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Fluorescence *in situ* hybridization (FISH) using non-commercial probes in the diagnosis of clinically suspected microdeletion syndromes

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***Background & objectives*: Microdeletion syndromes are characterized by small (<5 Mb) chromosomal deletions in which one or more genes are involved. These are frequently associated with multiple congenital anomalies. The phenotype is the result of haploinsufficiency of genes in the critical interval. Fluorescence *in situ* hybridization (FISH) technique is commonly used for precise genetic diagnosis of microdeletion syndromes. This study was conducted to assess the role of FISH in the diagnosis of suspected microdeletion syndrome.**

***Methods*: FISH was carried out on 301 clinically suspected microdeletion syndrome cases for the confirmation of clinical diagnosis using non-commercial probes. Of these, 177 cases were referred for 22q11.2 microdeletion, 42 cases were referred for William syndrome, 38 cases were referred for Prader Willi/Angelman and 44 cases were referred for other suspected microdeletion syndromes.**

***Results*: FISH was confirmatory in 23 cases only (7.6%). There were 17 cases of 22q11.2 microdeletion, four cases of Prader Willi syndrome and two cases of William syndrome.**

***Interpretation & conclusion*: We conclude that FISH should not be the method of choice for clinically suspected microdeletion syndromes. We propose to follow strict clinical criteria for FISH testing or preferably to follow better methods (genotype first approach). Whole genome screening may be used as first line of test and FISH may be used for confirmation of screening result, screening of family members and prenatal diagnosis.**

Key words Fluorescence *in situ* hybridization - molecular cytogenetics - suspected microdeletion syndrome

The microdeletion syndrome is characterized by hemizygous microdeletion (<5 MB) of chromosomes in which one or more genes are lost. It is mostly spontaneous, and is reported to occur in approximately 5 per cent of patients with unexplained mental retardation[1,2]. This is frequently associated with multiple congenital anomalies and developmental delay[3,4]. The known microdeletion syndromes are DiGeorge/Velocardiofacial (22q11.2), Prader-Willi/ Angelman (15q11-13), William (7q11.23), Smith-

Magenis (17p11.2), Cri-du-Chat (5p15.2), Miller-Dieker (17p13.3), WAGR (Wilms tumour, Aniridia, Genitourinary anomalies and mental Retardation; 11p13), HNPP (Hereditary Neuropathy with Liability to Pressure Palsy; 17p12), Wolff Hirschhorn (4p16.3), TRPS (Tricho-Rhino-Phalangeal; 8q24.1), ATR 16 (Alpha Thalassaemia mental Retardation-16; 16p13.3), *etc*.

G-banded karyotyping is the most common approach for the detection of genomic alterations. However, despite its indisputable success, this tool has limited resolution, usually being unable to detect genomic changes of less than 5 Mb[3,4]. In addition, most rearrangements of the ends of the chromosomes (telomere or sub-telomere) are too small to be detected using traditional banding technique. These limitations of conventional chromosome analysis have been overcome by FISH[5,6]. The ability of FISH to detect cryptic chromosomal rearrangements exceeds the resolution of any form of cytogenetic banding techniques. However, conventional FISH does not allow a comprehensive evaluation of the whole genome. Thus, FISH provides a high-resolution analysis of only targeted locations. This study was aimed to assess the role of FISH for the rapid detection of various clinically suspected microdeletion syndromes.

Material & Methods

This study was conducted in the department of Reproduction Biology and Pediatrics, All India Institute of Medical Sciences (AIIMS), New Delhi, India, from January 2005 to December 2011. A total of 301 consecutive cases of suspected microdeletion syndromes who required confirmation of diagnosis by FISH were prospectively enrolled in the study. All cases were referred from various States of northern India for management, including confirmation of clinical diagnosis. All patients had a normal karyotype before sending for FISH. The study protocol was approved by the Institutional Human Ethics Committee. All patients underwent clinical genetics evaluation as per specific microdeletion syndrome (Table I). The relevant morphological features were recorded. EDTA and heparinized blood samples were collected from the affected individuals (0.5-1 ml each). FISH study was carried out on both interphase and metaphase cells.

