Multiplex ligation dependent probe amplification (MLPA) for rapid distinction between unique sequence positive and negative marker chromosomes in prenatal diagnosis

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Abstract

Background: Small supernumerary marker chromosomes (sSMC) are extra structurally abnormal chromosomes that cannot be unambiguously identified with conventional chromosome banding techniques. These marker chromosomes may cause an abnormal phenotype or be harmless depending on different factors such as genetic content, chromosomal origin and level of mosaicism. When a sSMC is found during prenatal diagnosis, the main question is whether the sSMC contains euchromatin since in most cases this will lead to phenotypic abnormalities. We present the use of Multiplex Ligation Dependent probe Amplification (MLPA) for rapid distinction between non-euchromatic and euchromatic sSMC.

Results: 29 well-defined sSMC found during prenatal diagnosis were retrospectively investigated with MLPA with the SALSA MLPA centromere kits P181 and P182 as well as with the SALSA MLPA telomere kits P036B and P070 (MRC Holland BV, Amsterdam, The Netherlands). All unique-sequence positive sSMC were correctly identified with MLPA, whereas the unique-sequence negative sSMC had normal MLPA results.

Conclusions: Although different techniques exist for identification of sSMC, we show that MLPA is a valuable adjunctive tool for rapidly distinguishing between unique-sequence positive and negative sSMC. In case of positive MLPA results, genetic microarray analysis or, if not available, targeted FISH can be applied for further identification and determination of the exact breakpoints, which is important for prediction of the fetal phenotype. In case of a negative MLPA result, which means that the sSMC most probably does not contain genes, the parents can already be reassured and parental karyotyping can be initiated to assess the heritability. In the mean time, FISH techniques are needed for determination of the chromosomal origin.

Background

The finding of a sSMC presents a challenge in prenatal diagnosis particularly for prediction of the clinical consequences which will depend on its genetic content, familial occurrence, level of mosaicism and chromosomal origin [1-5] and parental origin of the sSMC related sister chromosomes [6]. According to the review of Liehr and Weise [7] sSMC are to be expected in 0.075% of all analysed prenatal cases. In case of fetal ultrasound abnormalities this frequency rises to 0.204%, which is 2.7x higher than in the general prenatal population.

Before the introduction of FISH for cytogenetics, identification studies involved the use of classical staining techniques such as GTG, QFQ, Ag-NOR, CBG and DAPI [1]. Nowadays, different molecular cytogenetic techniques have been developed for identification of sSMC, such as FISH techniques like cenM- and subcenM-FISH [8,9], multicolour banding (MCB)[10], microdissection followed by reverse FISH [11,12], spectral karyotyping (SKY) [13], M-FISH [14] and genomic microarray analysis [15,16]. These techniques are expensive and not available in all cytogenetic laboratories [17].
In this paper we present the use of Multiplex Ligation Dependent Probe Amplification (MLPA) (MRC Holland, Amsterdam, The Netherlands) as an alternative approach for identification of euchromatic sSMC. On the basis of 29 well characterised sSMC we show that MLPA can rapidly distinguish between unique sequence positive and negative sSMC, which is the most important task when finding a sSMC prenatally. However, other molecular cytogenetic techniques will remain necessary for determining the exact genetic content in case of a positive sSMC, whereas FISH techniques will still be indispensable for identification studies in case of an unique sequence negative sSMC.

Methods

Samples
We retrospectively tested the value of MLPA for sSMC identification on 29 well-defined sSMC found during prenatal diagnosis in amniotic fluid (AF) (n = 26) and chorionic villi (CV) (n = 3) (see table 1 and additional file 1). For routine cytogenetics GTG-banding was used in all cases according to standard techniques. Mostly, sSMC identification was done with FISH, sometimes after additional staining with DA-DAPI [18] (see additional file 1). In 23/29 cases the sSMC was present in all investigated cells. In 6/29 cases mosaicism was found in cultured CV or AF cells with the level of mosaicism varying between 30 and 89% (table 1).

