Detection of Y chromosome microdeletions in recurrent abortions among Egyptian females using SYBR Green Real Time PCR

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Abstract: Objectives: Many researches concerned with etiologies behind recurrent pregnancy loss (RPL) have focused mainly on female factors, with a lesser attention to male factors. Based on previous studies in different populations, Y chromosome microdeletions were the key words to unveil the real problem for many RPL couples. In contrast however, other studies reported no association between Y chromosome microdeletions and recurrent miscarriages. The current study aimed at determining the possible correlation between Y chromosome microdeletions and RPL Egyptian couples. Study Design: The study involved blood sample collection from male partners of 80 couples (40 couples with unexplained RPL and 40 healthy fertile couples as controls). DNA was extracted and purified and analyzed for Y chromosome microdeletions using PCR primers for the amplification of three critical loci AZF-1, AZF-b, and AZF-c regions on the long arm of the Y chromosome. Results and Conclusion: Our results showed 10% of the investigated RPL couples to have Y chromosome microdeletions. In contrast, the remaining RPL couples in addition to the entire control group did not show any microdeletions. In conclusion, Y chromosome microdeletions are potentially associated with unexplained RPL in 10% of Egyptian couples.
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Key Words: Recurrent Pregnancy Loss (RPL), Y chromosome, AZF regions, microdeletions

Introduction

Recurrent pregnancy loss (RPL) is defined by the spontaneous loss of a pregnancy up to the 20th week of gestation affecting 0.5-3% of couples trying to conceive [1, 2]. Different etiological factors can lead to spontaneous RPL including anatomical, endocrinologic, hematologic, immunologic and genetic abnormalities of the parents [2, 3]. Usually clinical investigations of couples suffering from RPL focus mainly on the female partner but rarely on male partners. Nevertheless, half of the RPL cases remain unexplained [4]. On the other hand, the contribution of males to RPL has been less investigated and limited to karyotyping analysis only. However, it was evidenced that male factors can potentially affect fertilization, embryo development, viability and placental proliferation and differentiation of trophoblast cells where paternally expressed genes were found to modulate the proliferation and invasiveness of trophoblast cells and placental proliferation [5-8].

Approximately 1-4% of couples with recurrent have cytogenetic abnormalities [2]; nevertheless, cytogenetic analysis cannot detect small deletions, substitutions, duplications and translocations. Miscarriages of unknown causes might be attributed to the presence of these small mutations in the genome of one of the parents. Y chromosome microdeletions were first hypothesized by Tiepolo and Zuffardi to be associated with male infertility. Later on, several researches confirmed the potential association between such microdeletions and RPL [9, 10]. There are three critical candidate regions on the long arm of the Y chromosome for male infertility [11]. The first region is azoospermic factor region a (AZFa) that contains DBY (DDX3y) encoding a RNA-helicase with a DEAD box [12]. The second region, AZFb, possesses several genes among which is RBMY that encodes a RNA-binding protein functioning in slicing [13, 14] and the third region is AZFc that has the DAZ family [15, 16]. AZFc is the most frequently deleted in males with azoospermia or oligozoospermia [17]. Those regions have never been studied in correlation with RPL among Egyptian couples. In the current study, we

investigated the potential association of Y chromosome microdeletions with RPL in Egyptian couples using SYBR Green Real Time PCR quantification for the first time.

Materials and Methods

Subjects

For this case-control genetics association study 80 couples were recruited. 40 couples suffering fromunexplained RPL, who were referred to the Maternity Hospital of the Ain Shams University in Cairo, Egypt, were included as cases in this study for further evaluation. The inclusion criteria for the case couples were women in the child-bearing period aged from 18 to 40 years who had experienced 2 or more first trimester abortions, and had no apparent causes of RPL. Careful history regarding the mode, number and timing of abortions was taken from each case. Oral glucose tolerance test and measurements of FSH, LH, prolactin, and TSH were performed for the females in the cases. Also, hysteroscopic evaluation and antiphospholipid markers were assessed. Exclusion criteria consisted of presence of uterine structural anomalies, endocrinal disorder, or positive antiphospholipid markers. The husbands of women fulfilling these criteria were used in the experiments; the selected study cohort consisted of males aging from 20-50 years not suffering any clinical disorders (Table 1).

