INTRODUCTION

The male factor can account for up to 50% of infertility when included in combination with the female factor. A majority of male infertility cases are related to the dysfunction of spermatogenesis. Moreover, genetic abnormalities are one of the obvious causes of testicular dysfunction. Y chromosome microdeletion and the numerical and structural abnormalities of chromosomes are clinically important genetic abnormalities associated with male infertility. The guidelines published by the American Society for Reproductive Medicine or European Academy of Andrology (EAA) and European Association of Urology state that "genetic tests for severe oligozoospermia and azoospermia are recommended." 1, 2 Previously, we had been examining Y chromosome microdeletion using classical methods; however, the sensitivity of the markers recommended by the EAA guidelines was low when assessed using these methods in Japanese cases. Thus, we developed a polymerase chain reaction-reverse sequence-specific oligonucleotide (PCR-rSSO) with microbead suspension array for Y chromosome microdeletion screening in the Japanese population that is currently being widely used in Japan. 3 Using this new method, minor deletions, including gr/gr deletions, can be detected along with major deletions using 21 sequence-tagged site (STS) markers. It is well known that gr/gr deletion is present in a third of the Japanese male population. To the best of our knowledge, this is the first multicenter study investigating the clinical data of Japanese male patients with infertility examined using the new Y chromosome microdeletion detection method.

PATIENTS AND METHODS

In this study, 1072 male patients with infertility who underwent testing using the GENOSEARCH™ AZF Deletion kit (MBL, Nagoya, Japan) for detecting Y chromosome microdeletions between April 2014 and December 2016 at eight hospitals in Japan (Kanazawa University Hospital in Kanazawa; Suzuki Lady’s Hospital in Kanazawa; Kyono ART Clinic Sendai in Sendai; Kyono ART Clinic Takanawa in Tokyo; Ebisu Tsuji Clinic in Tokyo; Tenjin Tsuji Clinic in Fukuoka; Dokkyo Medical University Kosigaya Hospital in Kosigaya; and Toho University Medical Center Omori Hospital in Tokyo) were enrolled. We analyzed hormone levels (e.g., luteinizing hormone [LH], follicle-stimulating hormone [FSH], and total testosterone), patient backgrounds (comorbidity, medical histories, surgical histories, and drug histories), chromosome karyotypes, semen parameters, and results of testicular sperm extraction (TESE). Patients with ejaculatory dysfunction, a history of chemotherapy or radiation therapy, or hypogonadism were excluded from the analysis.

Keywords: azoospermia factor; Japanese infertile men; sperm recovery rate; Y chromosome microdeletion

The azoospermia factor (AZF) region is important for spermatogenesis, and deletions within these regions are a common cause of oligozoospermia and azoospermia. Although several studies have reported this cause, the present research, to the best of our knowledge, is the first large-scale study assessing this factor in Japan. In this study, 1030 male patients with infertility who were examined for Y chromosome microdeletion using the polymerase chain reaction-reverse sequence-specific oligonucleotide (PCR-rSSO) method, a newly developed method for Y chromosome microdeletion screening, were included. The study enrolled 250 patients with severe oligospermia and 717 patients with azoospermia. Among the 1030 patients, 4, 4, 10, and 52 had AZFa, AZFb, AZFb+c, and AZFc deletions, respectively. The sperm recovery rate (SRR) of microdissection testicular sperm extraction in patients with AZFc deletions was significantly higher than that in those without AZF deletions (60.0% vs 28.7%, P = 0.04). In patients with gr/gr deletion, SRR was 18.7%, which was lower than that in those without gr/gr deletion, but was not statistically significant. In conclusion, our study showed that the frequency of Y chromosome microdeletion in male patients in Japan was similar to that reported in patients from other countries, and SRR was higher in patients with AZFc deletion.

