

ORIGINAL ARTICLES

The Use of Comparative Genomic Hybridization and Fluorescent In Situ Hybridization in Postmortem Pathology Investigation of Congenital Malformations

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ABSTRACT

Chromosomal abnormalities are an important cause of multiple congenital anomalies (MCA). However, conventional cytogenetic analysis using culture is unsuccessful in 10% to 40% of the cases. The purpose of this study was to examine if retrospective chromosomal analysis was possible on paraffin-embedded autopsy material with new techniques, including comparative genomic hybridization (CGH) and fluorescent in situ hybridization (FISH). We investigated 92 patients, including 71 patients with MCA, 17 patients with an isolated congenital anomaly, and 4 normal controls, by conventional CGH analysis and/or FISH. The karyotype was known in 52 cases, of which 26 patients were normal and 26 had chromosomal anomalies. Comparative genomic hybridization or FISH confirmed all but 2 cases, which were not interpretable. In 40 patients the karyotype was unknown but could be analyzed successfully in 36 cases (90%) by CGH. In this series, we found 1 additional chromosomal aberration, 45,X (Turner syndrome). Furthermore, we examined the postmortem material of 12 patients by FISH, confirming a known abnormal karyotype in 9 patients, an abnormal karyotype found by CGH in 1 case, and confirming DiGeorge syndrome (22q11 deletion) in twins. Comparative genomic hybridization and FISH are reliable techniques with which to perform retrospective genetic analysis on paraffin-embedded autopsy material.

Key words: autopsy, CGH, congenital malformation, FISH

INTRODUCTION

Chromosomal abnormalities are a well-known cause of multiple congenital anomalies (MCA). Karyotyping is a necessary step in the diagnostic approach in patients with MCA and can be performed on material from amniocentesis, skin fibroblasts, chorionic villi, cord blood, or blood from live-born children. Fibroblast culture fails in 10% to 40% of cases of early spontaneous abortion and in 30% of cases of spontaneous late abortion or intrauterine death [1–4]. Other problems with fibroblast culture include the poor quality of chromosomes; in chorionic villus culture maternal cell overgrowth can lead to an erroneous diagnosis. High-resolution chromosome banding on this material is difficult, and small abnormalities can be missed.

New techniques, such as comparative genomic hybridization (CGH), provide us with the ability to analyze the entire genome in frozen or paraffin-embedded tissue, as has been shown in tumor biology [5]. Thus, analysis might be performed on stored autopsy tissues if conventional karyotyping is unsuccessful or has not been done. In addition, fluorescent in situ hybridization (FISH) can be used for the confirmation of CGH results and for those instances in which CGH cannot detect chromosomal abnormalities, such as in cases involving triploidy, or when abnormalities are small, such as in cases involving microdeletions.

The purpose of our study was to examine the feasibility of CGH and FISH on paraffin-embedded autopsy material of children with MCA. To this end, we selected a series of 92 patients, most of whom had MCA. A proportion of these patients had a known normal or abnormal karyotype, allowing us to check the sensitivity of the CGH technique. The remaining patients constituted

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the study group, which yielded 1 hitherto unknown case of Turner syndrome and a twin pair that were concordant for DiGeorge syndrome.

MATERIALS AND METHODS

Patients and tissues

All patients analyzed were selected from the files of the Department of Pathology of the Erasmus MC in the period ranging from 1988 to 2004. Because our children's hospital is a tertiary referral center for pediatric surgery, we chose to study all cases ($n = 27$) of congenital diaphragmatic hernia, most of which ($n = 20$) fall into the MCA category. Further, we expanded this series with 51 patients with MCA, 10 patients with an isolated congenital anomaly, and 4 normal control patients, which were selected to represent cases with known normal and abnormal karyotypes, as well as cases with unknown karyotypes, as a result of technical failure of tissue culture. The clinical patient details are listed in Table 1. All studies were performed on paraffin-embedded thymic tissue, which is routinely fixed for 24 hours in buffered formalin, according to our local autopsy protocol, except in cases of thymic agenesis. The study was performed according to the guidelines for the proper use of patient material of the Erasmus MC Ethical Committee, which requires anonymization of the study material.