Interphase cell suspension was prepared by standard method[7]. In brief, blood nucleated cells were washed in phosphate buffer saline (PBS), three times before hypotonic treatment (50 mMol KCL) for 30 min and fixation in methanol:acetic acid solution (3:1). Cells were finally re-suspended in 100 µl of fresh fixative. Approximately 20 µl of cell suspension was used to prepare the slide. Metaphase spreads were prepared from phytohaemagglutinin stimulated human peripheral blood lymphocytes using standard cytogenetic technique[7]. Microdeletion status was determined by FISH using non-commercial DNA probes. Bacterial artificial chromosome (BAC)/ Phage artificial chromosome (PAC) clones (Table II) were obtained from European Resource Centre for Molecular Cytogenetics, University of Bari, Italy (*http://www.biologia.uniba.it/rmc/*; Courtesy: Prof. Mariano Rocchi) for the study. The clones were received as bacterial (LB) agar stab culture, which were sub-cultured on LB agar plate before growing in large amount in LB medium. Probe DNA was extracted using a commercial BAC extraction kit (Sigma, India). All probes were labelled using nick translation method[7] with FITC-12-dUTP (Roche, Germany) or TRITC-12-dUTP (Roche, Germany) or Cy3 (Amersham, UK). Working concentration of probe DNA was between 100-200 ng/µl. Slide was washed in acetic acid for two min, and dehydrated in 70, 90, 100 per cent ethanol, three min each. Nuclei on the slide were digested with pepsin (100 µg/ml) in 0.01 N HCl for 20 min at 37°C, rinsed in double distilled water and followed by PBS, and fixed in 1 per cent paraformaldehyde in PBS for 10 min at 4°C. Slides were then rinsed in PBS, twice in double distilled water and then dehydrated through ethanol series as before. The hybridization buffer (60% formamide, 2× SSC, 10% dextran sulphate, Sigma, USA) containing labelled probe was applied to the

Table I. List of common microdeletion syndromes

Syndrome	Micro deletion
DiGeorge/Velocardiofacial syndrome	22q11.2
Prader-Willi Syndrome and Angelman syndrome	15q11-ql3
William Syndrome	7q11.23
Miller-Dieker/Lissencephaly syndrome	17p13.3
Tricho-Rhino-Phalangeal (TRP) syndrome	8q24.11-q13
Wolff-Hirschhorn syndrome	4p
Cri-du-chat syndrome	5p15.2
ATR-16 syndrome	16p13.3
1p36 Deletion syndrome	1p36

Table II. Details of FISH probes used in the study

Microdeletion syndromes (MDS)	Disease locus	BAC/PAC clones	Clone locus
DiGeorge/Velo Cardio Facial syndrome	22q11.2	**RP5 882J5** RP11 22M5 CTA154 H4 CTA322 B1	**22q11.2** 22q11.22 22q11.21 22q11.23
Prader-Willi/Angelman syndrome	15q11-13	**RP11 20B10** RP11 26F02 RP11 37J13 RP11 456J20	**15q12** 15q11.2 15q13.1 15q13.3
Williams syndrome	7q11.23	**RP5 1127A24** RP5 1177A1 RP11 99J9	**7q11.23** 7q11.23 7q11.23
Tricho Rhino Pharyngeal syndrome	8q24.11 -q24.13	RP11 1082L8 RP11 494N20	8q24.13 8q24.11
Wolff Hirschhorn syndrome	4p16.3	RP5 860A13	4p16.3
Cri du chat syndrome	5p15.2-p15.3	RP5 982O4	5p15.2-p15.3
Miller-Dieker/Lissencephaly syndrome	17p13.3	RP11 22G12	17p13.3
Retinoblastoma	13q14.1-q14.2	RP11 540M5	13q14.12
Steroid Sulfatase Deficiency syndrome	Xp22.32	RP5 1129A6	Xp22.13
ATR 16 syndrome	16p13.3	dJ471F17	16p13.3
1p36 Deletion syndrome	1p36	RP5 902P8	1p36.33