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Y chromosome microdeletions were assessed at a clinical laboratory (SRL, Inc., Tokyo, Japan) using the GENOSEARCH™ AZF Deletion kit based on the PCR-rSSO method which combines multiplex PCR and hybridization. This kit was developed to solve the problem that the sensitivity of STS markers recommended by EAA is insufficient for the Japanese population, by reselecting STS markers and improving detection methods. Genomic DNA extracted from the patients’ peripheral blood lymphocytes was amplified using multiplex PCR and detected with a bead suspension array. In this method, the following 20 STS probes in the Y chromosome were selected: SY1324, SY1316, and SY1714 in the AZFa region; SY1024, SY1967, SY1309, SY1399, SY2333, SY3010, SY2990, SY1197, SY1911, SY1307, SY1291, SY2858, and SY1206 in the AZFb and AZFc regions; SY14 and SY3118 in the short arm of the Y chromosome; and SY1251 and SY3159 outside the AZF region in the long arm of the Y chromosome.

TESE was performed at each hospital by several doctors. The results of microdissection TESE (microTESE) for nonobstructive azoospermia (NOA) with an FSH level of >10 IU l⁻¹ and without Klinefelter syndrome (KS) were compared between patients with and without Y chromosome microdeletions.

Statistical analysis was performed using the SPSS 22.0 software (IBM Corp., Armonk, NY, USA). Continuous variables were analyzed using an unpaired t-test, and categorical variables were analyzed using the Chi-square test. P < 0.05 was considered statistically significant. The study was a retrospective study, and instead of obtaining informed consent, we informed the patient by posting a written description of the study. This study was approved by the Ethics Committee of the Kanazawa University (Kanazawa, Japan) and complies with the Code of Ethics of the World Medical Association (Declaration of Helsinki 1964, revised in 2013).

RESULTS

Among 1072 patients who were tested using the PCR-rSSO method for Y chromosome microdeletion screening, 42 patients with ejaculatory dysfunction, a history of chemotherapy or radiation therapy, or hypogonadism were excluded. Of these, 250 had severe oligozoospermia (sperm count <500 × 10⁶ ml⁻¹), 717 had azoospermia, and the results of semen analysis were unknown among 63 patients. Chromosomal karyotyping revealed that 797 patients had no abnormalities, 83 had KS, 16 had Y chromosome structural abnormalities, 6 had Robertsonian translocations, and 11 had other abnormalities. Y chromosome structural abnormalities included 46,X,del(Y)(q11.23); 46,X,+mar, 46,X,Y; 46,XY,inv(Y); 46,X,+mar/45X; and 46,XY,inv(Y). Table 1 shows the deletion patterns for all 1030 patients. Each deletion pattern in Y chromosome long arm is shown in Figure 1. In the 250 patients with severe oligospermia, AZFc deletion was observed in 20 (8.0%) patients. Among the 83 patients with KS, AZFc deletion was found in 1 (1.2%) patient. Table 2 shows the clinical data of 249 patients who underwent micro TESE (patients with KS and FSH level <10 IU l⁻¹ were excluded). Patients with AZFc deletion had significantly higher sperm recovery rate (SRR) compared with those without AZFc deletion (P < 0.05). The SRR of patients with gr/gr deletion tended to be lower than that of those without deletion, but without significant difference (P = 0.09). Table 3 shows the data of patients with oligozoospermia with AZFc deletions and all patients with azoospermia (including FSH level <10 IU l⁻¹) who underwent micro TESE. In addition, LH and FSH levels were significantly higher in patients with azoospermia than those in patients with oligospermia (P < 0.05); however, patients with azoospermia in whom sperms could not be recovered were significantly older than those with oligozoospermia (P < 0.05).

