Original Communication

Placental chimerism in early human pregnancy

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BACKGROUND : Human chimerism is rare and usually uncovered through investigations of ambiguous genitalia or blood grouping or prenatal diagnosis. Most of the publications on placental chimerism are mainly case reports. There is no systematic search with sensitive techniques for placental chimerism in human.
AIM : This study was aimed to asses placental chimerism through two sensitive molecular techniques i.e., interphase fluorescent in situ hybridization and quantitative fluorescent PCR.
MATERIAL AND METHODS : Placental chimerism was analyzed using X & Y dual color fluorescent in-situ hybridization onto 154 placentae from natural conceptions, obtained at termination of pregnancy between 7 to 16 weeks of gestation.
RESULTS : Three cases of placental sex chromosome chimerism were observed (1.95%). Exclusion of maternal contamination and diagnosis was confirmed later by quantitative fluorescent PCR.
CONCLUSION : This finding indicates that placental chimerism in early human pregnancy is not rare.

Key Words : Placental Chimerism, Interphase FISH & Quantitative Fluorescent PCR.

In biology, the word chimerism is used when an organism contains cell population from two or more zygote.[1] This may be of true chimerism or confined chimerism. In true chimerism admixture of 2 or more zygote occurs very early in embryonic life and every tissue of individual including placenta are chimeric. Typical example is true hermaphrodite with 46,XX/46,XY chromosome complement. In confined chimerism admixture of 2 or more zygote is limited to one type of tissue viz. blood (confined blood chimerism; CBC) or placenta (confined placental chimerism; CPC). Mechanism for chimerism could be due to placental vascular anastamosis or an admixture of trophoblastic cells during early blastocyst development. CBC frequently seen in monochorionic dizygotic twining.[2] CPC is common with twin pregnancy or with vanishing twin or as part of natural selection to rescue embryo proper.[3]

Chimerism (freemartin condition) is common in some animal viz. cattle (particularly in holstein cows[4] and callitrichid primates[5]. Freemartins arise when vascular connections form between the placentae of developing heterosexual twin foeti resulting XX/XY chimerism. It may lead to masculinization of the female offspring to varying degrees. It's prevalence is increasing following introduction of high fecundity genes into flocks leading to multiple pregnancy and placental vascular connection.[4] Callitrichid primates typically give birth to twin offspring that are somatic chimeras of cells derived from two products of conception.[5]

Chimerism in human considered as rare phenomenon.[6] However, some investigators observed this more frequently than previously recognized.[7-9] In recent years, due to interference through assisted reproductive techniques, chimerism either confined to placenta or blood or true is seen more frequently in human.[2,10] CBC is going to be more frequent and important in the near future due to more & more

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successful heterologous bone marrow transplantation.

CBC is risk factor for twin-twin transfusion syndrome and may mislead physician when genotyping for a disease-susceptibility test in medical (prenatal/postnatal) practice. CBC is often associated with complications like error in blood grouping/typing and HLA typing to fetal amelia/cutis aplasia.[8,11-13] CPC may lead to prenatal diagnostic error from chorionic villous sampling (CVS). The special situation of prenatal diagnosis from CVS and medico-legal aspects related to diagnostic error has led interest in the assessment of placental chimerism. Furthermore, there is no systematic prospective search for confined placental chimerism in human, to the best of knowledge. CPC requires both placenta and embryo for diagnosis, which is difficult to obtain. This study is an attempted to asses placental chimerism through two sensitive molecular techniques i.e., interphase fluorescent in situ hybridization (FISH) and quantitative fluorescent PCR (QF PCR) with short tandem repeats marker (STR) in a prospective manner.

Material and Methods

At termination of pregnancy (between 7-16 weeks of gestation) samples were collected from 154 placentae together with maternal peripheral blood from heterogenous group of patients (white, black, asian, etc) attending Elizabeth Garrett Anderson and Obstetric Hospital, UCL, London, UK. All terminations were carried out by intravaginal misoprostol (200 ug) 2-6 hours before suction evacuation under ultrasound monitoring. All cases were unwanted pregnancy and first ultrasound was carried out few days (1-3) before MTP. All 154 placentae, but one (case 3), were from singleton pregnancy (proven by ultrasonography before as well as during termination). All placentae washed three times in normal saline immediately during collection to prevent sticking maternal nucleated blood cells & hence maternal cell contamination. Placenta washed again in phosphate buffer saline (PBS; Sigma) in laboratory before preparation for FISH. Each placenta was cut into small pieces with the help of a scalpel and was treated with collagenase (Sigma; 2 mg/ml) at 37°C for 45 minutes to dissociate cells. After collagenase digestion the sample was pelleted, supernatant was removed and hypotonic KCl (Sigma, UK; 50 mMol) solution was added to swell nuclei at 37°C for 30 minutes. Without removing the KCl solution, an equal volume of fixative (3:1 methanol-acetic acid) was added. The cells resuspended and incubated for 5 minutes and spun. The pellet was resuspended in fixative, same repeated 3 times before resuspending in 50-100 uL fresh fixative. Approximately 10 - 15 uL of cell suspension were used for slide preparation.

