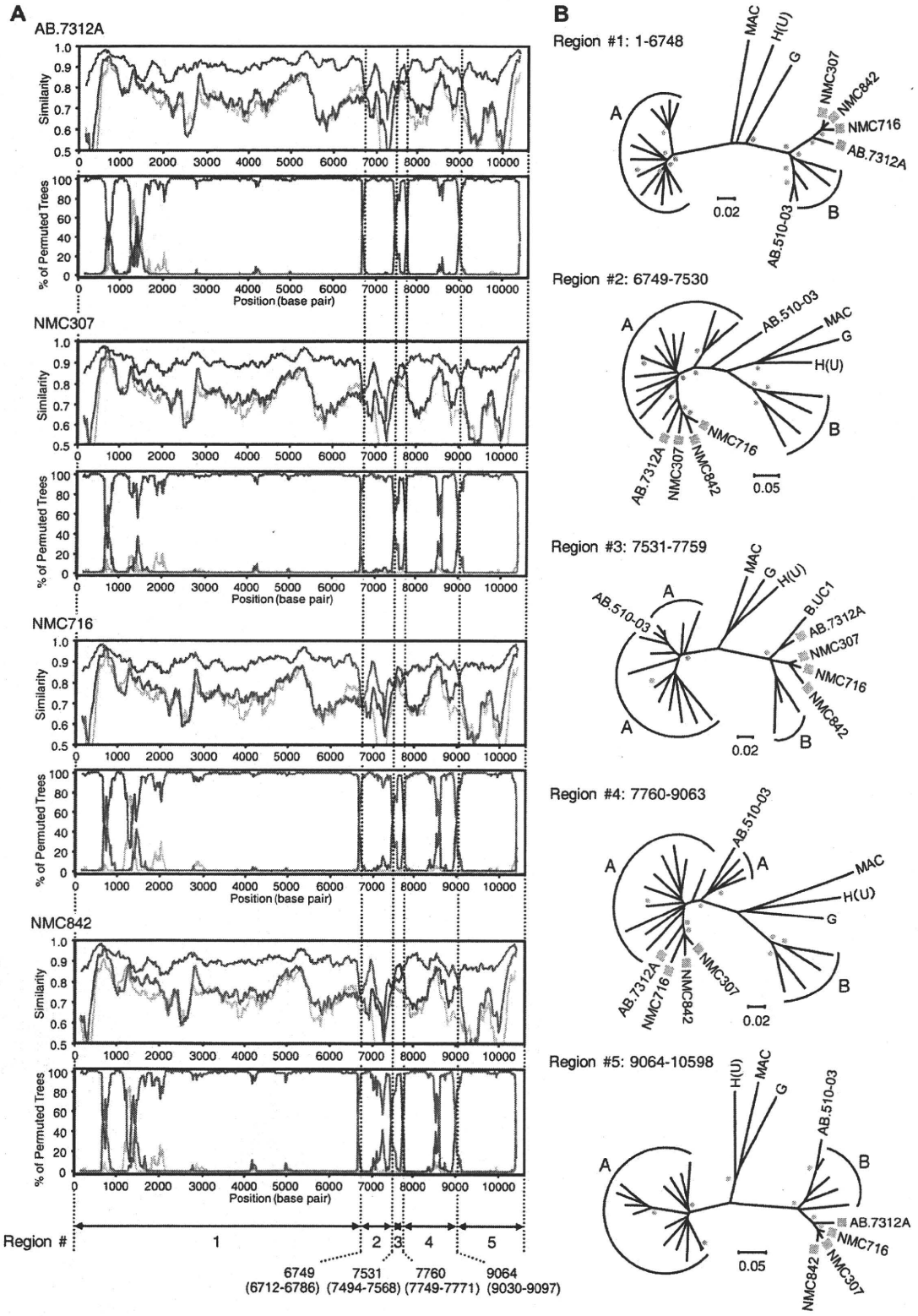

FIGURE 2. Determination of mosaic genome structures of HIV-2 AB recombinants. A, Similarity plotting (top) and bootscanning (bottom) data for each case of AB.7312A, NMC307, NMC716, and NMC842. Plots for consensus group A, consensus group B, and SIVmac239 are shown in red, blue, and gray, respectively. Both similarity plotting and bootscanning were performed with window and step sizes of 300 and 20 nucleotides, respectively. Bootscanning was performed using the neighbor-joining algorithm with 500 replicates. Each position of the 4 recombinant breakpoints is represented in the aligned sequence data set as the midpoint and

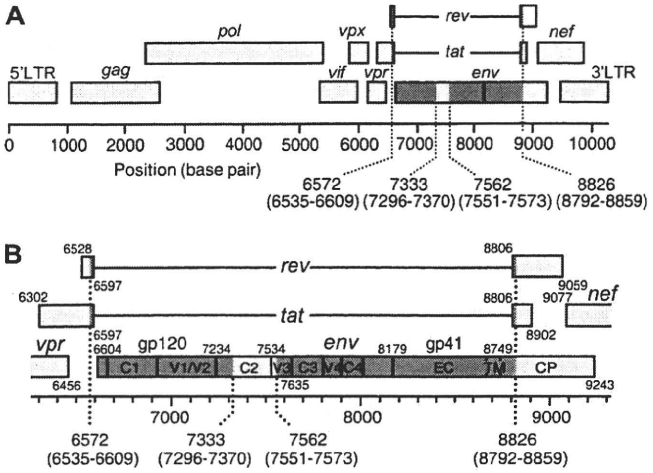

FIGURE 3. Schematic drawings for the genomic structure of HIV-2 CRF01_AB. A, Whole genomic structure; and B, Details around the *env* gene are represented. Regions belonging to group A and B are shown in red and blue, respectively. Numbering positions were adjusted to the reference SIVmac239 sequence.[35,36] Each position of 4 recombinant breakpoints is represented as the midpoint and range. C, constant region; CP, cytoplasmic domain; EC, extracellular domain; gp, glycoprotein; TM, transmembrane domain; V, variable region.

requirements are perfectly fulfilled with full-length genomic sequence information for 4 cases independently infected on different occasions with the AB recombinant identified by us and others.[12,13,19] Our data were carefully reviewed by editors of the Los Alamos HIV sequence database and confirmed as the first CRF discovered in HIV-2. They decided that the least confusing and most consistent way to name this new strain was to call it HIV-2 CRF01_AB.

The genomic structure of CRF01_AB is shown in Fig. 3. Interestingly, all 4 recombinant breakpoints of the CRF were located near or within the *env* gene (Fig. 3A). Further detailed analysis revealed that CRF01_AB possessed a chimeric gp120 containing a backbone of group A and a partial C2V3 fragment of group B and a chimeric gp41 containing extracellular and transmembrane domains of group A and a cytoplasmic domain of group B (Fig. 3B).

CRF01_AB Emerged Approximately in the Mid 20th Century

To estimate the time of CRF01_AB emergence, the time of the most recent common ancestor (tMRCA) of the recombinant was calculated by the Bayesian MCMC method. The mean substitution rates per year for the group A and B regions were estimated as 2.22×10^{-3} and 1.64×10^{-3}, respectively (Table 2), and the mean tMRCAs for groups A and B were estimated from 1921 to 1929, and from 1909 to 1948, respectively (Table 3). Similar results[31,37] validate our estimations. Finally, the mean tMRCA of CRF01_AB was estimated from 1964 to 1973. As the emergent times for groups A and B were estimated in the early 20th century, several decades seem to have been required for CRF01_AB to emerge. Concerning the geographical origin of the recombinant form, 3 of 4 isolates (7312A, NMC307, and NMC716) were identified in West Africans from Côte d'Ivoire and Nigeria. As these 2 countries were reported as sites of an epidemic in HIV-2 group A and B strains,[38,39] the most likely geographical origin of CRF01_AB is the south coastal area of West Africa.