FISH
Metaphase FISH was performed according to standard techniques. The probes that were used for identification were whole chromosome paints (wcp’s)(Kreatech Diagnostics, Ankeveen, The Netherlands and Euro-Diagnostica AB, Nijmegen, The Netherlands), centromere probes (Abbott Molecular Inc., Des Plaines, USA; Resources for Molecular Cytogenetics, Bari, Italy (https://www.biologia.uniba.it/rmc/) and partially received from several investigators), subtelomere-probes [19], locus-specific probes (SNRPN from Cytocell Ltd, Cambridge, UK; LSI TEL AML1 from Abbott Molecular Inc., Des Plaines, USA and others like 102D10 (CES-probe), Y41 and Y11H11 (15q11), r521 (rDNA-probe) were kindly provided by several investigators) and subcentromere-BAC clones that were selected from the University of California Santa Cruz (UCSC) genome browser (http://genome.ucsc.edu) (see additional file 1).

FISH slides were analyzed using the Axioplan 2 Imaging microscope (Zeiss), and images were collected using Isis Software System (Metasystems).

Sample preparation for MLPA and SNP array
DNA was isolated from 4 ml of uncultured AF or from cultured CV or AF cells. AF cells were cultured by the

| Case | sSMC | Euchromatin (based on GTG/FISH) | % of cultured cells with sSMC |
|------|------|---------------------------------|-------------------------------|
| 1    | der(3)(p12.2->cen)² | +                              | 100                           |
| 2    | min(4)(p11->q11)³  | -                              | 100                           |
| 3    | psu idic(9)(q12)     | +                              | 87.5                          |
| 4    | i(12)(p10)          | +                              | 89                            |
| 5    | i(12)(p10)          | +                              | 100                           |
| 6    | neo(12)(pter->p12.3) | +                              | 47                            |
| 7    | der(13)t(4;13)(q31.3q13) | -                              | 100                           |
| 8    | min(13 or 21)       | -                              | 100                           |
| 9    | min(13 or 21)       | -                              | 100                           |
| 10   | inv dup(14)(q11.2)  | -                              | 100                           |
| 11   | der(14)t(4;14)(q12q21) | +                              | 100                           |
| 12   | inv dup(15)(q12)    | +                              | 100                           |
| 13   | neo(15)[qtel->q24]  | +                              | 100                           |
| 14   | der(15)t(9;15)(p12q14) | +                              | 100                           |
| 15   | inv dup(15)(q11)    | -                              | 100                           |
| 16   | inv dup(15)(q11.2)  | -                              | 100                           |
| 17   | inv dup(15)(q11.2)  | -                              | 100                           |
| 18   | min(16)(p11.1->q11.1) | -                              | 30                            |
| 19   | min(17)(p11.1->q11.1) | -                              | 45                            |
| 20   | r(20)(q11.21q13.12) | +                              | 87                            |
| 21   | inv dup(22)(q11.21) | +                              | 100                           |
| 22   | inv dup(22)(q11.21) | +                              | 100                           |
| 23   | inv dup(22)(q11.21) | +                              | 100                           |
| 24   | inv dup(22)(q11.21) | +                              | 100                           |
| 25   | del(22)(q11.2)      | +                              | 100                           |
| 26   | inv dup(22)(q11.1)  | -                              | 100                           |
| 27   | inv dup(22)(q11.1)  | -                              | 100                           |
| 28   | inv dup(22)(q11.1)  | -                              | 100                           |
| 29   | inv dup(22)(q11.1)  | -                              | 100                           |

¹ For identification details, see additional file [38]. ² This case was previously published by Srebniak et al. [39]. ³This case was published earlier by Van Opstal et al. [40].
in situ method and CV were cultured using trypsin-EDTA and collagenase treatment as described previously [20]. DNA-isolation from uncultured AF cells was done with the Chemagic Magnetic Separation Module I (Chemagen, Baesweiler, Germany). DNA isolation from cultured cells was performed using the QIAamp DNA Mini Kit from Qiagen (Hilden, Germany) or Puregene DNA Purification Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions.

MLPA-reaction and data analysis
4 SALSA MLPA kits were used: two centromere kits, P181 and P182, and two telomere-kits, P036B and P070 (http://www.mlpa.com/WebForms/WebFormMain.aspx). Between 20 and 70 ng of DNA was used in a MLPA reaction which was performed on a PCR thermocycler with heated lid (Biometra Thermal Cycler, Westburg, The Netherlands). MLPA reaction and data analysis were performed as described earlier [21]. In order to enable the detection of chromosomal mosaicism as was seen in 6/29 cases, we calculated own cut off values (median ± 2x SD) for the different probes on the different chromosomes for all four MLPA-kits on the basis of 95 (P181), 91 (P182), 104 (P036B) and 105 (P070) normal samples (see table 2).

