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1 **Title**

2 Small supernumerary marker chromosomes derived from chromosomes 6 and 20 in
3 a woman with recurrent spontaneous abortions

4

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22

23 **Running title**

24 Multiple sSMC associated with recurrent abortions

25

26 **Abstract**

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

33 Karyotyping revealed 48,XX,+2mar[24]/47,XX,+mar[5]/46,XX[3] for the woman and
34 46,XY for her husband. FISH analysis showed that the two sSMC consisted of
35 chromosomes 6 and 20. Array CGH analysis showed gains of the 6p11.2q12 (9 Mb)
36 and 20p11.21 (3.3 Mb) chromosomal regions with a total of 42 genes present on both
37 sSMC. Our findings support also the hypothesis that the modification of the
38 expression of some genes involved in embryo implantation, like *THBD* gene, could
39 be responsible in the recurrent abortions.

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

43

44 **Key words**

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

47

48 **Introduction**

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

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

60 sSMC are defined as structurally abnormal chromosomes that cannot be identified or
61 characterized by conventional-banding cytogenetic techniques alone. sSMC are
62 generally equal in size or smaller than chromosome 20 of the same metaphase
63 spread (8). sSMC have been observed in cancer, congenital malformations and/or
64 intellectual disability, reproductive disorders and during prenatal diagnosis (9-11). In
65 a review published by Liehr et al. in 2007 (12), the sSMC frequency was estimated at
66 0.044% in newborns, 0.075% in prenatal cases, 0.288% in patients with intellectual
67 disability and 0.122% in infertile patients. The chromosomal origin of these sSMC
68 remains usually unknown by conventional cytogenetic techniques but the
69 development of molecular technologies based on fluorescence *in situ* hybridization
70 (FISH) and array comparative genomic hybridization (array CGH) has allowed for
71 important progress toward this goal. Here we describe the combined use of
72 conventional cytogenetic, FISH and array CGH for the detection and characterization
73 of multiple sSMC carried by a woman that has experienced two repeated
74 spontaneous abortions.

75

76 **Patient data**

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

87

88 **Methods**

89 **Conventional and FISH cytogenetic experiments**

90 Standard chromosome analyses were performed from cultured peripheral
91 lymphocytes of the couple using standard procedures (G-band by trypsin using
92 Giemsa (GTG), R-band after heat denaturation and Giemsa (RHG) banding
93 techniques and Giemsa staining).
94 FISH analyses were performed on lymphocyte metaphase spreads of the woman.
95 The following probes were used according to manufacturer's recommendations:
96 whole chromosome painting probes specific for chromosomes 6 and 20 (Kreatech,
97 Amsterdam, The Netherlands), chromosomes 6 and 20 centromeric probes (Vysis,
98 Downers Grove, USA), a pancentromeric probe specific for all chromosomes
99 (QBiogene, Illkirch, France) and a pantelomeric probe specific for all chromosomes
100 (Cambio, Cambridge, UK). Bacterial artificial chromosome (BAC) clones specific for

101 the 20p chromosomal region (RP4-580G13, RP1-234M6 and RP5-1025A1 located at
102 20p11.21 and RP4-760C5 located at 20p11.1) were used (Bluegenome, Cambridge,
103 UK).

104

105 **Array CGH analysis**

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

121

122 **Results**

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

126 cells: normal cells (9.4%), cells with one sSMC (15.6%) and cells with two sSMC
127 (75%) that define karyotype 48,XX,+2mar[24]/47,XX,+mar[5]/46,XX[3]. The first
128 sSMC had a larger size than the second sSMC (Fig. 1A). Giemsa staining showed a
129 symmetric and a non-satellited aspect of both sSMC (Fig. 1B(c)).

130

131 Further array CGH analysis showed a gain of 6p11.2q11.1 (5.4Mb, Fig. 2A), a gain of
132 6q11.1q12 (3.6Mb, Fig. 2A) and a gain of 20p11.21 (3.3Mb, Fig. 2B) chromosomal
133 regions. Thus, 9Mb and 3.3Mb chromosomal regions containing euchromatin were
134 amplified on chromosomes 6 and 20, respectively. Seven genes were amplified on
135 chromosomes 6 and thirty-five genes on chromosome 20 (Table 1). Analyses
136 revealed no other variations that were not known as polymorphisms according to the
137 Database of Genomic Variants.