Conventional karyotype analysis

Karyotyping was performed on GTG-banded metaphases obtained from fibroblasts or prenatal chorionic villi or amniocyte cultures using standard procedures. Reporting was performed in accordance with the International System for human Cytogenetic Nomenclature 2005 nomenclature [6].

DNA extraction and CGH

Paraffin-embedded tissue sections of 10 μm were treated with xylene, and the DNA was isolated with a DNeasy Tissue Kit (QIAGEN Benelux B.V., Leusden, The Netherlands), following the standard procedure. EcoR1-digested patient and control DNA were labeled (Random Prime labeling system, Invitrogen, Breda, The Netherlands) with Alexa Fluor 594-5-dUTP (Invitrogen) and SpectrumGreen-dUTP (Vysis, Hoofddorp, The Netherlands). Metaphase CGH was performed on standard, well-spread control metaphases. The 500 ng-labeled patient and control DNA and 50 μg Cot-1 DNA (Invitrogen) were precipitated and dissolved in hybridization mix (50% formamid, $2\times$ saline sodium citrate and 1% Tween-20), followed by a simultaneous denaturation of the probe mix on the target slide for 3 minutes at 72°C and a cooling down for 30 minutes, until a temperature of approximately 40°C was reached. After 72 hours at 37°C the slides were washed 3 times (5 minutes each time) in, respectively, $0.4\times$ saline sodium citrate/0.1% Tween-20 at 66°C, phosphate-buffered saline at 66°C, and phosphate-buffered saline at room temperature. Slides were

counterstained with 4',6-diamidino-2-phenylindole (Sigma, Zwijndrecht, The Netherlands) and mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA). Images were captured and analyzed with an Axioplan 2 Imaging microscope (Zeiss, Sliedrecht, The Netherlands) and Isis CGH-analysis software (MetaSystems, Altussheim, Germany). At least 10 recordings were made of 10 different metaphase spreads, and the average green-to-red fluorescence ratio was calculated. The resulting CGH profiles were interpreted by comparing this average ratio to the theoretical normal ratio of 1.0. Loss was defined as a ratio value under 0.8 (lower threshold) and gain as a ratio value over 1.2 (upper threshold) [7].

Confirmation of CGH data by FISH

BAC clones were selected from the University of California–Santa Cruz genome browser and purchased from BACPAC Resources (Oakland). Five to 10 μg of DNA was semi-automatically isolated with an AutoGen-Prep 3000 robot (Autogen), and after whole-genome amplification (REPLI-g, Molecular Staging), the DNA was digested and labeled indirectly with Bio-16-dUTP or Dig-11-dUTP (Roche) or directly with Alexa 594-5-dUTP (Molecular Probes) or SpectrumGreen-dUTP (Vysis/Sanbio) using the random prime labeling protocol (Random Prime labeling system, Invitrogen). The probes were validated on control metaphases. Tissue slides (10 μm) were deparaffinized and fixed in 70% to 100% ethanol washes. The slides were treated with pepsine (4000 U/0.2 M HCL); hybridization was allowed to occur overnight at 37°C, and slides were further treated as described above. For each probe, 100 interphase nuclei were scored for the presence of signal, following the criteria of Hopman and colleagues [8]. Fluorescent in situ hybridization slides were analyzed with an Axioplan 2 Imaging microscope (Zeiss), and images were captured using Isis software (MetaSystems).

RESULTS

Overview of CGH findings

Table 2 summarizes all chromosomal abnormalities identified in the 92 patients who were studied by CGH and/or FISH. Six patients (7%) were excluded from our series as a result of incomplete information or uninterpretable results of the CGH. The retrieved DNA was of sufficient quality to allow us to successfully perform CGH analysis in 93% of cases. Of 84 patients (45 males and 39 females) successfully studied by CGH, 23 were known to have a normal karyotype, all of which were confirmed by CGH. This was also true for the 25 patients with abnormal karyotypes (13 males and 12 females), except for the 2 cases involving triploidy and the patient with der(22)t(13;22) translocation, which cannot be detected by CGH analysis. The abnormal karyotypes included trisomy 18 ($n = 7$), trisomy 21 ($n = 9$), trisomy 13 ($n = 1$); 2 patients with an anomaly of chromosome 9