SNP array, data analysis and interpretation
In two cases (cases 13 and 25) a SNP array (HumanCytoSNP-12, Illumina) was performed because of discrepancies between the results of GTG/FISH and MLPA. 200 ng of DNA isolated from cultured cells was used in both cases. DNA amplification, tagging and hybridisation were performed according to the manufacturer’s protocol. Array slides were scanned on the iScan Reader (Illumina). Data analysis was performed using Genome Studio version 2010.1 (Illumina). The HapMap control set provided by the manufacturer was used as a control.

Results
Unique sequence positive sSMC
All unique sequence positive sSMC (Table 1) were correctly identified with MLPA with the centromere kits (cases 1 and 20), telomere kits (cases 6 and 13) or both (cases 3-5, 7, 11, 12, 14, 21-25) (see table 3) confirming GTG/FISH results. There were no false negative cases.

a. Non-mosaic cases
The relative probe signals in most non-mosaic cases (see table 1) correctly discriminated between 3 and 4 copies of the investigated probes, with relative probe signals > 1.3 and < 1.5 for a trisomy and > 1.6 for a tetrasomy (see table 3). In 3/23 non-mosaic cases a discrepancy was found.

Case 1: Although amplification of EPHA3 confirmed the results of FISH and DNA marker studies (sSMC=der(3)(p12.2->cen:)) in case 1, the relative probe signals of the 3p11.2 marker EPHA3 in both centromere-kits were not above 1.3 (1.178 in P181 and 1.226 in P182) as would be expected in a full blown case. However, they were clearly above the normal cut off value of 1.077 for both kits probably indicating loss of the sSMC in part of the cells and therefore mosaicism at the time that DNA for MLPA was isolated from the cell cultures.

Case 13 showed a full blown neo(15) in cultured AF cells (8 cell clones investigated) (see Figure 1a), which is an analphoid sSMC with a neocentromere and consisting of two copies of the distal end of chromosome 15q. However, the relative probe signals of the 15q-subtelomere probes were only 1.45 and 1.428 in respectively P036B and P070, indicative of a trisomy but not a tetrasomy as expected (Figure 1b).

In order to elucidate this discrepancy, genomic microarray analysis was performed. Investigations with the HumanCytoSNP-12 indicate that this case is more complex than initially thought which may explain the MLPA results. Based on Log R ratio and B-allele frequency (BAF), we expect mosaicism of different abnormal cell lines containing different sSMC. However, BAF’s of 0, 1, 0.333 and 0.667 and absence of a BAF of 0.5 at 15q26.3 (in contrast to the region 15q24.1-q26.2) indicate a trisomy at 15qtel which confirms the MLPA results (see Figure 1c).

In case 25 of an extra familial del(22)(q11.2) the results of MLPA with the 22q11 probes in the four kits were indicative for 4 copies of this chromosomal region: relative probe signals were 1.770 and 1.621 (P181), 1.760 and 1.869 (P182), 1.688 (P036B) and 1.806 (P070) (Figure 2b). This contradicts the FISH results with one signal on the sSMC for two different 22cen-probes, one for the rDNA-probe and one signal with the probe from the Cat Eye Syndrome (CES)-region (Figure 2a). HumanCytoSNP-12-analysis confirmed the sSMC to be at least a partial duplication of chromosome 22, indicated by a BAF of 0.5, resulting in 4 copies of the sequences detected by the proximal 22q-probes in the 4 MLPA kits (Figure 2c).

b. Mosaic cases
In one out of four mosaic cases (case 3), MLPA correctly identified four copies of 9ptel (P070, P036B), 9p11 (p182) and 9p13.2 (P181) with relative probe signals > 1.6 (table 3). However, in three out of four mosaic cases (cases 4, 6 and 20), the relative probe signals of some probes were below the expected values (1.3 for a trisomy and 1.6 for a tetrasomy). In these cases, the level of mosaicism, which was determined in cultured AF cells, was unknown in uncultured AF cells from which the DNA for MLPA was isolated. Nevertheless, the sSMC in all three cases could be identified by making use of our
Table 2 Cut off values (median probe signal ±2 SD) for the different probes in the MLPA kits P181, P182, P036B and P070

