CASE REPORT

Atsushi Akane,[1] M.D.; Kazuo Matsubara,[1] Ph.D.; Hiroshi Shiono,[1] M.D.; Isao Yuasa,[2] Ph.D.; Shin-Ichi Yokota,[3] B.S.; Masao Yamada,[3] Ph.D.; and Yasuo Nakagome,[3] M.D.

Paternity Testing: Blood Group Systems and DNA Analysis by Variable Number of Tandem Repeat Markers

---

REFERENCE: Akane, A., Matsubara, K., Shiono, H., Yuasa, I., Yokota, S.-I., Yamada, M., and Nakagome, Y., **"Paternity Testing: Blood Group Systems and DNA Analysis by Variable Number of Tandem Repeat Markers,"** *Journal of Forensic Sciences*, JFSCA, Vol. 35, No. 5, Sept. 1990, pp. 1217–1225.

ABSTRACT: Two recent paternity cases are reported. In the first case of paternity exclusion, deoxyribonucleic acid (DNA) restriction fragment length polymorphisms (RFLPs) on variable number of tandem repeat (VNTR) loci with multiple alleles were informative, as well as established systems of red blood antigens, red cell enzymes, serum proteins, and human leukocyte antigens. In the second case, in which both the alleged father and the first wife were deceased, the paternal genotype was determined by using genetic markers from the second wife and four children, which then were compared with the paternal alleles of the child in question, the plaintiff in this case. The high probability of paternity (0.999 998 7) made us conclude that the man probably was the actual father. The DNA analysis by VNTR probes appears to be quite valuable in the study of paternity cases.

KEYWORDS: pathology and biology, paternity, genetic markers, variable number of tandem repeat (VNTR) markers, paternity testing, deoxyribonucleic acid (DNA) analysis

Abbreviations of Genetic Markers

ACP1	acid phosphatase
AHSG	α_2HS (Heremans-Schmid) glycoprotein
C6, C7, C81, BF, and IF	complement components
ESD	esterase D

This work was supported in part by grants from the Ministry of Education, Science, and Culture and the Ministry of Health and Welfare of Japan. Received for publication 22 July 1989; revised manuscript received 8 Sept. 1989; accepted for publication 29 Sept. 1989.

[1]Forensic scientists and professor, respectively, Department of Legal Medicine, Shimane Medical University, Izumo, Japan.
[2]Assistant professor, Department of Legal Medicine, Tottori University, School of Medicine, Yonago, Japan.
[3]Medical geneticists, National Children's Medical Research Center, Taishido, Setagaya, Tokyo, Japan.

Copyright © 1990 by ASTM International

F13A and F13B	fibrin stabilizing factors
GC	group-specific component
GPT1	soluble glutamic-pyruvic transaminase
HP	haptoglobin
ORM1 and ORM2	orosomucoid phenotypes
PGD	phosphogluconate dehydrogenase
PGM1	phosphoglucomutase
PI	α_1-antitrypsin
PLG	plasminogen
TF	transferrin

Paternity is usually determined using blood group systems such as red blood antigens, red cell enzymes, serum proteins, and human leukocyte antigens (HLA). In recent years, molecular biological techniques have progressed sufficiently well that we can now analyze the variations of deoxyribonucleic acid (DNA) as restriction fragment length polymorphisms (RFLPs). However, most RFLP markers have only two alleles and are therefore not sufficiently informative for pedigree analysis in many families [*1*].

In 1980s, several DNA marker systems revealing highly variable RFLPs have been found [*1*]. These multiallelic RFLPs are the result of differences in the number of tandemly repeating oligonucleotides in the loci. Minisatellite DNA sequences reported by Jeffreys et al. reveal a complex and highly polymorphic pattern on Southern blots of genomic DNA [*2*]. This DNA "fingerprint" is constituted of alleles of a number of such loci that are partially homologous to the probe, and is expected to be useful for forensic science purposes. However, the spontaneous mutation rates of these minisatellite loci appeared to be too high (about 5% for the most unstable locus) to be useful for paternity testing [*3*]. On the other hand, each of the variable number of tandem repeat (VNTR) markers established by Nakamura et al. can detect a single locus revealing a multiallelic RFLP, which should greatly reduce the number of families without informative markers [*1*].

Case Reports

In Case 1, the genetic markers of the child, mother, and alleged father were analyzed to test the paternity.

