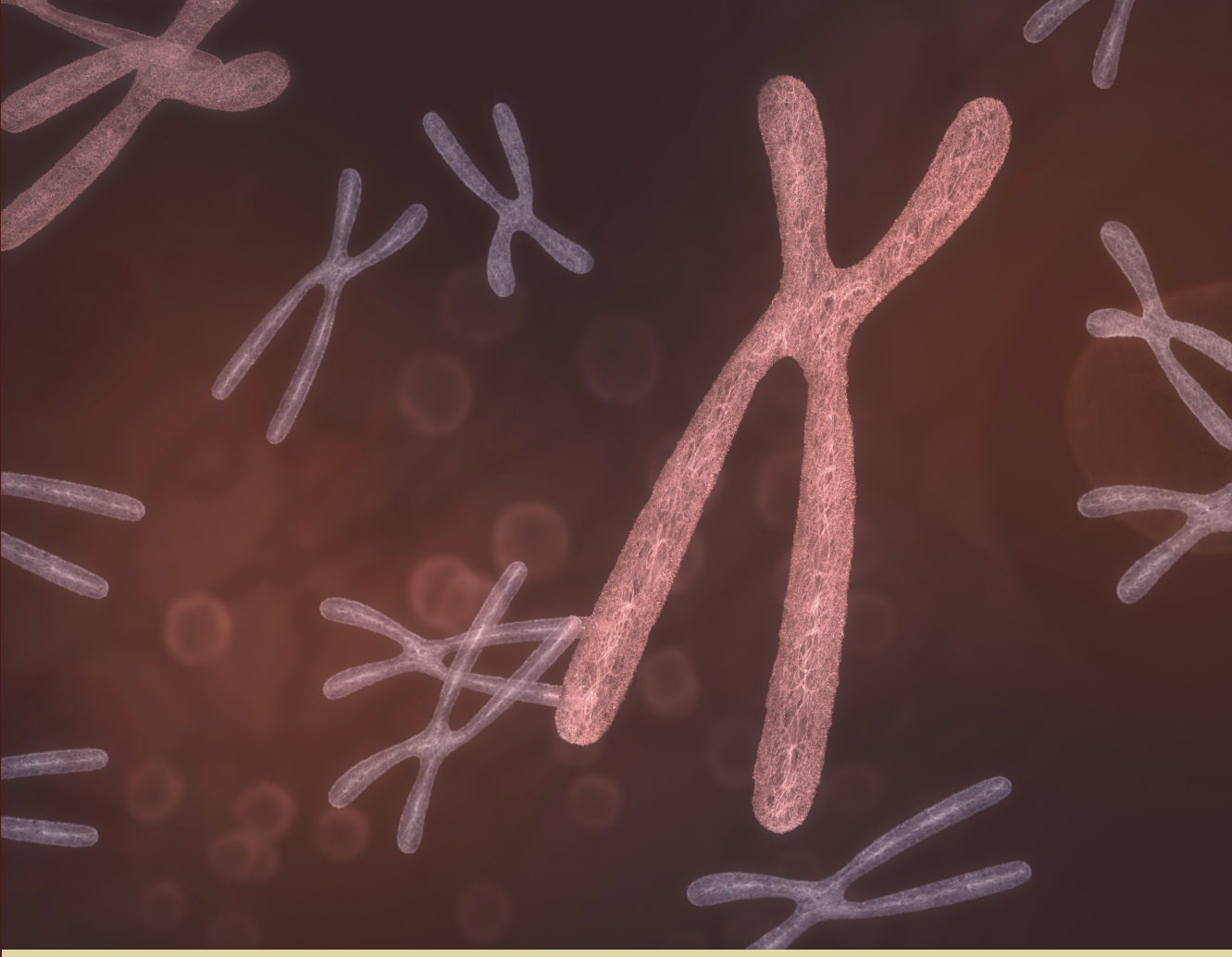

Maria Joana Lima Barbosa de Melo

CYTOGENETIC CHARACTERIZATION OF SMALL SUPERNUMERARY MARKER CHROMOSOMES
Towards a Genotype/Phenotype Correlation

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FACULDADE DE MEDICINA DA UNIVERSIDADE DE COIMBRA
2010

MARIA JOANA LIMA BARBOSA DE MELO

CYTOGENETIC CHARACTERIZATION OF SMALL SUPERNUMERARY MARKER CHROMOSOMES – TOWARDS A GENOTYPE/PHENOTYPE CORRELATION

Tese de Doutoramento em Ciências Biomédicas orientada pela Senhora Professora Doutora Isabel Marques Carreira e pela Senhora Professora Doutora Catarina Resende de Oliveira e apresentada à Faculdade de Medicina da Universidade de Coimbra.

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2010

"Tudo tem a sua ocasião própria e há tempo para todo propósito debaixo do céu. Há tempo de nascer e tempo de morrer; tempo de plantar e tempo de arrancar o que se plantou; tempo de matar e tempo de curar; tempo de derrubar e tempo de edificar; tempo de chorar e tempo de rir; tempo de prantear e tempo de dançar; tempo de espalhar pedras e tempo de ajuntar pedras; tempo de abraçar e tempo de abster-se de abraçar; tempo de buscar e tempo de perder; tempo de guardar e tempo de deitar fora; tempo de rasgar e tempo de coser; tempo de estar calado e tempo de falar; tempo de amar e tempo de odiar; tempo de guerra e tempo de paz."

Eclesiastes (capítulo 3); Antigo Testamento

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LIST OF ABBREVIATIONS

AC/ACH	Accessory Chromosomes
ALGS	Alagille Syndrome
Array CGH	Array Comparative Genomic Hybridization
AT-	Adenine and Thymine
BAC	Bacterial Artificial Chromosome
bp	base pair
BPs	Breakpoints
C-banding	Centromeric-banding
cen	centromere
CES	*Cat-Eye* Syndrome
CGH	Comparative Genomic Hybridization
CNV	Copy Number Variant
Del	deletion
der	derivative
DNA	Deoxyribonucleic Acid
DOP	Degenerated Oligonucleotides Primer
dr	Double ring
dup	duplication
EBV	Epstein Barr Virus
ESAC	Extra Structurally Abnormal Chromosome
FISH	Fluorescence *In Situ* Hybrydization
G-band	Giemsa band
h	hour
HPE	Holoprosencephaly
i(18p)	isochromosome 18p
i(19p)	isochromosome 19p
i(2p)	isochromosome 2p
idic	isodicentric
idic(15)	Isodicentric chromosome 15
inv dup	inverted duplication chromosome
inv dup(15)	inverted duplication chromosome 15
inv dup(22)	inverted duplication chromosome 22
ISCN	International System for Human Cytogenetic Nomenclature
Kb	Kilobase
LCR	Low Copy Repeat
LCRs	Low Copy Repeats
m	minutes
mar	marker chromosome
Mb	Megabase
MCB	Multicolour Banding
M-FISH	Multicolour Fluorescence *in situ* Hybrydization
min	minute chromosome
mos	mosaic
MTX	Methotrexate
NOR	Nucleolar Organizer Regions
OFC	Occipital Frontal Circumference
OMIM	Online Mendelian Inheritance in Man
p-arm	short arm of a chromosome
pcp	partial chromosome painting
PCR	Polymerase Chain Reaction
PHA	Phytohemaglutinin

PKS	Pallister-Killian Syndrome
PWS/ASCR	Prader Willi/Angelman Syndrome Critical region
q-arm	long arm of a chromosome
Q-band	Quinacrine band
r	ring chromosome
RNA	Ribonucleic acid
s	seconds
SAC	Small Accessory Chromosome
SMC	Supernumerary Marker Chromosome
SNP	Single Nucleotide Polymorphism
SNPs	Single Nucleotide Polymorphisms
SRC	Supernumerary Ring Chromosomes
SMC	Supernumerary Marker Chromosome
sSMC	small Supernumerary Marker Chromosome
sSMCs	small Supernumerary Marker Chromosomes
SubcenM-FISH	Subcentromeric Multicolour FISH
UBCAs	Unbalanced Chromosomal Abnormalities
UPD	Uniparental Disomy
wcp	whole chromosome painting

RESUMO

Cromossomas marcadores supranumerários (sSMCs, do inglês, *small Supernumerary Marker Chromosomes*) são pequenos cromossomas estruturalmente anormais que aparecem adicionalmente aos 46 cromossomas humanos e que não podem ser identificados ou caracterizados por citogenética convencional, tendo um tamanho inferior a um cromossoma 20 da mesma placa metafásica. Os sSMCs constituem um grupo heterógeneo de cromossomas anómalos que podem ter diferentes formas, como cromossomas duplicados invertidos (inv dup), cromossomas *minute* cêntricos (min) e cromossomas em anel (r).

