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Title

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Running title

Multiple sSMC associated with recurrent abortions

Abstract

In this report, we describe a case of multiple small supernumerary marker chromosomes (sSMC) presenting with recurrent abortions. Peripheral blood lymphocytes of a young, healthy and non consanguineous couple who asked for genetic evaluation after two spontaneous miscarriages were obtained for karyotypes. Lymphocytes of the woman were analyzed by FISH techniques and DNA was extracted and used for array CGH investigation.

Karyotyping revealed 48,XX,+2mar[24]/47,XX,+mar[5]/46,XX[3] for the woman and 46,XY for her husband. FISH analysis showed that the two sSMC consisted of chromosomes 6 and 20. Array CGH analysis showed gains of the 6p11.2q12 (9 Mb) and 20p11.21 (3.3 Mb) chromosomal regions with a total of 42 genes present on both sSMC. Our findings support also the hypothesis that the modification of the expression of some genes involved in embryo implantation, like *THBD* gene, could be responsible in the recurrent abortions.

This report underpins the necessity of array CGH for characterizing precisely sSMC and helping in genotype-phenotype correlations. Furthermore, a literature review on sSMC is included.

Key words

small supernumerary marker chromosomes (sSMC), array CGH, FISH, recurrent abortions

Introduction

Infertility and sterility are occurring in approximately 15% of the couples wishing to start a family (1). It has been suggested that infertility may be due to different causes

such as systemic infections, endocrine and immunology disorders or cytogenetic alterations. One important cause of infertility is the presence of a chromosomal aberration in one member of the couple. In the general population, there is a 0.85% frequency of chromosomal aberrations (2). However, it has been reported that in couples with repeated spontaneous abortions, this frequency is about 2.4%-6.8%, women being more frequently affected (3, 4). Cytogenetic analysis of aborted fetus showed that 50-70% of them had a chromosome rearrangement (5, 6). Among the parental karyotypes of the couples presenting with recurrent spontaneous abortions, a small supernumerary marker chromosome (sSMC) was found in less than 1% (7).

sSMC are defined as structurally abnormal chromosomes that cannot be identified or characterized by conventional-banding cytogenetic techniques alone. sSMC are generally equal in size or smaller than chromosome 20 of the same metaphase spread (8). sSMC have been observed in cancer, congenital malformations and/or intellectual disability, reproductive disorders and during prenatal diagnosis (9-11). In a review published by Liehr et al. in 2007 (12), the sSMC frequency was estimated at 0.044% in newborns, 0.075% in prenatal cases, 0.288% in patients with intellectual disability and 0.122% in infertile patients. The chromosomal origin of these sSMC remains usually unknown by conventional cytogenetic techniques but the development of molecular technologies based on fluorescence *in situ* hybridization (FISH) and array comparative genomic hybridization (array CGH) has allowed for important progress toward this goal. Here we describe the combined use of conventional cytogenetic, FISH and array CGH for the detection and characterization of multiple sSMC carried by a woman that has experienced two repeated spontaneous abortions.

Patient data

A non consanguineous 28-year-old couple was referred for genetic evaluation because they had two natural conceptions ending in spontaneous abortions after six weeks of gestation. Physical examinations, especially gynecological and urological examinations, were normal. Laboratory data showed no biological, hormonal, coagulation or semen anomalies. The couple had a normal phenotype and no developmental delay, learning problems or intellectual disability. The family history revealed five healthy children and four spontaneous miscarriages in the woman's mother. The parents and family members were unavailable for chromosome analysis. Cytogenetic analysis showed a normal karyotype 46,XY for the man but an abnormal one for his wife: 48,XX,+2mar[24]/47,XX,+mar[5]/46,XX[3].

Methods

Conventional and FISH cytogenetic experiments

Standard chromosome analyses were performed from cultured peripheral lymphocytes of the couple using standard procedures (G-band by trypsin using Giemsa (GTG), R-band after heat denaturation and Giemsa (RHG) banding techniques and Giemsa staining).

FISH analyses were performed on lymphocyte metaphase spreads of the woman. The following probes were used according to manufacturer's recommendations: whole chromosome painting probes specific for chromosomes 6 and 20 (Kreatech, Amsterdam, The Netherlands), chromosomes 6 and 20 centromeric probes (Vysis, Downers Grove, USA), a pancentromeric probe specific for all chromosomes (QBiogene, Illkirch, France) and a pantelomeric probe specific for all chromosomes (Cambio, Cambridge, UK). Bacterial artificial chromosome (BAC) clones specific for

the 20p chromosomal region (RP4-580G13, RP1-234M6 and RP5-1025A1 located at 20p11.21 and RP4-760C5 located at 20p11.1) were used (Bluegnome, Cambridge, UK).