DISCUSSION

In this study, we identified 3 HIV-2 AB recombinants with the same recombination pattern as 7312A, an isolate reported in Côte d'Ivoire in 1990.[12,13,19] These 4 isolates are determined as the first CRF of HIV-2, named CRF01_AB. It is noteworthy that all 3 of our cases infected with CRF01_AB were found at the AIDS stage. Considering that more than 75% of HIV-2–infected cases have a prognosis of remaining asymptomatic throughout their lifetimes[4] and that few HIV-2–seropositive cases were reported in Japan in the last 2 decades, 3 HIV-2 cases in the AIDS stage infected with the same CRF and identified in the past 5 years is highly unusual. Regarding the incubation periods for AIDS development in the 3 cases, not much information was available except for NMC842. This case was found to be seronegative for HIV-1/2 when tested in 2000. Thus, this case seems to have developed AIDS at most within 8 years, same as the median incubation period for AIDS development in HIV-1 infections (7.7–12.3 years).[40–45] As for the other 2 cases (NMC307 and NMC716), they developed AIDS at 28 and 36 years old (Table 1), which is significantly younger than age 65, reported as the peak of death by HIV-2 infections.[46,47] Though the number of cases identified is still small, we are concerned that the CRF01_AB might have acquired higher pathogenicity through recombination and adaptation to humans. As shown in Figure 3B, CRF01_AB has a recombination in the C2V3 region, the site of the major determinant for anti-envelope host immune responses and a functional domain for the chemokine receptor–binding site. The chimeric structure in the C2V3 region may confer advantages in host immune escape and viral replication capacity.

According to tMRCA analysis of the 4 isolates, CRF01_AB is estimated to have emerged sometime between 1964 and 1973. Interestingly, the mean tMRCA of the 3 isolates collected at NMC was estimated from 1982 to 1995 (Table 3), a later estimate than that of the 4 isolates, suggesting ongoing selection and evolution of CRF01_AB through transmission which has been taking place from the era of the 7312A isolate to the NMC isolates.

In conclusion, we report here the first CRF of HIV-2, CRF01_AB. Although national borders worldwide have

range (bottom). B, Subregion phylogenetic tree analyses. Phylogenetic trees were individually constructed by the neighbor-joining method using 5 subregion sequences. The HIV-2 isolates identified in this study (NMC307, NMC716, and NMC842) and AB.7312A are shown by green filled squares. Bootstrap values were calculated from 1000 analyses, and values greater than 95% are shown as orange dots at tree nodes. Scale bar represents 0.02 or 0.05 nucleotide substitutions per site. MAC, SIVmac239.

TABLE 2. Parameters in Bayesian MCMC Analysis for HIV-2/SIV Phylogenetic Inferences

	Substitution Rate Per Year		Coefficient of Variation		Population Size	
Data Set	Mean	95% HPD	Mean	95% HPD	Mean	95% HPD
Group A Region	2.22×10^{-3}	6.86×10^{-4}–3.68×10^{-3}	0.173	0.076–0.293	405.2	98.3–830.2
Group B Region	1.64×10^{-3}	5.99×10^{-4}–2.87×10^{-3}	0.269	0.170–0.395	341.2	93.3–668.9
Combined*	1.87×10^{-3}	6.39×10^{-4}–3.32×10^{-3}	0.235	0.088–0.382	357.9	93.3–709.2

*Combined data were produced from the 2 subsets, "group A region" and "group B region," using a LogCombiner program.
HPD, highest posterior density.

TABLE 3. Estimated TMRCAs of Monophyletic Clades in the HIV-2/SIV Lineage

	Group A region		Group B region		Combined	
Data set	Mean	95% HPD	Mean	95% HPD	Mean	95% HPD
Clade						
NMC isolates*	1982	1960–1996	1995	1987–2002	1990	1974–2002
CRF01_AB†	1964	1933–1985	1973	1956–1986	1971	1949–1986
Group A	1921	1864–1963	1929	1882–1964	1927	1879–1964
Group B	1909	1837–1962	1948	1915–1973	1934	1879–1973
HIV-2/SIV	1818	1670–1923	1821	1697–1930	1822	1693–1926

*This clade consisted of our 3 CRF01_AB isolates: NMC307, NMC716, and NMC842.
†This clade consisted of all 4 CRF01_AB isolates: 7312A, NMC307, NMC716, and NMC842.
HPD, highest posterior density;
SIV, simian immunodeficiency virus.

become more porous than ever, it is still surprising that the same recombinant strain was harvested in Japan, an island nation remote from the original endemic area, West Africa. This ectopic observation of the virus outside its endemic area suggests an ongoing global spread of HIV-2 CRF01_AB.

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Expression of human oocyte-specific linker histone protein and its incorporation into sperm chromatin during fertilization

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Objective: To investigate the expression of oocyte-specific linker histone protein (hH1FOO) in human ovaries and its incorporation into sperm chromatin after intracytoplasmic sperm injection (ICSI).
Design: Laboratory study.
Setting: University hospital.
Patient(s): Human ovarian tissues were obtained from patients at oophorectomy. Human oocytes and embryos were obtained from infertile patients undergoing IVF and ICSI.
Intervention(s): A polyclonal rabbit antibody targeting the predicted hH1FOO protein was used for immunohistochemical analysis. Western blot analysis and the reverse transcriptase–nested polymerase chain reaction were done to detect hH1FOO in chromatin of germinal vesicle–stage oocytes through to two-cell embryos.
Main Outcome Measure(s): The hH1FOO antibody reactivity of oocytes, ovarian tissues, and sperm chromatin after ICSI.
Result(s): hH1FOO protein was localized in all oocytes from primordial to Graafian follicles. In unfertilized oocytes after ICSI, the chromatin of injected sperm was condensed without hH1FOO incorporation in 45.5% of oocytes, condensed with hH1FOO incorporation in 9%, and decondensed with hH1FOO incorporation in 45.5%. None of the oocytes contained decondensed sperm chromatin without hHFOO incorporation.
Conclusion(s): hH1FOO protein was expressed by human oocytes from primordial follicles to early embryogenesis. Sperm nuclei that were still condensed after ICSI could be separated into two categories by hH1FOO incorporation, which might provide valuable information regarding failed fertilization. (Fertil Steril® 2010;93:1134–41. ©2010 by American Society for Reproductive Medicine.)

Key Words: H1FOO, oocytes, fertilization, histones, decondensation, human

Eukaryotic nuclear DNA associates with histone proteins to form chromatin, which is composed of nucleosomes connected by linker DNA. A nucleosome has a protein core, which is an octamer containing two molecules each of histones H2A, H2B, H3, and H4, with DNA wound around its surface. It is believed that histone H1, also termed a linker histone, binds to the linker DNA and plays an important role in the development and stabilization of higher-order chromatin structure and also possibly is involved in regulating gene expression (1–4).

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Although all histones are encoded by multiple genes, H1 histones show greater variability than any of the core histones. Eleven different linker histone variants have been identified in mammals, which include seven somatic variants (H1.1–H1.5, H10, and H1x), three spermatogenic variants (H1t, H1T2, and HILS1), and an oocyte-specific variant (H1FOO; H1 histone family, member O, oocyte-specific) (5). In mice, the oocyte-specific histone protein H1FOO is expressed by oocytes from the germinal vesicle (GV) stage through to the two-cell embryo stage (6). After intracytoplasmic sperm injection (ICSI) is performed in mice, H1FOO replaces protamine on sperm chromatin and then is replaced by somatic H1s at the two- or four-cell embryo stage (7). A similar developmental transition also occurs in *Xenopus laevis* (8). Such findings suggest a significant role of H1FOO in the regulation of gene expression during oogenesis and early embryogenesis (9–11).

We recently characterized the transcript encoding human oocyte–specific H1 (hH1FOO) protein, by using direct single-cell reverse transcription followed by a nested polymerase chain reaction (RT-nested PCR) (12). As well as being expressed exclusively by oocytes, this gene product has

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several distinctive features that are shared by oocyte-specific linker histones from other species. First, it encodes the longest H1 histone (347 amino acids). Second, it is located on chromosome 3q21-22 and contains five introns, whereas the somatic H1 genes are located as a large cluster on the short arm of chromosome 6 and lack introns. Third, its messenger RNA (mRNA) shows polyadenylation, whereas somatic H1 mRNA lacks a poly-A tail. However, the expression and localization of the protein encoded by this mRNA have not yet been determined.