DISCUSSION

Frequency of major AZF deletions

AZF is a region comprising numerous genes that are important for spermatogenesis and is located in the male-specific region of the Y chromosome long arm. Due to the palindrome structure in this region, chromosomal homologous recombination causes microdeletion, which cannot be detected in the G-band. Tiepolo and Zuffardi first reported AZF in 1976, and subsequently, the deletion patterns were classified into AZFa, AZFb, and AZFc by Vogt et al. in 1996. Many studies on AZF have been conducted; however, there are only a few reports involving approximately 1000 patients. The frequency of AZF deletions in large-scale studies with approximately 1000 patients is shown in Table 4, demonstrating that the frequency of deletion varies depending on the ethnic composition of the population. Among the 717 patients with azoospermia in the present study, AZFa, AZFb, AZFc, AZFb+c, and AZFa+b+c deletions were observed in 0.6%, 0.6%, 3.9%, 1.3%, and 1.1% of the patients, respectively, which were similar to those reported in previous studies. In patients with severe oligozoospermia, the AZFc deletion was observed in 8.0% of patients, which was higher than that in patients with azoospermia. To detect AZFc deletions, the EAA/European Molecular Quality Network (EMQN) guidelines recommended Y chromosome microdeletion screening at a sperm count of <5 × 10⁶ ml⁻¹, whereas Johnson et al. reported that even if this level was reduced to 0.5 × 10⁶ ml⁻¹, the sensitivity to detect AZFc deletion did not decrease. However, in the present study, one patient had a sperm count of 2 × 10⁶ ml⁻¹; therefore, the threshold <0.5 × 10⁶ ml⁻¹ is not considered appropriate for screening for the Japanese population. Y chromosome microdeletion screening is strongly recommended in cryptozoospermia because 12 out of 20 patients with severe oligozoospermia with AZFc deletions had cryptozoospermia in our study.

Frequency of minor AZF deletions

The PCR-rSSO method used in this study enabled the detection of minor deletions in addition to major deletions. In the present study, b1/b3, b2/b3, and gr/gr deletions, known as the partial deletions of the AZF region in the long arm of the Y chromosome.
Large-scale study of AZF deletion in Japan
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AZFc, were detected in 0.3%, 1.4%, and 33.3% patients, respectively. Analysis of approximately 20,000 patients revealed that the frequency of b1/b3 and gr/gr deletions was 0.25% and 4.1% and the odds ratio was 1.9 and 2.5 times higher than those without deletion in the severe spermatogenic failure (SSF) with the sperm concentration <5 × 10^6 ml^-1, b2/b3 deletion was not a risk factor of SSF. Meanwhile, influences of minor deletion differ depending on the population; Krausz and Casamonti reported that b2/b3 deletion has a strong correlation with male infertility. The prevalence of gr/gr deletion is less than 10% in many ethnic groups.

Figure 1: Deletion patterns in Y chromosome long arm detected by PCR-rSSO method. Major deletions: AZFa, AZFb, AZFb+c, and AZFc; minor deletions: Ym-3, Ym-6, Ym-8, Ym-9, Ym-11, and Ym-12; Y chromosome long arm terminal deletions: Yq I, Yq II, Yq III, Yq IV, Yq V, and Yq VI. All deletion patterns are defined in our previous study.3 AZF: azoospermia factor; PCR-rSSO: polymerase chain reaction-reverse sequence-specific oligonucleotide.