O risco de alterações fenotípicas associadas à presença de um sSMC depende de vários factores, como a hereditariedade, a origem do cromossoma, a morfologia, o conteúdo genético, a presença de dissomia uniparental ou o grau de mosaicismo. De facto, os fenótipos associados a um sSMC são variáveis, desde normais a severamente afectados.

Os sSMCs podem ser detectados em diagnóstico pós-natal ou em pré-natal constituindo, sobretudo no último caso, um problema complexo para a investigação citogenética e o aconselhamento genético. Tendo em conta dados publicados na literatura que estimam uma frequência de 0.044% recém-nascidos portadores de um sSMC e que a população mundial é estimada actualmente em cerca de 6 823 000 000 pessoas, pode-se extrapolar o número de aproximadamente 3 milhões de indíviduos no mundo portadores de um sSMC. A grande maioria destes indíviduos não tem manifestações fenotípicas aparentes, no entanto cerca de 26% dos casos de indivíduos com um sSMC terão manifestações fenotípicas que poderão ser mais ou menos severas.

A maioria dos cromossomas marcadores publicados na literatura não têm uma caracterização citogenética detalhada, quer da origem quer do conteúdo genético, que permita uma correlação robusta com o fenótipo esperado. Este facto dificulta o aconselhamento genético quando é encontrado um sSMC, sobretudo em diagnóstico pré-natal. Neste domínio é essencial sistematizar o conhecimento sobre o conteúdo

genético envolvido nos sSMC e a descriminação das manifestações fenotípicas expectáveis de um indivíduo ou grupo de indivíduos, sejam elas normais ou anormais.
Com este trabalho pretendeu-se contribuir para esta sistematização, procedendo-se à caracterização por citogenética molecular de um grupo de cromossomas marcadores supranumerários derivados de vários cromossomas, nomeadamente dos cromossomas 1, 2, 5, 11, 13, 15, 16, 17, 18 e 22. Pretendeu-se validar a técnica de hibridização Genómica Comparativa por array (array CGH, do inglês *array Comparative Genomic Hybrydization*) para caracterização da extensão genética do cromossoma marcador, após a sua microdissecção e amplificação. Concluiu-se que esta técnica será de grande utilidade para a correcta caracterização de sSMCs sobretudo quando o cromossoma marcador está presente em mosaico de baixa expressão. Após caracterização molecular do grupo de sSMC em estudo estabeleceu-se, na medida do possível, uma relação genótipo/fenótipo, comparando cada caso com outros casos reportados na literatura. Neste trabalho propõem-se ainda linhas de orientação para lidar com casos de sSMC num laboratório de diagnóstico em citogenética, tendo em consideração factores como por exemplo o tempo para a caracterização citogenética, o grau de mosaicismo e as técnicas disponíveis no laboratório. O trabalho incluído nesta dissertação reforça e clarifica a necessidade de uma caracterização citogenética molecular detalhada de cromossomas marcadores supranumerários, recorrendo nomeadamente a técnicas de M-FISH e de array CGH. Esta caracterização possibilita o estabelecimento de relações genótipo/fenótipo cada vez mais precisas e essenciais para um correcto aconselhamento genético, por parte do Geneticista Clínico.

ABSTRACT

Small supernumerary marker chromosomes (sSMCs) are small structurally abnormal chromosomes that occur in addition to the 46 human chromosomes, that cannot be identified or characterized unambiguously by conventional banding cytogenetics alone, and are (in general) equal in size or smaller than a chromosome 20 of the same metaphase spread. sSMCs are a morphologically heterogeneous group of structural abnormal chromosomes that can appear in different shapes: inverted duplicated chromosomes (inv dup), centric minute chromosomes (min) and ring chromosomes (r).

The risk for phenotypic abnormalities associated with a sSMC depends on several factors, including inheritance, chromosomal origin, morphology, genetic content, presence of uniparental disomy or grade of mosaicism. In fact, phenotypes associated with a sSMC are variable, from normal to severely affected.

sSMCs can be detected postnatally or prenatally, the last being a major problem for cytogenetic investigation and genetic counselling. Taking into account data in the literature that estimate a 0.044% frequency of newborns with a sSMC and that the estimated world population is 6 823 000 000 people, there will be at present approximately 3 million living sSMC carriers. The great majority of these carriers will not have apparent phenotypical consequences. However, about 26% of individuals with a sSMC will have phenotypical manifestations, more or less severe.

The majority of sSMCs published in the literature do not have an accurate cytogenetics characterization, of the origin and genetic content of the marker, which allows a robust correlation with the expected phenotype. This is a difficulty for genetic counselling when a sSMC is encountered, especially at prenatal diagnosis. It is essential to systematize the knowledge of the genetic content of markers and the phenotypical manifestations encountered, normal or abnormal.

With this work we want to contribute to this systematization, by doing a detailed molecular cytogenetic characterization of a group of sSMC derived from different chromosomes, namely chromosomes 1, 2, 5, 11, 13, 15, 16, 17, 18 and 22. We aimed to validate array CGH technique, after the microdissection and amplification of the marker, for the characterization of the genetic extension of the sSMC. We concluded

that this technique is of great value for an accurate characterization of the marker, especially when the sSMC is present in low levels of mosaicism.

After the characterization of the group of sSMCs cases in study, it was established a genotype/phenotype correlation, whenever possible, and a comparison of each case with those described in the literature was done. In this work, guidelines are proposed for the management of sSMCs in a diagnostic cytogenetic laboratory, taking into account several factors, like time available for the cytogenetic characterization, levels of mosaicism and techniques availability in the lab. This work reinforces and clarifies the need of an accurate molecular cytogenetic characterization of sSMCs, using particularly M- FISH techniques or array CGH. This characterization allows the establishment of more precise genotype/phenotype correlations, which are essential for an accurate genetic counselling by the Clinic Geneticist.

1. INTRODUCTION

1.1. CYTOGENETICS – *state of the art*

1.1.1. Historical Perspective

Cytogenetics is the field of genetics that concerns the study of chromosomes' structure and function. The discovery of the number of human chromosomes on embryonic lung tissue in 1955 by Tjio was the fundamental step for the birth of human cytogenetics (Fig. 1.1.). This finding, that the diploid number of chromosomes in humans is 46, was published by Tjio and Levan in 1956 [Tjio and Levan, 1956], and that same year the number was confirmed by examining meiotic chromosomes [Ford and Hamerton, 1956]. This milestone occurred at the moment the hypotonic chromosome spreading procedure described by Hsu [Hsu, 1952] was made possible thanks to significant advances in techniques, later being improved through the use of phytohemaglutinin [Moorhead et al., 1960]. Given the simplicity of the procedure to observe human chromosomes, the study of certain syndromes of genetic origin began immediately, revealing the chromosomal origin of some of them by the end of the 1950s [Garcia-Sagredo, 2008]. In fact in 1959, Lejeune and collaborators reported the first autosomal aneuploidy, trisomy 21, associated with a characteristic phenotype [Lejeune et al., 1959]. This marked the beginning of clinical cytogenetics and soon after other descriptions of aneuploidies associated with syndromes were published, like Edwards syndrome (trissomy 18), Patau syndrome (trisomy 13), Klinefelter syndrome (47,XXY) or Turner syndrome (45,X). In tumour cytogenetics, one of the most exciting findings was the identification in 1960 of a minute chromosome, later named the Philadelphia chromosome, which was regularly found in the peripheral blood of patients with chronic myeloid leukaemia [Nowell and Hungerford, 1960].