In Case 2, the alleged father (AF) had two wives (M1 and M2) and four acknowledged sons (C1 through C4). Sons, C1, C2, and C3 were born of their father's first marriage, and C4 was a son of the second marriage. AF and M1 had died before the analysis was performed. Another child (C5) was the son of a woman (M3) who claimed a relationship with AF. Whether C5 was actually the son of AF or not was the subject of the case. First, we tried to deduce the phenotype and genotype of AF by analysis of genetic markers from M2 and the four acknowledged children. The genetic markers of C5 and M3 were analyzed in the same way, to deduce paternal alleles within the genotype of C5. The alleles of each genotype of AF were then compared with the paternal allele of C5. Finally, the probability of paternity was calculated using the equation of Essen-Möller [*4*], based on gene frequencies in the Japanese population [*5*]. Allele frequencies of RFLPs were determined from the genotypes of 60 normal Japanese individuals.

Analysis of Genetic Markers

Red Blood Antigens—Secretor status (Sese type) was determined by a routine hemagglutination inhibition technique. Other red blood antigens were analyzed according to instructions from suppliers of the antisera.

Red Cell Enzymes—PGD typing was performed by electrophoresis. Other enzymes were typed by isoelectric focusing.

Serum Proteins—HP phenotypes were determined by electrophoresis on a polyacrylamide gradient gel. Others were determined by polyacrylamide gel electrophoresis and isoelectric focusing, followed by staining with Coomassie brilliant blue R-250 and by immunofixation or immunoblotting with commercially available antibodies.

HLA—The HLA types present at the A, B, and C loci were analyzed at the Hokkaido Red Cross Hospital Blood Center.

RFLPs—All probes used were obtained from the Japanese Cancer Research Resources Bank. Probes pYNH24 [*6*], pTHH59 [*7*], pEFD75.1 [*1*], and pYNZ2 [*8*] were used to determine VNTR loci, and the heterozygosities of pYNH24, pTHH59, and pYNZ2 were 97, 76, and 65%, respectively [*6–8*]. Probes pHF12-65, p9D11, p4.1H2, pHF12-62, and OS-4 were used to reveal diallelic polymorphisms. High-molecular-weight genomic DNAs were isolated from peripheral blood leukocytes according to the method of Blin [*9*]. DNA samples were digested with a restriction enzyme previously reported [*6–8*], electrophoresed in 0.7% agarose gel, and then transferred to nitrocellulose filter by the Southern blotting method [*10,11*]. The hybridization of a phosphorus-32 (^{32}P)-labeled probe onto its specific locus showed the RFLP alleles on the autoradiogram. Restriction fragment lengths of the alleles were obtained from the electrophoretic mobilities by comparison with known fragment lengths of ^{32}P-labeled, *Hin*dIII- digested bacteriophage λ DNA that had been electrophoresed in the same gel.

Results

The VNTR probe pYNH24 detected a multiallelic polymorphism in the Japanese population, with more than 20 alleles being recognized in 60 Japanese individuals (Fig. 1).

In Case 1, all three VNTR markers were informative, as well as the results of GC, AHSG subtype, and HLA-C typing, in the exclusion of paternity. Neither the two-allele RFLP markers nor the nine conventional markers, including HLA-A and HLA-B, were informative (Table 1). RFLP by pYNH24 (Fig. 2) showed that one allele of the child was inherited from his mother, but that another was different from both alleles of the alleged father.

In Case 2, the genotypes of the deceased AF had to be determined from those of M2 and the four children. Some markers were unfortunately useless for the testing, although the paternity was not excluded by any typing. The probabilities of paternity from the red

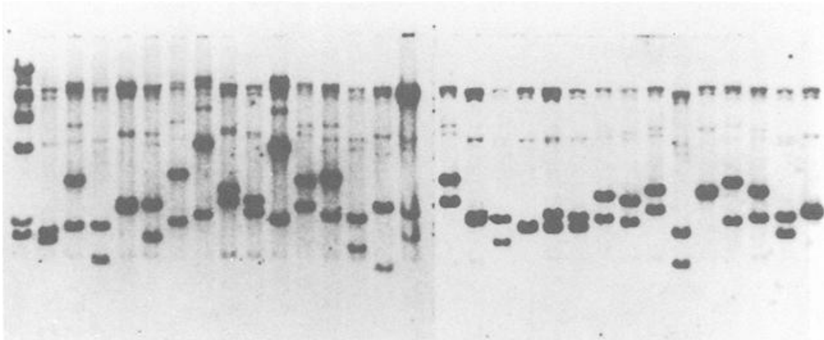

FIG. 1—*Autoradiogram of Southern transfer from 30 unrelated Japanese individuals. The DNA was digested with MspI. The filter was hybridized with pYNH24. The far left lane contains* HindIII-*digested bacteriophage λ DNA.*