Table 2: Clinical data of patients who underwent testicular sperm extraction

| Deletion pattern | Patient (n) | Age (year) | LH (IU l^-1) | FSH (IU l^-1) | TT (ng ml^-1) | TESE outcome | SRR (%) | P |
|------------------|-------------|------------|--------------|--------------|--------------|--------------|---------|---|
| AZFb + c         | 1           | 37         | 3.9          | 14.2         | 3.3          | 0            | 0.53    |   |
| AZFc             | 10          | 36.0±6.1 (26-43) | 8.3±3.1 (4.7-14.0) | 25.6±10.2 (15.7-48.7) | 4.4±1.8 (2.1-7.4) | 60.0 | 0.04* |
| Ym-6 (P3 + P2 + P1) | 1            | 34         | 7.3          | 13.3         | 8.5          | 0            | 0.53    |   |
| Ym-8 (b1/b3)     | 2           | 36.5±1.5 (35-38) | 16.3±3.9 (12.4-20.2) | 37.9±1.0 (36.9-38.9) | 5.5±1.5 (3.9-7.0) | 50.0 | 0.51 |
| Ym-11 (b2/b3)    | 5           | 29.0±2.1 (26-31) | 10.2±5.4 (4.3-18.5) | 22.6±7.7 (15.3-35.5) | 5.2±2.5 (1.3-8.0) | 40.0 | 0.58 |
| Ym-12 (gr/gr)    | 91          | 36.2±5.5 (23-50) | 8.5±3.7 (3.6-23.1) | 22.7±7.6 (11.2-53.3) | 4.0±1.7 (1.4-13.0) | 18.7 | 0.09 |
| Yq I (distal to AZFa) | 1          | 33         | 7.4          | 22.9         | 4.4          | 0            | 0.53    |   |
| Yq II (distal to AZFb) | 1          | 29         | 13.1         | 27.4         | 3.2          | 0            | 0.53    |   |
| Yq V (distal to AZFb) | 1          | 38         | 13.1         | 37.3         | 3.7          | 0            | 0.53    |   |
| No deletion      | 136         | 34.9±6.3 (24-62) | 9.6±6.7 (2.7-51.0) | 25.3±11.0 (10.0-71.2) | 4.3±2.1 (0.2-13.5) | 28.7 | -    |

Values are shown as means ± s.d. (range) and statistically compared between no deletion group and each deletion group. Patients with an FSH level of >10 IU l^-1 and KS were excluded.

*Significant difference (P<0.05). LH: luteinizing hormone; FSH: follicle-stimulating hormone; TT: total testosterone; SRR: sperm retrieval rate; s.d.: standard deviation; KS: Klinefelter syndrome; AZF: azoospermia factor; “no deletion” is the control group.

Table 3: Clinical data of patients with azoospermia factor c deletion

| Patient (n) | Age (year) | LH (IU l^-1) | FSH (IU l^-1) | TT (ng ml^-1) |
|-------------|------------|--------------|--------------|--------------|
| Oligozoospermia | 20        | 32.6±3.5 (27-41) | 4.7±1.6 (1.0-7.0) | 8.7±4.2 (2.5-16.9) |
| Azoospermia (TESE sperm positive) | 10        | 34.6±6.3 (26-44) | 7.2±4.0 (1.7-14.0) | 21.2±13.8 (3.6-48.7) |
| Azoospermia (TESE sperm negative) | 38        | 37.0±5.8 (30-43) | 8.2±3.3 (4.8-12.3) | 20.0±7.3 (10.3-30.1) |

Values are shown as means ± s.d. (range) and statistically compared between each group. *Significant difference (P<0.05) compared with patients with oligozoospermia. LH: luteinizing hormone; FSH: follicle-stimulating hormone; TT: total testosterone; SRR: sperm retrieval rate; s.d.: standard deviation; TESE: testicular sperm extraction.

Table 4: Prevalence of Y chromosome microdeletions in reported studies with azoospermia

| Study | Patients (n) | AZFa | AZFb | AZFc | AZFb+c | AZFa+b+c |
|-------|--------------|------|------|------|--------|----------|
| Present study | 717        | 0.6  | 0.6  | 3.9  | 1.3    | 1.1      |
| Johnson et al. | 405        | 0.2  | 1.0  | 7.9  | 2.0    | NA       |
| Akinsal et al. | 1043       | 0.1  | 0.4  | 1.6  | 0.6    | 0.7      |
| Zhu et al. | 984         | 0.7  | 0.6  | 6.7  | 1.7    | 1.0      |
| Stahl et al. | 1153        | 0.3  | 1.4  | 4.3  | 2.7    | 1.6      |
| Ferlin et al. | 625         | 1.0  | 1.1  | 4.0  | 1.9    | 0.3      |

Each study included over 1000 male patients suffering from infertility in total. NA: not available. AZF: azoospermia factor.