Intracytoplasmic sperm injection has proved to be an effective treatment for severe male factor infertility (13). However, fertilization is achieved for only 60% to 70% of injected oocytes, resulting in a significant wastage of oocytes (14, 15), and poor fertilization (failure to detect two pronuclei [PN]) sometimes occurs after ICSI (16, 17). Sperm chromatin is mainly associated with a sperm-specific nuclear protein, which is termed protamine, instead of with histones. It is six times more compact than metaphase chromosomes and represents the most highly condensed chromatin found in eukaryotes (18). To achieve fertilization, the penetrating sperm heads must undergo various morphologic and biochemical changes, including chromatin decondensation and the replacement of protamine by histones (19). Visualization of sperm chromatin decondensation along with protamine-histone replacement might provide useful information about the process of fertilization, as well as about the reasons for failure of PN formation after ICSI.

In this study, we investigated the expression and localization of hH1FOO protein in human oocytes, ovaries, and fertilized embryos by using a specific anti-hH1FOO antibody. The relationship between morphologic changes of the sperm head after ICSI and the dynamics of histone transfer into sperm chromatin also were investigated by immunohistochemical analysis with use of the same antibody.

MATERIALS AND METHODS

This study was approved by the Ethics Committee of Keio University Hospital. All samples used in this study were obtained from patients who had given written informed consent.

Human Oocytes, Embryos, and Ovarian Tissues

Human oocytes were retrieved from infertile women undergoing IVF or ICSI at the Infertility Clinic of Keio University Hospital. Oocytes that did not display 2PN and had not initiated cleavage by 48 hours after ICSI were considered to be unfertilized. The oocytes and embryos that served as samples for this study, including GV-stage oocytes, 3PN oocytes, arrested 2PN oocytes, and arrested two-cell embryos all were scheduled to be discarded. Specimens of normal human ovarian tissue were obtained from women of reproductive age who underwent oophorectomy during surgery for cervical cancer or uterine leiomyoma.

Polyclonal Anti-hH1FOO Antibody

Anti-hH1FOO antiserum was raised in rabbits that were immunized with a mixture of two synthetic oligopeptides corresponding to the amino acid sequences SRSPESEKPGPSHG and KASSSKVSSQRAEA of the predicted hH1FOO protein (12). These oligopeptides were selected by using Epitope Adviser and DNASIS software (Hitachi, Tokyo, Japan) and were conjugated to keyhole limpet hemocyanin. The antiserum thus obtained was purified by affinity chromatography (Takara, Shiga, Japan).

Western Blotting of Ovarian Tissue and Oocytes

To obtain samples for blotting, 10 mg of ovarian tissue was ground in sodium dodecyl sulfate (SDS) sample buffer, as described previously (20). After centrifugation at 800 × *g* for 5 minutes, the supernatant was applied to 15% SDS–polyacrylamide gel for electrophoresis (1.0 μg of protein per lane). To obtain oocyte protein samples, a total of 30 oocytes were subjected to three freeze-thaw cycles in SDS sample buffer and applied to 15% SDS–polyacrylamide gel for electrophoresis. Proteins were transferred from the gel to a polyvinylidene difluoride membrane, which was blocked overnight in TBST buffer (10 mmol/L tris[hydroxymethyl]aminomethane [Tris; Sigma Aldrich, St. Louis, MO] HCl, pH 7.5; 150 mmol/L NaCl; and 0.1% polysorbate 20 [Tween 20; Sigma Aldrich]) containing 5% skim milk powder (wt/vol). Next, the membrane was incubated with anti-hH1FOO antibody (diluted 1:500) for 1 hour at room temperature. After washing three times in TBST for 10 minutes each, the membrane was incubated with a peroxidase-conjugated antibody for rabbit immunoglobulin (diluted 1:30,000; GE Healthcare Bio-Sciences, Piscataway, NJ). Signals were detected by using an enhanced chemiluminescence Western blot detection kit (GE Healthcare Bio-Sciences).

Immunohistochemical Analysis of Ovarian Tissues

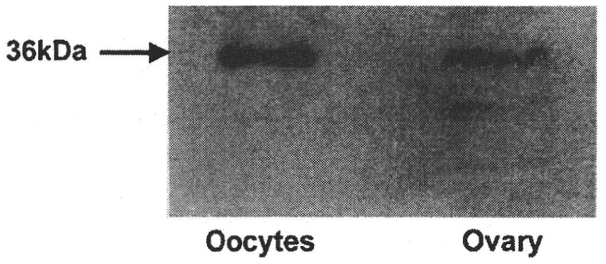
Ovarian tissue specimens were frozen in optimal cutting temperature compound, sections (6 μm thick) were cut on a cryostat, and these sections were fixed in acetone for 15 minutes at room temperature. After blocking with 5% normal swine serum for 30 minutes at room temperature, the sections were incubated with the anti-hH1FOO antibody (diluted 1:500) overnight at 4°C. As a negative control, the antibody was replaced by preimmune serum. Sections then were incubated with peroxidase-conjugated porcine anti-rabbit immunoglobulin (diluted 1:100; DakoCytomation, Glostrup, Denmark). Color was developed with 3,3′-diaminobenzidine tetrahydrochloride (0.2 mg/mL; Dojindo Laboratories, Kumamoto, Japan) in a solution containing 50 mmol/L Tris-HCl (pH 7.4) and 0.003% hydrogen peroxide, after which the sections were counterstained with hematoxylin.

Reverse Transcription–nested PCR of hH1foo mRNA

About 1 μL of phosphate-buffered saline solution (PBS) containing a single unfertilized oocyte that had been previously

FIGURE 1

Western blotting of extracts from unfertilized human oocytes (*left lane*) and human ovarian tissue (*right lane*). An identical band is seen at 36 kDa in each lane (*arrow*), corresponding to the molecular weight of hH1FOO protein.



Mizusawa. Expression of human oocyte-specific H1. Fertil Steril 2010.

washed three times in drops of PBS was added to 1.0 μL of 0.2% sarcosyl solution. After brief vortexing, the lysate was immediately frozen in liquid nitrogen and stored at −28°C until use. The lysate was subjected directly to RT-nested PCR as described previously (12), with use of primer pairs that flanked an intron to avoid amplification of genomic DNA.

Fluorescence Immunohistochemical Analysis of Oocytes and Embryos

Unfertilized oocytes and arrested embryos were fixed for 20 minutes at room temperature in a freshly prepared solution of 2% paraformaldehyde in PBS (pH 7.4). Fixed samples were then incubated for 1 hour at room temperature in blocking solution (PBS with 10% goat serum and 0.5% Triton X-100). After blocking, the oocytes or embryos were incubated for 1 hour at room temperature with the anti-hH1FOO antibody (diluted 1:1,500 with a solution of 0.5% Triton X-100 in PBS). Next, the specimens were washed three times in PBS for 15 minutes each at room temperature and incubated with Alexa Fluor 488 goat anti-rabbit IgG conjugate (diluted 1:1,000; Molecular Probes, Eugene, OR). After the specimens were washed again as above, DNA was stained with use of Hoechst 33342 dye (Molecular Probes). After another wash in PBS, each cell was pipetted into a drop of PBS and placed on a glass slide for examination with an Olympus AX70 microscope (Tokyo, Japan) that was equipped with the appropriate filter sets for epifluorescence studies.

RESULTS

Specificity of the Anti-hH1FOO Antibody

The specificity of the polyclonal anti-hH1FOO antibody was investigated by Western blotting of protein extracts from 30 unfertilized human oocytes and from ovarian tissues. In both the oocytes and the ovarian tissue samples, a single band was detected at 36 kDa (Fig. 1), which was consistent with the molecular weight calculated from the predicted amino acid sequence of hH1FOO.

Ovarian Localization of hH1FOO

Immunohistochemical analysis of ovarian tissue specimens with the polyclonal anti-hH1FOO antibody is shown in Fig. 2. The protein was localized in oocytes at all stages from primordial follicles to Graafian follicles, whereas preimmune serum did not react with any ovarian structures. There was no reactivity of the anti-hH1FOO antibody with granulosa cells or theca cells, confirming its specificity for hH1FOO as opposed to other histones. Although staining was more intense in the nucleus, the cytoplasm also was stained in all oocytes examined, indicating that cytoplasmic synthesis of hH1FOO is active from a very early stage of oogenesis and that this protein persists in the cytoplasm.