Besides the 40 case couples, another 40 couples were included in this study as controls. These couples have at least one healthy offspring with no history of any abortions in their marital life. The males from these couples were also aged from 20-50 years and did not suffer from any clinical disorders such as diabetes, hypertension and cardiac disorders. A written, informed consent was obtained from each couple in both the case and control groups. All procedures in this study were approved by the Ethical Committee of Ain Shams Maternity Hospital and Ain Shams University.

Table 1: Average age of the 40 couples suffering from spontaneous RPLs

Number of RPLs	Mean age (years)		Number of couples	Percentage of couples
	Male	Female		
2	28	25	15	37.5
3	30	26	11	27.5
4	35	28	8	20
5	35	33	4	10
More than 5	38	35	2	5
Total			**40**	**100%**

Methods

Sample Collection and DNA Extraction

From the 80 males, 10ml of peripheral blood was collected in the presence of an anticoagulant (EDTA) and genomic DNA was extracted using the QIAamp DNA blood mini extraction kit (Qiagen, Germany) according to the manufacturer's protocol. DNA quality and concentration were determined spectrophotometrically and by gel electrophoresis. The AZF regions, AZFa, AZFb and AZFc, in the Yq AZF region of the Y chromosome were screened for microdeletions using SYBR Green real time PCR amplification method. For each DNA sample amplification of DBY gene (in the AZFa region), RBMY gene (in the AZFb region), and DAZ gene (in the AZFc region) was performed to detect microdeletions at these loci. The SRY gene (sex-determining region on the short arm of the Y chromosome) and X-chromosomal AMELX were also amplified in each DNA sample as controls. The primers employed in this study belong to the sequence-tagged sites (STSs) specifically mapping to the corresponding target genes on the Y and the X chromosomes, as previously described [18].

Conventional PCR Amplification and Capillary Electrophoresis

Initially, successful amplification of the abovementioned X and Y chromosomal gene regions by the primer pairs was ensured using multiplex conventional PCR amplification followed by separation of amplicons by capillary electrophoresis. For these amplifications, the 20 µL reaction mix consisted of 2.5 units of AmpliTaq Gold DNA Polymerase, LD (Applied Biosystems, USA), 2 µL GeneAmp 10X PCR Gold Buffer (containing 150 mM Tris-HCl, 500 mM KCl, 15 mM $MgCl_2$, pH 8.0) (Applied Biosystems, USA), 0.25µM dNTP mix (Qiagen, Germany), 75 ng genomic DNA, and 0.5µM of each primer for DBY, RBMY, DAZ and SRY, and 1µM of each primer for AMELX. Amplification was carried out by an initial incubation for 10 minutes at 94°C, followed by 30 cycles of 30 seconds at 94°C, 60 seconds at 56°C and 60 seconds at 72°C, then a final step of 72°C for 10 min. The PCR reactions were prepared using DNA samples purified from a female as a negative control and using DNA samples from a fertile male as a positive control. The PCR amplification products were analyzed using the QIAxcel System for high-resolution capillary

electrophoresis and the BioCalculator Analysis software (Qiagen, Germany). This method is based on size-separation of nucleic acid molecules by applying an electrical current to a gel-filled capillary. A detector in the QIAxcel instrument detects the nucleic acid molecules as they migrate towards the positively charged terminus of the capillary. These data are then passed through a photomultiplier before being converted to an electropherogram and gel image by the BioCalculator Anaylsis software. Figure 1A shows the electropherogram of the negative control where only a peak corresponding to AMELX gene can be seen. Figures 1B and 1C are the electropherogram and the gel image of the positive control showing peaks and bands for each of the five genes amplified, respectively.

