Asian Journal of Andrology (2011) 13, 505–508
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www.nature.com/aja

LETTER TO THE EDITOR

Polymorphisms in microRNA targets: a source of new molecular markers for male reproduction

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Asian Journal of Andrology (2011) **13,** 505–508; doi:10.1038/aja.2011.4; published online 11 April 2011

Dear Editor,

Herein we discuss the impact of microRNA (miRNA) target genetic variability in male infertility genes, which can represent a source of novel molecular-genetic markers that can be used for the diagnosis of male infertility. Male-factor infertility accounts for 30%–40% of infertility cases. The causes of spermatogenetic failure found in most cases of non-obstructive azoospermia or severe oligozoospermia still remain idiopathic.[1]

Significant progress has recently been made by utilizing small RNAs, e.g., small interfering RNAs, miRNAs and piwi-interacting RNAs, to elucidate the molecular mechanisms that regulate spermatogenesis.[2] The miRNAs repress protein synthesis from targeted messenger RNAs (mRNAs) and control approximately 30% of human genes.[3] Single-nucleotide polymorphisms (SNPs) of miRNA precursors and their target sites, as well as the silencing machinery, interfere with miRNA function and are likely to affect phenotypic variation, including disease susceptibility.[4] The term miR-SNP refers to the variation that occurs in the miRNA gene sequence, whereas miR-TS-SNP refers to the SNP within the miRNA target site (TS) or binding site.[5] miR-TS-SNPs have been associated with many diseases, including tumour susceptibility;[6] however, mir-TS-SNPs have not yet been shown to affect male fertility.

The aim of this study was to explore *in silico* genetic variability of 3′-UTR miRNA target sites of the selected (in)fertility related candidate genes to determine whether miR-TS-SNPs could represent a starting point for the discovery of novel genetic markers that affect male fertility phenotypes *via* miRNA regulation. This analysis was performed on the 34 candidate genes associated with this trait most frequently.[7]

Using online tools, the selected candidate human and mouse orthologs were analyzed for the following: (i) testicular expression levels obtained using BioGPS (Genomic Institute of the Novartis Research Foundation; GNF) (http://biogps.gnf.org/); (ii) conserved miRNA target sites using TargetScan (http://www.targetscan.org/); and (iii) polymorphic miRNA target sites using Patrocles (http://www.patrocles.org/).

The results of the analyses are presented in **Table 1**. TargetScan, which predicts biological targets of miRNAs by searching for the presence of conserved sites across several species that match the miRNAs seed regions, revealed that more than two-thirds of the analyzed candidate genes in human (76%; 26/34) and mouse (68%; 23/34) genomes comprise conserved miRNA-binding sites. Candidate genes were further analyzed for miR-TS-SNPs within the 3′-UTRs of human and mouse orthologs using Patrocles. The search revealed 55 and 33 miR-TS-SNPs in humans and mice, respectively. More than half (62%; 21/34) of the analyzed human candidate genes contained miR-TS-SNPs; the most polymorphic were KIT ligand (*KITL*, 12 SNPs), actin beta (*ACTB*, 5 SNPs), angiotensin I converting enzyme (peptidyl-dipeptidase A) 1 (*ACE*, 4 SNPs), calcium/calmodulin-dependent protein kinase IV (*CAMK4*, 4 SNPs), oestrogen receptor 1 (*ESR1*, 4 SNPs) and 5,10-methylenetetrahydrofolate reductase (*MTHFR*, 4 SNPs) (**Table 1**). For example, the highly polymorphic 3′-UTR of *KITL* spans a region of only 3740 bp; therefore, sequencing such a region could be highly informative in the context of discovering novel genetic markers. In mice, nearly half (41%; 14/34) of the genes contained miR-TS-SNP; the most polymorphic were prolactin receptor (*PRLR*, 7 SNPs), oestrogen receptor 1 (*ESR1*, 6 SNPs) and KIT ligand (*KITL*, 5 SNPs). Despite the known problem of a low overlap between the target prediction tools, TargetScan and Patrocles predicted several overlapping miRNA-target pairs: *AKT1* (miR-25 and miR-32), *DAZL* (miR-1269), *ESR1* (miR-211 and miR-204) and *RAD23A* (miR-361-5p). However, predicted miRNA-mRNA pairs described in the present study have not been experimentally validated thus far, and the validation status of several SNPs is still unknown.