Bold clones indicate informative locus; BAC/PAC, bacterial artificial chromosome / phage artificial chromosome
FISH probe clones were obtained from European Resource Centre for Molecular Cytogenetics, University of Bari, Italy (*http://www.biologia.uniba.it/rmc*)

slides under a circular cover slip (11 mm in diameter). The probes and nuclear DNA were denatured together at 76°C for 6-7 min. Hybridization was performed in a dark moist chamber at 37°C for overnight. After hybridization, cover slips were removed and slides were washed with NP40 (0.03% solution) Sigma, India; also known as Tergitol/nonyl, phenoxypolyethoxylethanol), at 72°C for 2-3 min, followed by NP40 (0.01% solution; for 2 min at room temperature. Then slides were dehydrated in ethanol series, as before and mounted in antifade (Vector, USA) with 1 µg/ml 4,6 diaminidino-2- phenylindol (DAPI; Sigma, USA). The slides were screened under Olympus BX 51 fluorescent microscope (Japan) with 100 watt mercury bulb using 100× plan apochromatic objective and single band pass filter for DAPI, FITC and TRITC (Olympus Japan). FISH image was captured through spectral imaging system. A total of at least 500 interphase nuclei and at least 10 metaphase nuclei were scored from each case. Presence of two signals in 100 per cent metaphases and 90 per cent interphase cells was considered as normal whereas demonstration of one signal in 100 per cent metaphases or 85-90 per cent interphase cells was considered as microdeletion positive. When presence of both one and two signals in interphase (at least 15% deleted) as well as in metaphase (at least 10% deleted) cells were observed, the case was considered to have mosaicism[8].

Results & Discussion

A total of 301 consecutive cases of clinically suspected microdeletion syndromes were studied by FISH. The mean age of the patient was 4.47 yr (range 5 days to 29 yr). There were 197 males and 104 females. One hundred seventy seven patients had referred for 22q11.2 microdeletion, 42 patients for William Syndrome, 38 patients for Prader Willi/Angelman syndrome and remaining 44 cases for various microdeletions (Table III). Of the 301 patients, 23 (7.6%) had hemizygous microdeletion; 13 non-mosaic and 10 mosaic (Table III; Fig 1-3). All karyotypes were normal with conventional cytogenetic analysis.

Molecular probes for most microdeletion syndromes are now readily available. This leads to routine use of FISH for diagnosis of microdeletion syndromes. FISH is considered as the gold standard

Table III. Details of FISH results

MDS (number)	Locus	FISH positive (%)	Pure (%)	Mosaic (%)	Deleted clones
DiGeorge/Velo Cardio Facial syndrome (177)	22q11.2	17 (9.6)	11 (6.2)	6 (3.4)	RP05-882J5
Williams syndrome (42)		2 (4.76)	0	2 (4.76)	RP05-1127A24
Prader-Willi/Angelman syndrome (38)		4 (10.5)	2 (5.25)	2 (5.25)	RP11-20B10
Miller-Dieker/Lissencephaly syndrome (17)	17p13.3	0	0	0	
Retinoblastoma/RB1 (13)	13q14	0	0	0	
Tricho Rhino Pharyngeal syndrome (5)	8q24.11 - q24.13	0	0	0	
Cri du chat syndrome (2)	5p	0	0	0	
ATR 16 syndrome (1)	16p33	0	0	0	
1p36 Deletion syndrome (3)	1p36.33	0	0	0	
Steroid Sulfatase Deficiency syndrome (1)	Xp22.3	0	0	0	
Wolff Hirschhorn syndrome (2)	4p	0	0	0	
Total (301)		23 (7.6)	13 (4.3)	10 (3.3)	