Expression of hH1foo mRNA and Protein During Oogenesis and Early Embryogenesis

Unfertilized human oocytes and arrested embryos were analyzed by RT-nested PCR and by fluorescence immunohistochemical analysis. As a result, hH1foo mRNA was detected at all stages from GV-stage oocytes to two-cell embryos as specific bands with a similar intensity (Fig. 3A). We confirmed that the nucleotide sequence of these bands was identical to that previously reported for hH1foo mRNA (12) (data not shown).

The protein also was detected in the chromatin of oocytes from the GV stage through to two-cell embryos (Fig. 3B). In these experiments, at least three oocytes or embryos at each stage were examined by RT-nested PCR and immunohistochemical analysis, with consistent results being obtained.

Replacement of Protamine by hH1FOO in Unfertilized Oocytes After ICSI and Sperm Decondensation

Thirty-three unfertilized oocytes were obtained after ICSI and were classified according to the reactivity of sperm chromatin with the hH1FOO antibody and the occurrence of sperm chromatin decondensation (from slightly to totally decondensed) (Fig. 4): [1] no immunoreactivity for the hH1FOO antibody and persistence of condensed chromatin (15 oocytes, 45%), [2] immunoreactivity for hH1FOO with persistence of condensed chromatin (3 oocytes, 9%), and [3] immunoreactivity for hH1FOO with decondensed chromatin (15 oocytes 45%). Interestingly, none of the oocytes contained sperm that showed decondensation but were nonreactive to the hH1foo antibody.

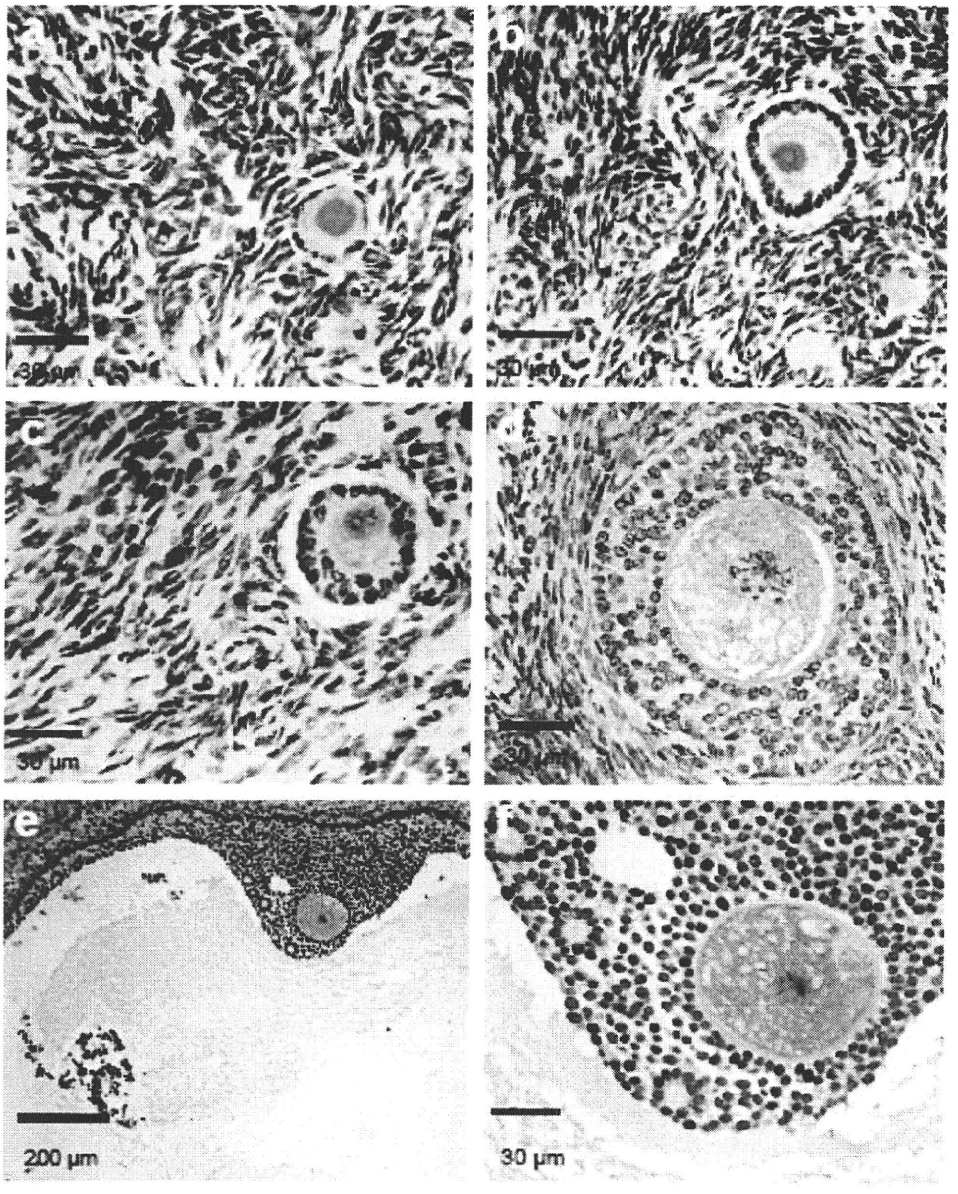
DISCUSSION

Role of H1FOO in Oocytes

H1FOO is thought to be the predominant H1 histone associated with chromatin during oocyte growth, oocyte maturation, and early embryogenesis. On entry of a sperm into an oocyte, protamine bound to sperm chromatin is replaced by maternal histones, including H1FOO, in a process that is referred to as protamine-histone replacement (18, 19). Concurrently, the highly condensed sperm chromatin undergoes

FIGURE 2

Immunohistochemical analysis for hH1fOO in ovarian sections. Human ovarian follicles are shown at various stages, including a primordial follicle (*a*), primary follicle (*b*), secondary follicle (*c*), preantral follicle (*d*), and Graafian follicle (*e, f*). Staining is detected in both the nucleus (orange or dark yellow) and the cytoplasm (yellow) of the oocytes, with its intensity being greater in the nucleus.