Interphase FISH was carried out on dissociated placental cells & umbilical cord (in some) cells using probes specific for human chromosome X & Y as described before.[14] Specificity of probes was checked in each experiment on lymphocyte metaphase spreads. Dual color FISH analysis with probes X (green, flurogreen labelled, Amersham) and Y (red, flurored labelled, Amersham) was performed on all samples.

DNA extracted from all placentae, umbilical cord (case 3) and maternal blood, according to the instruction of QIAamp blood and tissue kit method (QIAGEN). STR markers specific for chromosome 18 (D18S535) and 21 (D21S11, D21S1412, D21S1411 & D21S1414; Perkin Elmer) were tested using multiplex QF PCR with fluorochrome labelled forward oligonucleotide primers as previously described.[15] Extracted DNA first amplified for 23 cycles and then analyzed on automated genetic analyzer (Gene Scan, ABI Prism 310, Perkin Elmer) for size detection and quantification. This was based on the amplification of particular DNA repeat sequences formed by di, tetra or penta nucleotides specific for each pair of chromosome (one from father & other from mother). These STR markers were amplified with fluorescent labelled primer in PCR reaction (reaction stopped in exponential phase of PCR amplification so that the amount of DNA produced was proportional to the initial target sequence) using limited number of cycles. The amplified DNA was analyzed in DNA scanner. The products of two different alleles (one from mother & other from father) should amplify in identical manner and hence same fluorescent activity (in normal diploid state). Thus, if an individual was heterozygote for a chromosome specific STR, two fluorescent peak (one from mother & other from father) with a ratio 1:1 should be detected. When this test carried out along with parental DNA then inheritance pattern of chromosome (STR targets) of the individual could be obtained by tracing & comparing the STR products in the family.

Results

Chromosome X and Y dual color FISH was performed on uncultured dissociated interphase cells of placenta, umbilical cord (case 3) and maternal leukocytes using directly labelled probes. The prevalence of maternal cell contamination (XX cells in placental tissue of male i.e., XY fetuses) were between 0.5% to 4.8% (average 1.8%) even with above precautions. Due to unexpected results in three case i.e., admixture of XX & XY cells in very high concentration, repeat sample preparation from stored sample was carried out from different parts of placenta and umbilical cord. The repeat test gave more or less similar result (Table 1 & Fig. 1). Case 3, a twin pregnancy, showed mixture of two cell lines in placentae as well as in umbilical cord. The PCR result with different polymorphic marker has shown in Table 2 and Fig.2. Case 1 & 3 had two maternal and another two nonmaternal alleles with D18S535 markers i.e., tetra allelic or other way tetra gametic origin. Case 2 was not conclusive because of homozygosity between placenta & maternal blood with most of the markers.

Discussions

Findings of XX and XY cells admixture in placenta may be due to three reasons. Firstly, due to maternal (blood and /or decidual) cell contamination, secondly, due to mosaicism (XX & XY cell lines derived from XXY cell line) and finally, due to chimerism (derived from two distinct XX and XY zygotes i.e., tetragametic). It is not possible to differentiate maternal cell contamination by FISH analysis. However, the way placenta was prepared for FISH analysis, it is unlikely to give such high percentage (15% to 90% in chimeric cases Vs 0.5% to 4.8% in normal samples) of maternal cell (XX) contamination. Later, we excluded this possibility in two cases by QF PCR analysis, from the finding of presence of two non-maternal alleles (paternal) i.e., tetra gametic origin. Possibility of sex chromosomal mosaicism is unlikely, as there were no XXY cell lines. This excluded by QF PCR analysis by finding of more than two autosomal alleles (chromosomes 18 & 21). Presence of four alleles (2 maternal & 2 nonmaternal i.e. paternal alleles) in case 1 & 3 (table 2) proves chimerism (tetra gametic-dispermic). However, in case two, it was not possible to confirm chimerism by QF PCR in the absence of paternal DNA analysis, as markers were non-informative.

Table 1: Showing results of chromosome X & Y FISH on interphase cells (X probe specific to centromere of X chromosome & Y probe specific to q heterochromatic region of Y chromosome)

Cases	Sample Types	XX%	XY%	XXY%	XYY%
No.1	Maternal Leukocytes	99	0	0	0
	Placenta (Initial)	25	75	0	0
	Placenta (repeat)	21	79	0	0
No.2	Maternal Leukocytes	98	0	0	0
	Placenta (Initial)	90	10	0	0
	Placenta (repeat)	93	7	0	0
No.3	Maternal Leukocytes	0	0	99	0
	Cytogenetic analysis show karyotype 46,XX,t(14;Y)(pter;qh)				
Twin A	Placenta site a	0	0	85	15
	Placenta site b	0	0	93	7
	Umbilical cord	0	0	95	5
Twin B	Placenta site a	0	0	15	85
	Placenta site b	0	0	16	84
	Umbilical cord	0	0	31	69

