN3 (rs197388) in the patient group (Table 2). These deviations are important with regard to high parental consanguinity in populations such as that of south-east Turkey. High parental consanguinity could bring out genetic factors or provide a permissive background for complex disorders [2].

To investigate any potential epistatic interactions among these six gene polymorphisms and idiopathic azoospermia, the combined effects of the genotypes were evaluated by summing the genotypes of the six SNPs (Table 3). When all of the studied loci were evaluated together by linkage analysis, it was found that there was no linkage in any of the studied loci (data not shown). However, we observed statistically significant differences between the patient and control groups for 18 combined genotypes. For instance, the frequency of the AA/CA-CC-TT-AT compound genotype for XPOS-RAN-DICER1-GEMIN3 combined loci was found to be significantly higher ($p = 0.019$) in the control group (10.08%) than in the patients (1.37%), which could be interpreted to mean that these compound genotypes provide protection against spermatogenic failure (OR, 0.12; 95% CI, 0.02–0.96) (Table 3). Another compound genotype, TT-TA-AA for DICER1-GEMIN3 combined loci, was found in 12.99% of the patient group but in only 4.20% of the control group ($p = 0.029$); the risk confidence interval of these genotypes was found within significance limits, which might be regarded as a higher predisposition for spermatogenic failure (OR, 3.40; 95% CI, 1.12–10.32) (Table 3). The results obtained from the comparative analysis of combined genotypes indicate that there is a moderate association between the polymorphisms of DROSHA, DGCR8, XPOS, RAN, DICER1 and GEMIN3 genes and idiopathicazoospermia.

This preliminary study, however, had several limitations. First, not all biological and biochemical

### Table 3. Statistically significant differences in combined genotypes between azoospermic patients and control subjects.

| Combined loci (P/C) | Combined genotypes | Patient n (%) | Control n (%) | $P$-value | OR (95% CI) |
|---------------------|--------------------|---------------|---------------|-----------|-------------|
| XPOS-RAN-DICER1-GEMIN3 (73/119) | AA/CA-CC-TT-AT | 1 (1.37) | 12 (10.08) | 0.019 | 0.12 (0.02–0.96) |
| XPOS-DICER1-GEMIN3 (73/119) | AA/CA-TT-AT | 2 (2.74) | 19 (15.97) | 0.004 | 0.15 (0.03–0.65) |
| RAN-DICER1-GEMIN3 (75/119) | CC-TTAT-AA | 5 (6.85) | 0 | 0.007 | 19.19 (1.06–347.86) |
| RNASN-DGCR8 (105/125) | TT-GG | 6 (5.71) | 1 (0.80) | 0.049 | 7.52 (0.90–62.90) |
| RNASN-XPOS (106/125) | TT-CC | 6 (5.66) | 1 (0.80) | 0.050 | 7.44 (0.89–62.27) |
| RNASN-GEMIN3 (82/120) | CC/CT-AT | 9 (10.84) | 30 (25.00) | 0.012 | 0.36 (0.16–0.81) |
| DGCR8-XPOS (103/125) | TT-AA/AT | 5 (6.02) | 0 | 0.011 | 16.89 (0.93–305.79) |
| DGCR8-RAN (103/125) | TT-TT | 0 | 6 (4.80) | 0.034 | 0.09 (0.01–1.57) |
| XPOS-RAN (104/125) | TT-GT-CT | 2 (1.94) | 13 (10.40) | 0.014 | 0.17 (0.04–0.77) |
| XPOS-GEMIN3 (81/120) | AA/CA-TT | 5 (4.81) | 16 (12.80) | 0.041 | 0.34 (0.12–0.97) |
| RAN-GEMIN3 (80/120) | CC-AA | 5 (6.17) | 0 | 0.010 | 17.33 (0.96–313.85) |
| DICER1-GEMIN3 (77/119) | TT-TA-AA | 10 (12.99) | 5 (4.20) | 0.029 | 3.40 (1.12–10.32) |

Note: P/C (P, total number of patients; C, total number of control subjects) are given in brackets. n, total number of cases with combined genotypes.
interactions of the studied genes were examined. Therefore, it is difficult to say with certainty whether the observed statistical differences between patients and controls for the combined genotypes are random or due to real interactions. Second, the studied groups were relatively small; therefore, the results need to be confirmed by independent research groups working with larger sample sizes.

Within the limitations of this study, the obtained results indicated that the SNP in the \textit{GEMIN3} (rs197388) gene was significantly associated with risk of male infertility with idiopathic azoospermia, suggesting that it might be a genetic predisposing factor for idiopathic azoospermia in the studied population from south-east Turkey. The polymorphic variants of the \textit{GEMIN3} gene might contribute to the functional disorder of the apoptotic mechanism in germ cells, thus resulting in poor semen quality of the ejaculated sperm. Furthermore, the results from this study suggest that combined genotypes of SNPs at the miRNA biogenesis pathway genes occurring in a dose-dependent manner may affect idiopathic azoospermia susceptibility. This finding strengthens the concept that idiopathic azoospermia is a polygenic process. Thus, a combined analysis of multiple variants might be better able to characterize high-risk populations. As a result, the results from this study suggest that SNPs in the miRNA biogenesis pathway genes, individually and jointly, might be a genetic predisposing factor for male infertility with idiopathic azoospermia. Although these results provide critically important data, further studies with larger sample sizes and different ethnic populations are needed to better understand the role of microRNA biogenesis pathway gene polymorphisms in susceptibility to idiopathic azoospermia.

Conclusions

In the present study, individual analysis of SNPs revealed no associations between the polymorphisms at five individual \textit{DROSHA}, \textit{DGCR8}, \textit{XPOS}, \textit{RAN} and \textit{DICER1} genes and male infertility. However, the genotype frequencies of SNP at \textit{GEMIN3} (rs197388) were found to differ significantly between the patient and control groups, indicating that the \textit{GEMIN3} AA genotype might be regarded as a factor for higher predisposition to idiopathic azoospermia. Further studies including larger cohorts and different ethnicities would help to throw light on the involvement of microRNA-related gene SNPs in the susceptibility to idiopathic azoospermia.

Disclosure statement

The authors declare that there are no conflicts of interest.

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