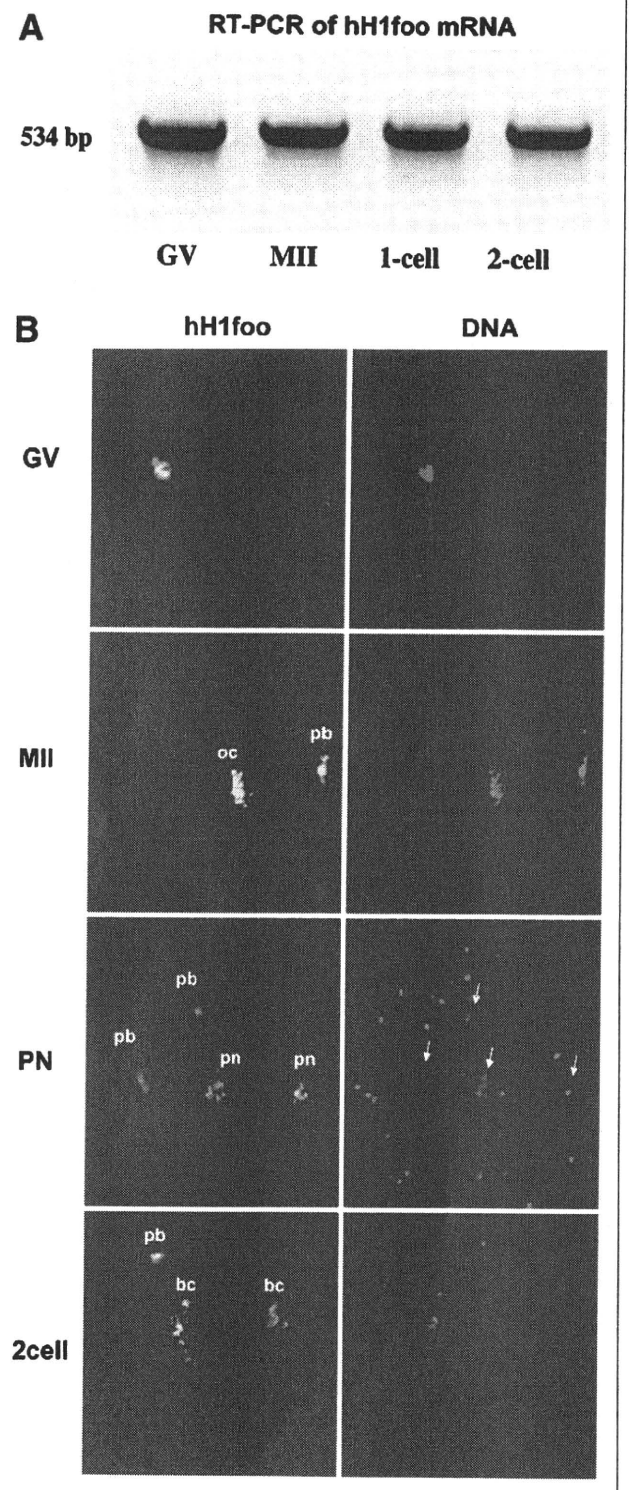


Mizusawa. Expression of human oocyte-specific H1. Fertil Steril 2010.

extensive decondensation to form the male PN. In fact, animal studies have shown that development of the male PN from sperm chromatin requires the presence of maternal histones (21). In mice, H1FOO then is replaced by somatic H1 at the two- to four-cell stage of embryogenesis (6). Because this transition of H1 subtypes takes place simultaneously with the initiation of zygotic development, H1FOO is thought to be involved in the transition from maternal to embryonic gene expression, resulting in remodeling of the two PN and the onset of DNA replication (7).

To our knowledge, the present study is the first to demonstrate the expression of H1FOO protein in human oocytes. Immunohistochemical analysis of human ovarian tissues revealed hH1FOO protein expression in oocytes throughout oogenesis, even as early as the primordial follicle stage. This finding differs from a previous report that H1FOO immunoreactivity was limited to oocytes after the primary follicle stage in mice, with no immunoreactivity being detected in the oocytes of primordial follicles (although a few H1foo transcripts were noted in these oocytes) (22).

FIGURE 3



Mizusawa. Expression of human oocyte-specific H1. Fertil Steril 2010.

FIGURE 3 Continued

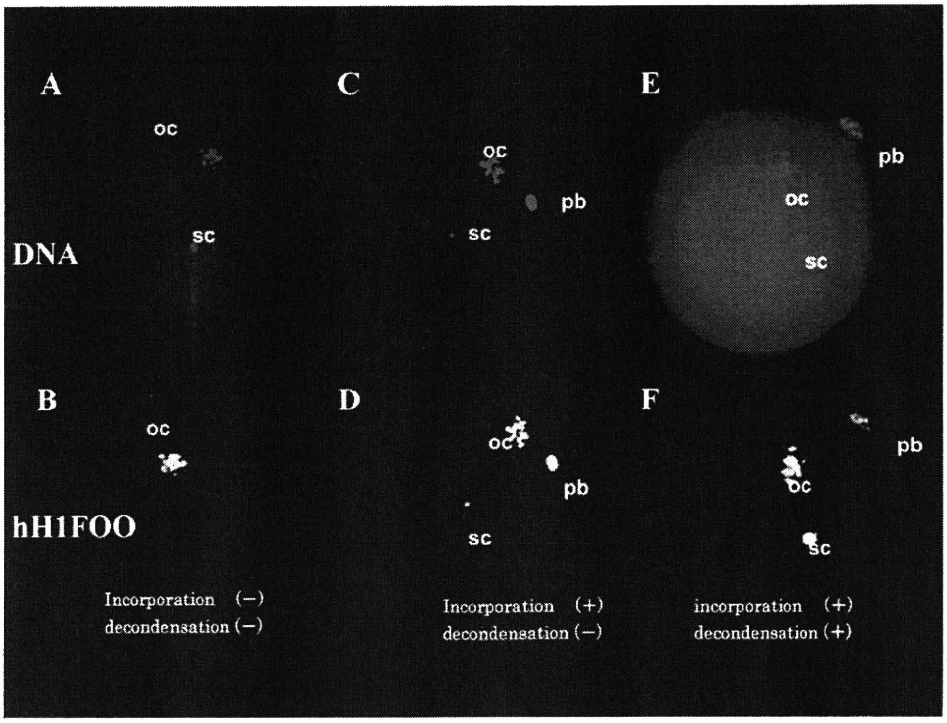
(**A**) Reverse transcription–nested PCR of hH1foo mRNA during oogenesis and early embryogenesis. hH1foo mRNA was detected at all stages from GV-stage oocytes to two-cell embryos as specific bands with a similar intensity. bp = base pair. (**B**) Fluorescence immunohistochemical analysis of an oocyte at the GV stage, an oocyte in MII, a PN oocyte, and a two-cell embryo (*2-cell*). The PN oocyte was obtained from IVF and ICSI, and the arrested 2PN oocyte was obtained after ICSI. The GV and MII oocytes were not exposed to sperm by either conventional IVF or ICSI. Deoxyribonucleic acid was visualized by Hoechst 33342 staining, and hH1FOO was detected by indirect immunofluorescence. hH1FOO protein can be detected clearly in the nucleus of the GV oocyte, MII oocyte, PN oocyte, and two-cell embryo. All polar bodies also were positive for both anti-hH1FOO antibody and Hoechst 33342. All of the sperm surrounding the PN oocyte, four-cell embryo, and morula were unstained by the anti-hH1FOO antibody (some are indicated by *arrows*). oc = oocyte chromatin; pb = polar body; pn = pronucleus; bc = blastomere chromatin.

On the basis of such findings, those authors proposed that expression of the H1foo gene might be coupled to the process of primordial follicle recruitment. On the other hand, Clarke et al. (23) reported that somatic H1 immunoreactivity, which is absent in growing oocytes, was very weak in nongrowing oocytes within the primordial follicles of adult mouse ovaries. Although the report of Clarke et al. suggested that the subtype transition from somatic H1 to oocyte-specific H1 may occur shortly after birth in all oocytes of the ovary (including those in the primordial follicles), further studies will be necessary to clarify the ontogeny and role of hH1FOO during oogenesis.

In the present study, preovulatory oocytes in human ovarian tissue samples showed hH1FOO immunoreactivity in both the nucleus and cytoplasm (Fig. 2), although staining of the nucleus was much stronger than that of the cytoplasm. It has been suggested that mouse H1FOO is mainly localized inside the germinal vesicles, because mouse H1FOO protein contains nuclear localization sequences but has no nuclear export sequences (22). On the other hand, McGraw et al. (24) found that H1FOO immunoreactivity not only was associated with the chromatin in bovine GV-stage oocytes but was also detected in the ooplasm, although staining of the cytoplasm was less intense compared with that of the chromatin. It also has been reported that after removal of the GV nucleus, H1FOO was detected in the somatic nucleus of oocytes after nuclear transfer (7), suggesting the existence of H1FOO in the ooplasm. On the basis of these findings, it seems that H1FOO protein is primarily localized in the nucleus in oocytes, although there is also a relatively abundant cytoplasmic pool.

FIGURE 4

Fluorescence immunohistochemical analysis of oocytes that were not fertilized by ICSI. These oocytes display three patterns of sperm chromatin immunoreactivity with the hH1foo antibody: [1] no immunoreactivity and no decondensation (*A, B*), [2] immunoreactivity without decondensation (*C, D*), and [3] immunoreactivity with decondensation (*E, F*). oc = oocyte chromatin; pb = polar body; sc = sperm chromatin.



Mizusawa. Expression of human oocyte-specific H1. Fertil Steril 2010.