Array CGH analysis

Genomic DNA of the patient was isolated from peripheral blood using a DNeasy Blood and Tissue Kit (Qiagen, Courtaboeuf, France). The extracted DNA concentration was estimated using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Genomic imbalances were analyzed by array CGH using a 244k oligonucleotide array (Hu-244A, Agilent Technologies, Massy, France). Hybridization was performed according to the manufacturer's recommended protocol and as previously described (13). Captured images were processed with Feature Extraction software (10.7.3.1) and data analysis was performed with Genomic Workbench V5.0.14 (Agilent Technologies). The genomic positions were determined using the version 18 of the Human Genome (http://genome.ucsc.edu/). The ADM2 algorithm was used for statistical analysis. Copy number variations (CNV) were considered significant if they were defined by three or more contiguous oligonucleotides spanned at least 40 kb, contained at least one gene and were not identified in the Database of Genomic Variants at the Centre for Applied Genomics (http://projects.tcag.ca/cgi-bin/variation/gbrowse/hg18/).

Results

Cytogenetic analyses of cultured lymphocytes using GTG, RHG banding techniques and Giemsa staining revealed a normal karyotype for the man (46,XY). For the woman, a mosaicism containing three cell lines was observed among 32 analyzed

cells: normal cells (9.4%), cells with one sSMC (15.6%) and cells with two sSMC (75%) that define karyotype 48,XX,+2mar[24]/47,XX,+mar[5]/46,XX[3]. The first sSMC had a larger size than the second sSMC (Fig. 1A). Giemsa staining showed a symmetric and a non-satellited aspect of both sSMC (Fig. 1B(c)).

Further array CGH analysis showed a gain of 6p11.2q11.1 (5.4Mb, Fig. 2A), a gain of 6q11.1q12 (3.6Mb, Fig. 2A) and a gain of 20p11.21 (3.3Mb, Fig. 2B) chromosomal regions. Thus, 9Mb and 3.3Mb chromosomal regions containing euchromatin were amplified on chromosomes 6 and 20, respectively. Seven genes were amplified on chromosomes 6 and thirty-five genes on chromosome 20 (Table 1). Analyses revealed no other variations that were not known as polymorphisms according to the Database of Genomic Variants.

FISH analysis using WCP probe specific for chromosome 6 showed hybridization on both normal chromosomes 6 and on the larger sSMC (Fig. 1B(d), 1C). FISH analysis using WCP probe specific for chromosome 20 showed hybridization on both normal chromosomes 20 and on the smaller sSMC (Fig. 1B(d), 1C). No additional hybridization signal was detected on other chromosomes, eliminating an insertion or a translocation elsewhere. The chromosome 6 centromeric probe hybridized on both normal chromosomes 6 and on the sSMC(6) (Fig. 1B(e), 1C). The chromosome 20 centromeric probe hybridized on both normal chromosomes 20 but no signal was detected on the sSMC(20) (Fig. 1B(e), 1C). Further analysis with the pancentromeric mixture specific for alpha-satellite common sequences of all chromosomes showed a fluorescent signal on the 46 normal chromosomes and on both sSMC (Fig. 1B(f)). The pantelomeric probe specific for all chromosomes showed signals on all

chromosomes but not on the two sSMC. These results could suggest a ring structure of both sSMC. This might explain the mosaïcism observed in the analyzed cells and also, because of the mitotic formation of double rings, the difference in size of the sSMC(6) (Fig. 1B(a) and (c)). BAC clones RP4-580G13, RP1-234M6, RP5-1025A1 located at 20p11.21 and RP4-760C5 located at 20p11.1 gave one signal on each normal chromosomes 20 and one signal on sSMC(20) and three signals when analyzed on interphasic nuclei.

Thus, the patient carried an abnormal karyotype with two sSMC in the majority of the cells; a larger sSMC derived from chromosome 6 with an original centromere and a smaller sSMC derived from chromosome 20 with a centromere without specific sequences of the centromere of chromosome 20. Based on the FISH and array CGH analyses, the patient's karyotype was defined as 48,XX,+2mar[24]/47,XX,+mar[5]/46,XX[3].ish der(6)(wcp6+,D6Z1+),der(20)(wcp20+,RP4-580G13+,RP1-234M6+,RP5-1025A1+,RP4-760C5+,D20Z1-).arr 6p11.2q11.1(57,354,689-62,746,115)x3,6q11.1q12(62,757,919-66,400,962)x3,20p11.21p11.1(22,833,806-26,156,226)x3 according to ISCN 2009 nomenclature.

Discussion

Our report described a woman carrying two sSMC derived from chromosomes 6 and 20 presenting with recurrent abortions without further clinical symptoms. Array CGH showed that these sSMC corresponded to the 6p11.2q12 and 20p11.21 chromosomal regions, resulting in partial trisomies.