Furthermore, predicted miR-TS-SNPs and conserved target sites were compared to an miRNA expression study performed in non-obstructive azoospermic patients and compared to fertile controls.[8] Notably, it was possible to determine infertility-related miRNA-target pairs; Patrocles revealed miR-TS-SNPs in the analyzed candidates that are targeted by miRNAs previously reported as being either up- or downregulated in azoospermic patients. There were miR-TS-SNPs for three upregulated miRNAs, miR-129-5p (having predicted polymorphic target site in *DAZL*), miR-302a (*ESR1*) and miR-557 (*PRM2*), as well as for 10 downregulated miRNAs, mir-31* (*ACE*), miR-32 (*AKT1*), miR-199b-5p (*APRT*), miR-153, miR-425, miR-448 (all in *CREM*), miR-515-5p (*KITL*), miR-363* (*MTHFR*), miR-31 (*PRM1*) and miR-374a* (*RAD23A*). Additionally, TargetScan predicted conserved target sites in human orthologs of infertility candidate genes for three upregulated miRNAs, miR-302a (predicted target in *AKT1*), miR-129-5p (*CAMK4*) and miR-557 (*DAZL*), as well as for 14 downregulated miRNAs, miR-145 (*ACTB*), miR-302d, miR-372, miR-373 (all in *AKT1*), miR-301b and miR-454 (both in *AR*), miR-1 (*CREM*), miR-548a-3p (*DAZ*-family), miR-548c-3p (*DAZ*-family and

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Received: 5 January 2011; Revised: 7 January 2011; Accepted: 16 January 2011; Published online: 11 April 2011

Table 1 Selected genes associated with a male reproduction phenotype: level of expression in testes, conserved target site analysis and polymorphisms at miRNA target sites[a]