Early Incorporation of H1FOO Into Sperm Chromatin After Penetration of the Oocyte

Studies in animals have shown that H1FOO is assembled on sperm chromatin almost immediately after fertilization. For example, H1FOO was detected on condensed sperm chromatin only 5 minutes after ICSI in metaphase II (MII)–arrested mouse oocytes (6). In pigs, incorporation of H1FOO into the sperm chromatin has been reported to precede decondensation after penetration of the ooplasm (25, 26). The assembly of H1FOO on sperm chromatin during decondensation also seemed to occur very quickly in humans, because all swollen sperm heads (even those that were only slightly swollen) revealed hH1FOO immunoreactivity in this study. We found a small number of unfertilized oocytes that contained condensed sperm heads with hH1FOO immunoreactivity (3/18 unfertilized oocytes containing condensed sperm heads), which also indicated that hH1FOO assembly might occur very early in the decondensation process.

A previous cytogenetic study of unfertilized human oocytes after ICSI revealed various outcomes of the injected sperm: [1] the entire sperm was missing or was ejected outside the ooplasm, [2] sperm chromatin was undecondensed, or [3] there was variable premature chromosome condensation (27). Among these outcomes, failure of decondensation may arise from incomplete immobilization of sperm due to insufficient damage to the sperm plasma membrane. However, there has been no way to determine whether sperm have reacted with the ooplasm. Therefore, we have been unable to assess whether condensed sperm heads had not reacted because the plasma membrane was not sufficiently damaged or had reacted but then failed to decondense. This latter situation is thought to arise from chromatin anomalies (28) or intrinsic oocyte defects. In this context, the performance of immunostaining for hH1FOO might provide valuable information about the cause of failed fertilization after ICSI. Because hH1FOO exclusively exists in oocytes and not in the sperm nucleus, hH1FOO immunoreactivity might be able to distinguish sperm that have started to react with the ooplasm and then failed to proceed to decondensation from membrane-intact and totally unreactive sperm, although our sample size was too small to draw a firm conclusion.

There are two possible explanations for the existence of hH1FOO-positive, condensed sperm nuclei. The first is that hH1FOO has been assembled, but decondensation has not occurred because the fertilization process was arrested at a very early stage. The second possibility is that these nuclei

underwent decondensation, but then recondensed. In mice, natural fertilization leads to initial enlargement and dispersion of protamine, after which the decondensed sperm head decreases in size (recondensation) and then gradually enlarges to form the male PN. It has been proposed that the recondensation of histone-containing sperm chromatin is due to a high level of metaphase-promoting factor activity and that oocyte activation causes this activity to decline to its basal level, resulting in PN formation (29). Indeed, in nonactivated oocytes of pigs, there is a time-dependent increase in the percentage of recondensed sperm nuclei (30). Sperm may fail to decondense because the oocyte lacks decondensing activity. It has been shown that mammalian oocytes possess sperm decondensing activity for only a short period after activation, and sperm introduced into mouse oocytes at any time later than the first cell cycle do not undergo decondensation (31–33). Presumably, the ability of human oocytes to promote the decondensation of sperm is also time limited, because Dozortsev and De Sutter (34) reported a high incidence of slightly swollen sperm in the cytoplasm of 1PN (i.e., activated) oocytes. Although there currently is no way to decide which of these two explanations is correct, further observation of sperm chromatin in nonactivated (MII) oocytes, activated (1PN) oocytes, and chemically (e.g., puromycin) activated unfertilized oocytes might help to clarify the reason for the existence of condensed and histone-positive sperm nuclei.

In the present study, we did not find any decondensed sperm head without H1FOO immunoreactivity. However, it would be interesting to search for such an alternative mechanism of failed fertilization after human ICSI in a study with a larger sample size, along with performing experiments on H1FOO knockout mice. So far, there have been no reports about the detection of decondensed sperm heads without H1FOO immunoreactivity in animals, but such sperm might represent an alternative mechanism of failed fertilization after ICSI.

In conclusion, we demonstrated the exclusive expression of H1FOO protein in human oocytes from those in primordial follicles through to early embryogenesis. During the process of fertilization after ICSI, hH1FOO seemed to be rapidly incorporated into the injected sperm nucleus. Because sperm that remained condensed after ICSI could be separated into two categories based on H1FOO incorporation or nonincorporation, immunostaining for H1FOO might provide valuable information about the factors leading to failed fertilization after ICSI.

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臨床研究の進歩　診断，検査

HIV検査
—最近のスクリーニング検査と遺伝子検査の進歩—

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Recent progress in HIV screening tests and nucleic acid tests

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Abstract

HIV testing plays a crucial role in detecting and monitoring HIV infection. Diagnosis of HIV infection is basically made by sequential two tests : a screening test with an enzyme immunoassay(EIA) and a confirmatory test with Western blot. The most recent EIAs, used in commercial laboratories, identify HIV infection earlier because they detect both HIV-1 antibody and antigen. Rapid tests represent another advance for HIV screening. They are widely used in voluntary counselling and testing at public health centers and private clinics. An assay for detection of HIV-1 RNA was approved as a confirmatory test of reactive screening tests to diagnose early infection. These new testing technologies offer more accurate, reliable, and convenient diagnosis of HIV infection.

Key words: HIV, screening test, antibody, nucleic acid test, real-time PCR

はじめに

HIV感染の有無を調べるための検査は，基本的にスクリーニング検査と確認検査の二段階により行われる．スクリーニング検査には抗HIV抗体を調べる方法と，抗HIV抗体とHIV抗原(p24抗原)を同時に調べる方法がある．現在，保健所などで即日検査に使用されている迅速検査キット(ダイナスクリーン)は抗HIV抗体を検出し，民間検査センターや病院などで行っている通常の酵素免疫測定法(EIA)などの検査試薬のほとんどは，抗HIV抗体と同時にHIV抗原を検出できる．また，最近は，抗HIV抗体とHIV抗原の双方を同時に検出できる迅速検査キットも開発され，近々発売が予定されている．確認検査としては，抗体の陽性を確認する方法としてウエスタンブロット(WB)が最も確実で現在も広く用いられている．ただし，感染初期の抗体の弱陽性の時期や抗体検出前の抗原陽性期については，WBによる確認は困難であり，血中のウイルス量を測定するために開発されたHIV遺伝子検査法が，非常に重要な役割を果たしている．

本稿の前半では，免疫学的検査法を中心に，後半では遺伝子検査を中心に，HIV検査法の最近の進歩と問題点や活用法について紹介する．

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1. 免疫学的検査

a. HIVスクリーニング検査法の進歩

HIVスクリーニング検査法は，1984にAIDSの病原体がHIVと確認されて以来，年々改良が重ねられ目覚ましい進歩を遂げてきた．最初のキットは培養ウイルス(HIV-1)由来の抗原を用いたEIA法でIgG抗体のみの検出であった．その後，抗原としてリコンビナントタンパクや合成ペプチド(HIV-1，HIV-2)を用い，また，抗原-抗体-抗原のサンドイッチ法などによりIgM抗体の検出も可能になった．このため，感染初期のウインドウ期間も大幅に短縮され，また，HIV-1に加えてHIV-2に対する抗体の検出も可能となった．

また，抗体検査に加え，抗原検査(HIV-1)も同時にできる抗原抗体同時検出キットも開発され，抗体の陽転前の抗原陽性期の検出も可能となり，ウィンドウ期は更に短縮された．現在，我が国で市販されているほとんどのHIVスクリーニング検査キット(**表1**)は抗原抗体同時検出キット(HIV-2については抗体のみ検出)である．

一方では，血液または血清を1滴加え，15分間静置するだけで，結果の得られる，簡易迅速キット(イムノクロマト法など)が開発されるなど，検査法の簡易化と迅速化に関しても急速な進歩がみられた．現在，我が国で市販されている迅速検査キットは抗HIV抗体を検出する1種類(ダイナスクリーン)だけであるが，抗原と抗体の両方を一つのキットで個別のラインとして検出可能な簡易迅速キットが開発され，認可もされており，近々，利用が可能になると思われる．

b. 確認検査法の進歩

HIVスクリーニング検査では，感染初期のウィンドウ期間内にある場合を除き，HIV感染者はすべて陽性となる．ただし，検査キットで使用している抗原や抗体との交差反応により，およそ0.3％程度の人が，感染していなくとも陽性(偽陽性)となる．