technique to confirm the diagnosis of microdeletion syndromes. FISH is also used to screen the ends of all human chromosomes to uncover terminal deletions and unbalanced translocations which are common with malformation syndromes. However, the drawback of conventional FISH is its failure to detect other than the targets, leading to negative result if clinical diagnostic criteria are not strict, as seen with previous study[9]. In this prospective study, FISH test was carried out in 301 clinically suspected microdeletion syndromes and detectable microdeletions were found in only 7.6 per cent patients. Poor clinical accuracy was found leading to negative FISH result in most of referrals. This frequency is extremely low. The possible reasons for this low positivity in our study were as follows: *(i)* Majority of the microdeletion cases were from 22q11.2 microdeletion syndrome (177 of 301 cases) and criteria for the FISH test were conotruncal cardiac abnormality requiring surgery with or without other abnormalities. We did not find any FISH positive 22q11.2 microdeletion with isolated conotruncal cardiac anomaly (72 of 177 cases). *(ii)* Prader Willi/Angelman syndrome (PWS/AS 38/301 cases; locus 15q11-13) could be due to microdeletion (~70% cases) or uniparental disomy or imprinting centre deletion or point mutations. We have tested only for microdeletion, hence deletion negative cases could still be PWS/AS due to non microdeletion mechanisms. *(iii)* Clinical features, in particular facial dysmorphism were observed to be less distinct in infancy leading to inappropriate referral for FISH test. Often cases do not fit with any known microdeletion syndrome. These cases were screened for several possible microdeletions one by one without any success. *(iv)* Furthermore, it is expected to have low (~5%) microdeletion rate in retinoblastoma (congenital bilateral cases). *(v)* In fact, we were unable to follow strict clinical criteria for FISH test due to various non-technical reason *viz.*, request from parents or referring doctors leading to low pick up rate.

Other studies[10-12] also observed importance of accurate clinical criteria before FISH test for microdeletion syndrome. They have also observed that clinical diagnosis of microdeletion syndrome is difficult due to the large phenotypic variability. FISH provides specific and limited information thus requires clinician to anticipate a specific microdeletion syndrome accurately in order to determine what kind of FISH testing is needed for the patient. If such strict criteria are not followed, then FISH will not provide optimal result. However, it is difficult to follow strict clinical criteria as often microdeletion positive cases presented with minimal clinical features[13,14] and at the same time, cases with strong clinical features were observed without microdeletion. This has also been recognized in our study. FISH is still highly labour-intensive and time consuming procedure with non-commercial FISH probes, although inexpensive.

Recent technologies, such as microarray and multiplex ligation-dependent probe amplification (MLPA), are efficient methods for screening copy number imbalances in multiple genomic regions simultaneously[15,16]. Following use of microarray