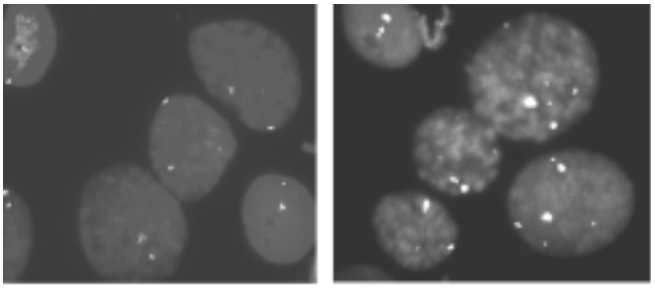

Figure 1: Showing X (green) and Y (red) dual color interphase FISH (mixture of XX and XY cells) on placental nuclei of Cases 1 and 2

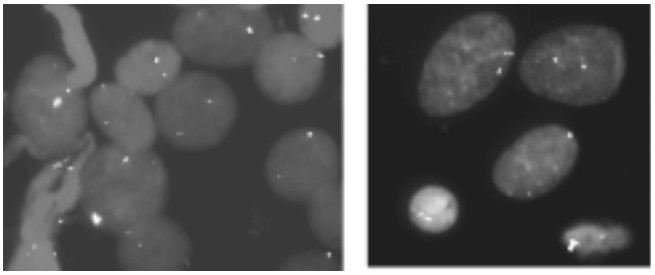

Figure 2 : showing QF PCR results (four peaks, i.e., alleles) with D18S535 STR of Case one (A), Case three twin A (B) & twin B (C). Differences in peaks (amplifications) were due to different amount of cell admixtures (more the initial concentration better is the peak height and area; red peaks are internal sizers; A1, A2, A3 & A4 are allele 1, 2, 3 & 4). Axis X denotes PCR product size in base pair (allele differentiation on size) and axis Y denotes quantification of PCR product through peak height and area (more peak height & area more is the initial DNA).

Table 2: Results of QF PCR with STR polymorphic markers on maternal blood and placentae/umbilical cord of chimeric cases (case 1 & 3 tetra allelic/gametic at D18S535 locus; case 2 non informative)

	CASE 1			CASE 2			CASE 3				
STRs	**Mat.Blood**	**Placenta**	**Remarks**	**Mat.Blood**	**Placenta**	**Remark**	**Mat.Blood**	**Cord/TwA**	**Remark**	**Cord/TwB**	**Remark**
D18S535											
A1	145	145	2 maternal	137	137	2 maternal	141	141	2 maternal	141	2 maternal
A2	150	150	alleles	145	145	alleles	155	155	alleles	155	alleles
A3		141	2 paternal			**non infor-**		130	2 paternal	130	2 paternal
A4		137	alleles			**mative**		151	alleles	151	alleles
			chimeric						**chimeric**		**chimeric**
D21S11											
A1	228	228	absence	221	221	**non infor-**	222	222	**non infor-**	222	**non infor-**
A2	230		of one mat.		225	**mative**	240	240	**mative**	240	**mative**
A3		238	allele					(2:1ratio)		(1:1 ratio)	
A4			**excludes** mat cell contam*								
D21S1412											
A1	294		absence	289	289	absence	277	277	absence	277	absence
A2	306	306	of one mat.	310		of one mat	281		of one mat		of one mat
A3		286	allele		344	allele		292	allele	292	allele
A4			**excludes** mat cell contam			**excludes** mat cell contam			**excludes** mat cell contam		**excludes** mat cell contam
D21S1411						no peaks		no peaks		no peaks	
A1	386	386	absence	389	389	**same**					
A2	395		of one mat		399						
A3		399	allele								
A4			**excludes** mat cell contam								
D21S1414	**not tested**	**not tested**						not tested	not tested		not tested
A1				341	341	**non infor-**					
A2				345	345	**mative**					

*mat cell contam = maternal cell contamination

This finding indicates that placental sex chromosomal chimerism is not a rare event in early pregnancy (1.95%). Similar findings also observed by some investigators.[7,8] It is expected that true incidence of chimerism will be much more if we look for chimerism of sex chromosome and autosomes. The incidence may be more with pregnancies resulted from assisted reproduction due to close proximity of placentae following multiple embryo transfer.[2] It is possible that chimerism more frequently happens in placenta than in fetus proper where early admixture of blastomeres or vascular connection between fetuses is essential. Placental chimerism may be frequent due to partial fusion of placentae in twin pregnancies in particular early in pregnancy; as high as 40%[9] or co-occurrence of vanishing twins. Placental chimerism, in particular confined to placenta, sometimes may interfere with prenatal diagnosis from CVS[16] and some think[7] that approximately 2% of discordant CVS results may be due to confined placental chimerism. This discordance not only restricted to CVS but also extends to amniotic fluid where, presence of XX cells along with XY cells is explained as maternal contamination, which may be wrong in some situation.[17] In view of medico-legal problems related to prenatal diagnosis, one should not forget this in case of ambiguity.

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