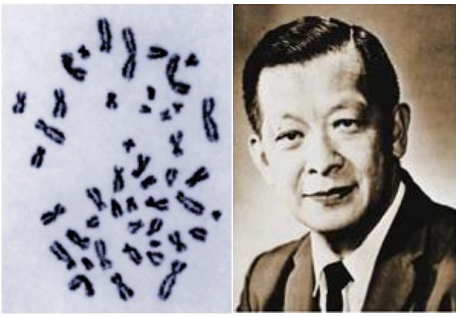


Figure 1.1. - A human metaphase plate, from the original Tjio (in the photo) and Levan paper, shows 46 chromosomes [from Gartler, 2006].

The discovery of chromosome banding techniques and structural chromosomal rearrangements soon followed. As revised by Garcia-Sagredo [Garcia-Sagredo, 2008], at the end of the 1960s, several banding techniques were discovered which allowed chromosomes to be individually identified. Using fluorescence or digestion with trypsin, it was possible to obtain the characteristic pattern of Q and G band, producing a karyotype with a pattern of 500 bands [Caspersson et al., 1970; Seabright, 1971]. Over time, higher resolution became necessary and in 1976, a high-resolution band method using prometaphase chromosomes that can reach 1000 bands was published, allowing cytogenetic anomalies to be better identified [Yunis, 1976].

To facilitate the identification of chromosomes and to prevent confusion in reporting cytogenetic results, an International System for Human Cytogenetic Nomenclature (ISCN) was created. The first steps for this system were done at "The Denver's Conference", USA, in 1960, when a group of researchers joined to propose a standard system for the nomenclature of human mitotic chromosomes. In 1978 the first "International System for Human Cytogenetic Nomenclature" was published. This Nomenclature System has been periodically revised, namely in 1981, 1985, 1995, 2005, being the last update done in 2009 [ISCN 2009 - Shaffer et al., 2009].

1.1.2. Conventional Cytogenetics

Traditionally, the term cytogenetics has referred to studies of the cellular aspects of heredity, especially the description of chromosome structure and the identification of genomic aberrations that cause disease. Cytogenetics has been used for many years for various applications, from clinical diagnostics to basic genomic research. Conventional chromosome analysis, which is based on banding and was developed in the 1970s is still widely used [Speicher and Carter, 2005].

Numerous procedures have been reported for producing banding patterns on metaphase spreads. A band is a part of a chromosome that is clearly distinguishable from its adjacent segments by appearing darker or lighter with one banding technique [Shaffer et al., 2009]. Chromosome banding methods are based on staining methods with a dye. The banding techniques fall into two principle groups: (1) those resulting in

bands distributed along the length of the whole chromosome, such as G-, Q- and R-bands, including techniques that demonstrate patterns of DNA replication; (2) those that stain specific chromosome structures and give rise to a restricted number of bands, including C- and NOR-banding. Bands that show staining are referred to as positive bands; weakly staining bands are negative bands. However the staining patterns are not always black and white, different bands stains to different intensities [Francke, 1994; Bickmore, 2001].

Nearly all methods of chromosome banding rely on harvesting chromosomes in mitosis. This is usually achieved by treating cells with tubulin inhibitors, such as colcichine or colcemide, which depolymerise the mitotic spindle and arrest the cell at this stage [Bickmore, 2001]. In 1976, Yunis published a method for obtaining high-resolution chromosomes, visualizing human chromosomes in mid-prophase, instead of metaphase [Yunis, 1976] (Fig. 1.2.).

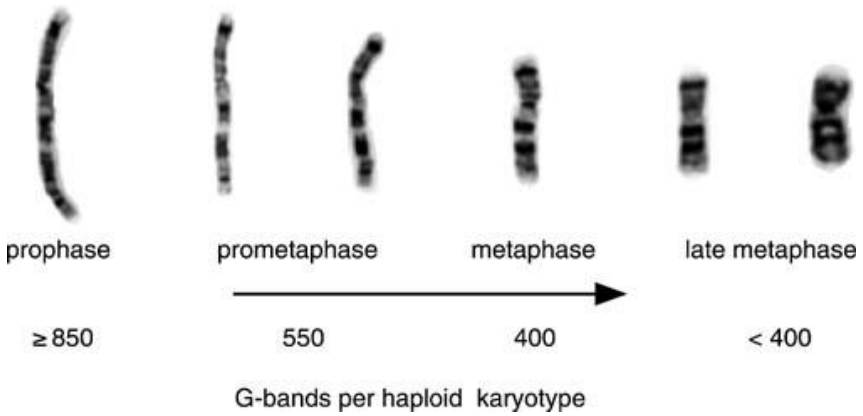


Figure 1.2. A partial karyotype of G-banded chromosome 7 from cells at successive stages of mitosis, illustrating the coalescing of sub-bands into larger, less defined, and less informative landmark bands. Cells captured in late prophase may show 850 to 1000 bands per haploid karyotype, i.e., high resolution. By mid-metaphase, fine band detail is lost as chromosomes condense and bands fuse, and only 400 or fewer bands per haploid karyotype may be observed [from Bangs and Donlon, 2005].

After staining to induce banding patterns, chromosome preparations are examined under a light microscope (1000x magnification). Images are captured and stored, nowadays with computerized image capture. They are then analysed by identifying

each chromosome pair, to form what is called a karyotype or karyogram[1] (Fig. 1.3.). Chromosomes are identified by their size, position of the centromere and the patterns of bands along their arms. The chromosomes are counted in several metaphase plates and looked for abnormalities in banding patterns. This is a non-automated and labour-intensive procedure [Warburton, 1995].

G-Banding and R-Banding

Techniques that use Giemsa dye mixture as the staining agent are termed G-staining methods and the resulting bands G-bands (Fig. 1.3.). Chromosomes are treated with trypsin and then with Giemsa stain to produce dark heterochromatic regions and light euchromatic regions [Seabright, 1971]. Giemsa is specific for the phosphate groups of DNA and attaches itself to regions of DNA where there are high amounts of adenine-thymine (AT) bonding.

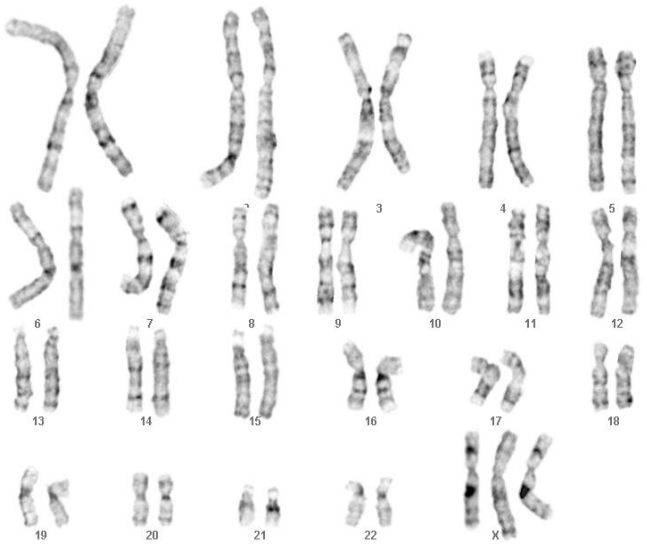


Figure 1.3. – High resolution karyogram with G-banding showing a female with an extra X chromosome (47,XXX) [photo from *Laboratório de Citogenética da Faculdade de Medicina da Universidade de Coimbra*].

