Rapid detection of aneuploidy using FISH in uncultured amniocytes for prenatal diagnosis : 8-year experience

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Purpose : FISH is suggested as a useful tool for rapid detection of specific aneuploidy in uncultured amniocytes abnormality in interphase nucleus. In this study, we are going to share our experience using FISH in prenatal diagnosis and suggest the criteria for the diagnosis of aneuploidy by analyzing the results of FISH test.
Methods : From January, 1999 to May, 2006, 8,613 tests in amniotic fluids obtained from 7,893 pregnant women were performed by using FISH for prenatal diagnosis of trisomy 21, trisomy 18 and trisomy 13. The indications of chromosome study were a screen positive for Down syndrome or Edwards syndrome in maternal serum marker screening test and an advanced maternal age (≥35 years old).
Results : We have the 8,502 informative results from 8,613 tests (98.7%) which is submitted our criteria and the sensitivity is 98.2%.
Conclusion : FISH on uncultured amniocytes is a rapid, clinically useful tool for prenatal diagnosis, with informative specimens being highly accurate. But the limitation of FISH is both expensive and labor-intensive.

Key Words : Trisomy 21, Trisomy 18, fluorescence in situ hybridization (FISH), uncultured amniotic fluid cells, criteria.

Introduction

Conventional cytogenetic techniques allow the diagnosis of number and structure of chromosomal abnormalities. They require a culture period for the amniocytes which varies from 1 to 3 weeks. When preliminary screening results indicate a greater risk of chromosomal abnormality, even a short wait for results can increase the emotional burden on the patient and/or physician. Rapid analysis using fluorescence in situ hybridization (FISH) probe on direct amniocytes offers an opportunity to reduce anxiety through earlier decision-making[2, 12].

FISH using probes specific for chromosome 13, 18, 21, X and Y has potential to detect more than 80% of clinically significant chromosome abnormality within 24h-48h[3, 5-7, 9]. As the method is not applicable for the identification of structural chromosome aberration, it is complementary to, but does not replace, fetal karyotype analysis. In this study, we are going to share our experience using FISH in prenatal diagnosis and suggest the criteria for the diagnosis of aneuploidy by analyzing the results of FISH test.

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Materials and Methods

1. Materials

From January, 1999 to June, 2006, FISH was performed in amniocytes from 8,613 tests in amniotic fluids obtained from 7,893 pregnant women. The indications of chromosome study were a screen positive for Down syndrome or Edwards syndrome in maternal serum marker screening test, an advanced maternal age (≥35 years old) and abnormal ultrasound findings. The results of FISH test were compared with chromosomal results.

2. Treatment of uncultured amniotic fluid cells and slide preparation

The basic steps for slide and cell preparation were as follows. 3-5 mL of fresh amniotic fluid was used for *in situ* hybridization studies and the remained fluid was allocated for conventional cytogenetic analysis. Cells were initially acquired by centrifugation of amniotic fluid and washed with phosphate buffered saline (PBS). These cells were then incubated with 75 mM KCL at 37℃ for 30 minutes and fixative (3:1 v/v methanol : glacial acetic acid) were added for prefixation. After centrifugation, the 1st fixation was done with 3 mL fixative at 4℃ for more than 20 minutes. After centrifugation, the 2nd fixation was done with 3 mL fixative at 4℃ for more than 15 minutes. After centrifugation again, slide preparation was done by air-drying method and these slides were stained for counting the number nuclei that were able to be use for analysis. Ethanol-dehydration was done and stored at -20℃ until used.

3. Probe preparation

A locus specific probe LSI-21 (Vysis, IL, USA) for chromosome 21 hybridizing to 21q22.13-q22.2, a centromeric probe CEP-18 (Vysis, IL, USA) for chromosome 18 hybridizing to 18p11.1-q11.1 and LSI-13 (Vysis, IL, USA) for chromosome 13 hybrididizing to 13q14 were used.