Table 1. Continued

Patient No. ^a	Sex	GA	Birth	Structural cardiac defects	Respiratory system	Urogenital system	Digestive tract	CNS/eye	Dysmorphic features	Skeletal system	CDH	Others
78	m	34	LB								+	
79	m	Term	LB								+	
80	f	Term	LB				+				+	
81	m	Term	LB								+	
82	f	Term	LB								+	
83	m	Term	LB								+	
84	f	Term	LB								+	
85	f	34	SB						+			
86	f	21	TOP							+		
87	f	34	SB				+			+		
88	f	21	TOP									
89	m	31	LB		+	+		+				
90	f	31	SB		+		+					Thymic agenesis
91	m	27	LB						+		+	Thymic agenesis
92	m	27	LB			+			+			Thymic agenesis

CNS indicates central nervous system; CDH, congenital diaphragmatic hernia; f, female; GA, gestational age; LB, live born; m, male; SA, spontaneous abortion; SB, stillborn; TOP, termination of pregnancy.

^aItalic text indicates no congenital anomalies; bold text indicates isolated congenital anomaly.

Table 2. Detected karyotypes by several methods

Patient No.	Karyotype	CGH	FISH
1	47,XY,+21	rev ish XY,enh(21)	+
2	47,XX,+21	rev ish XX,enh(21)	
3	47,XY,+21	rev ish XY,enh(21)	
4	47,XY,+21	rev ish XY,enh(21)	
5	47,XY,+21	rev ish XY,enh(21)	+
6	47,XX,+21	rev ish XX,enh(21)	
7	47,XY,+18	rev ish XY,enh(18)	+
8	47,XY,+18	rev ish XY,enh(18)	+
9	46,XY	rev ish XY	
10		rev ish XX	
11	46,XX	rev ish XX	
12		rev ish XY	
13		rev ish XY	
14		rev ish XX	
15	45,XY,-13, der(22)t(13;22)(q12.1;q13.3)	rev ish XY	+
16	47,XX,+13	rev ish XX,enh(13)	
17	46,XY	rev ish XY	
18	46,XX,der(7)(7pter->7q36::10q11.2->10qter),idic(10)(q11.2)	rev ish XX,dim(7q),enh(10p)	
19		rev ish XY	
20	46,XY	rev ish XY	
21	46,XX	rev ish XX	
22	46,XY	rev ish XY	
23		rev ish XX	
24	46,XX	rev ish XX	
25	46,XY	rev ish XY	
26		rev ish XX	
27	Not successful	rev ish X	+
28		rev ish XY	
29		rev ish XX	
30		rev ish XX	
31		rev ish XY	
32	47,XX,+i(12)(p10)	rev ish XX,enh(12p)	+
33	69,XXX	rev ish XX	+
34	46,XX	rev ish XX	
35	69,XXY	rev ish XY	
36	46,XY	rev ish XY	
37		rev ish XY	
38		rev ish XY	
39	47,XX,+9	rev ish XX,enh(9)	+
40		rev ish XX	
41		rev ish XY	
42		rev ish XY	
43	47,XY,inv(9)(p12q13),+i(9)(p10)	rev ish XY,enh(9p)	
44		rev ish XY	
45	46,XY	rev ish XY	
46		rev ish XX	
47		rev ish XY	
48		rev ish XX	
49	46,XX	rev ish XX	
50	46,XY	rev ish XY	
51	46,XX	rev ish XX	
52		rev ish XY	
53	47,XX,+18	rev ish XX,enh(18)	
54	47,XY,+18	rev ish XY,enh(18)	
55	47,XY,+18	rev ish XY,enh(18)	
56		rev ish XX	
57		rev ish XY	
58		rev ish XY	