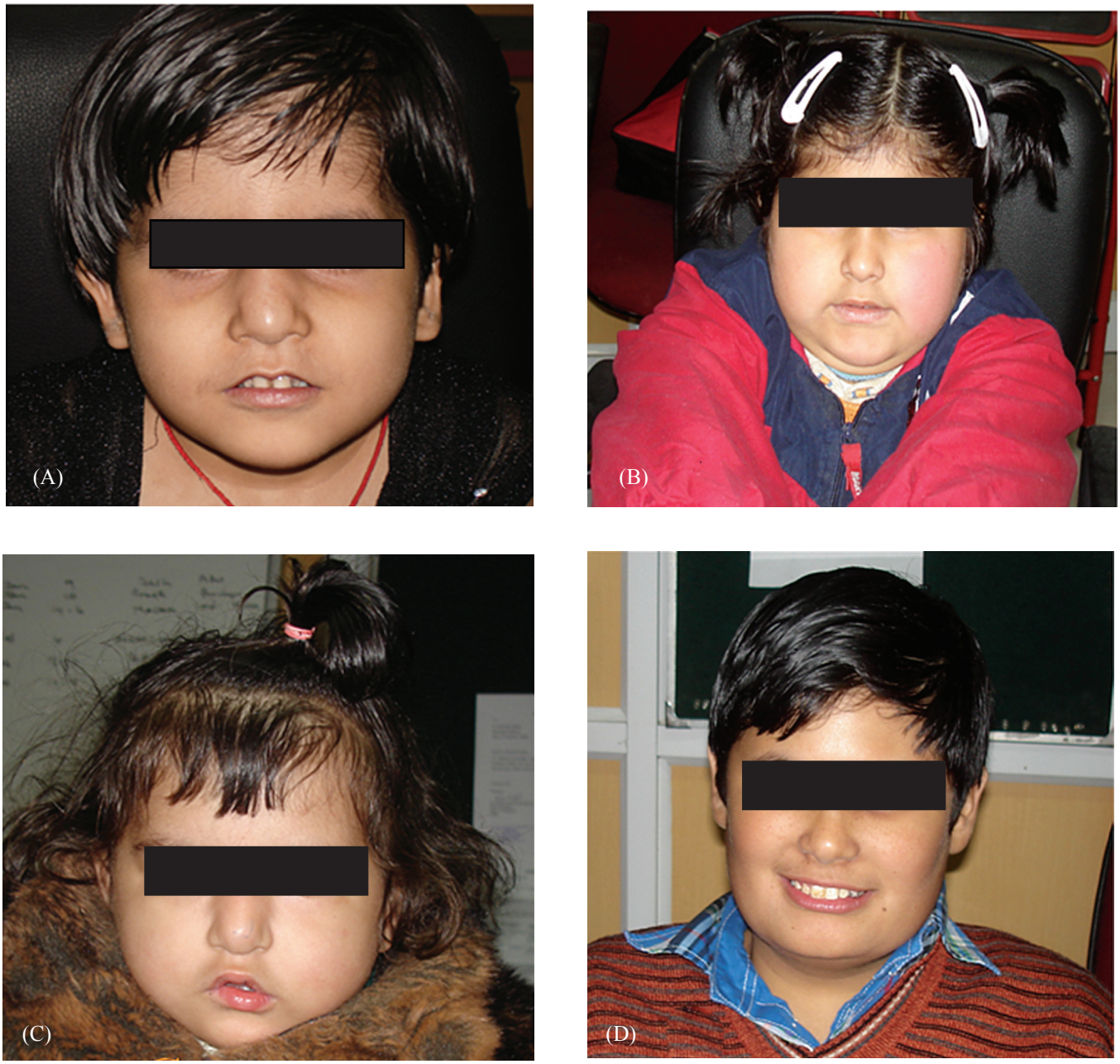

Fig. 1. Shows facial profile of microdeletion positive cases (A: 22q11.2 microdeletion; B: Prader-Willi syndrome; C: mosaic 22q11.2 microdeletion; D: mosaic Prader-Willi syndrome).

technique we now know that microdeletions are frequently co-occurred with a second deletion or duplication[17] elsewhere in the genome. These second hits are missed by the FISH assay. In our recent study to find out reason for phenotypic discordance in a pair of monozygotic twins, microarray study was able to detect alteration in deletion size between the twins as a possible cause for the phenotypic differences[14]. This indicates that microarray technique should be carried out not only for diagnostic screening but also for research work. We have carried out microarray analysis in two more cases, one found to be William syndrome (FISH test was carried out for 22q11.2 microdeletion 4 years ago in infancy due to wrong diagnosis) and other had multiple gains (3) and losses (2) in chromosomes other than suspected microdeletion. These factors warrant relooking of routine use of FISH assay for diagnosis of microdeletion syndrome and/or multiple

