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·*Original Article*·

Associations of homologous RNA-binding motif gene on the X chromosome (*RBMX*) and its like sequence on chromosome 9 (*RBMXL9*) with non-obstructive azoospermia

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Abstract

Aim: To investigate the associations of autosomal and X-chromosome homologs of the RNA-binding-motif (RNA-binding-motif on the Y chromosome, *RBMY*) gene with non-obstructive azoospermia (NOA), as genetic factors for NOA may map to chromosomes other than the Y chromosome. **Methods:** Genomic DNA was extracted using a salting-out procedure after treatment of peripheral blood leukocytes with proteinase K from Japanese patients with NOA (n = 67) and normal fertile volunteers (n = 105). The DNA were analyzed for *RBMX* by expressed sequence tag (EST) deletion and for the like sequence on chromosome 9 (*RBMXL9*) by microsatellite polymorphism. **Results:** We examined six ESTs in and around *RBMX* and found a deletion of *SHGC31764* in one patient with NOA and a deletion of *DXS7491* in one other patient with NOA. No deletions were detected in control subjects. The association study with nine microsatellite markers near *RBMXL9* revealed that *D9S319* was less prevalent in patients than in control subjects, whereas *D9S1853* was detected more frequently in patients than that in control subjects. **Conclusion:** We provide evidence that deletions in or around *RBMX* may be involved in NOA. In addition, analyses of markers in the vicinity of *RBMXL9* on chromosome 9 suggest the possibility that variants of this gene may be associated with NOA. Although further studies are necessary, this is the first report of the association between *RBMX* and *RBMXL9* with NOA. *(Asian J Androl 2006 Mar; 8: 213–218)*

Keywords: spermatogenesis; homologous *RBMY*; polymorphism; microsatellite marker; azoospermia; Y chromosome

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1 Introduction

Approximately 15%–20% of infertile men exhibit azoospermia, which may be caused by failure of spermatogenesis or obstruction of the seminal tract [1]. Con-

genital dysfunction in spermatogenesis, referred to as non-obstructive azoospermia (NOA), might, in many cases, be the result of genomic abnormalities. Many men who have NOA of presumed genetic origin might possess some spermatozoa by testicular sperm extraction (TESE). Furthermore, a microdissection TESE procedure that uses direct visualization with an operating microscope to target the large whitish tubules that presumably contain the greatest number of spermatogenetically active germ cells was recently reported [2–6]. We previously reported that the spermatozoa retrieval rate was improved by up to 42.9% using microdissection TESE in patients with NOA [6]. These technical improvements and expanded indications for TESE with intracytoplasmic sperm injection are advantageous for patients with NOA. However, such success also means that the genetic abnormalities in NOA can be transmitted to the next generation. Therefore, it is important to understand the genetic basis of NOA.

Microdeletions of the Y chromosome at the azoospermia factor (AZF) locus have been suggested as a major cause of NOA. The AZF locus was recently shown to contain four loci, AZFa, b, c and d [7]. Strong candidate genes for *AZF* belong to two gene families that encode testis-specific RNA-binding proteins: the RNA-binding-motif (*RBMY*) gene family from the AZFb region [8]; and the deleted in azoospermia (*DAZ*) gene from the AZFc region [9]. Although numerous studies have reported a relation between deletions of *AZF*, including *RBMY* and *DAZ*, and azoospermia and severe oligospermia [10–12], fewer than 10% of cases of NOA involve these deletions. Therefore, additional genetic factors that cause NOA are thought to be located on the X chromosome or aoutosomes. The *DAZ* gene in the AZF region has been reported to have a homolog, which may be one of the genes responsible for NOA, on chromosome 3 in humans [13]. In contrast, there are no data supporting a relation between the *RBMY*-related gene and NOA, although a homolog of the *RBMY* gene on Xq26 (*RBMX*) and a homologous *RBMX*-like sequence on chromosome 9 (*RBMXL9*), which is specifically expressed in testis, were recently described [14, 15].

In the present study, we examined the associations of expressed sequence tags (ESTs) in and near *RBMX* and polymorphic microsatellite markers near *RBMXL9* in NOA patients and fertile control subjects.