[1] - According to Shaffer *et al.* (2009) – ISCN 2009, the term *karyogram* should be applied to a systematized array of chromosomes prepared either by drawing, digitized imaging, or by photography, typifying the chromosomes of an individual or a species. The term *karyotype* should be used to describe the normal or abnormal, constitutional or acquired, chromosomal complement of an individual, tissue or cell line. The term *ideogram* is reserved for the diagrammatic representation of a karyotype.

R-banding includes a pre-denaturation of chromosomes with hot acidic saline followed by Giemsa staining. The AT-rich DNA is preferentially denatured, being stained under-denatured GC-rich regions. This way the banding patterns are opposite in staining intensity to those obtained by the G-staining method [Dutrillaux et al., 1973; Shaffer et al., 2009].

Q-Banding

Q-banding (Fig. 1.4.) involves staining with quinacrine, which reacts specifically with certain bases. Quinacrine intercalates into chromosomal DNA irrespective of sequence, but fluoresces brighter in regions of AT-rich DNA [Caspersson et al., 1970]. In addition to quinacrine, other fluorescent dyes, like DAPI, Hoechst 33258, are specific for AT-rich DNA. The fluorescence of DAPI and Hoeschst is not quenched by guanine, like quinacrine is, and the banding patterns are less distinct than those produced by quinacrine [Bickmore, 2001].

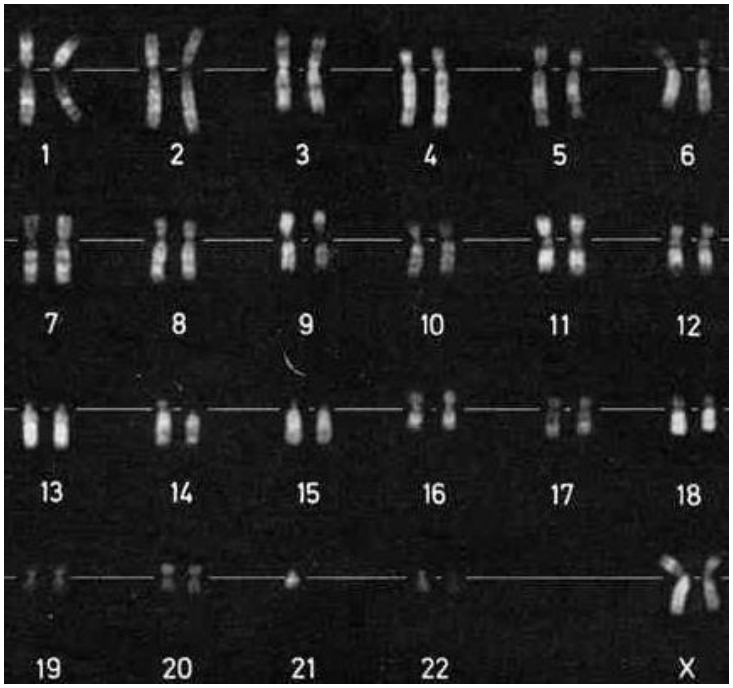


Figure 1.4. - Quinacrine stained karyotypes showing a 45,XX,-21 chromosome constitution [from Dutrillaux et al., 1973].

C-Banding

Centromeric DNA and pericentromeric heterochromatin, composed of alpha-repetitive DNA and various families of repetitive satellite DNA are detected by C-banding (Fig. 1.5.). The patterns obtained with C-banding, revealing constitutive heterochromatin, do not allow the identification of every chromosome in somatic cell complement, being used only to identify specific chromosomes [Eiberg, 1973]. The C-bands on chromosome 1, 9, 16 and Y are morphologically variable. The short-arms of the acrocentric chromosomes also demonstrate variations in size and staining intensity, not only with C-banding, but also with the other banding techniques [Shaffer et al., 2009].

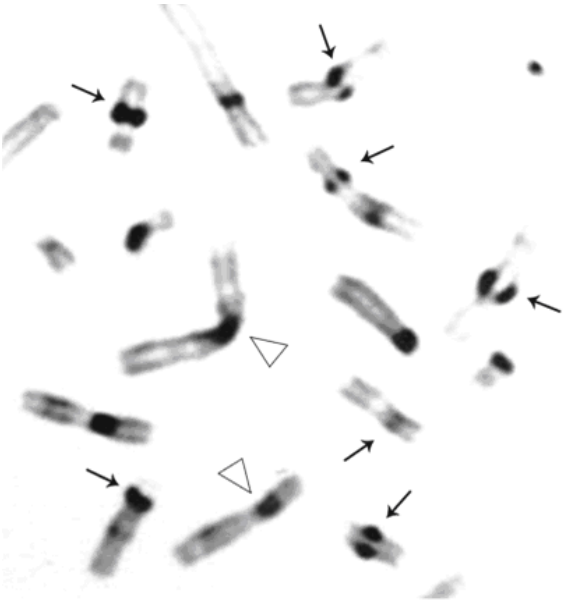


Figure 1.5. - C-banding of metaphase chromosomes of a patient with Roberts syndrome. Arrows show selected chromosomes with premature centromere separation. Open arrows show selected chromosomes with normal C-banded regions [from Vega et al., 2005].

NOR-banding

NOR-banding, with silver staining, detects the 18S and 28S ribosomal RNA genes that are clustered together in large areas containing about 40 copies of each gene, located at the acrocentric short arms, at the nucleolar organizer regions (NORs) (Fig. 1.6.) [Tantravahi et al., 1977; Shaffer et al., 2009].

Figure 1.6. - Ag-NOR staining of human chromosomes for detection of NOR regions. The short arms of acrocentric chromosomes have a positive staining [photo from *Laboratório de Citogenética da Faculdade de Medicina da Universidade de Coimbra*].

Replication Banding

Replication banding is a function-based banding method and is based on the fact that different bands replicate their DNA at different times during S phase of the cell cycle. This relationship between timing of replication and chromosome banding is usually studied by incorporating pulses of 5-bromo-2'-deoxyuridine (BrdU) into cells during defined stages of S phase and then examining chromosomes in the subsequent metaphase [Latt, 1973; Bickmore, 2001].

Chromosome Band Nomenclature

Each chromosome in the human somatic cell complement is considered to consist of a continuous series of bands, with no unbanded areas. A band is a part of a chromosome clearly distinguishable from adjacent parts, having lighter or darker staining intensity. The bands are allocated to various regions along the chromosome arms, and the regions are delimited by specific landmarks, including the centromeres, the telomeres

and certain bands characteristic of different chromosomes [Shaffer et al., 2009]. The designation of the regions, bands and sub-bands for each chromosome are described in the International System for Human Cytogenetic Nomenclature [ISCN(2009) - Shaffer et al., 2009]. Briefly, regions and bands are numbered consecutively from the centromere outward along each chromosome arm. The symbols **p** (*petit*) and **q** are used to designate, respectively, the short and long arms of each chromosome (Fig. 1.7.). The centromere (cen) itself is designated 10; the part facing the short arm is p10, the part facing the long arm is q10. The two regions adjacent to the centromere are labelled as 1 in each arm, the next more distal regions as 2, and so on [Shaffer et al., 2009].

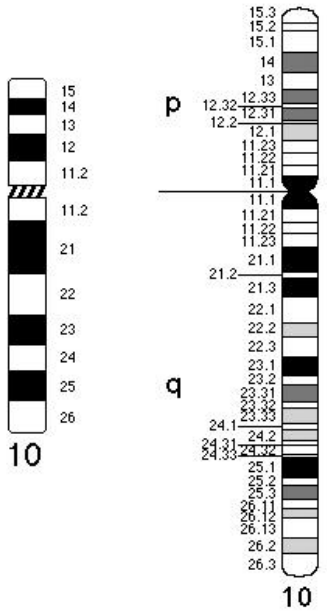


Figure 1.7. – G-band ideograms of human chromosome 10 at 400- (left) and 850- (right) band resolution [adapted from ISCN 2009].