TABLE 1—*Genetic markers in Case 1.*

System	Alleged Father	Child	Mother
Red blood antigens			
ABO	B	A	A
MNSS	MNss	Mss	Mss
Rh	CcDEe	CcDEe	CcDEe
P	$P_1(-)$	$P_1(-)$	$P_1(-)$
Duffy	a+b+	a+b−	a+b−
Lewis	a−b+	a+b+[a]	a−b+
Red cell enzymes			
PGM1	1A	1A-2A	1A-2A
Serum proteins			
HP	2-2	0-0[a]	2-2
GC[b]	1F	1F-2	1F-1S
AHSG[b]	1	2	2-1
HLA			
A	24,31	2,31	2,31
B	35,w52	35,51	51,w61
C[b]	w4	w9,w10	w10
RFLPs			
pYNH24[b,c]	3.35,2.65	4.25,2.45	4.25,3.45
pTHH59[b,c]	1,4	3,3	2,3
pYNZ2[b,c]	1,1	5,5	5,5
pHF12-65	1,1	1,1	1,2
p9D11	1,1	1,2	1,2

[a]Tested as a neonate.
[b]Paternity was excluded.
[c]Probe for VNTR locus.

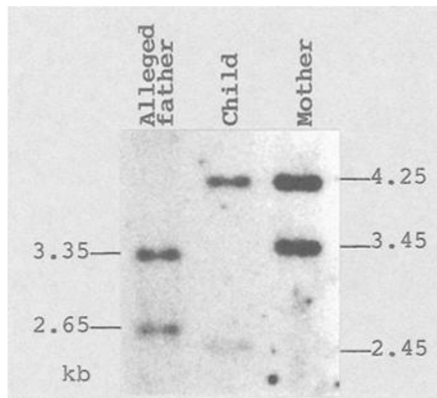

FIG. 2—*The DNA alleles detected by pYNH24 in Case 1. The numbers are the restriction fragment length (kb = kilobases).*

blood antigens and red cell enzymes were 0.586 and 0.661, respectively; these values were too low to be significant (Table 2). The probability calculated from the typing of serum protein groups was high (0.997), since the rare PLG*M5 was common to both C5 and AF (Table 2). The HLA A26-Bw61 haplotype was thought to be inherited by C5 from AF (Table 3), and the probability of paternity was 0.950.

RFLPs revealed by pYNH24, a VNTR marker that has more than 20 alleles, gave a high probability of paternity, 0.866 (Table 4 and Fig. 3). The probability determined using pEFD75.1, which has 5 alleles, was 0.657. The paternal alleles at VNTR loci detected by pTHH59 and pYNZ2 were obscure, since C5 and M3 had the same heterozygous genotype. Analysis of diallelic RFLPs by pHF12-65, p9D11, p4.1H2, pHF12-62, and OS-4 gave probabilities in the range from 0.502 to 0.614. The combined probability, according to DNA analyses, was 0.974.

In consequence, the total probability of paternity in Case 2 was 0.999 998 7.

Discussion

Genetic polymorphisms studies have usually been based on the analysis of protein isozymes. However, protein coding sequences represent only a part of human genomic DNA. Most of the DNA consists of non-coding regions, which are thus expected to be more variable and more polymorphic [*12*]. There may be a variation for every 100 base pairs in human genomic DNA.

Most of the known differences in DNA are attributed to base-pair changes that create or destroy a cleavage site for a specific restriction enzyme [*12*]. With such variants, the RFLP will have only two alleles because of the presence or absence of a cleavage site. On the other hand, VNTR markers reveal multiple alleles as a result of the different numbers of copies of tandemly repeating sequences in VNTR loci. Since one of the probes detects a single specific VNTR locus, personal identity or paternity can be checked at every locus, whereas the complex DNA "fingerprint" of Jeffreys et al. [*2*] is the sum of a number of RFLPs at the hypervariable loci. With such a multiallelic RFLP, the frequency of each allele is so low that a high probability of paternity can be given, or else paternity can be easily excluded.

