Bull Environ Contam Toxicol (2012) 89:1111–1114
DOI 10.1007/s00128-012-0827-0

A Microarray for MicroRNA Profiling in Spermatozoa from Adult Men Living in an Environmentally Polluted Site

Yan Li · Mingcai Li · Yuxin Liu · Gaihuan Song · Ningsheng Liu

Received: 12 July 2012 / Accepted: 12 September 2012 / Published online: 25 September 2012

Abstract We compared the miRNA expression profiles of spermatozoa from adult men living in an environment contaminated with electronic waste with those living in a normal environment, using a miRNA microarray. There were 73 significantly upregulated and 109 downregulated miRNAs in spermatozoa from men in the polluted environment compared with controls. Ten miRNAs were expressed significantly differently between the control and polluted samples and this result were further confirmed through quantitative real-time polymerase chain reaction amplification. A bioinformatics pipeline was developed to distinguish relationship with spermatogenesis. Our data indicated specific miRNAs expression of spermatozoa in men living in environmentally polluted sites and suggested that miRNAs have a role in regulating spermatogenesis. We also propose a subclassification scheme for miRNAs for assisting future experimental and computational functional analyses.

Keywords Microarrays · MicroRNA · Sperm · Environment pollution

Environmental pollution is one factor contributing to a recent decrease in human sperm quality (Sharpe 2010). Evidence for declining sperm counts, especially in young men in recent decades, indicates that the environmental impact on spermatogenesis is becoming an important health issue (Hammoud et al. 2010). Spermatogenesis produces mature male germ cells within the seminiferous tubules of the testis of the sexually mature man. Some researchers have reported that lead pollution might affect sperm chromatin by altering sperm zinc availability (Hernández-Ochoa et al. 2005). It was also reported that low levels of cadmium accumulation in semen might contribute to male infertility by reducing sperm quality (Wu et al. 2008). There is also evidence indicating that some pesticides affect sperm count. Thus, polychlorinated biphenyls are detrimental to sperm motility. Studies also suggested that ambient air pollutants might affect seminal characteristics. Mobile phones might affect the quality of semen, mostly by decreasing sperm mobility but also by decreasing viability, morphology and sperm counts (Jurewicz et al. 2009).

Rapid development of modern science and technology has lead to newest period of electronic products, thus large quantities of waste electrical and electronic equipment (e-waste) are generated. The e-waste recycling in China is often performed by family-run workshops using uncontrolled methods that damage the environment and threaten local people's health (Li et al. 2008a; Li et al. 2008b; Li et al. 2011). There are more than 1,000 different substances in e-waste, many of which are highly toxic. Several studies have reported pollution area residents to have high levels of toxic heavy metals (Wong et al. 2007) and the highest documented levels of atmospheric polychlorodibenzo-p-dioxins (PCDDs), polychlorodibenzofurans (PCDFs) and polybrominated diphenyl ether (PBDE) in the world (Li et al. 2007). Therefore, we have assumed that male reproductive health might be influenced by local e-waste environmental pollution.

Y. Li (✉) · M. Li · Y. Liu · N. Liu
Department of Anatomy, Histology and Embryology, Medical School of Ningbo University, 818 Fenghua Rd., Ningbo 315211, Zhejiang, China
e-mail: liyan@nbu.edu.cn

G. Song
Department of Gynaecology and Obstetrics, Affiliated Hospital of Ningbo University, Ningbo, Zhejiang, China

There has been much research on DNA, mRNAs and proteins in attempting to determine the effects of environmental pollution on spermatogenesis and in finding ideal biomarkers for the diagnosis and prevention of male reproduction disorders. Here we employed a microarray technique to explore the expression pattern of miRNAs and used the latest bioinformatics approaches to discover and analyze the miRNA expression profiles in spermatozoa from adult men living in an environmentally polluted site and in a control site. A miRNA is small non-coding RNAs that usually consist of 19–23 nucleotides and they play important roles in regulating posttranscriptional translation. During spermatogenesis, miRNAs regulate the post-transcriptional control of spermatid differentiation, such as miRNA-targeting sequences, in the 5′- and 3′-untranslated regions of miRNAs (Dadoune 2009). Studies of miRNA expression profiles have identified a number that are enriched in the mammalian testis (Ro et al. 2007; Yan et al. 2007), including a suite of novel miRNAs in human spermatozoa and some miRNAs that are expressed preferentially in mouse male germ cells, such as spermatogonia, pachytene spermatocytes, spermatids and spermatozoa (He et al. 2009). These observations suggest that miRNAs are likely involved in the regulation of gene expression during almost every developmental stage of spermatogenesis. Therefore, studying miRNA expression profiles can help in understanding how environmental pollution impacts on sperm quality and sperm counts.