2 Materials and methods

2.1 Subjects

The study subjects were 67 infertile Japanese men with NOA who had undergone microdissection TESE. The remaining subjects were 105 fertile men who had fathered children delivered at affiliated maternity clinics and who served as the control subjects. All study participants provided informed consent. The 67 infertile patients were diagnosed with NOA on the basis of a complete history, physical examination and endocrinologic profile. NOA was confirmed by a Johnsen score of less than 7 in a histological specimen from the testis obtained after the participant was enrolled in the study. The mean patient age was (33.7 ± 5.0) years, and preoperative serum concentrations were (7.3 ± 5.1) mIU/mL luteinizing hormone, (24.7 ± 14.6) mIU/mL follicle-stimulating hormone, (3.5 ± 1.4) ng/mL total testosterone, (13.0 ± 3.7) pg/mL free testosterone, (10.5 ± 9.7) ng/mL prolactin, (25.1 ± 13.0) pg/mL estradiol, and (39.0 ± 45.6) pg/mL inhibin B. We excluded any patients with Klinefelter syndrome or obstructive azoospermia from the study.

2.2 Genomic DNA extraction

Genomic DNA was extracted with a salting-out procedure after treatment of peripheral blood leukocytes with proteinase K, as described by Tsujimura *et al*. [16].

2.3 Detection of deletion of ESTs in and around RBMX

We performed polymerase chain reaction (PCR) to detect deletions of X-chromosome ESTs, *W11365*, *DXS7491*, *G42696*, *SHGC31764*, *D11S2422* and *RH45175*, which are all located in and around the *RBMX* gene (Figure 1). The PCR primers for each EST are shown in Table 1. After initial denaturation for 12 min at 95ºC, amplification was carried out in an automated thermal cycler for 35 cycles at 95ºC for 30 s, 58ºC for 60 s and 72ºC for 60 s, with a final extension of 72ºC for 10 min for *W11365* and *DXS7491*. The annealing temperature was changed to 55ºC for *D11S2422* and 60ºC for *G42696*, *SHGC31764* and *RH45175*. After electrophoretic separation on 2.0% agarose gels, PCR products were visualized with ethidium bromide staining and ultraviolet transillumination.

2.4 Genotyping microsatellites around RBMXL9

To determine the number of repeats of nine polymorphic microsatellite loci near *BBMXL9* (Figure 2), we synthesized unilateral primers by labeling the 5' ends with a fluorescent reagent, 6-FAM, HEX, or TET (PE Biosystems,

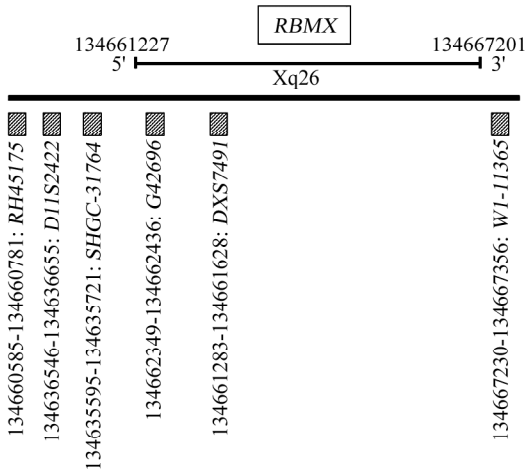

Figure 1. Locations of expressed sequence tags in and around the homologous RNA-binding-motif gene on the X chromosome (*RBMX*).

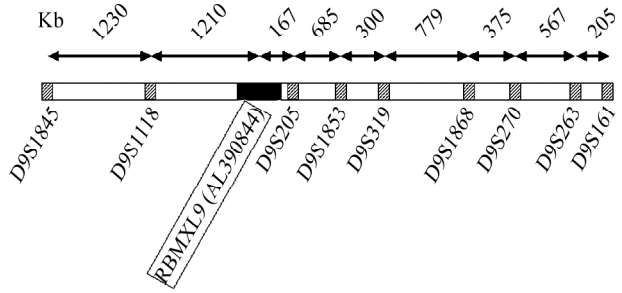

Figure 2. Locations of microsatellite markers near the homologous RNA-binding-motif gene on the X chromosome (*RBMX*)-like sequence on chromosome 9 (*RBMXL9*).