In addition, singlet conventional PCR amplifications were performed on these DNA samples and the amplicons were separated on a 2.5% agarose gel impregnated with ethidium bromide, and visualized under UV light (Figure 1D).

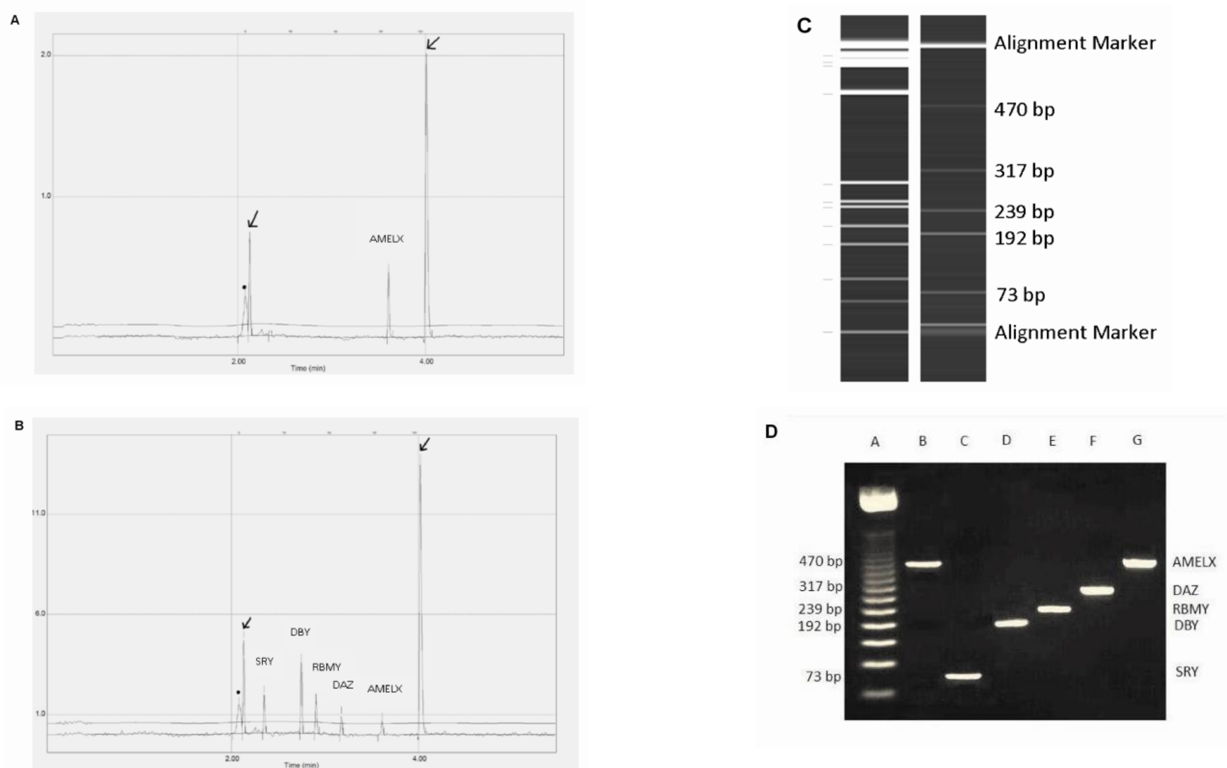

Figure 1: Analysis of amplification products from negative and positive controls by high-performance capillary electrophoresis and gel electrophoresis.

(A) The electropherogram of the multiplex reaction using DNA from a female (negative control). The arrows indicate the Alignment Marker. The peak marked by a dot (•) represents primer dimers. The electropherogram shows only one peak corresponding to AMELX gene.

(B) The electropherogram of the multiplex reaction using DNA from a fertile male (positive control). The arrows indicate the Alignment Marker and the peak marked by a dot (•) represents primer dimers. The electropherogram shows five peaks corresponding to each of the five amplified genes.