Although the RFLPs shown by VNTR markers or DNA fingerprints are useful for forensic science purposes, they are too hypervariable to distinguish the alleles only by their restriction fragment lengths. The difference in length caused by the addition or loss of one tandemly repeating sequence may be so small that the DNA samples for comparison must always be electrophoresed in the same gel. In contrast, it is easy to distinguish alleles of diallelic RFLP; we need note only whether an allele is distinctive by being longer or shorter. For this reason, diallelic RFLPs were also analyzed in these cases. However, the analysis of such polymorphisms proved to be not very informative either for determining paternity (Case 2) or for paternity exclusion (Case 1).

A popular hypothesis suggests that hypervariable loci should be generated by highly frequent mutations, such as unequal exchanges between homologous chromosomes. In fact, Jeffreys et al. recently reported that the spontaneous mutation rate could be as high as 5% for the most unstable hypervariable minisatellite locus [*3*]. The total rate of the DNA fingerprint would thus be 0.2, which is too high for paternity testing.

A mutation occurring at one of the VNTR loci has also been reported [*13*]. Sequencing of the locus showed that the maternal allele had lost one of the tandem repeating units and generated a *de novo* allele. However, when genotypes were analyzed within the family by 600 other DNA markers, including 100 VNTR probes, all of them showed Mendelian inheritance. This suggests that the analysis of a number of VNTR loci should allow us to distinguish the mutations occurring at a specific locus to some extent. The

TABLE 2—*Blood group systems in Case 2.*

System	Phenotype [Genotype]								Paternal Allele	Probability of Paternity[a]
	C1	C2	C3	M2	C4	AF	M3	C5		
Red blood antigens										(0.5856)
ABO	AB	AB	AB	A	A	[?,?]	A	A	?	...
MNSs	Mss	Mss	Mss	Mss	Mss	[Ms,?]	MNss	Mss	Ms	0.576
Sese	Se	Se	Se	Se	Se	[?,?]	Se	Se	?	...
Rh	CCDee	CCDee	CCDee	CCDee	CCDee	[CDe,?]	ccDEE	ccDEE	cDE	...
Kell	K−k+	K−k+	K−k+	K−k+	K−k+	[k,?]	K−k+	K−k+	k	0.500
P	$P_1(+)$	$P_2(-)$	$P_2(-)$	$P_1(+)$	$P_1(+)$	[P_2,?]	$P_2(-)$	$P_2(-)$	P_2	0.507
Duffy	a+b−	a+b−	a+b−	a+b−	a+b−	[$Fy,^a$?]	a+b−	a+b−	Fy^a	0.503
Red cell enzymes										(0.6612)
PGM1	1A-2A	1A-2A	1A-2A	1B-2B	1A-1B	[1A,?]	1B-2B	1A-1B	1A	0.524
ACP1	B	B	B	B	B	[B,?]	B	B	B	0.512
ESD	7-1	2-1	7-1	1	2-1	[2,?]	1	2-1	2	0.627
GPT1	2-1	2	2	1	2-1	[2,?]	2-1	1	1	...
PGD	A	A	A	AC	AC	[A,?]	A	A	A	0.502
Serum proteins										(0.9974)
HP	2-1	2-1	2-1	2	2	[2,?]	2	2	2	0.518
GC	2	2-1F	2-1F	2-1S	1S	[2,1S]	2-1F	2	2	0.694
TF	C1	C2-1	C1	C2	C2	[C1,?]	C2-1	C1	C1	0.517
PI	M1	M2	M2	M1	M1M2	[M1,M2]	M1	M1M2	M2	0.727
AHSG	1	1	1	1	1	[1,?]	2-1	2-1	?	...
ORM1	2=1	2<1	2<1	5<2	2>1	[2.1,?]	2=1	2=1	1	...
ORM2	1	1	1	1	1	[1,?]	3-1	1	1	0.500
C6	B	B	B	AB	B	[A,?]	AB	A	A	0.575
C7	5-1	5-1	1	1	1	[1,?]	1	5-1	5	...
C81	A	A	A	AB	AB	[A,?]	AB	A	A	0.538
BF	FaS	Fa	S	S	FaS	[Fa,S]	S	S	S	0.507
IF	B	B	B	B	B	[B,?]	B	B	B	0.503
PLG	AM5	AM5	AM5	A	AM5	[M5,A]	A	AM5	M5	0.972
F13A	1A-1B	2B-1A	1A-1B	2B-1B	2B-1B	[?,?]	2B-1B	1B	1B	...
F13B	3-1	3-1	3-1	3	3	[3,?]	3-1	3-1	?	...