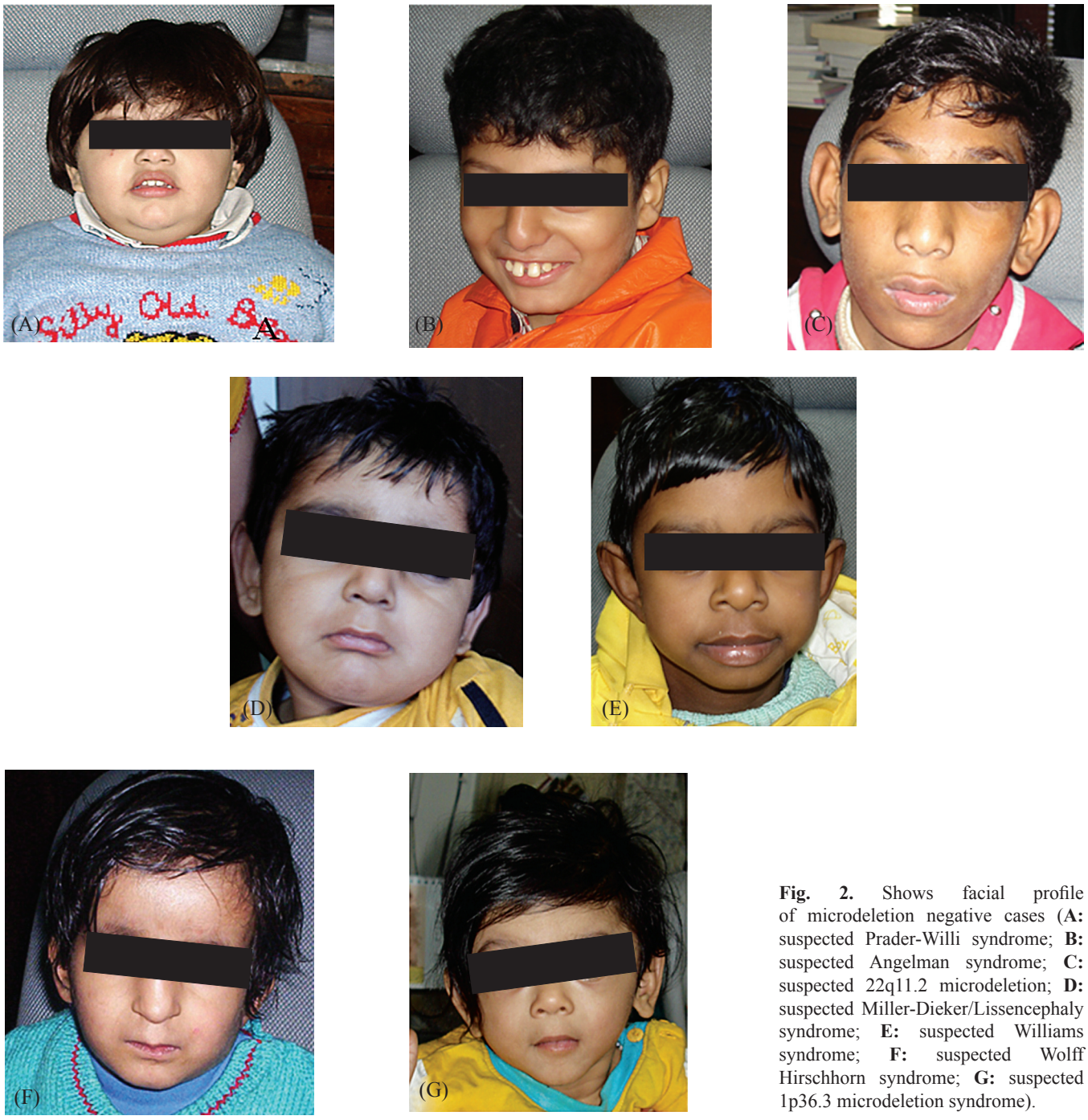

Fig. 2. Shows facial profile of microdeletion negative cases (**A:** suspected Prader-Willi syndrome; **B:** suspected Angelman syndrome; **C:** suspected 22q11.2 microdeletion; **D:** suspected Miller-Dieker/Lissencephaly syndrome; **E:** suspected Williams syndrome; **F:** suspected Wolff Hirschhorn syndrome; **G:** suspected 1p36.3 microdeletion syndrome).

malformations. This is also recommended by a recent study[18] as well as consensus statement[19].

The advances in molecular cytogenetic techniques, in particular microarrays have the capacity for accurately estimating the extent of the deletion[14]. The genome-wide view of microarray is the leading current mechanism for identifying unsuspected anomalies as well as reasons for clinical variability[14]. The 'genotype-first' approach will allow delineation of new syndromes based on the genomic alteration, rather than the clinical presentation. Such an approach will lead to the grouping of patients, sometimes with discordant phenotype. It is proposed that microarray should be used as the first approach to all suspected (non classical)