| Probes P181 | Cut off values (N = 95) | Probes P182 | Cut off values (N = 91) |
|-------------|------------------------|-------------|------------------------|
| Minimum     | Maximum                | Minimum     | Maximum                |
| 3p11.2 EPHA3| 0.923                  | 1.077       | 3p11.2 EPHA3           | 0.923                  |
| 3q11.2 PROS1| 0.907                  | 1.093       | 3q11.2 PROS1           | 0.913                  |
| 4p11 OCIAD1 | 0.880                  | 1.114       | 4p11 OCIAD1            | 0.880                  |
| 4q12 SGC8   | 0.946                  | 1.054       | 4q12 USP46             | 0.919                  |
| 9q13.2 IGFBL1| 0.902                | 1.098       | 9p11 EXOSC3            | 0.940                  |
| 9q13 TJP2   | 0.933                  | 1.067       | 9q13 TJP2              | 0.909                  |
| 12p11.21 PKP2| 0.888                | 1.112       | 12p11.21 PKP2          | 0.894                  |
| 12q12 KIF21A| 0.899                  | 1.101       | 12q12 KIF21A           | 0.886                  |
| 13q11 HSMPP8| 0.881                  | 1.119       | 13q11 HSMPP8           | 0.924                  |
| 13q11 ZNF198| 0.930                  | 1.066       | 13q11 ZNF198           | 0.881                  |
| 14q12 ADPRTL2| 0.863                 | 1.115       | 14q11.2 ADPRTL2        | 0.913                  |
| 14q12 APEX  | 0.917                  | 1.083       | 14q11.2 APEX           | 0.932                  |
| 15q12 NIPA2 | 0.883                  | 1.097       | 15q11.2 NIPA2          | 0.864                  |
| 15q12 NDN   | 0.916                  | 1.078       | 15q11.2 MKRN3          | 0.874                  |
| 16q12 TFG811| 0.882                  | 1.098       | 16p11.2 ERAF           | 0.927                  |
| 16q12 ORC6L | 0.914                  | 1.086       | 16q12 VPS35            | 0.899                  |
| 17p11.2 MAP2K3| 0.934                | 1.064       | 17p11.2 MAP2K3         | 0.828                  |
| 17q11.1 WSB1| 0.927                  | 1.073       | 17q11.1 WSB1           | 0.931                  |
| 20p11.2 PYGB  | 0.894                 | 1.102       | 20p11.21 ZNF337        | 0.914                  |
| 20q11.21 DUSP15| 0.912              | 1.088       | 20q11.21 REM1          | 0.893                  |
| 21q11 STHC  | 0.911                  | 1.089       | 21q11 STHC            | 0.896                  |
| 21q11 SAMSNI| 0.861                  | 1.153       | 21q11 SAMSNI          | 0.889                  |
| 22q11.2 CECR5| 0.872                 | 1.086       | 22q11.2 CECR1         | 0.877                  |
| 22q11.2 CECR1| 0.901                  | 1.089       | 22q11.2 SLC25A18      | 0.916                  |