(C) The size marker (left) and gel image (right) of the multiplex reaction using DNA from a fertile male (positive control). The uppermost and lowermost bands are the Alignment Markers; in between a band for SRY (73bp), DBY (192bp), RBMY (239bp), DAZ (317bp), and AMELX (470 bp) can be identified.

(D) Electrophoresis of the amplification products from singlet conventional PCR reactions for the negative and positive controls on a 2.5% agarose gel. Lane A is the molecular weight marker (100 bp ladder). Lane B is the AMELX (470bp) amplification product from the female negative control. Lanes C to G are the SRY (73bp), DBY (192bp), RBMY (239bp), DAZ (317bp), and AMELX amplification products from the fertile male positive control, respectively.

Detection of Y Chromosome Microdeletions Using Qualitative SYBR Green PCR

Finally, amplification using the SYBR Green PCR reaction was carried out on all case and control DNA samples using 25µL reaction mixes containing 850ng of genomic DNA, 0.5µM (for DBY, RBMY, DAZ and SRY) or 1µM (for AMELX) of each primer, 12.5µL 2X Brilliant II SYBR Green QPCR master mix (containing SureStart® Taq DNA polymerase, dNTP mix, 2.5mM $MgCl_2$, and SYBR Green I dye) (Stratagene, Agilent Technologies, USA), and 0.375 µL ROX reference dye. Amplification conditions began with an initial incubation for 10 minutes at 94°C for DNA Polymerase activation, followed by 40 cycles of 30 seconds denaturation at 94°C, 60 seconds of annealing at 56°C and 60 seconds elongation at 72°C. The amplification was completed by a final extension step at 72°C for 10 min.

After the amplification process, melting curves were obtained for each reaction by the following dissociation conditions: incubation of the reaction for 15 seconds at 94°C to denature the PCR amplification products, ramping down to 56°C, followed by ramping up the temperature from 56°C to 94°C at +0.3°C/second, collecting the fluorescence continuously on the 56-94°C ramp. Therefore for each patient, a total of 5 reaction mixes were prepared; for each reaction mix an amplification plot indicating the cycle threshold (CT) value for the gene of interest, and a melting curve demonstrating the melting point ($T_{melting}$) of the amplification product were obtained. When gene amplification is successful, $T_{melting}$ is high, usually ranging between 75-85˚C. In cases of microdeletions, no amplification of the gene occurs therefore CT value is undetermined, and $T_{melting}$ is lower, usually ranging between 60-65˚C.

Lack of non-specific amplification using SYBR Green PCR was confirmed for each primer set with a no-template control using reaction mixes where DNA was substituted with sterile nuclease-free PCR-grade water; no amplification curves was observed for these reactions (Figure 2A and 2B). Moreover, the ability of the primer sets to successfully amplify each gene was confirmed using a DNA sample from a fertile male as a positive control (Figure 2C and 2D).