For the designation of a particular band four items are required:

a) the chromosome number;

b) the arm symbol;

c) the region number;

d) the band number.

These items are given in order without spacing or punctuation. The bands can be subdivided in sub-bands, being numbered consecutively from the centromere outward. For example 18q22.1, is the band located at the long arm of chromosome 18, region 2, band 2, sub-band 1. The visualization of these sub-bands depends on the resolution of the karyotype (Fig. 1.2.).

It is important to emphasize that on routine banding of chromosomes, still used in most laboratories to this day (specially G-banding), only rearrangements and genomic imbalances (deletions and duplications) greater than 5 megabase (5 Mb) in size, and often 10 Mb, are detected.

1.1.3. Molecular Cytogenetics and Cytogenomics

The first application of molecular techniques to chromosome cytology was based on the observation that complementary nucleotide sequences could anneal or hybridize to each other to form more stable complexes. The first *in situ* hybridization analysis was reported in 1969 by Joe Gall and Mary Lou Pardue, who used DNA–RNA radioactive hybridization [Gall and Pardue, 1969]. The first successful multicolour FISH experiments were done in 1989 by Nederlof and co-workers by visualizing three differently labelled nucleic acid sequences, simultaneously, in blue (amino methyl coumarin acetic acid – AMCA), red (tetramethylrhodamine isothiocyanate – TRITC) and green (fluorescein isothiocyanate – FITC) [Nederlof et al., 1989; Liehr et al., 2009]. Over the past years molecular cytogenetic techniques of increasingly higher resolution have been developed. The cytogenetic use of these new technologies is designed to provide a description of chromosome structure at a resolution that exceeds that of microscopic analysis. Therefore, these technologies bridge the gap between cytogenetic and molecular approaches. The molecular cytogenetics techniques include: Fluorescence *in situ* hybridization (FISH); Comparative Genomic Hybridization (CGH) and Array - Comparative Genomic Hybridization (array CGH). Beside these cytogenetic techniques, there are also molecular biology techniques that have been used to study chromosome imbalances, such as Multiplex Ligand Probe Amplification (MLPA) and Quantitative Fluorescence Polymerase Chain Reaction (QF-PCR).

1.1.3.1. FISH – Fluorescence *in situ* Hybridization

Molecular cytogenetics is usually based on fluorescence *in situ* hybridization (FISH). In this technique a labelled DNA probe is hybridized to cytological targets such as metaphase chromosomes, interphase nuclei or even extended chromatin fibres.

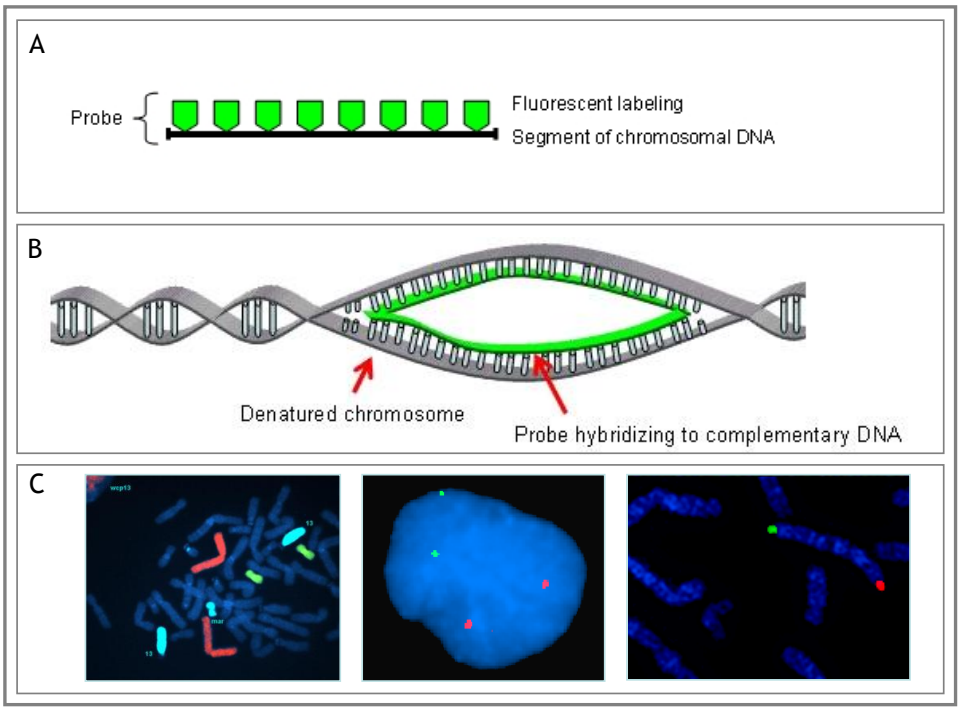


Figure 1.8. – Principal steps of a FISH experience. A) A fluorescently-labeled segment of DNA complementary to a chromosomal region of interest is used; **B)** Hybridization between the probe and the complementary chromosomal DNA occur; **C)** The slides can be visualized in a fluorescent microscope. If the DNA complementar to the probe is present a signal with the color of the emission wavelength of the fluorochrome of the probe is seen [A and B - adapted from Medical Genetics Information Resource database; C - photos from *Laboratório de Citogenética da Faculdade de Medicina da Universidade de Coimbra*].

FISH is a technique used to identify the presence of specific chromosomes or chromosomal regions through hybridization of fluorescently-labelled DNA probes to denatured chromosomal DNA. Every FISH experience has 3 fundamental steps:

1) Preparation of the probe – A probe is a fluorescently-labeled segment of DNA complementary to a chromosomal region of interest (Fig 1.8. A);

2) Hybridization – Denatured chromosomes fixed on a microscope slide are exposed to the fluorescently-labeled probe. Hybridization occurs between the probe and complementary chromosomal DNA (Fig 1.8.B);
3) Visualization – Following hybridization, the slide is examined under a microscope using filtered light (depending on the fluorochrome used). Fluorescent signals indicate the presence of the complementary chromosomal DNA for that probe (Fig 1.8.C).

All types of human DNA sequences have been used as probes for molecular cytogenetic studies. These include unique sequences, repetitive sequences such as α-satellite and telomere DNA, locus specific DNA obtained by PCR amplification, large genomic DNA sequences cloned into cosmids, bacterial artificial chromosomes (BACs), P1-derived artificial chromosomes (PACs), yeast artificial chromosomes (YACs), chromosomes band or arm specific sequences generated by microdissection and DNA libraries established by chromosome flow sorting. Following the sequencing of the human genome with the Human Genome Project, large-insert clones that have been mapped and sequenced can be used as probes and are now readily available for almost all genomic regions. Probes can be selected using internet-browsers such as Ensembl Cytoview or the UCSC genome browser [Speicher and Carter, 2005]. To make the probes, DNA sequences are labeled directly with fluorescent dyes or indirectly with biotin or digoxigenin, which are then detected with immunofluorescent staining [Fan, 2002].

Many FISH techniques have been developed, including FISH with unique sequences; FISH with multiple subtelomeric probes; multiplex or multicolour FISH (M-FISH), Combined Binary Ratio Labelling (COBRA) FISH [Tanke et al., 1999] and spectral karyotyping (SKY); color banding; primed *in situ* labeling (PRINS), fiber FISH, reverse FISH and comparative genomic hybridization (CGH) [Fan, 2002; Liehr et al., 2009]. With these techniques, deletions or rearrangements of a single gene can be detected, cryptic chromosome translocations can be visualized (except with CGH), copy number can be assessed and very complex rearrangements can be characterized. FISH can be used in metaphase spreads, but also in interphase nuclei. The later allows the study of

all types of human tissues at any stage of cell division, without the need of cell culture and chromosome preparation.

FISH with unique sequences

FISH with unique DNA sequences is the most basic molecular cytogenetic technique [Lichter and Ward, 1990]. The probe used could be specific of a single gene, a particular chromosomal region or locus, being the technique used, for example, for the confirmation of microdeletion syndromes (Fig. 1.9.).