[a]Calculated by the Essen-Möller equation.

TABLE 3—*HLA markers in Case 2.*

HLA Type	C1	C2	C3	M2	C4	AF	M3	C5	Paternal Allele
A	26,w33	1,w33	2,26	11,24	1,24	(1,26)	2,24	24,26	26
B	44,w61	37,44	51,w61	w60,w62	37,w60	(37,w61)	7,w46	7,w61	w61
C	. . .[a]	w6	. . .	w4,w10	w6,w10	(w6,. . .)	w7,w11	w7	?

[a]No defined allele was detected.

TABLE 4—*RFLPs in Case 2.*

RFLP Type	C1	C2	C3	M2	C4	AF	M3	C5	Paternal Allele	Probability of Paternity[a]
RFLPs (overall)										(0.9736)
pYNH24[b]	2,4	2,4	4,4	3,5	3,4	(4,?)	1,4	4,4	4	0.866
pTHH59[b]	3,3	3,4	2,4	2,2	2,4	(3,4)	1,4	1,4	?	. . .
pEFD75.1[b]	3,5	3,3	3,5	2,5	2,3	(3,?)	3,4	3,3	3	0.656
pYNZ2[b]	4,5	4,5	4,5	3,5	3,4	(4,?)	4,5	4,5	?	. . .
pHF12-65	1,2	1,2	1,1	1,2	1,2	(1,?)	1,1	1,1	1	0.502
p9D11	2,2	2,2	1,2	2,2	1,2	(1,2)	1,2	1,1	1	0.614
p4.1H2	2,2	2,2	2,2	ND[c]	2,2	(2,?)	1,2	2,2	2	0.539
pHF12-62	2,2	2,2	2,2	ND	1,2	(2,?)	2,2	2,2	2	0.541
OS-4	2,2	2,2	2,2	2,2	2,2	(2,?)	1,1	1,2	2	0.575

[a]Calculated by the Essen-Möller equation.
[b]Probe for VNTR locus.
[c]No defined allele was detected.

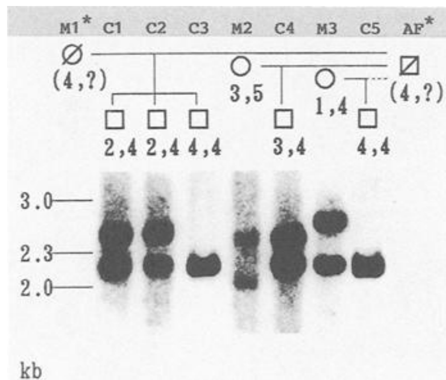

FIG. 3—*The DNA alleles detected by pYNH24 in the family of the alleged father (AF) in Case 2. The alleles were arbitrarily numbered for this family. Allele 4 was considered to be common to both the dead father (AF) and the child (C5). Asterisks indicate deceased individuals.*

fact that a VNTR probe detects only a specific locus should thus be of value in paternity testing. These probes were established for linkage analysis so that they would also be useful in determining the usual crossing-over that occurs between marker loci [*14*].

Conclusions

We have confirmed that VNTR markers can be actually useful for paternity testing. Although several hundreds of VNTR probes have been reported, many appear not to be of value. Some of the markers might not be polymorphic by analysis under usual conditions, but might only show polymorphism with the use of a high-resolution gel system [*1*]. Half of the markers contain human repetitive sequences, so that the filters have to be prehybridized with a large quantity of human placental DNA to eliminate background hybridization [*1*]. At the hypervariable loci, the distinction between alleles might be a delicate procedure, and mutations appear to occur easily. The paternity cases described here were investigated with probes that are relatively easy to use. To eliminate the possible mistake due to mutation at one of the hypervariable loci, the use of several VNTR markers at the same time may be advisable in paternity testing.