| Probes P036B | Cut off values (N = 104) | Probes P070 | Cut off values (N = 105) |
|--------------|--------------------------|-------------|--------------------------|
| Minimum      | Maximum                  | Minimum     | Maximum                  |
| 3p CHL1      | 0.890                    | 1.110       | 3p CHL1                  | 0.916                  |
| 3q BDH       | 0.905                    | 1.092       | 3q KIAA0226              | 0.933                  |
| 4p FLJ20265  | 0.900                    | 1.101       | 4p ZNF141                | 0.828                  |
| 4q FRG1      | 0.744                    | 1.239       | 4q FRG1                  | 0.865                  |
| 9p DMRT1     | 0.897                    | 1.103       | 9p FLJ00026              | 0.915                  |
| 9q MRPL41    | 0.847                    | 1.148       | 9q EU-HMTase1            | 0.935                  |
| 12p SLC6A12  | 0.947                    | 1.053       | 12p RBBP2                | 0.937                  |
| 12q ZNF10    | 0.858                    | 1.126       | 12q ZNF10                | 0.909                  |
| 13p PSPC1    | 0.914                    | 1.074       | 13p PSPC1                | 0.900                  |
| 13q F7       | 0.883                    | 1.116       | 13q CDC16                | 0.923                  |
| 14p HEI10    | 0.919                    | 1.081       | 14p ADPRTL2              | 0.929                  |
| 14q MTA1     | 0.859                    | 1.143       | 14q MTA1                 | 0.906                  |
| 15p CYFIP1   | 0.925                    | 1.075       | 15p NDN                  | 0.908                  |
| 15q ALDH1A3  | 0.890                    | 1.110       | 15q FLJ22604             | 0.938                  |
| 16p POLR3K   | 0.894                    | 1.108       | 16p DECR2                | 0.828                  |
| 16q GNAS1/GAS8| 0.894                | 1.106       | 16q GNAS1                | 0.936                  |
| 17p RPH3AL   | 0.901                    | 1.099       | 17p RPH3AL               | 0.843                  |
| 17q TBCD     | 0.888                    | 1.108       | 17q SECTM1               | 0.890                  |
| 20p SOX12    | 0.843                    | 1.157       | 20p FLJ22115             | 0.888                  |
| 20q OPRL1    | 0.866                    | 1.134       | 20q FLJ20517             | 0.883                  |
| 21p RBM11    | 0.879                    | 1.121       | 21p STHC                 | 0.894                  |
| 21q HMT1     | 0.853                    | 1.147       | 21q S100B                | 0.921                  |
| 22p BD       | 0.894                    | 1.094       | 22p IL17R                | 0.837                  |
| 22q RABL2B   | 0.875                    | 1.127       | 22q ARSA                 | 0.904                  |

Only the chromosomes from which the sSMC in this paper are derived, are indicated.
### Table 3 MLPA results of 16 prenatal cases with a unique sequence positive sSMC

| No | sSMC | DNA source for MLPA | MLPA-results: copy number of sequence(s) in the kit, genes that they target and their abnormal relative probe signal(s) |
|----|------|---------------------|------------------------------------------------------------------------------------------------------------------|
|    |      | LTC-CV              |                                                                                                                 |
| 3  | psu idic(9)(q12) | LTC-CV              | 4 IGFBL1: 2.046 4 EXOSC3: 1.9013 4 DMRT1: 1.854 4 FLJ00026: 1.835                                                  |
| 4  | i(12)(p10)      | uAF                 | 4 PKP2: 2.1807                                                      |
| 6  | neo(12)(pter->p12.3) | uAF               | 2 PKP2: 1.006 2 KIF21A: 1.015 2 SGCG: 1.086 2 RBBP2: 1.5197          |
| 20 | r(20)(q11.21)(q13.12) | uAF               | 3 DUSP15: 1.461 3 REM1: 1.1837 2 SOX12: 1.086 2 OPRL: 1.009 2 FLJ22115: 0.989 2 FLJ20517: 1.004 |
|    |      | LTC-CV              |                                                                                                                 |
| 1  | der(3)(p12.2->cen) | LTC-CV             | 3 EPHA3: 1.178 3 EPHA3: 1.2265 3 CHL1: 0.974 3 CHL1: 0.965                                                          |
| 5  | i(12)(p10)      | uAF                 | 4 PKP2: 1.795                                                      |
| 7  | der(13)(q14.13)(q31.3)(q13) | cAF           | 3 HSMP8P: 1.470 3 ZNF198: 1.322 3 PSCP1: 1.352 3 FRG1: 1.2354                                                                   |
| 11 | der(14)(q14.16)(q12q21) | cAF          | 3 APEX: 1.358 3 ADPRTL2: 1.371 3 CHL1: 0.974 3 CHL1: 0.965 3 FLJ22604: 1.485 |
| 12 | inv dup(15)(q12) | cAF                 | 4 NDN: 1.702 4 MKRN3: 1.635 4 CYFIP1: 1.714 4 NDN: 1.668                                                          |
| 13 | neo(15)(qtel->q24) | cAF                 | 2 NDN: 1.007 2 MKRN3: 0.932 2 ALDH1A3: 1.450 2 FLJ22604: 1.485                                                          |
| 14 | der(15)(q15.1)(q12q14) | uAF           | 3 NDN: 1.324 3 NDN: 1.335 3 ADPRTL2: 1.314 3 FLJ22604: 1.485                                                          |
| 21 | inv dup(22)(q11.21) | cAF                 | 4 CECR1: 1.831 4 CECR1: 1.572 4 CECR5: 1.830 4 BID: 1.825 3 IL17R: 1.548 |
| 22 | inv dup(22)(q11.21) | cAF                 | 4 CECR1: 1.781 4 CECR1: 1.789 4 CECR5: 1.654 4 BID: 1.907 3 IL17R: 1.881 |
| 23 | inv dup(22)(q11.21) | uAF                 | 4 CECR1: 1.791 4 CECR1: 1.791 4 CECR5: 1.724 4 BID: 1.940 3 IL17R: 2.080 |
| 24 | inv dup(22)(q11.21) | cAF                 | 4 CECR1: 1.840 4 CECR1: 1.790 4 CECR5: 1.819 4 BID: 1.940 3 IL17R: 1.527 |
| 25 | del(22)(q11.2)   | LTC-CV              | 4 CECR1: 1.770 4 CECR1: 1.760 4 CECR5: 1.821 4 CECR5: 1.869 4 BID: 1.688 3 IL17R: 1.806 |