Materials and Methods

Semen samples were collected from individual from an electronic waste (e-waste) environmentally polluted area. These were workers and other staff in factories engaged in dealing with e-waste. Control samples were collected from a Reproduction and Health Center; this is 100 km away from environmentally polluted area and does not have an e-waste factory. We excluded any men with congenital diseases, hypertension, diabetes mellitus, and symptoms of infection or reproductive diseases. All volunteers gave signed informed consent. The study was approved by the Human Ethics Committee of the Medical School of Ningbo University.

Participants were directed to abstain from ejaculation for 3–5 days before providing the semen. Semen was collected via masturbation into separate sterile glass beakers and incubated at 37°C for 30 min, allowing the semen to liquefy. The samples were subjected to 1,000×*g* centrifugation for 25 min to separate the seminal plasma. This was followed by gradient centrifugation through 60 % and 80 % (Percoll) at 1,000×*g* for 25 min. Smears were stained with propidium iodide to check for any contamination with leukocytes and to evaluate the integrity of the sperm membrane. Spermatozoa were stored at –80°C before extracting RNA.

Total RNA was extracted from pooled semen samples in each group using TRIzol according to the manufacturer's instructions. RNA quantification and quality assurance was evaluated using NanoDrop ND-1000 kits, and RNA integrity and genomic (g) DNA contamination were tested using denaturing agarose gel electrophoresis. Total RNA extracts were used for miRNA isolation and verification by quantitative real-time polymerase chain reaction (QRT-PCR).

miRNA microarrays were obtained from Exiqon or Agilent Corporation (Shanghai, China). After having passed RNA measurement on the Nanodrop instrument, the samples are labeled using the miRCURY^{TM} Hy3^{TM}/Hy5^{TM} Power labelling kit and hybridized on the miRCURY^{TM} LNA Array (v.14.0). Scanning is performed with the Axon GenePix 4000B microarray scanner. GenePix pro V6.0 is used to read the raw intensity of the image. The ratio of red signal to green signal was calculated after background subtraction and normalization using the global Lowess (Locally Weighted Scatter plot Smoothing) regression algorithm (MIDAS, TIGR Microarray Data Analysis System).

To verify the accuracy of the miRNA results we used QRT-PCR to measure the expression levels of individual miRNAs. Gene-specific PCR primers were designed according to miRNA sequences and a universal PCR reverse primer. The expression of the U6 small nuclear RNA gene was used as an internal control. RNA was reverse transcribed to cDNA with gene-specific primers. QRT-PCR cycle parameters for the reaction were 95°C for 15 min followed by 40 cycles of a 10 s denaturing step at 95°C and a 60 s annealing/extension step at 60°C. All reactions were run in triplicate. The relative amount of each miRNA to U6 RNA was calculated using the equation $2-\Delta Ct$, where $\Delta Ct = CtmiRNA - CtU6$.

Results and Discussion

For spectrophotometer, the OD A260/A280 ratio should be close to 2.0 for pure RNA (ratios between 1.8 and 2.1 are acceptable). The OD A260/A230 ratio should be more than 1.8. RNA quantification and quality assurance was verified using NanoDrop ND-1000 kits and the OD 260/280 ratio were 1.85, 1.98 respectively, the OD 260/230 were 2.05, 2.14 respectively in expose and control sample. RNA integrity and cDNA contamination testing were assessed using denaturing agarose gel electrophoresis.

From the study and control samples we identified 183 miRNAs with significantly different expression levels,

Table 1 Results carried out by QRT-PCR

miRNA name	Fold changes ($2^{-\triangle\triangle CT}$)[a]	miRNA name	Fold changes	miRNA name	Fold changes
U6	1	hsa-miR-183	1.85	hsa-miR-363	0.66
hsa-miR-208a	13.45	hsa-miR-205	1.65	hsa-let-7d	0.30
hsa-miR-155	9.32	hsa-miR-106a	1.80	hsa-miR-33b	5.24
hsa-miR-222	5.98	hsa-miR-10b	1.45	hsa-miR-223	3.84