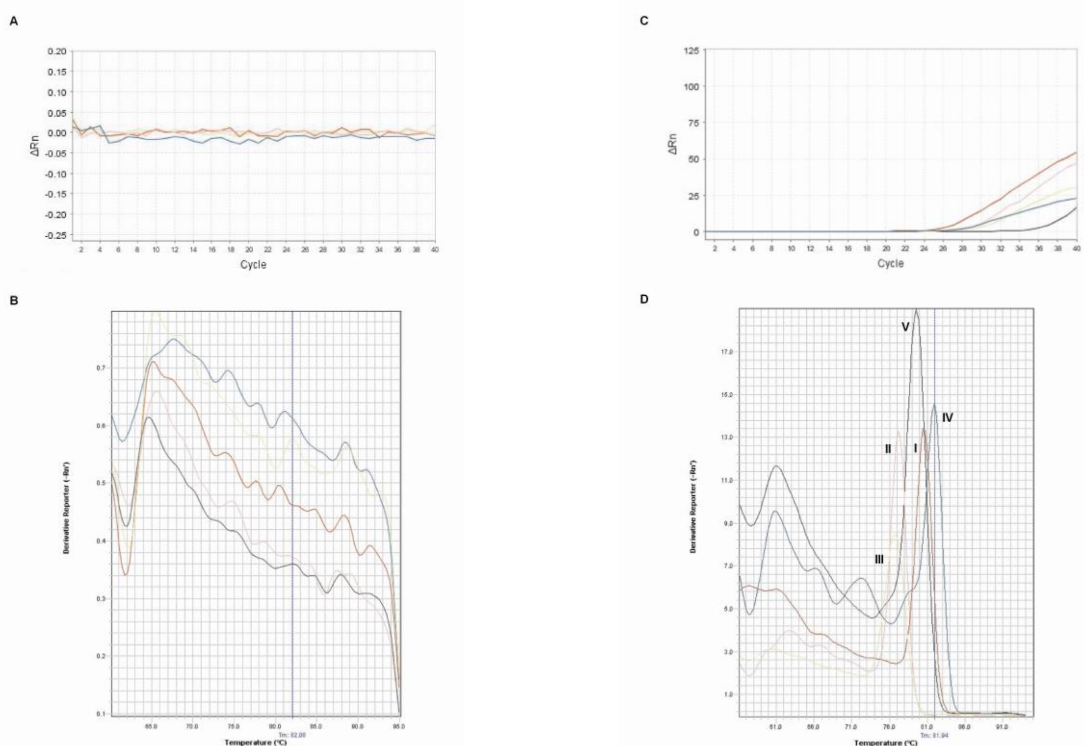

Figure 2: Amplification plots and melting curves of the controls. A no-template control was prepared for each primer set to ensure the lack of non-specific amplifications; in these reaction mixes DNA was replaced with sterile nuclease-free PCR-grade water. Also, positive controls using DNA from a fertile male were utilized to confirm the ability of the primer sets to successfully amplify each gene. All graphs show 5 curves: DBY, RBMY, DAZ, SRY, and AMELX; the 5 reactions were pipetted into

separate reaction tubes however the results of all reactions have been superimposed onto one graph.

(A) The five superimposed amplification curves of the no-template control. The absence of any amplification curve indicates that none of the primer sets led to non-specific amplification.

(B) The five superimposed melting curves of the no-template control. The absence of any amplification curve indicates that none of the primer sets led to non-specific amplification.

(C) The five superimposed amplification curves of the positive control, where I is RBMY, II is DBY, III is SRY, IV is DAZ, and V is AMELX. This fertile male has no Y chromosome microdeletions therefore an amplification curve is detected for each gene, indicating successful amplification by the primer sets.

(D) The five superimposed melting curves of the positive control, where I is RBMY, II is DBY, III is SRY, IV is DAZ, and V is AMELX. The Tm of all products is in the range of 76-82°C, signifying target gene amplification products and not primer-dimers.

Results

Of the 40 case samples obtained from males of couples with unexplained RPLs, only 4 samples (10%) showed single microdeletions in the Y chromosome. Two of these microdeletions were in RBMY gene in the AZFb region; one microdeletion was in DBY of the AZFa region, and the other in DAZ gene in the AZFc region (Table 2 and Figure 3). None of the 40 control samples obtained from fertile males displayed any deletions in the Y chromosome among the 5 genes tested (0%).

Table 2: Cycle Threshold (CT) values and Melting Temperatures ($T_{melting}$) for the five amplified genes in 40 males from couples suffering spontaneous RPLs