AZFc, were detected in 0.3%, 1.4%, and 33.3% patients, respectively. Analysis of approximately 20,000 patients revealed that the frequency of b1/b3, b2/b3, and gr/gr deletions was 1.1%, 0.1%, and 2.4%, respectively. In this report, although the frequency of b1/b3 and gr/gr deletions was 0.25% and 4.1% and the odds ratio was 1.9 and 2.5 times higher than those without deletion in the severe spermatogenic failure (SSF) with the sperm concentration <5 × 10^6 ml^-1, b2/b3 deletion was not a risk factor of SSF. Meanwhile, influences of minor deletion differ depending on the population; Krausz and Casamonti reported that b2/b3 deletion has a strong correlation with male infertility. The prevalence of gr/gr deletion is less than 10% in many ethnic groups.
but about 33% in the Japanese population with haplogroup D as one of the major groups. In the present study, the frequency of gr/gr deletion was 33.3%, which is similar to that reported above.

**Spermatogenesis in each AZF deletion**

Okuyama et al. reported that the SRR of Japanese patients with NOA was 28.9%, which was similar to that observed in the present study. SRR was 0 for patients with AZFa, AZFb, and AZFb+c deletions; however, the number of patients analyzed was small. In addition, the SRR of patients with AZFc deletion tended to be as high as approximately 50%–70%. Similarly, in the present study, a significantly higher SRR was observed in patients with AZFc deletion than that in those without deletion (P = 0.04). For AZFc deletion, Hops et al. demonstrated that there was no significant difference in age between males with oligozoospermia and those with azoospermia; similarly, there was no significant difference in age between men with azoospermia in whom sperm retrieval was not successfully performed using TESE and men with azoospermia in whom sperm retrieval was not successfully performed using TESE. Our results showed that patients with oligozoospermia were significantly (P < 0.05) younger than those with azoospermia in whom sperm retrieval was not performed using TESE (Table 3). In comparison with the results of intracytoplasmic sperm injection (ICSI) using testicular and ejaculated sperms in patients with AZFc deletion, the result of ICSI with ejaculated sperm is slightly better. These results indicate that an early diagnosis of AZFc at younger age can achieve pregnancy without artificial technology. Patients with AZFc deletion have a good chance of fertilizing an egg. Moreover, male offspring of such patients inherit this deletion. Therefore, genetic counseling should be performed, so that the second generation of men with AZFc deletions can receive early treatment.

Sin et al. reported that the frequency of gr/gr deletion was not significantly different between the control group and the infertile male group in the Japanese population. However, when analyzed according to the gr/gr deletion subtype in the normozoospermic group, the sperm concentration in subjects with CDY1b deletion, which is abundant in haplogroup O, was significantly lower than that with CDY1a deletion. Ghorbel et al. also reported that CDY1b deletion was significantly more frequent in infertile patients than that in fertile men and is a risk factor of male infertility in Tunisian men.

In the present study, no significant difference (P = 0.09) was observed in SRR using TESE between men with and without gr/gr deletions. Subgroup analysis by CDY1 copy number might lead to different results.

This is the first large-scale study assessing Y chromosome microdeletions in Japanese males. The frequency of AZF deletions was similar to that reported in previous studies. Besides, SRR determined using TESE was significantly higher in men with AZFc deletion than that in those without the deletion. Further studies are needed to reveal the effect of gr/gr deletion on SRR. Sperms could not be retrieved from the patients with complete AZFa, AZFb, and AZFb+c deletions.

**AUTHOR CONTRIBUTIONS**

MI conceived the study and coordinated and drafted the manuscript. HI, KK, YS, YT, YK, and HK collected data and created databases. KS performed statistical analysis. AM supervised the study. All authors read and approved the final manuscript.