INTRODUCTION

Human chromosome Y is often lost in the somatic cells of elderly men. Recent studies have shown that the frequency of recognizable mosaic loss of chromosome Y (mLOY), that is, LOY involving ≥10% of peripheral blood cells, continuously increases with age in men over 40 years of age. Aging-related mLOY has been associated with shorter life expectancy and increased risks of cancer and other disorders. The frequency of mLOY in men under 40 years of age remains unknown, but is predicted to be extremely low.
Human chromosome Y encodes several spermatogenic genes. Hemizygous interstitial deletions of chromosome Y, particularly those in the azoospermia factor (AZF) region, are known as the risk factor of non-obstructive azoospermia. Thus, mLOY, if it occurs in testis of young men before or during reproductive ages, may cause spermatogenic failure. In 2016, Shin et al. performed standard karyotyping for blood samples from 1,354 reproductive-aged men with non-obstructive azoospermia and detected a mosaic 45,X/46,XY karyotype in eight. Similarly, Yatsenko et al. identified the same karyotype in one of 629 samples from men with severe spermatogenic failure. These results provided the first indication that early onset mLOY in leukocytes is present in a small percentage of young men with spermatogenic failure. Given that standard karyotyping subjects only about 20 leukocytes, this method may miss some cases with mLOY. Hence, the frequency of mLOY among patients may have been underestimated in the previous studies. The present study aimed to clarify the frequency of cryptic mLOY in young men with spermatogenic failure. Given that standard karyotyping subjects only about 20 leukocytes, this method may miss some cases with mLOY. Hence, the frequency of mLOY may have been underestimated in the previous studies. The present study aimed to clarify the frequency of cryptic mLOY in young men with spermatogenic failure. To this end, we screened 198 samples using a semi-quantitative multiplex PCR method, whose sensitivity has been confirmed in previous studies.

2 | MATERIALS AND METHODS

2.1 | Patients

The study group consisted of 198 Japanese patients with non-obstructive azoospermia at ages 24-55 years (median 34 years). The patients were numbered in order of their age. These patients visited our clinic because of infertility and were diagnosed with non-obstructive azoospermia of unknown etiology. Prior to the present study, all patients underwent conventional G-banding analysis for 20 peripheral leukocytes and were found to have a normal 46,XY karyotype. In addition, the patients were subjected to copy-number analysis of the AZF regions, which showed the lack of AZF deletion in 132 of 198 patients. The common gr/gr, AZFb, AZFc, and AZFb+c deletions were detected in 49, 1, 15, and 1 patient(s), respectively. The frequencies of these AZF deletions were comparable to previous data in Japan.

2.2 | Screening of mLOY by semi-quantitative multiplex PCR

We performed semi-quantitative multiplex PCR for all samples to screen mLOY. Genomic DNA samples were obtained from peripheral blood of each patient. We utilized a previously established method with slight modifications. In brief, copy numbers of chromosome Y relative to chromosome X were assessed by comparing the area under the curve of PCR products for AMELY at Yp11.2 to that for AMELX at Xp22.2. The PCR products were analyzed using the 3130 Genetic Analyzer with LIZ500 (Applied Biosystems). The area under the curve was calculated using the GeneMapper 3.7 software (Applied Biosystems). Samples which showed AMELY/AMELX ratios of ≤0.89 in two independent assays were subjected to the second analysis by droplet digital PCR. This cutoff value, 0.89, was determined based on the previous report and our own data on 42 fertile men aged between 31 and 39 years (range, 0.89-1.04; mean, 0.97).

2.3 | Detection of copy-number alterations involving AMELY

Low AMELY/AMELX ratios are indicative of mLOY; however, they can also result from interstitial deletions involving the AMELY locus. To exclude such Y-linked interstitial deletions, we performed array-based comparative genomic hybridization (CGH) for samples with a low AMELY/AMELX ratio. We used a human catalog array (4 x 180 k format; Agilent Technologies, Palo Alto, CA), which contains three probes for the AMELY gene. Copy number of the AMELY locus was
assessed using the Genomic Workbench (version 7.0.4.0, Agilent technologies) with the default settings of the aberration detection algorithm.