4. In situ hybridization and detection

Direct method using probe directly combined with fluorescence was used for hybridization. After denaturation of prepared slide in 70% FA (Qbiogen, USA)/2XSSC at 72℃, it was hybridized with probe solution for 18-24 hours at 37℃. After hybridization, coverslip were removed by rinsing in 2XSSC. Slide were washed in 50% formamide/1XSSC solution at 40-42℃ for 2 minutes and in 1x SSC solution. Slides were observed after counterstaining with DAPI solution. All the procedures were processed carefully in order not to be exposed to light and kept in light protection box.

5. Microscopy

At least 30 nuclei were scored from each case, usually from a single slide, using a fluorescent microscopy equipped with cooled CCD camera with ×1,000 magnification to collect images for documentation and further analysis. Round, well-preserved nuclei free from cytoplasm or overlapping material and exhibiting one, two, three, or four signals were selected for scoring; these comprised the majority of the nuclei retained on the slide. The signals appeared well embedded in the nucleus and were of regular or irregular shape. Often they appeared as characteristic twin spots if the chromosomes had replicated. It was also noticed that occasionally an additional set of signals (two in normal, three in abnormal cased) could be seen at a different level of focusing, probably due to a burst nucleus superimposed on the one analysed, Samples contaminated with maternal blood were occasionally received for analysis; most of the maternal cells could be excluded on the basis of size, shape, and different contrast properties.

6. Interpretation

In order to interprete FISH results, the criteria of pilot study in 2001 was used. Among 1,177 cases, over 30 nuclei could be counted in 1,155 cases (98.1%). FISH could not be performed in 22 cases because there were few nuclei to scan. Among 1,155 cases, definitive FISH results could not be obtained in only 3 cases.

By the criteria, a case with 2 signals in over 75% among over 30 countable nuclei was regarded as normal. In con-

trast, a case with 3 signals in over 70% was regarded as abnormal (trisomy). Other cases were interpreted as mosaicism, in which over 100 nuclei were examined (Table 1).

Results

Among 8,613 cases were enrolled, 8,502 (98.7%) cases could be counted over 30 nuclei. 8,490 (99.9%) was informative and only 12 (0.1%) cases were uninformative. 111 (1.3%) cases were less than 30 countable nuclei (Table 2). Fig. 1 shows FISH pictures of Down syndrome and Edwards syndrome.

In the samples confirmed as disomy 21, 18 and 13 by karyotype with definitive FISH result, mean percentage of 2 signals was 94.58% (range 65.7-100%) in FISH 21, 96.21 % (range 82.4-100%) in FISH 18 and 95.21% (range 87.1-100%). The lowest value with two signals in both FISH 21 and 18 was 65.70% (Table 3). Concerning the samples confirmed as trisomy 21, 18 and 13 by karyotype with definitive FISH result, mean percentage of 3 signals was 87.02% (60-98%) in FISH 21 and 79.0% (70.5-89.0%) in FISH 18. The lowest percentage showing 3 signals in both FISH 21 and 18 was 60% . There was no trisomy detected in this study (Table 4).

When compared with the karyotype, the accuracy of FISH in identifying disomy 21 and trisomy 21 was 99.9% and 98%, respectively. The accuracy of FISH in identifying disomy 18 and trisomy 18 was 100% in both. 1 case of mosaicism of trisomy 18 was suspected by FISH and was confirmed by karyotype. Also the accuracy of FISH in

Table 1. The Criteria in FISH Signals in our System. The Results were Analyzed with our own Criteria Established from Pilot Study in 2001

	2 signals 75%	3 signals 70%	2 signals <75% 3 signals >70%
30< No. cells 50 No.cell > 100	Disomy	Trisomy	? Mosaicism

Table 2. FISH of Uncultured Amniocytes Total 8,613 Cases were Enrolled in this Study. 8,502 (98.7%) Could be Counted Over 30 Nuclei and 111 (1.3%) which were Less than 30 Countable Nuclei was not Reported

FISH	NO.(%)
Total	8,613
No. of ≥30 nuclei	8,502 (98.7)
Informative	8,490 (99.9)
Unformative	12 (0.1)
No. of <30 nuclei	111 (1.3)