[a] Fold changes displayed above had been all normalized by U6 as reference gene

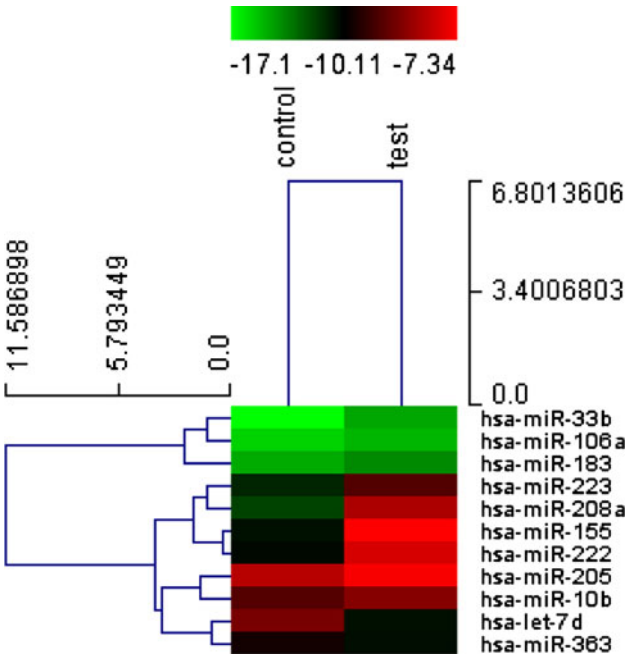

Fig. 1 MicroRNA's heat map: expression levels of the eleven miRNAs in the test and control samples. Calculated the miRNA relative expression levels normalized against *U6*

11 of which were effective probes and have conservative property in species. Thus only 11 miRNAs were analyzed and verified. These miRNAs included hsa-miR-205, hsa-miR-10b, hsa-miR-155, hsa-miR-208a, hsa-miR-222, hsa-miR-223, hsa-miR-33b, hsa-miR-106a, hsa-miR-183, hsa-let-7 d and hsa-miR-363. We compared the expression levels of the study and control samples. Using the equation above, we calculated the miRNA relative expression levels normalized against *U6*. The results are shown in Table 1 and Fig. 1. The QRT-PCR confirmed the downregulated expression of the two miRNAs. Two miRNAs were greatly upregulated in expression, namely hsa-miR-208a and hsa-miR-155. Seven other miRNAs increased their expression from 1.45- to 5.98-fold.

Cluster analysis was applied for all upregulated miRNAs; there were 25 signal pathways. The most significant signal was for the Notch pathway ($p = 2.01 \times 10^{-5}$) followed by the semaphorin interaction pathway ($p = 6.74 \times 10^{-5}$). GO enrichment analysis was used to analyze target genes, and showed that hsa-miR-10b and hsa-let-7d had a relationship with spermatogenesis (Table 2). The target genes of hsa-miR-10b were *BCL2L11* (−1.059), *DAZAP1* (−0.959) and *BCL6* (−0.323) and the target genes of hsa-let-7d were *YBX2* (0.184) and *STRBP* (1.668).

In studying the effects of environmental pollution on spermatogenesis, researchers have examined DNA, mRNA and protein, expecting to illustrate the mechanism of spermatogenesis and to find ideal biomarkers for the diagnosis and prevention of male reproductive disorders (Ehrmann and Elliott 2010; Bao et al. 2010; Buchold et al. 2010; Bouhallier et al. 2010). miRNAs are the regulators of mRNA, playing major roles in the control of gene expression (Meng et al. 2011; Papaioannou and Nef 2010; Marcon et al. 2008). The link between miRNAs and spermatogenesis affected by environmental pollution has not been proven. Therefore, in this study, we applied microarray chip analysis to test the possible relationship between spermatogenesis affected by environmental pollution and miRNAs. We identified 182 miRNAs, of which 73 were upregulated and 109 were downregulated in spermatozoa from men living in an environmentally polluted site. Cluster analysis and GO were used to evaluate the miRNA microarray results and 11 miRNAs were verified using QRT-PCR. In this study, all RNA from each sample group was pooled, so that individual differences were eliminated. The aim of our study was to test the relationship between environmental pollution and miRNAs and bring it to public attention with the aim of attracting further research. There were 182 miRNAs identified that

Table 2 Each miRNA regulated GO

miRNA name	Go name	Target genes	Fisher *p*
hsa-miR-10b	Spermatogenesis	BCL2L11(−1.059)||DAZAP1(−0.959)||BCL6(−0.323)	0.043065568
hsa-let-7d	Spermatid development	YBX2(0.184)||STRBP(1.668)	0.044788863

were differentially expressed in spermatozoa from adult men living in an environmentally polluted site that could help in the further study of spermatogenesis. Our data indicated specific miRNAs expression of spermatozoa in men living in environmentally polluted sites and suggested that miRNAs have a role in regulating spermatogenesis. We plan further research on the functional significance of these miRNAs.