このため，スクリーニング検査で陽性となった場合には，感染による真の陽性か交差反応などによる偽陽性かを鑑別するため確認検査が必要となる．

抗体の確認検査法としては，HIVの構成タンパクそれぞれに対する抗体の有無を調べるWB法が広く使われており，WB法で典型的な陽性パターンを示す場合はHIV感染が確実となる．ただし，WB法で1-2本のバンドが出て判定保留となるケースや，陰性であっても感染初期が疑われるケースでは，遺伝子検査により感染初期か否かを確認する必要がある．

感染初期で抗体が弱陽性のケースや抗体の陽転前で抗原が陽性のケースでは，血液中のウイルスを鋭敏に検出できる遺伝子検査で陽性となる．このため，スクリーニング検査陽性で，WBの結果が保留あるいは陰性のため結論の得られないケースについては，遺伝子検査を行うことで，感染初期かスクリーニング検査の偽陽性例かを確定することが可能である．

c. 偽陽性を除外するための追加検査法

保健所などで行っている即日HIV検査(迅速検査)では，現在迅速検査キット(ダイナスクリーン)を使用しているが，このキットではほぼ1％の偽陽性がある．これら比較的頻度の高い偽陽性を除外するため，迅速検査キットで陽性となった場合には，より検出感度の優れている他の検査キット(抗原抗体検査キットやPA法)で追加のスクリーニング検査を行い，追加検査陰性であれば，スクリーニング検査陰性と判断する．追加検査で陽性であればスクリーニング検査陽性として，確認検査を行う．

この追加検査法で迅速検査の偽陽性例のほとんどを陰性と判定できる．

通常のHIVスクリーニング検査法の偽陽性率0.3％に比べて，感染率の著しく低い集団(妊婦など)を対象にHIV検査を行う場合にも，この追加検査により偽陽性例をスクリーニング検査段階で除外することにより，偽陽性によりもたらされる多くの無用な負担が避けられる．

d. HIV-2の検査

現在，我が国で使用されているHIVスクリーニング検査キットのすべてが，抗HIV-2抗体

表1 HIV検査試薬(2009年9月現在)

スクリーニング検査試薬

検査法	キット名	メーカー名	測定方法
抗体検査	ダイナスクリーン・HIV-1/2	インバネス・メディカル・ジャパン	イムノクロマト
	ジェンスクリーンHIV1/2	バイオ・ラッド ラボラトリーズ	ELISA
	ジェネディア HIV-1/2 ミックスPA	富士レビオ	PA
	セロディア・HIV-1/2(HIV型別用)	富士レビオ	PA
	ルミパルスオーソ HIV-1/2	オーソ・クリニカル・ダイアグノスティックス	CLEIA
	ビトロス HIV-1/2抗体	オーソ・クリニカル・ダイアグノスティックス	CLEIA
	ランリーム HIV-1/2	シスメックス	ラテックス定量
抗原抗体同時検査	アキシム HIV Ag/Ab コンボアッセイ・ダイナパック	アボットジャパン	MEIA
	アーキテクト・HIV Ag/Ab コンボアッセイ	アボットジャパン	CLIA
	ジェンスクリーンHIV Ag-Ab	バイオ・ラッド ラボラトリーズ	ELISA
	エンザイクノストHIVインテグラルII	シーメンス	ELISA
	バイダスアッセイキットHIVデュオII	シスメックス・ビオメリュー	ELFA
	エスプラインHIV-Ag/Ab	富士レビオ	イムノクロマト
抗原検査	ルミパルスI HIV-1p24(感染初期検出)	富士レビオ	CLEIA

確認検査試薬

検査法	キット名	メーカー名	測定方法
抗体検査	ラブブロット1	バイオ・ラッド ラボラトリーズ	WB
	ラブブロット2	バイオ・ラッド ラボラトリーズ	WB
	ペプチラブ1, 2(HIV型別用)	バイオ・ラッド ラボラトリーズ	イムノブロット
遺伝子検査	アンプリコアHIV-1モニター Ver.1.5	ロシュ・ダイアグノスティックス	RT-PCR
	コバスアンプリコアHIV-1モニター Ver.1.5	ロシュ・ダイアグノスティックス	RT-PCR
	コバスTaqMan HIV-1「オート」	ロシュ・ダイアグノスティックス	RT-PCR
	コバスTaqMan HIV-1「マニュアル」	ロシュ・ダイアグノスティックス	RT-PCR

を検出できるが，日本国内で見いだされたHIV-2感染例はいまのところ10例に満たない．しかしながら，HIVスクリーニング検査で陽性となりHIV-1感染の可能性が否定されたケースについてはHIV-2感染の可能性を考慮し，抗HIV-2抗体の確認が必要となる．通常HIV-2のWBを行い陽性であればHIV-2感染，陰性であればHIV-2の感染は否定される．ただし，WB-2の検査で1-2本のバンドが検出され判定保留となった場合，他の方法による確認が必要となる．一つはHIV-2のPA検査であり，もう一つは，最初のスクリーニング検査に用いたのとは異なるスクリーニング検査キットを用いた再検査である．これらの検査で陰性であれば，抗HIV-2抗体は陰性と判定できる．もし，これらの方法でも陽性の場合や，HIV-2の初期感染

表2 コバスTaqMan HIV-1とアキュジーンm-HIV-1の性能比較表

	コバスTaqMan HIV-1	アキュジーンm-HIV-1
方法	リアルタイムPCR	リアルタイムPCR
プローブ	TaqManプローブ	部分的2本鎖プローブ
検体量	0.85mL	0.6 mL, 0.2mL
検体前処理	自動処理(AmpliPrep)	自動処理(m2000sp)
検体種	血清・血漿(EDTA)	血漿(ACD-A, EDTA)
測定下限	<40コピー/mL	40コピー/mL(0.6mL使用時) 150コピー/mL(0.2mL使用時)
測定上限	10^7コピー/mL	10^7コピー/mL
サブタイプ検出能	グループM	グループM, O, N
ターゲット領域	*gag*遺伝子	*pol* integrase遺伝子
データ管理	QS(内部標準)により抽出・増幅効率を補正	ICにより増幅阻害を確認
コンタミネーションのモニタリング	不要(AmpEraseあり)	定期的なモニタリング(AmpEraseなし)
試薬調製の要否	自動処理	要用手調製
試薬保存	冷蔵(2-8℃)	冷凍(<-10℃)

の可能性が高いと思われる場合には，HIV-2の遺伝子検査などでHIV-2感染の確認を行う必要がある．

2. 遺伝子検査

a. PCRによるHIV遺伝子の検出・定量

レトロウイルスの一種であるHIVは，遊離ウイルス粒子の状態ではRNAゲノムをもつが，ヒトの細胞に感染するとDNA(プロウイルス)となり染色体DNAの中に組み込まれる．したがって，血漿中のHIV RNAを測定することによりウイルスの存在やその量を，末梢血単核球DNA中のHIV DNAを測定することにより感染細胞の存在やその量を調べることができる．

HIV-1が最初に分離されたのは1983年のことであるが，この年はPCRが考案された年でもある．当初，HIV-1のDNAとRNAの検出にはそれぞれサザンブロットとノーザンブロットが使われていたが，臨床検体からの検出には感度が不足していることがすぐに判明した．そこで，はるかに検出感度が高く，操作性にも優れたPCRが，HIV-1のDNAとRNAの検出・定量に応用され，感染者の診断と病態把握のため大きな貢献を果たした．HIV-1の発見とPCRはその後どちらもノーベル賞を受賞している．

先にも述べたように，HIV-1のDNAとRNAはHIV感染の異なった側面を反映するものであり，どちらも病態把握のためには重要な指標である．しかし，血中HIV-1 RNA量(遊離ウイルス量)が病期の進行や抗HIV治療により大きく変動するのに対し，末梢血単核球中DNA量(感染細胞数)はその変動が小さく，測定誤差や日内変動以上の有意な変化を得ることが困難であることがわかった．そのため，発症予測や抗ウイルス効果判定の指標として血中HIV-1 RNA量が主に使われることになった．