LTC-CV = long-term cultured chorionic villi; uAF = uncultured amniotic fluid cells; cAF = cultured amniotic fluids cells.

1. Level of mosaicism was determined in cultured cells, whereas DNA from uncultured cells was used for MLPA in cases 4, 6 and 20.
2. Although relative probe signals indicate 3 copies (<1.6) we interpreted these results as 4 copies because of mosaicism in the cell cultures.
3. Relative probe signal is clearly above the normal cut-off but less than 1.3, probably indicating mosaicism.
4. The relative probe signal of 4qtel probe (FRG1) is in fact below the cut-off value of 1.239. The FRG1 specific probes in the P036 kits were found not to be reliable by the manufacturer due to the presence of population specific SNPs in FRG1 (see website of MRC-Holland).
5. The probe for 9p in the P070 kit was found to be duplicated in some healthy individuals (see MRC Holland-website) which may explain the relative probe signal of 1.82 indicating 4 instead of 3 copies of 9pter.
6. The relative probe signal of the sequence targeting CECR5 in case 21 and IL17R in cases 21 and 24 is < 1.8 and therefore indicating 3 instead of 4 copies. However, on the basis of the relative probe signals of the probes in the same and/or other kits, we assume the presence of 4 copies of CECR5 and/or IL17R on the sSMC in cases 21 and 24.
own calculated cut off values (table 2) with relative probe signals of the involving probes above the normal cut off values in all kits.

**Unique sequence negative sSMC**

The unique sequence negative sSMC (cases 2, 8-10, 15-19, 26-30) all showed normal MLPA results with relative probe signals between the normal cut off values 0.7 and 1.3 confirming GTG/FISH results. There were no false positive cases.

**Discussion**

sSMC, when detected with conventional cytogenetic banding techniques, are still a problem as they often are too small or without a specific banding pattern to be considered for their chromosomal origin by traditional...
banding techniques. Therefore, molecular cytogenetic techniques are often needed for further characterisation. Since the phenotypic consequences of a sSMC will greatly depend on its genetic content and chromosomal origin, it is of particular clinical importance to rapidly distinguish unique-sequence-negative from unique-sequence-positive sSMC because the former are less likely to be associated with an abnormal fetal outcome.

For a collection of all available reported sSMC cases, see the sSMC database [22].

Different papers describe the use of genomic array analysis for identification of sSMC [15,16,23,24]. The big advantage of this technique is that the exact chromosomal content of the unique-sequence positive sSMC can be determined in one reaction, although targeted FISH is often used following an abnormal array result and is
sometimes necessary to determine the structure of the sSMC [24]. However, genomic array analysis is labour intensive, time-consuming and expensive. The use of the MLPA technique for characterisation of some specific sSMC has already been described in postnatal cytogenetics [25–28]. In this paper, we show that MLPA with centromere and telomere kits may be a quick initial approach for sSMC characterisation in prenatal diagnosis when time is limited. From every AF sample that we receive in our laboratory 4 ml is used for direct DNA isolation. As soon as a sSMC is found, this DNA can be used for MLPA and results are available within less than 24 hours. In case of a positive result, targeted FISH and/or array on (un)cultured AF cells may be used for confirmation and further identification. However, as with array analysis, MLPA will give normal results in case of an unique sequence-negative sSMC or in case of low level mosaicism [16]. Therefore, other techniques such as cenM-FISH [8] or sequential targeted FISH [29], although labour-intensive and frequently time-consuming, will be necessary for determining the chromosomal origin. Since such a sSMC most probably does not contain euchromatin, in the meanwhile, the parents can be karyotyped after reassurance.