Acknowledgments This work was supported by the Natural Science Foundation of Ningbo City, China (2011A610060); the Scientific Innovation Team Project of Ningbo (No. 2011B82014); the Scientific Research Fund of Zhejiang Provincial Education Department (Y201120665); the Academic Discipline Project of Ningbo University (XKL11D2112); the Natural Science Foundation of Zhejiang Province, China (LY12H04002); and Student Research and Innovation Program of Ningbo University. Microarray experiments were performed by Kang Chen Bio-tech, Shanghai, China. The research was sponsored by K.C. Wong Magna Fund in Ningbo University.

References

Bao J, Zhang J, Zheng H, Xu C, Yan W (2010) UBQLN1 interacts with SPEM1 and participates in spermiogenesis. Mol Cell Endocrinol 327:89–97

Bouhallier F, Allioli N, Lavial F, Chalmel F, Perrard MH, Durand P, Samarut J, Pain B, Rouault JP (2010) Role of miR-34c microRNA in the late steps of spermatogenesis. RNA 16: 720–731

Buchold GM, Coarfa C, Kim J, Milosavljevic A, Gunaratne PH, Matzuk MM (2010) Analysis of microRNA expression in the prepubertal testis. PLoS ONE 5:e15317

Dadoune JP (2009) Spermatozoal RNAs: what about their functions? Microsc Res Tech 72:536–551

Ehrmann I, Elliott DJ (2010) Expression and functions of the star proteins Sam68 and T-STAR in mammalian spermatogenesis. Adv Exp Med Biol 693:67–81

Hammoud A, Carrell DT, Gibson M, Sanderson M, Parker-Jones K, Peterson CM (2010) Decreased sperm motility is associated with air pollution in Salt Lake City. Fertil Steril 93:1875–1879

He Z, Kokkinaki M, Pant D, Gallicano GI, Dym M (2009) Small RNA molecules in the regulation of spermatogenesis. Reproduction 137:901–911

Hernández-Ochoa I, García-Vargas G, López-Carrillo L, Rubio-Andrade M, Morán-Martínez J, Cebrián ME, Quintanilla-Vega B (2005) Low lead environmental exposure alters semen quality and sperm chromatin condensation in northern Mexico. Reprod Toxicol 20:221–228

Jurewicz J, Hanke W, Radwan M, Bonde JP (2009) Environmental factors and semen quality. Int J Occup Med Environ Health 22:305–329

Li H, Yu L, Fu J, Peng P (2007) Severe PCDD/F and PBDD/F pollution in air around an electronic waste dismantling area in China. Environ Sci Technol 41:5641–5646

Li Y, Xu X, Liu J, Wu K, Gu C, Shao G, Chen S, Chen G, Huo X (2008a) The hazard of chromium exposure to neonates in Guiyu of China. Sci Total Environ 403:99–104

Li Y, Xu X, Wu K, Chen G, Liu J, Chen S, Gu C, Zhang B, Zheng L, Zheng M, Huo X (2008b) Monitoring of lead load and its effect on neonatal behavioral neurological assessment scores in Guiyu, an electronic waste recycle town in China. J Environ Monit 10:1233–1238

Li Y, Huo X, Liu J, Peng L, Li W, Xu X (2011) Assessment of cadmium exposure for neonates in Guiyu, an electronic waste pollution site of China. Environ Monit Assess 177:343–351

Marcon E, Babak T, Chua G, Hughes T, Moens PB (2008) miRNA and piRNA localization in the male mammalian meiotic nucleus. Chromosome Res 16:243–260

Meng YN, Meng LJ, Song YJ, Liu ML, Zhang XJ (2011) Small RNA molecules and regulation of spermatogenesis. Yi Chuan 33:9–16

Papaioannou MD, Nef S (2010) microRNAs in the testis: building up male fertility. J Androl 31:26–33

Ro S, Park C, Sanders KM, McCarrey JR, Yan W (2007) Cloning and expression profiling of testis-expressed microRNAs. Dev Biol 311:592–602

Sharpe RM (2010) Environmental/lifestyle effects on spermatogenesis. Philos Trans R Soc Lond B Biol Sci 365:1697–1712

Wong CS, Wu SC, Duzgoren-Aydin NS, Aydin A, Wong MH (2007) Trace metal contamination of sediments in an e-waste processing village in China. Environ Pollut 145:434–442

Wu HM, Lin-Tan DT, Wang ML, Huang HY, Wang HS, Soong YK, Lin JL (2008) Cadmium level in seminal plasma may affect the pregnancy rate for patients undergoing infertility evaluation and treatment. Reprod Toxicol 25:481–484

Yan N, Lu Y, Sun H, Tao D, Zhang S, Liu W, Ma Y (2007) A microarray for microRNA profiling in mouse testis tissues. Reproduction 134:73–79