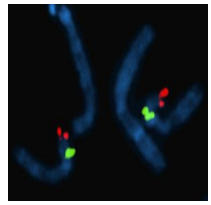


Figure 1.9. – Partial metaphase hybrydized with locus-specific probes for Smith Magenis Syndrome (17p11.2) (green) and Miller Dicker Syndrome (17p13.3) (red) [photo from *Laboratório de Citogenética da Faculdade de Medicina da Universidade de Coimbra*].

FISH for multiple subtelomeric regions

This technique uses 41 subtelomeric probes for all 24 different chromosomes (not including the short arms of acrocentrics) [Knight et al., 1997]. Each of these probes is composed of unique sequences in the subtelomeric regions (~300 kb from the chromosome end). The probes for the short arms and the long arms are dual labelled with a green and a red fluorochrome respectively (Fig. 1.10.) [Fan, 2002]. This technique is particularly important in the detection of chromosomal imbalances in these regions, especially deletions that are associated with mental retardation.

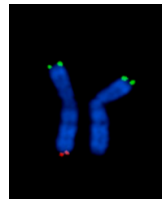


Figure 1.10. – Hybridization with subtelomeric probes for 5p and 5q showing a deletion of 5q subtelomeric region [photo from *Laboratório de Citogenética da Faculdade de Medicina da Universidade de Coimbra*].

Chromosome Painting

Chromosome painting allows the labeling of an entire chromosome using probes generated from specific chromosome libraries or chromosome microdissection (wcp, whole chromosome painting) [Cremer et al., 1988; Guan et al., 1994]. Partial chromosome painting (pcp) identifies the short or the long arm of a specific chromosome (Fig. 1.11.). This technique is extremely useful for the characterization of several chromosomal imbalances, like insertions and translocations.

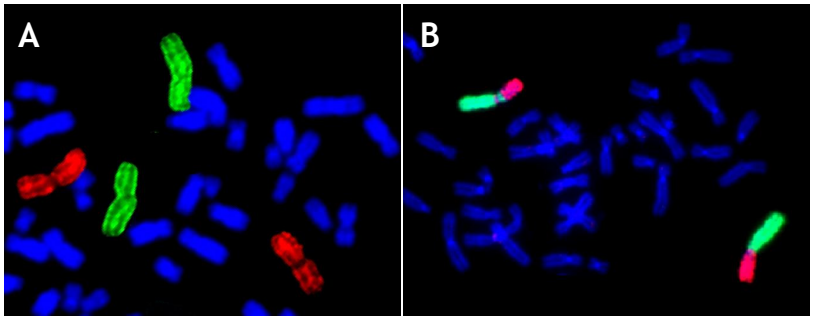


Figure 1.11. – A) Whole chromosome painting for chromosomes 2 (green) and 3 (red); **B)** Partial chromosome painting of chromosome 2, being the short arm red and long arm green. [photo from Molekulare Zytogenetik Labor in Jena].

SKY, COBRA and M-FISH

Combinatorial labelling uses the calculation of a spectral signature for each probe — each probe is identified by its unique combination of absences and presences of each fluorochrome. The required 24 colour combinations (for the 22 human autosomes plus the 2 sex chromosomes) can also be achieved using ratio labelling. In ratio labelling, different probes can be labelled with the same fluorochrome combinations, but are distinguished by the different proportions of the fluorochromes used. Using this approach, only four fluorochomes are necessary to achieve 96 possible colour combinations or pseudocolours. These labelling strategies allow the simultaneous visualization of all 24 human chromosomes, each in a different colour, in a single hybridization (Fig. 1.12.). The principle of combinatorial labelling is applied in almost cases. Specific technologies that use these approaches include Multicolour-FISH (M-FISH), Spectral Karyotyping (SKY-FISH) and Combined Binary Ratio Labelling (COBRA)

[Schröck et al., 1996; Speicher et al., 1996; Tanke et al., 1999; Speicher and Carter, 2005; Liehr et al., 2009].

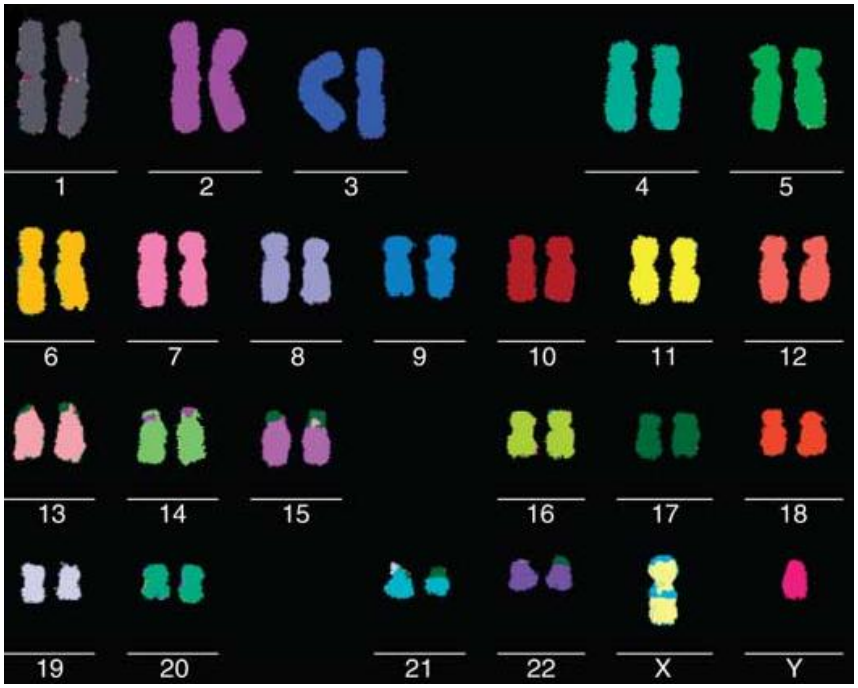


Figure 1.12. Human male karyogram after hybridization of a seven-fluorochrome M-FISH mix [adapted from Geigl et al., 2006].

Multicolour-FISH (M-FISH) is defined as the simultaneous use of at least three different ligands or fluorochromes for the specific labelling of DNA (excluding the counterstain). Camera-based image acquisition and computer-based image analysis are required for the analysis of SKY, COBRA and M-FISH experiments. Indeed, in combinatorial labeling, at least one of the fluorochromes used has its emission wavelength in a spectral region that is invisible to the human eye and in ratio labeling the computer is essential to detect slight colour differences [Liehr et al., 2009]. Using these multicolour approaches the detection sensitivity for small interchromosomal rearrangements (involving <3 Mb of sequence) is poor [Speicher and Carter, 2005].

Subcentromeric FISH (subcenFISH) is a M-FISH technique that allows the characterization of the pericentromeric regions of all human chromosomes. SubcenFISH includes the most proximally available single-copy probes,

microdissection-derived partial chromosome probes specific for the long and the short arm, and a centromere specific probe. For the acrocentric chromosomes, no proximal single copy probes and no chromosome specific pcp probes of the short arms are available. For all the acrocentric chromosomes set, a probe that stains all p-arms of the acrocentric chromosomes (midi54) is used (Fig.1.13.) [Starke et al., 2003].