Foster City, CA, USA). The PCR primers for amplifying *D9S161*, *D9S263*, *D9S270*, *D9S1868*, *D9S319*, *D9S1853*, *D9S205*, *D9S1118*, and *D9S1845* are shown in Table 2. After initial denaturation for 12 min at 95ºC, amplification was carried out in an automated thermal cycler for 35 cycles of 95ºC for 30 s, 58ºC for 45 s and 72ºC for 60 s, followed by a final extension of 72ºC for 10 min for *D9S161*, *D9S263*, *D9S270*, *D9S1868*, *D9S319* and *D9S1853*. The annealing temperature was changed to 55ºC for *D9S205*, 52°C for *D9S1118* and 60ºC for *D9S1845*. The PCR products were denatured for 5 min at 100ºC, mixed with formamide-containing stop buffer and loaded together with a size standard (GS500 TAMRA; PE Biosystems, Foster City, CA, USA) on a 4% polyacrylamide denaturing gel containing 8 mol/L urea. The gels were run in a Model 377 Automated DNA Sequencer (PE Biosystems, Foster City, CA, USA). Fragment sizes were determined automatically with GeneScan software (PE Biosystems, Foster City, CA, USA).

Table 1. Primers for expressed sequence tags in and around RNA-binding-motif gene on the X chromosome. F, forward; R, reverse.

Marker		5'	3'
WI11365	F	GGAATAGTATTACCTCACTGCAAAA	
	R	CTTAGCTCGTTATCGTTTCCTTT	
DXS7491	F	CCACTTCCTATCTGATTTTTCCC	
	R	ATGGTCTTATTGGGGGAAGG	
G42696	F	CCGTCTTCAGTCTTTTCAAACA	
	R	CCATTGCTGGCCATAACAG	
SHGC31764	F	ATAGCCACTTTGTTTCAGCCA	
	R	GCCTTTACCACCCTCTGTGA	
D11S2422	F	CTCCCGAGTAGTTGGGCTAC	
	R	GTCAAGAGATCAAGACCATC	
RH45175	F	GAGCTGTTGTTTACCAATGGC	
	R	AAATGTCAAGTTTGCAATGGC	

Table 2. Primers for microsatellite loci near RNA-binding-motif gene on the X chromosome-like sequence on chromosome 9. F, forward; R, reverse.

Marker		5'	3'	Label
D9S161	F	TGCTGCATAACAAATTACCAC		FAM
	R	CATGCCTAGACTCCTGATCC		-
D9S263	F	TCATTTGGGCAGAGGATCA		HEX
	R	TACCTGGGTGGTGACCAGT		-
D9S270	F	AGGTGTAGTCCTTCTGGAATTT		TET
	R	GATGTGACTGCTGTTAAAACTAGAG		-
D9S1868	F	CAAATAAACGTCAACTGATTC		-
	R	ACATAGTCAAGAGCGCC		FAM
D9S319	F	GCCAGTGTTCTCCAGAGAAA		HEX
	R	TGGGATATGTCAGCCAAAAT		-
D9S1853	F	GATCCAGCCTCACTGAA		TET
	R	TTGGGCATAGAATTTTTACTTT		-
D9S205	F	TGAGGCAGGAAAATCACTTG		FAM
	R	CCAGTTATACATGTATGGGT		-
D9S1118	F	CAGGATATTATGTGATGGAATCC		-
	R	CTGCTGACTCCAAAAATATGC		HEX
D9S1845	F	CAACTTGGCAAAGGTAGGTG		-
	R	AGCTGGAGCCTCACACTG		TET

2.5 Statistical analysis

Significance of differences between patients with NOA and fertile control subjects in the distribution of microsatellite markers around *RBMXL9* was assessed by the χ^2 method with continuity correction and by Fisher's exact probability test. *P* was corrected by multiplication by the number of alleles observed at each locus. $P < 0.05$ was considered statistically significant.

3 Results

3.1 Deletion of ESTs in and around RBMX

Of the six ESTs in and around *RBMX*, a deletion of *SHGC31764* was found in one patient with NOA, and a deletion of *DXS7491* was found in one other patient with NOA. No deletions were detected in control subjects (Table 3). The clinical characteristics of patients with deletions of ESTs did not differ substantially from those of the other NOA patients (Table 4).

3.2 Genotyping of microsatellite markers around RBMXL9

Association analysis of susceptibility to NOA with nine polymorphic markers near *RBMXL9* revealed the presence of two markers with significantly low *P* values (*D9S319*: $\chi^2 = 4.59$, $P = 0.03$; *D9S1853*: $\chi^2 = 5.28$, $P = 0.02$; Table 5). Interestingly, the frequency of *D9S319* was lower in NOA patients than in control subjects (relative risk, 0.51), whereas the frequency of *D9S1853* was higher in patients than in control subjects (relative risk, 3.85).