Gene	*GNF BioGPS*	*TargetScan analysis*		*Patrocles analysis*			
	Expression in testis (mouse/human)	No. of conserved (poorly conserved) miRNA target sites in mouse/human	miRNAs binding to conserved octamers[b]	No. of SNPs in miRNA target sites (mouse/human)	miRNAs binding to polymorphic target sites	NCBI or ENSEMBL SNP ID	
						Mouse	Human
ACE	++/++	0/0 (13/35)		0/4	hsa-miR-: 657, **31***	—	rs10853044 rs1125574 rs35974478 rs5821393
ACR	+++/++	0/0 (1/8)		1/0	NA	rs13482700	—
ACTB	+/+	2/1 (0/0)	mmu-miR-: 132, 212 hsa-miR-**145**	0/5	hsa-miR-: 486-5p, 1274a	—	rs11266786 rs3210647 rs11546927 rs11546941 rs11546891
AKT1	+/+	6/12 (0/0)	mmu-miR-: 25, 92b, 92a, 363, 367, 32 hsa-miR-: 520a-3p, 520d-3p, 520b, 520c-3p, 302e, 302b, **302a**, 302c, **302d**, 520e, **373**, **372**	1/2	hsa-miR-: 25, **32**	rs13467069	rs17846821 rs41307094
APOB	+/−	0/1 (15/26)	hsa-miR-548p	0/1	hsa-miR-615-3p	—	rs12720763
APRT	++/+	0/0 (15/29)		1/2	mmu-miR-31* hsa-miR-: 1275, 625, 199a-5p, **199b-5p**	rs31780616	rs3177509 rs4695
AR	+/+	5/5 (0/0)	miR-: 130a, 130b, 301a, **301b** mmu-miR-721 **hsa-miR-454**	0/1	mmu-miR-: 25, 92a, 92b, 367	rs29084377	—
BRCA2	++/+	0/1 (39/87)	hsa-miR-1245	0/2	hsa-miR-653	—	ENSSNP10730884 ENSSNP10730885
CAMK4	+/++	2/2 (0/0)	**miR-129-5p**	1/4	mmu-miR-16* hsa-miR-: 1252, 892b, 1290	rs31658022	rs13175122 rs13157105 ENSSNP12893204 ENSSNP12893205
CREM	++/+	2/3 (0/0)	mmu-miR-: 1, 206 hsa-miR-: **1**, 206, 613	1/2	hsa-miR-: **153**, **425**, **448**, 1197	rs29802966	rs34513310 rs11545325
DAZ-family[c]	NA/+	NA/40 (NA/1425)	hsa-miR-: 526b, **548c-3p**, 607, 1269, 509-3p, 577, 488, 186, 548f, **548a-3p**, 548e	NA/0	—	NA	—
DAZL	+++/+++	4/11 (111/172)	mmu-miR-: 694, 695, 590-5p, 488 hsa-miR-: 1305, 607, 513a-3p, **548c-3p**, 629, 1269, **590-3p,** 507, **557**, 488	1/2	mmu-miR-: 125a-5p, 125b-5p, 351, 670 hsa-miR-: **129-5p**, 1269	rs29502108	rs1048958 rs10510454
DDX25	+++/+++	0/2 (9/14)	hsa-miR-: 342-3p, 1229	0/0	—	—	—
ESR1	+/+	2/1 (2/0)	mmu-miR-: 211, 204 hsa-miR-122	6/4	mmu-miR-: 186*, 204, 211, 692, 542-5p, 467f hsa-miR-: **302a***, 1182	rs29349619 rs16783368 rs16783370 rs51239151 rs29315100 rs51700320	rs3020386 rs9341075 rs9341078 rs9341084
FHL5	+++/+	1/3 (35/62)	mmu-miR-450a-3p hsa-miR-: 450b-5p, 921, 922	0/1	hsa-miR-511	—	rs4839688
FSHR	+/+	0/0 (15/22)	NA	0/0	—	—	—
GNRHR	++/+	1/5 (4/314)	mmu-miR-129-5p hsa-miR-: 1246, **651**, 938, 129-3p	3/1	mmu-miR-: 683, 374*	rs33631357 rs3090530 rs31666210	rs1038427
GSTM1	++/++	0/0 (24/46)	NA	2/1	mmu-miR-695 hsa-miR-149*	rs8261721 rs8261727	rs17672
HSPA2	+++/+++	1/2 (0/0)	miR-96 hsa-miR-1271	0/0	—	—	—
KIT	+/+	1/1 (1/0)	miR-218	1/1	hsa-miR-603	rs13469910	rs17084736

To be continued

Table 1 (Continued) Selected genes associated with a male reproduction phenotype: level of expression in testes, conserved target site analysis and polymorphisms at miRNA target sites[a]

Gene	*GNF BioGPS*	*TargetScan analysis*		*Patrocles analysis*			
	Expression in testis (mouse/human)	No. of conserved (poorly conserved) miRNA target sites in mouse/human	miRNAs binding to conserved octamers[b]	No. of SNPs in miRNA target sites (mouse/human)	miRNAs binding to polymorphic target sites	NCBI or ENSEMBL SNP ID	
						Mouse	Human
KITL (KITLG)	+/+	2/2 (0/4)	mmu-miR-: 27a, 27b hsa-miR-: 132, 212	5/12	mmu-miR-: 103, 107, 485, 881*, 671-5p hsa-miR-: 496, 921, 620, 1270, 299-5p, 586, **515-5p**, 519e*, 335*, 618, 203	rs51068821 rs51436505 rs51005420 rs47027334 rs46343331	rs12344 rs3203697 rs41311951 rs41307009 rs41309421 rs41307046 rs41300052 rs4842625 rs35072453 rs41306433 rs41300048 rs995030
LHB	+/+	1/1 (4/6)	mmu-miR-325 hsa-miR-1300	0/0	—	—	—
MTHFR	++/+	1/1 (0/4)	miR-22	0/4	hsa-miR-: **363***, 1228*	—	rs35134728 rs34762557 rs3737966 ENSSNP11488330
POLG	++/+	0/0 (16/23)		0/0	—	—	—
PRDM9	++/+	NA	NA	0/0	—	—	—
PRLR	++/+	1/1 (1/1)	miR-142-3p	7/0	mmu-miR-: 205, 207, 26b*, 29a, 29b, 29c, 743a, 743-b-3p, 374	rs32240442 rs47952152 rs45986226 rs46045208 rs46505738 rs49089943 rs32350023	—
PRM1	+++/+++	3/3 (3/9)	miR-484 mmu-miR-: 669g, 325 hsa-miR-: 1256, 1305	0/1	**hsa-miR-31**	—	rs11544791
PRM2	+++/+++	0/1 (11/14)	hsa-miR-1266	0/1	hsa-miR-: 507, **557**	—	rs1042801
RAD23A	+/++	3/11 (26/41)	miR-423-5p mmu-miR-: 703, 361 hsa-miR-: **548a-3p**, 548e-3p, 548f-3p, 1302, 361-5p, 548o, 1323, 548e, 548f	2/2	mmu-miR-665 hsa-miR-: 29a-2*, **374a***, 361-5p	rs33102324 rs13470179	rs41558417 rs1059298
RBP4	−/−	1/1 (10/13)	mmu-miR-466l hsa-miR-577	0/0	—	—	—
SYCP3	++/++	2/0 (10/13)	mmu-miR-: 409-3p, 325	0/0	—	—	—
TNP1	+++/+++	4/4 (0/0)	miR-: **181a**, 181b, 181c, **181d**	1/0	mmu-miR-434-5p	rs3022818	—
TNP2	+++/+++	1/0 (1/6)	mmu-miR-153	0/0	—	—	—
UBE2B	+/++	1/2 (0/9)	miR-124 hsa-miR-506	0/2	hsa-let-7c*, hsa-miR-424	—	rs36072121 rs11538104