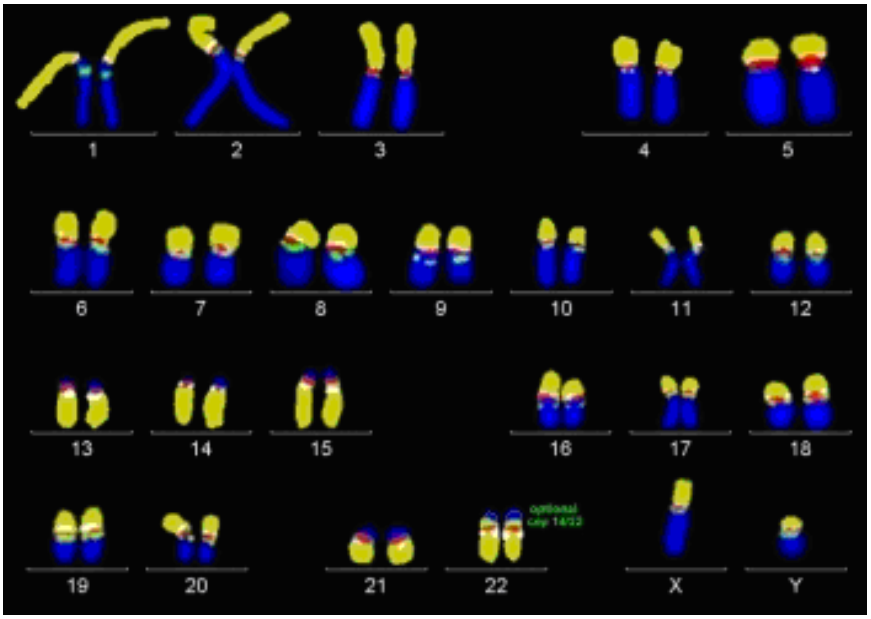


Figure 1.13. – Human male karyogram with subcentromeric FISH [adapted from Starke et al., 2003].

Multicolour Banding (MCB)

Multicolour Banding (MCB) is performed using overlapping microdissection libraries that are differentially labelled, producing reproducible and unique patterns of fluorescence ratios along chromosomes. These fluorescence ratios can be transformed into pseudocolour banding using specific software (Fig. 1.14.) [Chudoba et al., 1999]. MCB allows the differentiation of chromosome region specific areas at the band and sub-band level and provides the possibility to analyse chromosomes irrespective of their condensation grades [Liehr et al., 2009].

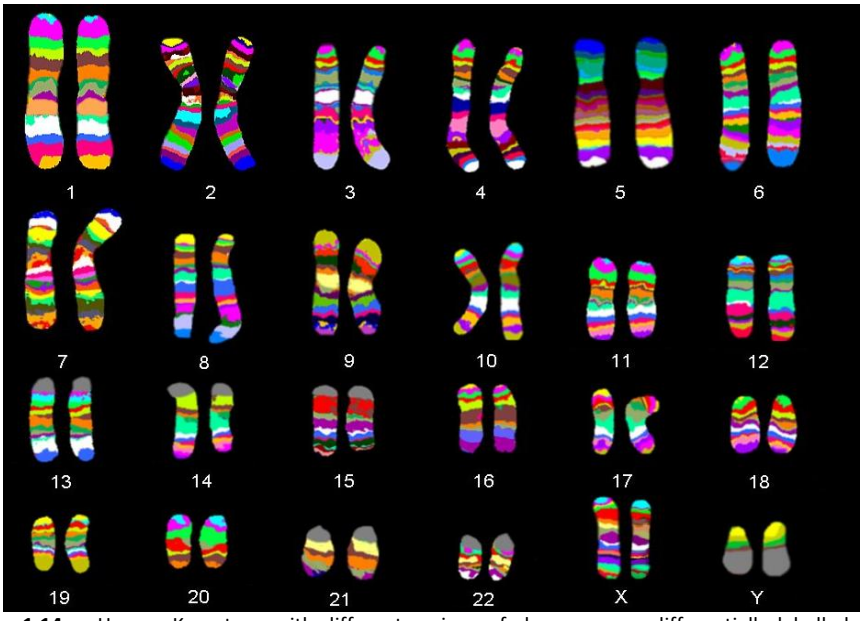


Figure 1.14. – Human Karyotype with different regions of chromosomes differentially labelled using probes obtained by microdissection – MCB [from Liehr et al., 2009].

Reverse FISH

A whole chromosome or a specific band is microdissected from a metaphase spread, amplified by PCR and labelled with a fluorochrome. The hybridization of this probe in a metaphase spread will reveal the chromosome or region microdissected (Fig.1.15.) [Su et al., 1994].

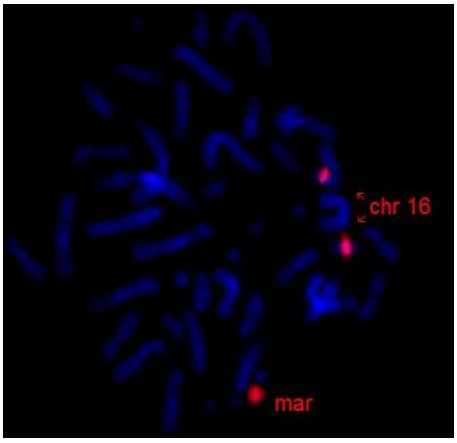


Figure 1.15. – Metaphase plate showing the presence of a marker chromosome by reverse FISH [from Melo et al., 2009].

Fiber-FISH

Fiber FISH is a powerful tool for mapping DNA sequences onto specific regions of the genome, because it allows accurate sizing of gaps and overlaps between probes (Fig. 1.16.). Overlaps between two differentially labelled probes are sized by measuring the 'length' of the signal on the DNA fibers that show mixed fluorescence (fluorescence from both probes). Gaps are sized by measuring the length of non-hybridized regions between two hybridization signals. Fiber FISH has been particularly useful for measuring the size of regions of the human genome that have been impossible to sequence [Florijn et al., 1995; Speicher and Carter, 2005].

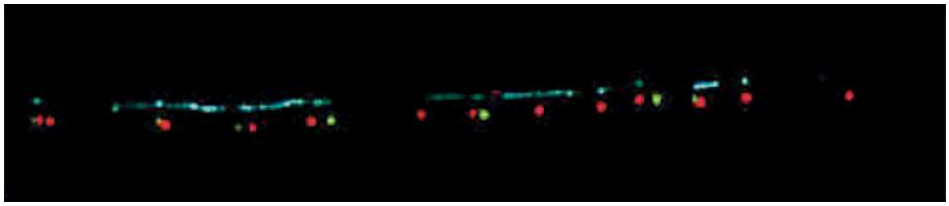


Figure 1.16. – Fiber-FISH image showing the overlaps and gaps between probes [adapted from Iafrate et al., 2004].

1.1.3.2. Comparative Genomic Hybridization

In Comparative Genomic Hybridization (CGH) the DNA is extracted from a test sample and a normal reference sample [Kallioniemi et al., 1992]. The two DNA samples are differentially labelled — for example, with the test labelled in green and the reference in red. The combined probes are then applied to target metaphase chromosomes and compete for complementary hybridization sites. Therefore, if a region is amplified in the test sample the corresponding region on the metaphase chromosome becomes predominantly green. Conversely, if a region is deleted in the test sample the corresponding region becomes red [Speicher and Carter, 2005]. By measuring the ratio of green to red colour, gains or losses of chromosomes can be detected (Fig. 1.17.). The size of DNA segments that CGH can detect is >~5-10 Mb, because it is dependable on the resolution of the metaphase spread used as a template [Kirchhoff et al., 2001]. By using dynamic standard reference intervals in high-resolution CGH, the resolution can be improved to ~3 Mb [Kirchhoff et al., 2004]. Although CGH can be applied to

constitutional cytogenetics, its main application is in tumour samples. Using DNA, and not metaphase spreads of the patient, CGH can be used for the study of cells in the different stages of cell cycle. For rearrangements that do not involve genomic imbalances, such as balanced chromosome translocations and inversions, the use of CGH is useless. In addition, whole-genome copy number changes (ploidy changes) cannot be detected. Furthermore, CGH provides no information about the structural arrangements of chromosome segments that are involved in gains and losses [Speicher and Carter, 2005]. For example, when a gain is observed it is not possible to ascertain whether it is a duplication or a marker chromosome.