4 Discussion

Mammalian spermatogenesis is a developmental process in which male germ cells undergo meiosis and complex morphological changes to form mature sperm. Many genes affecting male fertility have been identified in mice, and these genes are located both on autosomes and sex chromosomes. However, the search for human genes involved in spermatogenesis has so far focused only on the long arm of the Y chromosome. The relation between NOA and genes located on the X chromosome or on autosomes has not been clarified. We previously reported that a gene associated with NOA may be located on human chromosome 6 (6p21.3) near the DRB1 and the DQB1 loci [16, 17]. We also reported that a single nucleotide polymorphism in the protamine-2 gene on

Table 3. Deletions in and around RNA-binding-motif gene on the X chromosome.

	EST					
	SHGC31764	*D11S2422*	*RH45175*	*DXS7491*	*G42696*	*W11365*
Patient (n = 67)	1 (1.5%)	0	0	1 (1.5%)	0	0
Control (n = 105)	0	0	0	0	0	0

Table 4. Clinical characteristics of patients with deletion of expressed sequence tags in and around RNA-binding-motif gene on the X chromosome. JSC, Johnsen's score count; LH, luteinizing hormone; FSH, follicle stimulating hormone; TT, total testosterone; FT, free testosterone; PRL, prolactin; E2, estradiol.

		Testicular volume		Testicular histology	Endocrinologic profile						
	Deleted EST	Rt. (mL)	Lt. (mL)	Mean JSC	LH (mUI/mL)	FSH (mUI/mL)	TT (ng/mL)	FT (pg/mL)	PRL (ng/mL)	E2 (pg/mL)	Inhibin-B (pg/mL)
Patient 1	*DXS7491*	4	4	1.6	9.70	20.4	3.90	-	15.1	19.0	8.2
Patient 2	*SHGC31764*	14	14	2.0	6.47	22.6	2.65	15.9	14.9	23.6	21.4

Table 5. Markers associated with NOA in Japanese patients. NOA, non-obstructive azoospermia; RR, relative risk. *Pc*, corrected *P value*.

Marker	No. of alleles	Allele	Control (n = 105)	Patient (n = 67)	RR	χ^2	*P*	*Pc*
D9S319	10	170	63 (60.0%)	29 (43.3%)	0.51	4.60	0.03	0.32
D9S1853	9	253	4 (3.8%)	9 (13.4%)	3.85	5.28	0.02	0.19

human chromosome 16 (16p13.3) was present in 1 of 153 patients with NOA and absent in 270 fertile control subjects [18]. Furthermore, it was recently reported that a 1 bp deletion of the synapto-neal complex protein 3 (*SYCP3*) gene located on human chromosome 12 (12q23.3) was present in 2 of 19 patients with NOA [19]. These findings indicate that the genes associated with spermatogenesis in humans are located on chromosomes other than the Y chromosome [20].

The *RBMX/RBMY* gene family comprises a complex set of genes with multiple copies in the human genome. *RBMY*-like sequences were found on chromosomes 1, 4, 6, 9 and 11 as well as on the X chromosome by fluorescence *in situ* hybridization [15]. Of these *RBMY*-like sequences, the one on chromosome 9 is expressed specifically in testis and, to a lesser extent, in brain. The possibility that *RBMXL9* represents a novel gene involved in testicular function has been reported [14]. Thus, we examined the association of *RBMXL9* with NOA using nine polymorphic microsatellite markers located near *RBMXL9* in patients with NOA and in normal fertile volunteers. We found that 2 of 9 of these markers (*D9S319* and *D9S1853*) displayed strong associations with NOA. Interestingly, *D9S319* was found less frequently in patients than in control subjects, whereas *D9S1853* was detected more frequently in patients than in control subjects. Because *D9S20*, which is the nearest microsatellite marker to *RBMXL9*, did not show significant association with NOA, *RBMXL9* can be excluded as a candidate gene for NOA. However, it is possible that one or more gene(s) responsible for idiopathic azoospermia are localized in the narrow segment between *D9S319* and *D9S1853*. The possibility that a marker within this segment may affect the function of *RBMXL9* in spermatogenesis remains.

We report associations between genetic variations in *RBMX* in Japanese patients with NOA. Several ESTs in or near *RBMX* were deleted in a subset of patients with NOA. Although we did not find an association between *RBMXL9* and NOA, two markers near this gene were associated with NOA. The study described here is the first to investigate the possibility that *RBMX* or *RBMXL9* is responsible for NOA. Finally, we emphasize the importance of understanding the genes on the X and autosomal chromosomes that underline NOA.