Table 2. Continued

Patient No.	Karyotype	CGH	FISH
59		rev ish XY	
60	46,XX	rev ish XX	
61	46,XX	rev ish XX	
62	47,XX,+21	rev ish XX,enh(21)	
63	46,XX	rev ish XX	
64	Not successful	rev ish XY	
65	46,XY	rev ish XY	
66	46,XY,r(15)	rev ish XY,dim(15q25qter)	+
67	47,XX,+18	rev ish XX,enh(18)	
68	47,XX,+18	rev ish XX,enh(18)	
69	46,XY	rev ish XY	
70	47,XY,+21	rev ish XY,enh(21)	
71	47,XX,+21	rev ish XX,enh(21)	
72	46,XX	rev ish XX	
73		rev ish XX	
74	46,XX	rev ish XX	
75		rev ish XY	
76		rev ish XX	
77	46,XY	rev ish XY	
78		rev ish XY	
79		rev ish XY	
80		rev ish XX	
81		rev ish XY	
82		rev ish XX	
83		rev ish XY	
84	46,XX	rev ish XX	
85	47,XX,+21	Not successful	
86		Not successful	
87		Not successful	
88		Not successful	
89		Not successful	
90	46,XX	Not successful	
91	46,XY	Not done	22q11 deletion
92	46,XY	Not done	22q11 deletion

CGH indicates comparative genomic hybridization; FISH, fluorescent in situ hybridization.

[47,XX,+9 and 47,XY,inv(9)(p12q13),+i(9p)]; and 1 case each of a ring chromosome 15, a translocation of the q-arm of chromosome 13 to 22qter [45,XY,-13,der(22)t(13;22)(q12.1;q13.3)], tetrasomy 12p [47,XX,+i(12p)], and a complex anomaly apparently resulting in loss of part of the 7q36 and a net gain of chromosome 10p [46,XX,der(7)(7pter->7q36::10q11.2->10qter),idic(10)(q11.2)]. The karyotype was unknown in 36 patients (21 males and 15 females, 43%), of which 2 cases had a previous fibroblast culture/amniotic fluid culture that was not successful. In these, CGH showed no chromosomal abnormalities in 35 of 36 patients. In the remaining patient, a girl, we were able to detect a lack of one of the sex chromosomes, compatible with Turner Syndrome (45,X). This patient represents 1 of the 2 cases with an unsuccessful fibroblast culture.

Comparison of CGH and FISH results

We could confirm the CGH results in all cases that were analyzed by FISH. Fluorescent in situ hybridization was

only performed on selected cases, because it is not a genome-wide screening tool [2–9]. Thus, only selected regions of the genome are routinely explored, including the centromeres of chromosomes 18, 21, and X [2]. Representative cases are shown in Figure 1. This technique would also be useful in the determination of translocation breakpoints, as in our case of 45,XY,-13,der(22)t(13;22)(q12.1;q13.3). In addition, we used FISH in the detection of a 22q11 deletion in twins with cardiac abnormalities and thymic agenesis, known to have DiGeorge syndrome (22q11 deletion). Finally, we confirmed the ring chromosome 15 with a partial deletion of chromosome 15q by FISH analysis (Fig. 2).

DISCUSSION

In this study, we have investigated DNA from postmortem tissue in a group of 90 patients with congenital anomalies with CGH to confirm previously found karyotypic abnormalities and to detect hitherto unknown chromosomal anomalies. We could confirm all known chromosomal