138

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

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

158

159 Thus, the patient carried an abnormal karyotype with two sSMC in the majority of the
160 cells; a larger sSMC derived from chromosome 6 with an original centromere and a
161 smaller sSMC derived from chromosome 20 with a centromere without specific
162 sequences of the centromere of chromosome 20. Based on the FISH and array CGH
163 analyses, the patient's karyotype was defined as

164 48,XX,+2mar[24]/47,XX,+mar[5]/46,XX[3].ish

165 der(6)(wcp6+,D6Z1+),der(20)(wcp20+,RP4-580G13+,RP1-234M6+,RP5-

166 1025A1+,RP4-760C5+,D20Z1-).arr 6p11.2q11.1(57,354,689-

167 62,746,115)x3,6q11.1q12(62,757,919-66,400,962)x3,20p11.21p11.1(22,833,806-

168 26,156,226)x3 according to ISCN 2009 nomenclature.

169

170 **Discussion**

171 Our report described a woman carrying two sSMC derived from chromosomes 6 and
172 20 presenting with recurrent abortions without further clinical symptoms. Array CGH
173 showed that these sSMC corresponded to the 6p11.2q12 and 20p11.21
174 chromosomal regions, resulting in partial trisomies.

175

176 The interpretation of the clinical significance of sSMC is extremely problematic as
177 sSMC have heterogeneous phenotypic consequences. Their effects seem to depend
178 on the origin, size, content and the structure of the sSMC as well as the degree of
179 mosaicism, the varying amounts of euchromatin and their parental origin when the
180 marker contains imprinted genes (14, 15). To date, only two studies using array CGH
181 have been performed on sSMC in relation with spontaneous abortions (16, 17)
182 (Table 2). Whether the sSMC is a cause or a coincidental finding is still questionable
183 since the mechanism by which sSMC influence fertility has not yet been understood
184 (18). In almost 50% of cases the etiology of recurrent abortions is unknown. The
185 causes are heterogeneous and include endocrine dysfunction, autoimmune
186 disorders, genetic abnormalities, maternal and paternal age, infectious diseases,
187 environmental toxins and congenital or structural uterine anomalies (7). Almost 15-
188 20% of all pregnancies end up as spontaneous abortions, out of which the
189 contribution of chromosomal abnormalities is as high as 70%. Frequency of sSMC
190 detected in infertile patients is higher than that in general population (0.125% *versus*
191 0.044%) and it is also different between male (0.165%) and female infertility
192 (0.022%) (12). An enhanced rate of recurrent abortions in sSMC carriers or their
193 partners has been observed in 26-37% of the cases (18). Kumar et al. (1997) (35)
194 showed that 4.4% of sSMC pregnancies end in stillbirth or spontaneous abortion.
195 The presence of two sSMC in our patient could disturb correct chromosome pairing
196 by an unequal crossing over during meiosis, which can result in gametes with
197 unbalanced chromosomes like duplications or deletions. The clinical consequences
198 of such imbalances usually are lethal to the developing embryo leading to
199 spontaneous abortions or early neonatal deaths. Also, the consequences could be
200 more serious if the sSMC is present in a non-mosaic state in the fetus.

201

202 To date, some reports about recurrent abortions with sSMC have already been
203 published (Table 2) (5, 11, 16-34). Usually, sSMC derived from chromosomes 6 and
204 20 are rare and comprise 0.54% and 1.2% of all sSMC respectively. About 37% of
205 carriers of sSMC derived from chromosomes 6 and 20 are clinically normal (36). In
206 our case, a normal phenotype was observed for the woman. Among the 20 cases of
207 sSMC(6) described in the literature including our study, our patient is the first case of
208 female described with recurrent abortions. sSMC(20) seems to be more frequent as
209 46 cases were previously described. Among these cases, one woman presented an
210 unexplained infertility, two women a primary amenorrhea (18, 37) and a man an
211 azoospermia (18). All these cases were not studied at a molecular level with array
212 CGH, so the exact size of sSMC and gene content remained unknown (38). In the 59
213 published cases of multiple sSMC (i.e. more than one sSMC in cells), sSMC(6) is
214 present in near 18% of them. sSMC(6) and sSMC(8) are the more frequently markers
215 represented in cases of multiple sSMC and chromosome 6 seems to be over-
216 represented in multiple sSMC cases reported to date compared to their contribution
217 to single sSMC (39). This might point towards a specific way of formation of multiple
218 sSMC during meiosis (40).

219

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

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

242

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

247

248 **Acknowledgement**

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250 chromosome probes. They thank all the technical team for help with array CGH

251 experiments.

252

253 **Figure titles and legends**

254 Figure 1: Conventional and FISH cytogenetic findings.

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

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

257 (a) G-band by trypsin using Giemsa.

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

259 (c) Giemsa staining.

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

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

262 (f) Pancentromeric probe specific for all chromosomes

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

265

266 Figure 2: Array CGH using 244k oligonucleotide arrays showing a global 9Mb gain in
267 6p11.2-q11.1 and 6q11.1-q12 (A) and a 3.3Mb gain in 20p11.21 (B).

268

269 **Tables**

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

272

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

274

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