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References

1 Matsumiya K, Namiki M, Takahara S, Kondoh N, Takada S, Kiyohara H, *et al*. Clinical study of azoospermia. Int J Androl 1994; 17: 140–2.
2 Amer M, Ateyah A, Hany R, Zohdy W. Prospective comparative study between microsurgical and conventional testicular sperm extraction in non-obstructive azoospermia: follow-up by serial ultrasound examinations. Hum Reprod 2000; 15: 653–6.
3 Schlegel PN, Li PS. Microdissection TESE: sperm retrieval in non-obstructive azoospermia. Hum Reprod Update 1998; 4: 439.
4 Schlegel PN. Testicular sperm extraction: microdissection improves sperm yield with minimal tissue excision. Hum Reprod 1999; 14: 131–5.
5 Silber SJ. Microsurgical TESE and the distribution of spermatogenesis in non-obstructive azoospermia. Hum Reprod 2000; 15: 2278–84.
6 Tsujimura A, Matsumiya K, Miyagawa Y, Tohda A, Miura H, Nishimura K, *et al*. Conventional multiple or microdissection testicular sperm extraction: a comparative study. Hum Reprod 2002; 17: 2924–9.
7 Friel A, Houghton JA, Maher M, Smith T, Noel S, Nolan A, *et al*. Molecular detection of Y chromosome microdeletions: an Irish study. Int J Androl 2001; 24: 31–6.
8 Ma K, Inglis JD, Sharkey A, Bickmore WA, Hill RE, Prosser EJ, *et al*. A Y chromosome gene family with RNA-binding protein homology: candidates for the azoospermia factor AZF controlling human spermatogenesis. Cell 1993; 75: 1287–95.
9 Reijo R, Lee TY, Salo P, Alagappan R, Brown LG, Rosenberg M, *et al*. Diverse spermatogenic defects in humans caused by Y chromosome deletions encompassing a novel RNA-binding protein gene. Nat Genet 1995; 10: 383–93.
10 Tsujimura A, Matsumiya K, Takao T, Miyagawa Y, Koga M, Takeyama M, *et al*. Clinical analysis of patients with azoospermia factor deletions by microdissection testicular sperm extraction. Int J Androl 2004; 27: 76–81.
11 van der Ven K, Montag M, Peschka B, Leygraaf J, Schwanitz G, Haidl G, *et al*. Combined cytogenetic and Y chromosome microdeletion screening in males undergoing intracytoplasmic sperm injection. Mol Hum Reprod 1997; 3: 699–704.
12 Vogt PH, Affara N, Davey P, Hammer M, Jobling MA, Lau YF, *et al*. Report of the Third International Workshop on Y Chromosome Mapping 1997. Heidelberg, Germany, April 13–16, 1997. Cytogenet Cell Genet 1997; 79: 1–20.
13 Yen PH, Chai NN, Salido EC. The human autosomal gene

DAZLA: testis specificity and a candidate for male infertility. Hum Mol Genet 1996; 5: 2013–7.
14 Lingenfelter PA, Delbridge ML, Thomas S, Hoekstra HE, Mitchell MJ, Graves JA, *et al*. Expression and conservation of processed copies of the *RBMX* gene. Mamm Genome 2001; 12: 538–45.
15 Delbridge ML, Lingenfelter PA, Disteche CM, Graves JA. The candidate spermatogenesis gene *RBMY* has a homologue on the human X chromosome. Nat Genet 1999; 22: 223–4.
16 Tsujimura A, Takahara S, Kitamura M, Miura H, Koga M, Sada M, *et al*. HLA-DR antigen and HLA-DRB1 genotyping with nonobstructive azoospermia in Japan. J Androl 1999; 20: 545–50.
17 Tsujimura A, Ota M, Katsuyama Y, Sada M, Miura H, Matsumiya K, *et al*. Susceptibility gene for non-obstructive azoospermia located near HLA-DR and -DQ loci in the HLA class II region. Hum Genet 2002; 110: 192–7.
18 Tanaka H, Miyagawa Y, Tsujimura A, Matsumiya K, Okuyama A, Nishimune Y. Single nucleotide polymorphisms in the protamine-1 and -2 genes of fertile and infertile human male populations. Mol Hum Reprod 2003; 9: 69–73.
19 Miyamoto T, Hasuike S, Yogev L, Maduro MR, Ishikawa M, Westphal H, *et al*. Azoospermia in patients heterozygous for a mutation in SYCP3. Lancet 2003; 362: 1714–9.
20 Truong BN, Moses EK, Armes JE, Venter DJ, Baker HW. Searching for candidate genes for male infertility. Asian J Androl 2003; 5: 137–47.