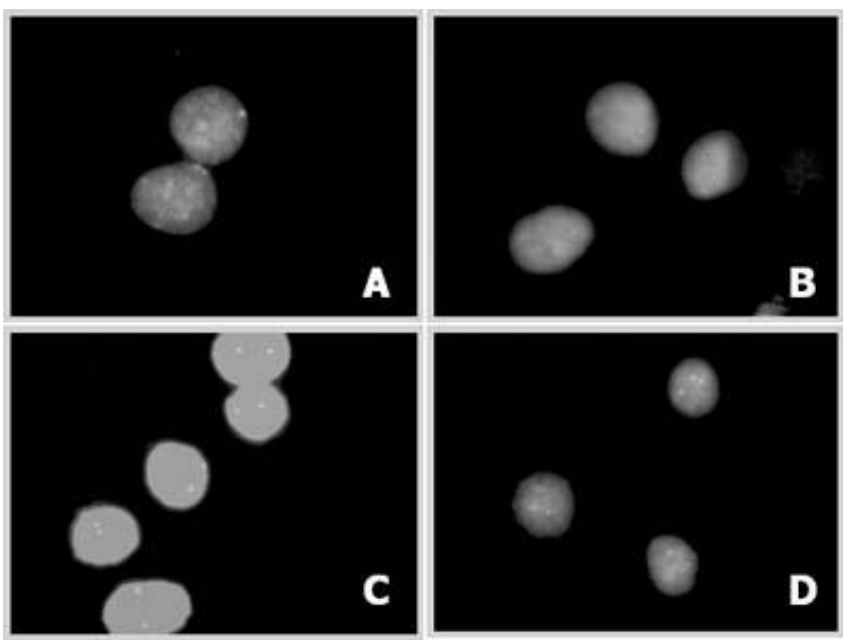

Fig. 1. Picture 1. Photographs of FISH : A. Disomy 21, B. Trisomy 21, C. Disomy 18, D. Trisomy 18.

Table 3. The Relationship of 2 Signals and Disomy 13/18/21 in Normal Karyotype

	시행건수	mean % (range) of nuclei showing					
		0	1	2	3	4	multi
FISH 21	7,613	0 (0-0)	0.02 (0-8)	94.58 (65.7-100)	5.37 (0-40.0)	0.01 (0-6.3)	0.02 (0-8.9)
FISH 18	983	0 (0-0)	0.04 (0-8)	96.21 (82.4-100)	3.73 (0-17.6)	0.01 (0-3)	0.01 (0-3)
FISH 13	34	0 (0-0)	0 (0-0)	95.21 (87.1-100)	4.79 (0-12.9)	0 (0-0)	0 (0-0)

Table 4. The Relationship of 3 Signals and Trisomy 18/21 in Abnormal Karyotype

	시행건수	mean % (range) of nuclei showing					
		0	1	2	3	4	multi
FISH 21	101	0 (0-0)	0 (0-0)	12.9 (2.0-40.0)	87.0 (60.0-98.0)	0 (0-0)	0.1 (0-9)
FISH 18	20	0 (0-0)	0 (0-0)	20.1 (11-29.5)	79.0 (70.5-89)	0 (0-0)	0 (0-0)

Table 5. The Accuracy of FISH Compared by Karyotypes

Final results	No. of tested FISH	FISH results	
		=Culture (%)	? (%)
Disomy 21	7,363	7,353 (99.9)	10 (0.1)
Trisomy 21	101	99 (98.0)	2 (2.0)
Disomy 18	983	983 (100)	0 (0.0)
Trisomy 18	20	20 (100)	0 (0.0)
Mosaicism 18	1	1 (100)	1 (0.0)
Disomy 13	34	34 (100)	0 (0.0)
Trisomy 13	0	0 (0.0)	0 (0.0)

identifying disomy 13 was 100% (Table 5).

12 cases did not meet our criteria in interphase FISH signals (Table 1). However, in disomy 21 and trisomy 21 cases, the lowest percentage showing 2 and 3 signals was 60% respectively (Table 6).