Abbreviation: microRNA, miRNA.

[a] Human miRNAs highlighted in bold were experimentally confirmed to be up- or downregulated in patients with non-obstructive azoospermia.[8]

[b] Mouse and human unless specifically specified.

[c] *DAZ* gene family contains genes *DAZ1*, *DAZ2*, *DAZ3* and *DAZ4*; +, below median; ++, median–10×median; +++, above 10×median; NA, data not available.

* Product from the opposite arm of the miRNA precursor.

DAZL), miR-590-3p (*DAZL*), miR-651 (*GNRHR*), miR-548a-3p (*RAD23A*), and mir-181a and miR-181d (both in *TNP1*).

Genes with high levels of testis-specific expression, polymorphic 3′-UTRs, and/or conserved miRNA target sites represent promising candidates for targets involved in miRNA-regulated pathogenesis of male infertility. Future analyses can now be directed toward sequencing the 3′-UTRs of the suggested candidate genes and identifying the genetic variability underlying miRNA regulation. However, the prediction and validation of such miRNA target genes represents a critical step, especially because there is no agreement about the experimental procedures that should be followed to demonstrate miRNA–mRNA interactions.[9] For example, negatively correlated coexpression of mRNA–miRNA pairs may indicate possible interactions; therefore, differential transcriptomic analyses of candidate genes and their putative miRNA target pairs in testes could be the first step in identifying such pairs. Putative miRNA–mRNA pairs can now be further experimentally confirmed,

and miR-TS-SNPs can be validated and analyzed for the effect of a gain or loss of miRNA binding sites. Our results imply that miR-TS-SNPs and miR-SNPs, in combination with expression patterns of miRNA-targeted genes, could influence the post-transcriptional regulation of fertility-associated genes and therefore contribute to male fertility problems.

In our opinion, this miRNA-related literature and database integration could be beneficial for researchers studying the involvement of miRNAs in male fertility and could present a starting point for the development of novel male infertility-associated genetic markers.

AUTHOR CONTRIBUTIONS

JO performed database searches and extracted information from publications. He participated in data analysis and draft writing. TK initiated and coordinated the project, supervised data collection and participated in data analysis and draft writing. PD participated in draft writing and final editing of the text. He is also mentor of JO and PI in the research programme from which the project was supported.

COMPETING FINANCIAL INTERESTS

The authors disclose any financial and personal relationships with third persons or organizations that could inapproproately bias or influence their research work.

ACKNOWLEDGMENTS

This work was supported by the Slovenian Research Agency, through the Comparative Genomics and Genome Biodiversity research program (P4-0220).

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