Acknowledgments

The authors are grateful to Dr. Yutaka Nakahori for his guidance and valuable criticism. We are also indebted to Prof. S. Robert Young, of the Department of Obstetrics/Gynecology, University of South Carolina, for reading the manuscript. All DNA probes were obtained from the Japanese Cancer Research Resources Bank.

References

[*1*] Nakamura, Y., Leppert, M., O'Connell, P., Wolff, R., Holm, T., Culver, M., Martin, C., Fujimoto, E., Hoff, M., Kumkin, E., and White, R., "Variable Number of Tandem Repeat (VNTR) Markers for Human Gene Mapping," *Science,* Vol. 235, 1987, pp. 1616–1622.

[*2*] Jeffreys, A. J., Wilson, V., and Thein, S. L., "Hypervariable 'Minisatellite' Regions in Human DNA," *Nature,* Vol. 314, 1985, pp. 67–73.

[*3*] Jeffreys, A. J., Royle, N. J., Wilson, V., and Wong, Z., "Spontaneous Mutation Rates to New Length Alleles at Tandem-Repetitive Hypervariable Loci in Human DNA," *Nature,* Vol. 332, 1988, pp. 278–281.

[4] Shiono, H., *Legal Medicine Manual,* Shinko Igaku Shuppan, Tokyo, 1987, pp. 195–199.
[5] Yada, S., Oya, M., Sekiyama, S., Haba, K., Sagisaka, K., Ishimoto, G. and Yamada, T., *Shin-Kiso-Houigaku, Ijihou,* Nankodo, Tokyo, 1989, p. 183.
[6] Nakamura, Y., Gillilan, S., O'Connel, P., Leppert, M., Lathrop, G. M., Lalouel, J.-M., and White, R., "Isolation and Mapping of a Polymorphic DNA Sequence pYNH24 on Chromosome 2 (D2S44)," *Nucleic Acids Research,* Vol. 15, 1987, p. 10073.
[7] Nakamura, Y., Holm, T., Gillilan, S., Leppert, M., O'Connel, P., Lathrop, G. M., Lalouel, J.-M., and White, R., "Isolation and Mapping of a Polymorphic DNA Sequence (pTHH59) on Chromosome 17q (D17S4)," *Nucleic Acids Research,* Vol. 16, 1988, p. 3598.
[8] Nakamura, Y., Culver, M., Sergeant, L., Leppert, M., O'Connel, P., Lathrop, G. M., Lalouel, J.-M., and White, R., "Isolation and Mapping of a Polymorphic DNA Sequence (pYNZ2) on Chromosome 1p (D1S57)," *Nucleic Acids Research,* Vol. 16, 1988, p. 4747.
[9] Blin, N., "Isolation of High-Molecular-Weight DNA," *Nucleic Acids Research,* Vol. 3, 1975, p. 2303.
[10] Yamada, M., Lewis, J. A., and Grodzicker, T., "Overproduction of the Protein Product of a Nonselected Foreign Gene Carried by an Adenovirus Vector," *Proceedings of the National Academy of Sciences of the U.S.A.,* Vol. 82, 1985, pp. 3567–3571.
[11] Kurosawa, H., Yamada, M., and Nakagome, Y., "Restriction Fragment Length Polymorphisms of the Human N-*myc* Gene: Relationship to Gene Amplification," *Oncogene,* Vol. 2, 1987, pp. 85–90.
[12] Fowler, J. C. S., Burgoyne, L. A., Scott, A. C., and Harding, H. W. J., "Repetitive Deoxyribonucleic Acid (DNA) and Human Genome Variation—A Concise Review Relevant to Forensic Biology," *Journal of Forensic Sciences,* Vol. 33, No. 5, Sept. 1988, pp. 1111–1126.
[13] Wolff, R. K., Nakamura, Y., and White, R., "Molecular Characterization of a Spontaneously Generated New Allele at a VNTR Locus: No Exchange of Flanking DNA Sequences," *Genomics,* Vol. 3, 1988, pp. 347–351.
[14] Boonlayangoor, P. W., "True Paternity or Exclusion: Analysis in the Case of a Deceased Party," *Journal of Forensic Sciences,* Vol. 34, No. 3, May 1989, pp. 703–707.

Address requests for reprints or additional information to
Atsushi Akane, M.D.
Department of Legal Medicine
Shimane Medical University
Izumo 693, Japan