1996年，商業的HIV-1 RNA定量検査試薬として，RT-PCRとハイブリダイゼーションを原理とするロシュ・ダイアグノスティックス社(ロシュ社)のアンプリコアHIV-1モニター(以下，アンプリコア)が，米国で初めて認可・販売された．その後，この検査法はサブタイプB以外のHIV-1の定量に問題のあることがわかり，2002年に改良が加えられた．また，PCR以外にも核酸を高感度検出する方法が開発され，転写介在増幅(TMA)あるいは枝分かれDNA(bDNA)を原理とするHIV-1 RNA定量検査法が実用化されている．

b. 我が国におけるHIV-1 RNA定量の現状

我が国においては，先述のアンプリコアが唯

一のHIV-1 RNA定量検査試薬として長らく使用されてきたが，2007年暮れから，リアルタイムPCRを原理とするコバスTaqMan HIV-1(以下，コバスTaqMan)が同じロシュ社から発売された．また，アボット社からも2009年1月，同じくリアルタイムPCRを原理とするアキュジーンm-HIV-1が発売されている．両社の製品の性能比較を表2に示す．リアルタイムPCRは，蛍光プローブを用いて得られたPCR産物の増幅曲線を解析することにより目的のDNAあるいはRNAを定量するものであり，従来の方法に比べて自動化が進み，迅速で定量範囲が広いなどの特徴がある．

コバスTaqManの発売に伴い，国内のほとんどの検査センターにおけるHIV-1 RNA定量は，2008年3月頃からアンプリコアからコバスTaqManに移行していったが，その過程でコバスTaqManによる患者の血中HIV-1 RNA測定値がアンプリコアによる測定値よりも2-3倍高くなることがわかってきた．また，それまでアンプリコアで検出限界以下(<50コピー/mL)を保っていた症例において，コバスTaqManで測定されるようになると50コピー/mL以上の値で検出される例も少なからずあることがわかった．

一方，ヨーロッパでは，逆にコバスTaqManの測定値がアンプリコアの測定値より有意に低い例のあることが報告されている[1,2]．最近，その原因の一つが，PCRに使われている下流プライマーと一部のHIV-1変異株との間の特定の1塩基ミスマッチにあり，定量値を1/100以下に低下させるという論文が発表された[3]．HIVデータベースを調べると，報告されたHIV-1の約2%で同種のミスマッチが見つかった．国内においても，このミスマッチを原因とするHIV RNA定量値の低下例が観察されている(近藤ら，第23回エイズ学会学術集会発表予定)．ロシュ社では，以上のような問題を解決するため，コバスTaqManの更なる改良を検討中である．

c. 診断法としてのHIV核酸検査

HIV-1 RNA定量法を確定診断に使用することでHIV-1感染をより早期に診断することが可能となった．しかし，HIV-1 RNA定量法はあくまでも定量を主な目的として作られたものであることを忘れてはならない．すなわち，感染者のウイルス量の動態把握のためのウイルス定量を目的に設計されており，非特異的シグナル増幅やコンタミネーションなどによる偽陽性反応の抑制を最優先課題として考慮して設計されたものではない．実際，EIAに比べてその特異度は必ずしも高くないとの報告もある[4]．現在，米国のFDAで認可されている診断目的のHIV-1 RNA検査法は，TMAを原理とする1機種だけである．このため，HIV-1 RNA定量法を実際に診断で用いる場合，定量値が非常に低いときには注意が必要である．例えば，EIA陽性，WB陰性で，コバスTaqManの結果が'<40コピー/mLで検出'の場合，感染初期かPCRの偽陽性かを再検査などにより慎重に判断することが必要と思われる．このような低いレンジにおける特異度は今後，更に詳細に検討すべき課題である．

また，HIV-2についても，その感染初期における診断をより確実に行うためには，EIAやWBなどの免疫学的検査法だけでなく，遺伝子検査法が利用できることが望ましい．日本赤十字社では，血液製剤の安全確保のため，スクリーニング検査が陰性であった全血液について検査試薬TaqScreen s401を用いて20プールでHIV-1とHIV-2のRNAを検査している．将来，このようなHIV-1/2の同時RNA検出法が，HIVの確認検査法の一つとして利用できることが望まれる．

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第4章　治療と管理・対応

HIV検査と検査相談体制

要旨

HIV 検査技術は飛躍的に進歩し，スクリーニング検査には抗原抗体同時検査や迅速検査，確認検査にはウェスタンブロット（WB）法に加えて遺伝子検査法が導入され，HIV 感染をより早期に診断できるようになった．このような技術的進歩のもと，保健所や病院での HIV 検査は順調に広がりつつある．しかしながら，新規感染数は増加の一途をたどっており，HIV 感染の早期診断・早期治療の普及と感染拡大の抑制のためには，より一層の受検しやすい検査相談体制の整備・拡充が重要となっている．

● キーワード

EIA
迅速検査
リアルタイム PCR
VCT
PITC

はじめに

HIV 感染を調べるための検査は，基本的にスクリーニング検査と確認検査の2段階により行われる．スクリーニング検査には抗原抗体同時検出法やイムノクロマト法などが使用されている．確認検査にはウェスタンブロット（WB）法が広く使用されているが，急性期感染の確認には遺伝子検査法が重要な役割を果たしている．

本稿の前半では，HIV に関する最新の免疫学的検査法と遺伝子検査法を紹介した後，さまざまな医療の場におけるこれらの活用法と問題点について解説する．後半では，我が国における検査相談体制の現状と今後の取り組むべき課題について述べる．

免疫学的検査法

HIV スクリーニング検査に用いる酵素免疫測定法（EIA）は，HIV が AIDS の原因であると確認されて以来，飛躍的な進歩を遂げてきた．現在，我が国の検査センターなどでは，HIV-1 と HIV-2 に対する抗体（IgG と IgM）と HIV-1 の p24 抗原を同時に検出できる，いわゆる HIV-1/2 抗原抗体同時検出スクリーニング検査法（HIV-

2 については抗体のみ検出）が標準的に使用されている．初期の頃の検査法に比べると，検出の感度と特異度が高まっただけでなく，ウインドウ期間が大幅に短縮され，HIV-1 に加えて HIV-2 感染にも対応できるようになった．

一方，検査法の簡易化と迅速化に関しても急速に進歩し，血液または血清を1滴加えて 15 分間静置するだけで結果の得られる迅速検査法（イムノクロマト法）が開発された．現在，国内で市販されている迅速検査キットはインバネス社のダイナスクリーン® だけであるが，近々，抗原と抗体の両方を1つのキットで個別のラインとして検出可能な迅速キットが販売される予定である．米国などでは唾液を用いて検査のできる迅速検査キットが市販されており，国内でも利用可能となることが期待される．

HIV スクリーニング検査では，抗原-抗体の交差反応や反応試薬の非特異的反応により，およそ 0.3 % 程度の人で，感染していないにもかかわらず陽性反応が出る場合がある（偽陽性）．このため，スクリーニング検査で陽性となった場合には，感染による真の陽性か偽陽性かを鑑別するため確認検査が必要となる．抗体の確認検査法としては，HIV の構成タンパクそれぞれに対する抗体の有無を調べる WB 法が広く使われている．ただし，WB 法の結果が判定保留となる場合や，陰性であっても感染初期が疑われる場合では，遺伝子検査により感染初期か否かを確認する必要がある．

遺伝子検査法

血漿 HIV-1 RNA 量は感染個体内のウイルス複製レベルを反映しており，病期の進行予測や抗 HIV 治療の効果判定の重要な指標であることから，HIV 感染のモニタリングのために広く使用されている．血漿 HIV-1 RNA 定量法の原理としては，PCR／ハイブリダイゼーション，リアルタイム PCR，転写増幅法（TMA），枝分かれ DNA（bDNA）などがある．国内で現在利用可能なのはロシュ社のコバス TaqMan HIV-1® とアボット社のアキュジーン m-HIV-1® で，どちらもリアルタイム PCR を原理としており，その特性を生かして広範囲の定量性，迅速性，コンタミネーション防止のために重要な密封性を実現している．以前は PCR／ハイブリダイゼーションを原理とす