Despite the development of molecular techniques for sSMC identification, conventional staining techniques are still valuable. Since 35% of the marker chromosomes with a known chromosomal origin are derived from chromosome 15 [30], the first thing to do in case of a satellited sSMC is a DA-DAPI staining. If positive, targeted FISH with chromosome 15 specific probes can be applied for further identification. If negative, FISH with a 13/21 and 14/22 centromere probe in conjunction with subcentromere probes for these 4 chromosomes can quickly elucidate the chromosomal content of the sSMC. In case of a non-satellited sSMC, MLPA might be a rapid and rather non-expensive technique for distinguishing between an unique sequence-positive and -negative sSMC.

The centromere kits P181 and P182 are presented by MRC Holland as kits for identification of sSMC. However, we recommend using the telomere kits in addition to these centromere kits for sSMC identification for three reasons. Firstly, by using only the centromere kits, the neo(12) and neo(15) would both have been missed. This type of sSMC, first described by Blennow [31] is rare. Up till now about ~90 neocentric acentric marker chromosomes have been described in patients with idiopathic mental retardation but also in cancer cells [32,33]. Secondly, the sSMC in cases 7 and 11 could be correctly identified as being unbalanced translocations by using centromere concomitant with telomere kits. Although rare, this type of sSSMC, the so-called unique complex sSMC which are derived from more than one chromosome, may be underdiagnosed as suggested by Trifonov et al. [34], and MLPA with centromere and telomere kits may enhance their detection rate. And finally, since most sSMC are derived from the acrocentric chromosomes, the simultaneous use of centromere and telomere kits allows for more markers to be tested in the subcentromeric region since the telomere kits also contain probes in the proximal long arm of the acrocentric chromosomes instead of a specific short arm subtelomere probe which they lack.

In 5/6 mosaic cases with the level of mosaicism determined in cultured cells, DNA isolated from uncultured AF cells was used for MLPA hampering the interpretation of the results. For instance, normal MLPA results in cases 18 and 19 are most probably explained by absence of unique sequences on both sSMC. However, due to low-level mosaicism at least in the AF cell cultures and therefore probably also in the uncultured AF cells used for MLPA, a normal result caused by low-level mosaicism can never be excluded. It is known that discrepancies may exist concerning mosaicism level between cultured and uncultured AF cells since these are not subjected to selection, mostly in favour of normal cells as seen in cell cultures. However, the reverse has also been observed in some cases of tissue specific mosaicism if the contribution of the affected organ or organsystem to the total AF cell population is small [35], but also in cases of generalised mosaicism [36]. From this experience, we learned that it is important to make FISH-slides at the time of DNA isolation necessary for determination of the level of mosaicism in the DNA sample enabling a proper interpretation of molecular results.

In cases 13 and 25 we show that the use of molecular techniques such as MLPA and array analysis may show that some sSSMC are much more complex than initially thought on the basis of conventional banding techniques and FISH. At the moment we are performing further FISH studies in order to elucidate the exact structure of the sSSMC in both cases. Tsuchya et al. [24] already published the uncoverage of unexpected complexity in the form of complex rearrangements of some sSMC when they used array CGH. This will ultimately lead to a more accurate sSMC-phenotype correlation which is important for proper counselling of the prospective parents when the sSMC is found prenatally.

**Conclusion**

In this paper we show that MLPA with centromere (P181 and P182)-and telomere (P036B and P070)-kits allow for the rapid differentiation between unique sequence positive and negative sSMC. As compared to multicolour FISH techniques and microarray analysis, MLPA is a
rather non-expensive and easy to perform technique in most clinical cytogenetic laboratories for the rapid elucidation of the harmfulness of a prenatally detected sSMC with results available within 24 hours.

Additional material

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Authors’ contributions

DVO co-ordinated the study and wrote the paper. MB performed the MLPA and microarray analyses. PN performed cytogenetic and FISH analyses. MIS, DVO, GH and R-JHG were responsible for the final (molecular) cytogenetic diagnoses and reports. GH and DVO studied the literature. R-JHG coordinated the genetic counselling of the parents. All authors read and approved the manuscript.

Competing interests

The authors declare that they have no competing interests.

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