Patient #	DBY		RBMY		DAZ		SRY		AMELX	
	CT value	$T_{melting}$	CT value	$T_{melting}$	CT value	$T_{melting}$	CT value	$T_{melting}$	CT value	$T_{melting}$
1	27.93	77.16	24.03	80.31	27.94	81.65	29.59	76.26	36.99	78.81
2	33.90	77.30	30.65	80.60	34.92	81.94	34.27	76.41	37.04	79.40
3	27.50	77.30	25.93	80.75	26.04	81.94	28.56	76.70	25.84	79.70
4	31.25	77.29	*undetermined*	*61.73*	36.00	81.66	32.89	76.27	35.93	79.71
5	28.10	77.17	25.90	80.61	28.05	81.81	30.94	76.57	29.44	79.71
6	29.94	76.27	25.73	80.46	27.51	81.66	29.86	76.42	28.12	79.71
7	24.68	77.17	22.51	80.31	24.94	81.81	24.83	76.17	24.12	79.71
8	26.71	77.02	23.82	80.31	26.44	81.51	28.08	76.42	26.87	79.71
9	26.67	77.44	21.76	78.15	*undetermined*	*64.23*	26.68	76.69	27.49	79.98
10	27.93	77.29	24.92	80.58	26.94	81.62	29.98	76.54	27.94	79.98
11	28.59	77.01	26.77	80.31	28.64	81.50	30.95	76.41	35.37	78.81
12	29.75	77.30	26.31	80.60	27.87	82.09	30.16	76.56	29.13	79.55
13	25.28	77.15	22.62	80.60	22.30	81.94	26.95	76.70	24.37	79.55
14	28.97	77.02	26.60	80.16	27.49	81.66	31.60	76.27	30.58	79.56
15	30.94	77.02	28.94	80.46	30.25	81.51	32.39	76.12	30.73	79.56
16	28.42	77.02	25.46	80.31	27.22	81.51	28.51	76.42	27.97	79.71
17	29.73	77.17	27.83	80.16	30.13	81.66	31.34	76.27	31.97	79.71
18	24.18	77.29	21.69	80.58	22.24	81.77	25.47	76.69	24.31	79.83
19	28.84	77.44	25.05	80.58	25.06	81.77	27.93	76.39	28.02	79.83
20	28.09	77.29	23.75	80.43	26.74	81.62	28.24	76.54	28.72	79.98
21	31.54	77.29	28.18	80.58	29.40	81.77	32.07	26.39	33.13	79.83
22	30.59	77.29	*undetermined*	*62.33*	28.46	81.62	29.80	76.24	29.04	76.68
23	29.81	77.29	26.37	80.43	26.14	81.62	29.09	76.39	29.56	79.83
24	31.60	77.29	26.75	80.43	29.86	81.77	30.32	76.39	31.81	79.83
25	*undetermined*	*61.24*	31.94	80.43	34.90	67.30	34.94	76.40	35.96	79.83
26	31.73	77.29	28.96	80.43	32.47	81.63	32.24	76.25	32.61	79.83
27	34.90	77.14	30.60	80.43	33.92	81.48	33.29	76.25	35.95	79.83
28	31.40	77.14	29.97	80.43	32.87	81.48	34.93	76.25	33.15	82.82
29	25.93	77.17	23.64	80.47	24.92	81.81	27.96	76.57	26.98	79.72
30	24.58	77.01	22.96	80.31	23.99	81.50	26.76	76.41	30.87	78.81
31	25.93	76.25	31.40	81.48	29.97	82.82	33.15	77.17	29.97	78.81
32	25.93	82.82	33.15	80.43	31.40	77.14	32.87	76.25	26.76	77.17
33	29.97	78.81	33.15	82.82	25.93	77.14	26.76	80.43	29.97	80.31

34	32.87	82.82	22.96	81.48	26.76	77.14	33.15	76.25	25.93	77.14
35	26.76	76.25	29.97	80.43	31.40	78.81	32.87	81.48	22.96	80.31
36	33.15	77.14	33.15	78.81	31.40	80.31	22.96	80.43	31.40	77.17
37	25.93	78.81	22.96	81.48	32.87	76.25	31.40	82.82	25.93	77.17
38	32.87	77.14	29.97	76.25	29.97	80.43	25.93	81.48	29.97	77.17
39	22.96	77.14	32.87	82.82	33.15	80.43	33.15	80.43	31.40	81.48
40	33.15	78.81	32.87	77.14	25.93	76.25	22.96	82.82	31.40	76.25