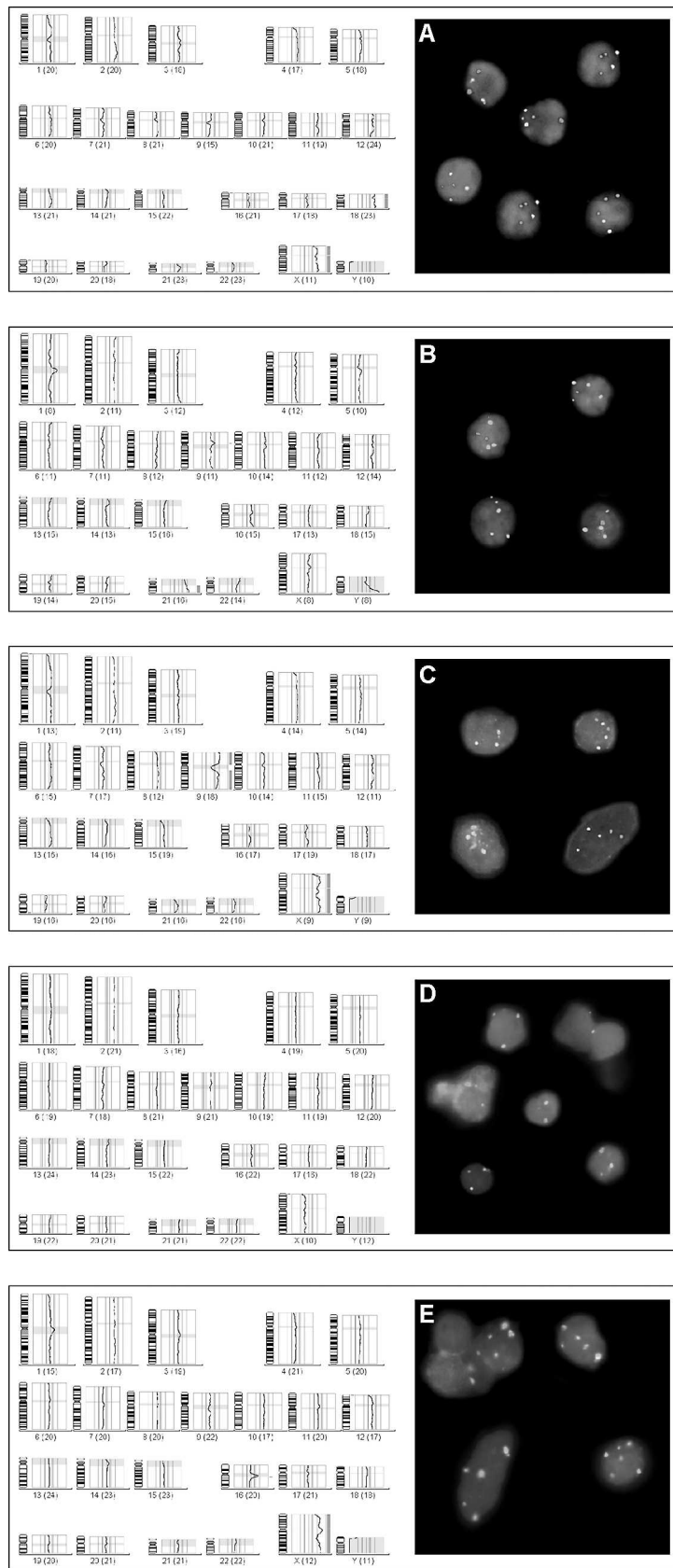


Figure 1. Examples of constitutional chromosome aberrations observed with comparative genomic hybridization and confirmed by fluorescent in situ hybridization (FISH) on nuclei from isolated formalin-fixed and paraffin-embedded tissue sections **A.** rev ish XX enh(18); **B.** rev ish XY enh(21); **C.** rev ish XX enh(9); **D.** rev ish X; **E.** rev ish XX. The probes used for FISH are in panels **A** and **B:** green (21q21, RP11-15H6) and red (18q11.2, RP11-79F3); panel **C:** green (9q22, CTD-236112) and red (cen6, D6Z1); panels **D** and **E:** green (cenX, pXB12) and red (cen6, D6Z1).

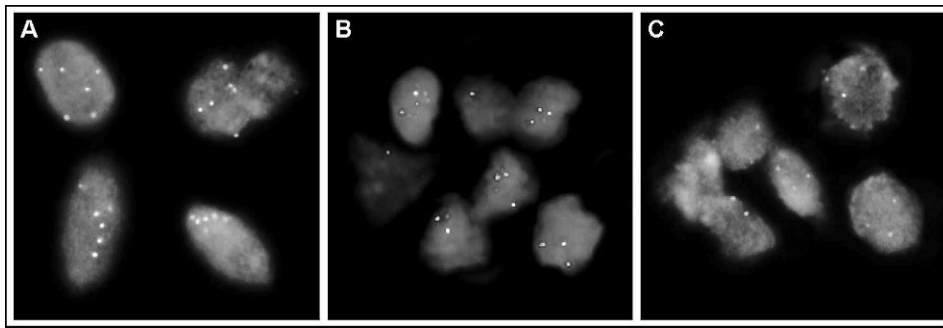


Figure 2. To confirm structural anomalies detected by comparative genomic hybridization [panels A, C: patient 32 (+i12p) and 66 (r15), respectively] or to verify a known del 22q11 phenotype (panel B: patient 91), we performed interphase fluorescent in situ hybridization on isolated nuclei from paraffin sections. The probes used are as follows: (A) RP11-784K12(12p13); (B) RP11-152E2 (22q11); and (C) RP1-154P1 (15qter) in green and in red the control probe cen6 (D6Z1).

aberrations. In addition, we have shown that CGH yields an interpretable result in 93% of cases after formalin fixation and paraffin embedding. In selected instances, FISH has been used to confirm CGH results or has been used to detect those abnormalities that cannot be found by CGH, such as triploidy. We conclude that these techniques are valuable additional tools in the postmortem analysis of fetuses and infants with (multiple) congenital anomalies and can be considered as an expansion of the diagnostic armamentarium of the pathologist.