Table 6. Cases which Could not be Determined by FISH

No. Nuclei	2 signals (%)	3 signals (%)	Final results
45	27 (60.0)	18 (40.0)	Disomy 21
100	67 (67.0)	33 (33.0)	Disomy 21
30	22 (73.3)	8 (26.7)	Disomy 21
35	23 (65.7)	12 (34.3)	Disomy 21
50	35 (70.0)	15 (30.0)	Disomy 21
39	29 (74.4)	10 (25.6)	Disomy 21
31	23 (74.2)	8 (25.8)	Disomy 21
30	22 (73.3)	8 (26.7)	Disomy 21
33	24 (72.7)	9 (27.3)	Disomy 21
92	71 (74.7)	24 (25.3)	Disomy 21
30	12 (40.0)	18 (60.0)	Trisomy 21
90	28 (31.1)	62 (68.9)	Trisomy 21

DISCUSSION

FISH is now widely used as adjunct method with conventional cytogenetics test. Especially, there have been a number of recent reports demonstrating the feasibility of aneuploidy detection in interphase nuclei of uncultured amniotic fluid cells[4, 6, 8, 10]. In order to use this technique, every center should has its own diagnostic criteria for accurate diagnosis according to difference in laboratory environment and handling technique. For this reason, it is known to be the first step to set up its criteria. In our study, according to our criteria the sensitivity is 99.9% which is targeted to Down syndrome, and the sensitivity is 100% which is targeted to Edwards syndrome. Also we analyze at least 30 cells each case to obtain informative results (Table 1-3).

During procedure of FISH, it was difficult to get an optimal condition for good signals on observation. Especially, temperature and time were very important factors. When temperature was high, the signal was weak due to damage of nucleus. On the contrary, when temperature was low and time was short, the signal was also weak. This might be due to the fact that a binding of probe was not enough[13, 16].

Simultaneous denaturation of this probe and target DNA at 80℃, without annealing of the probe to the competitor prior to hybridization, was effective and more convenient that alternative methods of separate slide and probe denaturation, because it results in a high hybridization effici-

ency, a low background, and short time procedures.

Provided that aneuploidy detection in uncultured amniotic fluid cells is always followed by definitive fetal karyotype analysis of cultured cells, little harm will result from false-negative results due to the occurrence of such structural chromosome aberrations[1, 11, 14, 15].

Sample quality is the major parameter which can not be controlled. Fluids of dark brown color on receipt, or samples of late gestational age, tend to contain a high proportion of degenerative cells, resulting in increased non-specific back-ground signals. The contamination with maternal blood cell also affects the quality of the specimens and is a potential source of error, although such cells can often be recognized and excluded from analysis. Samples heavily contaminated with maternal blood should be excluded from interphase analysis.

We conclude that FISH test must be a useful method to detect chromosomal numerical abnormality in interphase nucleus according to the criteria from this study, compared to the result of chromosome study. But the limitation of FISH is both expensive and labor-intensive.

한글요약

목 적: 산전진단에 있어 빠른 진단을 위해 그 유용성이 널리 알려져 있는 FISH 방법을 미배양 세포에 적용할 때, 그 민감도를 높이기 위해 본 연구소의 경험과 기준을 소개하고자 한다.

방 법: 1999년 5월부터 2006년 6월까지 본연구소에서 다운증후군 고위험군, 에드워드 증후군 고위험군, 고령산모, 초음파 이상소견 등의 적응증을 주소로 시행한 7,893례의 양수검체를 대상으로 빠른 진단을 위해 8,613례의 미배양 양수세포에 FISH 검사를 시행하였다. 분석은 함춘유전연구소의 기준에 따랐으며, 기존의 세포유전학적 결과와 최종 비교하였다.

결 과: 8613례의 FISH 검사 결과, 30개 이상의 세포관찰이 가능하고, 정상인 경우 정상세포의 비율이 75%, 비정상의 경우 비정상 세포의 비율이 70%에 해당하는 8,502례의 결과를 얻었으며, 세포유전학적 결과와도 일치하였다.

결 론: 산전진단 시 빠른 진단을 위한 FISH검사는 매우 유용하며, 정확한 분석을 위해 그 기준을 마련하는 것은 매우 중요하다 하겠다. 그러나 비용과 인력이 많이 소요되는 한계점을 가지고 있다.

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