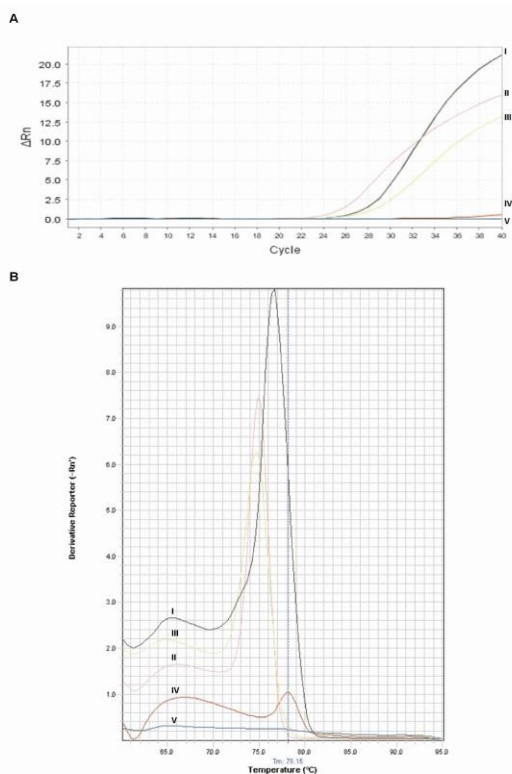

Figure 3: Amplification plots and melting curves of the five amplified genes in patient 9 with DAZ microdeletion. Each graph shows curves for DBY, DAZ, RBMY, SRY, and AMELX; the 5 reactions were pipetted into separate reaction tubes however the results of all reactions have been superimposed onto one graph. In both graphs I is AMELX, II is SRY, III is DBY, IV is RBMY, and V is DAZ. (A) The five superimposed amplification curves of patient 9. Amplification is detected for DBY, RBMY and the two control genes SRY and AMELX; however no amplification curve can be detected for DAZ. (B) The five superimposed melting curves of patient 9. The Tms of the four successfully amplified gene products is high (75-80°C) while for DAZ the Tm curve peaks at a low temperature (around 65°C) indicating primer-dimers and no gene amplification.

Discussion

Recurrent pregnancy loss affects approximately 0.5-3% of all couples [1, 2], with unknown causes for half of the presented cases [7]. Routine clinical investigations mostly consider the female partner whereas the male factor is neglected to a large extent [2]. In the present study, we focused on investigating the presence of microdeletions in some critical regions within the long arm of the Y chromosome, the AZF regions. These regions are typically responsible for normal spermatogenesis [17], consequently, presence of microdeletions in such essential regions affects the sperm quality negatively and this is highly suggested to be associated with RPL [19-21]. Therefore, this study was designed to explore the correlation of these microdeletions with RPLs among Egyptian couples using SYBR Green Real Time PCR analysis for the first time. According to previous researches, complete deletion of AZFb and AZFc showed a direct impact on early prophase and decreased the rate of normal pairing in pachytene stage of spermatocytes which then increase chromosomal abnormalities; these chromosomal abnormalities could be related to recurrent miscarriage [22, 23]. In addition, recent studies reported a high incidence of RPLs among couples with at least one AZF region deletion on the long arm of the Y chromosome [9]. These findings stand in agreement with our results. In this study, we showed that 10% (4/40) of the investigated RPL couples had AZF region microdeletions on the long arm of the Y chromosome. However, healthy fertile males did not show any microdeletions. Two out of the four males had AZFb microdeletions (RBMY) while the other two males possessing microdeletions each had one deletion in either the AZFa (DBY) or AZFc (DAZ) regions. Nevertheless, our findings contradict other studies that found no association between AZF region microdeletions and recurrent miscarriages in Sinhalese and Iranian populations [24, 25]. These contradicting results might be explained by the possibility that the presence of Y chromosome microdeletions and its association with RPL is demographic or ethnic-specific. Therefore, the recommendation of performing this test for RPL couples needs to be extensively tested in a wide pool of patients.

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