In the documentation of MCA, autopsy is an important tool [10]. Even after a full examination it is often still not possible to make a firm syndrome diagnosis. Karyotyping on autopsy tissue often fails because of the poor quality of the chromosomes, maternal cell contamination, or culture failure. However, new molecular techniques, such as CGH, can circumvent this unsuccessful karyotyping. Although CGH has been used in cancer research for the past 2 decades, few studies were performed on postmortem tissue [1–5,7,9–12]. Comparative genomic hybridization analysis is based on DNA and not on metaphase cultures, such as the traditional G-banded chromosome analysis. For this reason, the known problems of culture failure, maternal overgrowth, and poor quality of the G-banded chromosome morphology are overcome [1]. In retrospect, we could detect the chromosome pattern in 36 patients in which karyotyping had never been performed (94%) or was unsuccessful (6%). Also, we confirmed 22 of the 25 abnormal karyotypes, but 3 cases were not confirmed by CGH because of the fact that triploidy and balanced translocations cannot be detected with this technique.

Comparative genomic hybridization and FISH analysis can be regarded as complementary techniques, each with its own indications and limitations. Thus, CGH can be employed if genome-wide information is needed, with the exception of cases of triploidy and balanced translocations. Fluorescent in situ hybridization analysis, however, is specifically directed at certain chromosomal regions that may be suspected to be abnormal. Lomax and colleagues [3] used flow cytometry in combination with CGH to overcome the limitations of the latter technique in

ploidy determination. They achieved accurate information on the chromosomal complement in 99.7% of the analyzable cases of spontaneous abortion. However, flow cytometry cannot be used for the detection of balanced translocations, for which reason we confirmed selected cases with FISH, which is appropriate for this purpose. Additionally, we used FISH in 2 patients with known DiGeorge syndrome, which is characterized by cardiac abnormalities, abnormal facies, thymic hypoplasia, cleft palate, and hypocalcemia [11]. DiGeorge syndrome shows overlap with velo-cardio-facial syndrome and conotruncal-face syndrome.

In our study we selected autopsy material from children with MCA because one would expect to find more chromosomal aberrations in these children. However, we only found 1 additional chromosomal defect, a monosomy X in a stillborn child, in which the skin fibroblast culture had not been successful. Daniely and colleagues [12] also detected a 45,X karyotype in a patient in whom conventional cytogenetic analysis was not successful because of tissue culture failure. In the study of Schaeffer and colleagues [1], using CGH, 9.8% more chromosome abnormalities were detected on fresh material from early abortions, in comparison with G-banded chromosome analysis. This was due to the fact that CGH allows for the detection of chromosome abnormalities in cases in which culture failed. It still remains a subject of further research if this success can be confirmed on paraffin-embedded material from cases involving miscarriage or autopsies. Although we showed that retrospective genetic analysis with FISH and CGH is possible on postmortem formalin-fixed, paraffin-embedded material, we would like to emphasize that analysis on fresh material is preferable. We would like to encourage the storage of fresh-frozen material in liquid nitrogen in every autopsy for which parental consent has been obtained.

Since the studies described in this manuscript were performed, several new techniques have been introduced, not only in a research setting, but also for diagnostic purposes. These include array-CGH and single nucleotide polymorphism array analysis. Both techniques allow for the analysis of the entire genome at high resolution, which

has an advantage over conventional CGH analysis, which was employed in the present study. These techniques may or may not give a higher yield of chromosomal abnormalities in tissues from patients with MCA.

In summary, we have shown that CGH analysis, followed by FISH, in selected cases, is a suitable technique for the detection of chromosomal abnormalities in postmortem paraffin-embedded tissues in cases in which regular karyotype analysis by fibroblast culture was unsuccessful or was not performed.

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