2.4 Detection of mLOY by droplet digital PCR

To examine the copy number of chromosome Y in the samples with possible mLOY, we performed droplet digital PCR. Genomic DNA samples were analyzed using the QX200 system (Bio-Rad Laboratories). We examined copy numbers of three loci, that is, SRY (Bio-Rad Laboratories, Assay ID: dHsaCP2500472), USP9Y (Assay ID: dHsaCP2506328), and UTY (Assay ID: dHsaCNS782024066). RPP30 at 10q23.31 (Assay ID: dHsaCP2500350) was utilized as the internal reference. For each locus, two independent assays were performed.

3 RESULTS

3.1 Screening of mLOY by semi-quantitative multiplex PCR

Among the 198 patients examined, three (patients 7, 147, and 164) exhibited low AMELY/AMELX ratios. The three patients had no AZF microdeletion. Average AMELY/AMELX ratios of patients 7, 147, and 164 were 0.85, 0.89, and 0.87, respectively (Figure 1A,B), whereas the ratios of the remaining 195 samples ranged between 0.90 and 1.26.

3.2 Detection of copy-number alterations involving AMELY

Array-based CGH for patients 7, 147, and 164 detected no copy-number alterations at the AMELY locus (Figure S1). Thus, the low AMELY/AMELX ratios in these patients were not ascribable to an interstitial deletion of chromosome Y.

3.3 Detection of mLOY by droplet digital PCR

Droplet digital PCR for patients 7, 147, and 164 showed apparently normal copy numbers for the three tested loci (Figure 1C). Thus, these patients were unlikely to have mLOY.

4 DISCUSSION

None of the 198 men with non-obstructive azoospermia and normal karyotype had recognizable mLOY. Whereas semi-quantitative multiplex PCR showed low AMELY/AMELX ratios in three patients, droplet digital PCR for these individuals suggested normal copy numbers of three loci on chromosome Y. The semi-quantitative multiplex PCR method have been used in several previous studies showing that its detection limit of mLOY is as low as 4.6%. Thus, it is unlikely that this method failed to detect leukocyte mLOY in our subjects. In this regard, the false-positive results of patients 7, 147, and 164 were not ascribable to interstitial deletion of the AMELY locus, because array-CGH demonstrated a normal copy number for this locus. The low AMELY/AMELX ratios of these patients may reflect the relatively low specificity of this screening method. Actually, the ratios in patients 7, 147, and 164 were only slightly lower than the cutoff value.

We cannot completely exclude the possibility that some of our 198 subjects carried mLOY exclusively in the testis. Indeed, previous studies detected mLOY not only in blood cells, but also in buccal samples. Considering that strong expression of the sex-determining gene SRY was observed in the fetal testis but not in the postnatal testis, postnatal mLOY of young men may impair spermatogenesis but likely permits normal male-type sexual development. However, the probability of testis-specific occurrence of mLOY is low, because mLOY is predicted to occur predominantly in high-turnover cells such as peripheral leukocytes.

The results of this study, in conjunction with the previous reports by Shin et al and Yatsenko et al in which standard karyotyping detected mLOY in 0.60% and 0.15% of azo/oligozoospermia patients, respectively, indicate that leukocyte mLOY accounts for an extremely small fraction of young men with spermatogenic failure. Since no data are currently available for the frequency of leukocyte mLOY in healthy men under 40 years of age, it remains unknown whether early onset mLOY is more common in patients with spermatogenic failure than in the general population. Furthermore, there are no data whether mLOY in young men is associated with early death and the risk of various disorders, as is the case for elderly people. In this context, Thompson et al raised questions about the pathogenicity of mLOY in blood cells. It is possible that leukocyte mLOY itself has no deleterious effects on men’s health, but reflects genomic instability in other tissues. Large-scale studies are needed to clarify the clinical significance of leukocyte mLOY in young men.

In summary, the results of this study demonstrated the rarity of mLOY in reproductive-aged men with spermatogenic failure. Thus, early onset mLOY is unlikely to play a major role in the development of spermatogenic failure. In addition, our data imply that standard karyotyping is sufficient to screen early onset mLOY.

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CONFLICT OF INTEREST
The authors declared that they have no competing interests.

HUMAN RIGHTS STATEMENTS AND INFORMED CONSENT
This study was approved by the Institutional Review Board Committee at the National Center for Child Health and Development. All procedures followed were performed in accordance with the Helsinki Declaration of 1964 and its later amendments. Written informed consent was obtained from all participants.

ANIMAL STUDIES
No animals were used in this study.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.

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