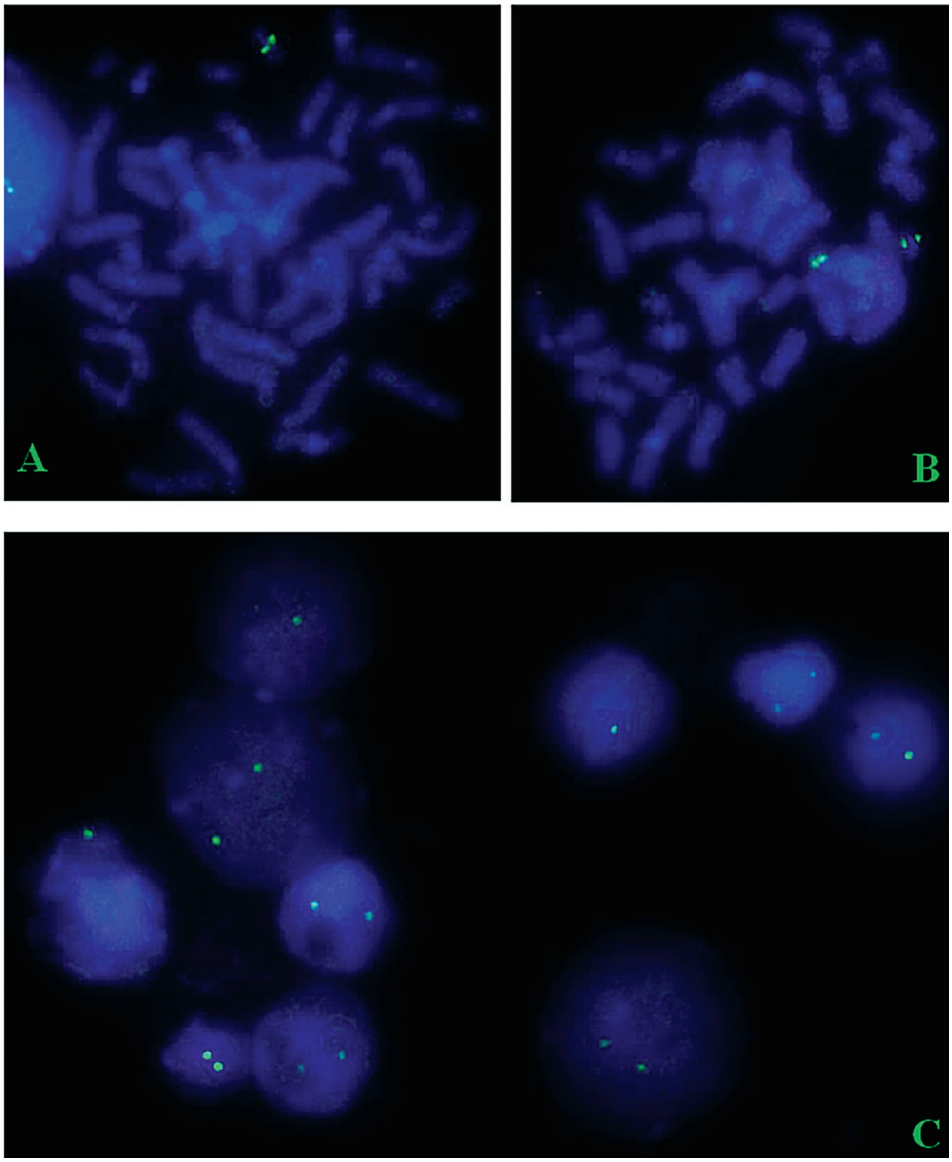

Fig. 3. Shows FISH image of clinically suspected microdeletion cases (**A:** hemizygous microdeletion *i.e.*, only one chromosome shows FISH signal; **B:** normal dizygous state *i.e.*, both chromosomes show FISH signal; **C:** mosaic hemizygous microdeletion *i.e.*, interphase cells with one as well as two FISH signals).

microdeletion syndrome or multiple malformation syndromes. This approach will provide the highest chance of making a diagnosis and sparing the patient unnecessary diagnostic testing from many places. FISH testing should be limited to use for confirmation of diagnosis as well as to study for mosaicism followed by subsequent screening of family members, prenatal diagnosis and preimplantation diagnosis.

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