The interpretation of the clinical significance of sSMC is extremely problematic as sSMC have heterogeneous phenotypic consequences. Their effects seem to depend on the origin, size, content and the structure of the sSMC as well as the degree of mosaicism, the varying amounts of euchromatin and their parental origin when the marker contains imprinted genes (14, 15). To date, only two studies using array CGH have been performed on sSMC in relation with spontaneous abortions (16, 17) (Table 2). Whether the sSMC is a cause or a coincidental finding is still questionable since the mechanism by which sSMC influence fertility has not yet been understood (18). In almost 50% of cases the etiology of recurrent abortions is unknown. The causes are heterogeneous and include endocrine dysfunction, autoimmune disorders, genetic abnormalities, maternal and paternal age, infectious diseases, environmental toxins and congenital or structural uterine anomalies (7). Almost 15-20% of all pregnancies end up as spontaneous abortions, out of which the contribution of chromosomal abnormalities is as high as 70%. Frequency of sSMC detected in infertile patients is higher than that in general population (0.125% *versus* 0.044%) and it is also different between male (0.165%) and female infertility (0.022%) (12). An enhanced rate of recurrent abortions in sSMC carriers or their partners has been observed in 26-37% of the cases (18). Kumar et al. (1997) (35) showed that 4.4% of sSMC pregnancies end in stillbirth or spontaneous abortion. The presence of two sSMC in our patient could disturb correct chromosome pairing by an unequal crossing over during meiosis, which can result in gametes with unbalanced chromosomes like duplications or deletions. The clinical consequences of such imbalances usually are lethal to the developing embryo leading to spontaneous abortions or early neonatal deaths. Also, the consequences could be more serious if the sSMC is present in a non-mosaic state in the fetus.

To date, some reports about recurrent abortions with sSMC have already been published (Table 2) (5, 11, 16-34). Usually, sSMC derived from chromosomes 6 and 20 are rare and comprise 0.54% and 1.2% of all sSMC respectively. About 37% of carriers of sSMC derived from chromosomes 6 and 20 are clinically normal (36). In our case, a normal phenotype was observed for the woman. Among the 20 cases of sSMC(6) described in the literature including our study, our patient is the first case of female described with recurrent abortions. sSMC(20) seems to be more frequent as 46 cases were previously described. Among these cases, one woman presented an unexplained infertility, two women a primary amenorrhea (18, 37) and a man an azoospermia (18). All these cases were not studied at a molecular level with array CGH, so the exact size of sSMC and gene content remained unknown (38). In the 59 published cases of multiple sSMC (i.e. more than one sSMC in cells), sSMC(6) is present in near 18% of them. sSMC(6) and sSMC(8) are the more frequently markers represented in cases of multiple sSMC and chromosome 6 seems to be over-represented in multiple sSMC cases reported to date compared to their contribution to single sSMC (39). This might point towards a specific way of formation of multiple sSMC during meiosis (40).

A detailed molecular cytogenetic characterization using array CGH is needed to evaluate the size and the genomic constitution of sSMC with precision. The varying degrees of phenotypic abnormality observed in several patients are most probably due to the different DNA sequences and thus gene content of the sSMC. Our patient presented two sSMC. In the 9Mb DNA sequences present on the sSMC(6), 7 genes are mapped (Table 1). To our knowledge, none of them were described associated

with infertility or pregnancy impairment. In the 3.3Mb DNA sequences located on the sSMC(20), 35 genes are mapped (Table 1). Among them, the *THBD* gene codes for the thrombomodulin, an endothelial-associated anticoagulant protein involved in the control of hemostasis and inflammation at the vascular beds (41). This protein is also a cofactor of the protein C anticoagulant pathway and is expressed mainly on the endothelial surface of blood vessels and in the placental syncytiotrophoblast cells (42). Various components of the coagulation and fibrinolytic pathways are involved in normal embryonic implantation, trophoblast invasion and placentation. Recurrent abortions are characterized by defective placentation and microthrombi in the placental vasculature (43). Although recurrent spontaneous abortions are a heterogeneous condition, the relationship between abnormalities in the hemostatic pathways and pregnancy outcome is increasingly recognized (44). Considering the crucial role of thrombomodulin in coagulation and in embryonic development, we hypothesize that a modification of its expression reveals an increase in procoagulant activity, which could be secondary to endothelial damage or coagulation activation and then involved in the pathogenesis of pregnancy loss.

In conclusion, the behavior of both sSMC(6) and sSMC(20) in relation to fetal loss of our patient has been a subject of scrutiny and debate. Our findings support also the hypothesis that the modification of the expression of some genes, like *THBD*, could be directly responsible in the repeated spontaneous abortions.

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experiments.

Figure titles and legends

Figure 1: Conventional and FISH cytogenetic findings.

A: Metaphase with R-banding showing the two sSMC (arrows).

B: Results for sSMC(6) and sSMC(20).

(a) G-band by trypsin using Giemsa.

(b) R-band after heat denaturation and Giemsa.

(c) Giemsa staining.

(d) WCP 6 (d1) and WCP 20 (d2) specific probes.

(e) CEP 6 (e1) and CEP 20 (e2) specific probes.

(f) Pancentromeric probe specific for all chromosomes

C: FISH on metaphase spread using WCP 6 (green), WCP 20 (red), CEP 6 (red) and CEP 20 (green) showing chromosomes 6 and 20, and the two sSMC (arrows).

Figure 2: Array CGH using 244k oligonucleotide arrays showing a global 9Mb gain in 6p11.2-q11.1 and 6q11.1-q12 (A) and a 3.3Mb gain in 20p11.21 (B).

Tables

Table 1: Genes present on respective sSMC, protein encoded and their function (Pubmed [Internet]: http://www.ncbi.nlm.nih.gov/pubmed/).

Table 2: sSMC associated with recurrent miscarriages described in the literature.

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