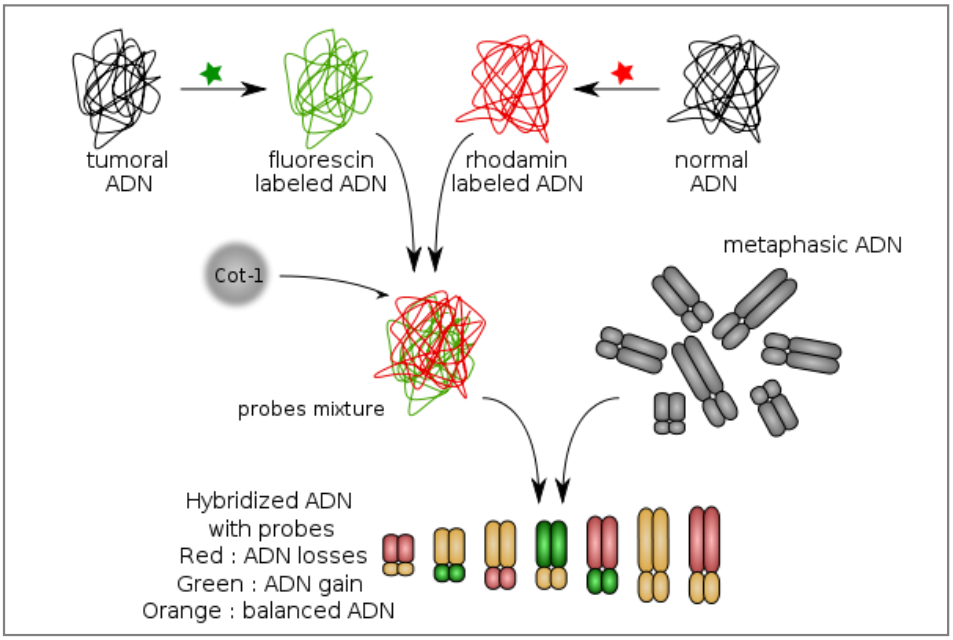


Figure 1.17. – Schematic representation of a CGH experiment. Patient DNA is labelled in green and a reference DNA is labelled in red. After mixture, the probes are hybridized in a metaphase plate. By measuring the ratio of green to red colour, gains or losses of chromosomes can be detected [downloaded from http://commons.wikimedia.org].

1.1.3.3. Array Comparative Genomic Hybridization – Array CGH

Array Comparative Genomic Hybridization (array CGH) was developed on the same principle as CGH on chromosomes. In array CGH, DNA probes are arrayed on a chip and CGH is used to test for increased or decreased dosage of chromosomal regions of interest. This technology allows the screening in one experiment of a great number of

DNA genomic sequences. Solinas-Toldo and co-workers were the first to report that it was possible to substitute the chromosome target by a glass where an ordered set of defined nucleic acid sequences are spotted [Solinas-Toldo et al., 1997; Sanlaville et al., 2005].

Genomic DNA of the patient is extracted from peripheral blood lymphocytes, skin fibroblasts or other available tissue and labelled with one fluorescent dye (usually Cy3-labelled dCTPs). The labelled patient DNA, together with an equal amount of control DNA labelled with another fluorescent dye (usually Cy5-labelled dCTPs), are co-hybridised to a selected set of pre-spotted genomic fragments. The spot intensities are measured at 532 nm (Cy3) and 635 nm (Cy5). If the amount of Cy3 and Cy5 fluorescent intensities are equal in one spot, this region of the patient DNA is interpreted as being normal/balanced; if a threshold of increased ratio of Cy3 to Cy5 is detected, a duplication of the patient DNA is suspected, and inversely if a deletion is present (Fig 1.18.) [de Ravel et al., 2007].

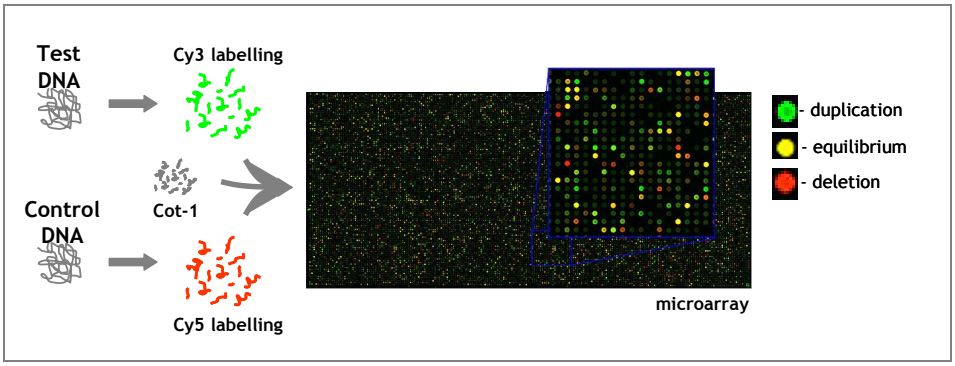


Figure 1.18. - Principle of array CGH. Test DNA and control DNA are differentially labelled with Cy3 and Cy5 respectively. The two labelled products are combined and hybridized onto the spotted slide. Images from hybridized slides are obtained by scanning in two channels and Signal intensity ratios from individual spots can be displayed as a simple plot. In the presence of a deletion the correspondent spot will appear red and in the presence of a duplication green.

With a single test, array CGH can detect genomic errors for disorders that are usually identified by cytogenetic analysis and multiple FISH tests. The detection limits of copy-number differences by array CGH depend on the probe density and the resolution of the platform used. The DNA probes commonly used range from genomic clones, most often BAC clones (80–200 kb), to oligonucleotides (25–85 bp) (Fig. 1.19.) [Emanuel and

Saitta, 2007]. A special type of oligonucleotide array can distinguish Single Nucleotide Polymorphisms (SNPs), allowing recognition of the parental origin of each DNA copy and enabling the detection of uniparental disomy.

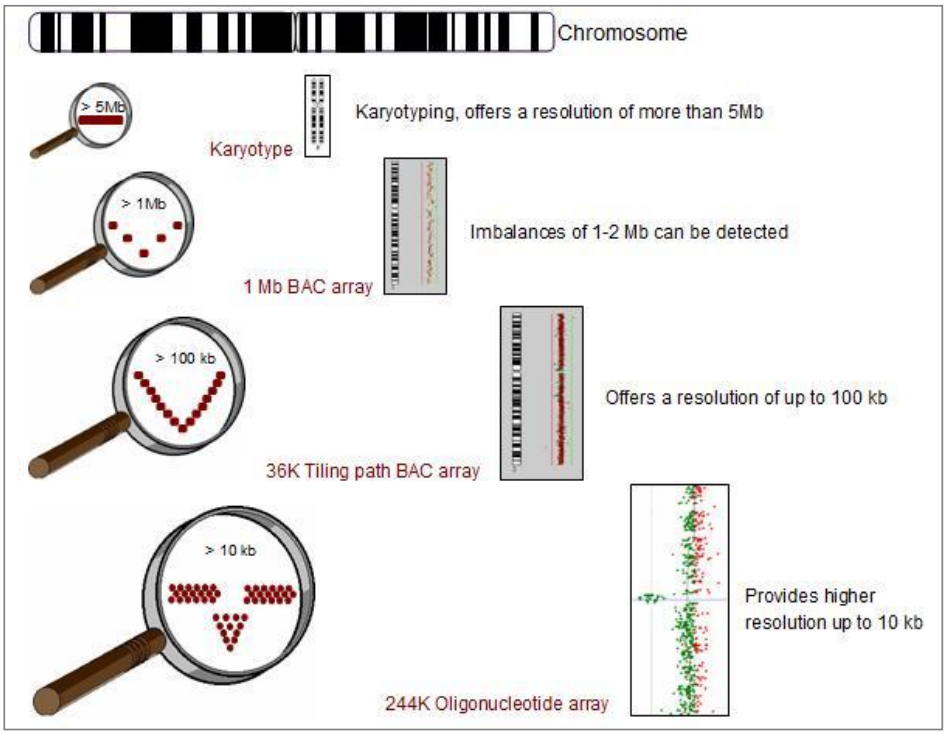
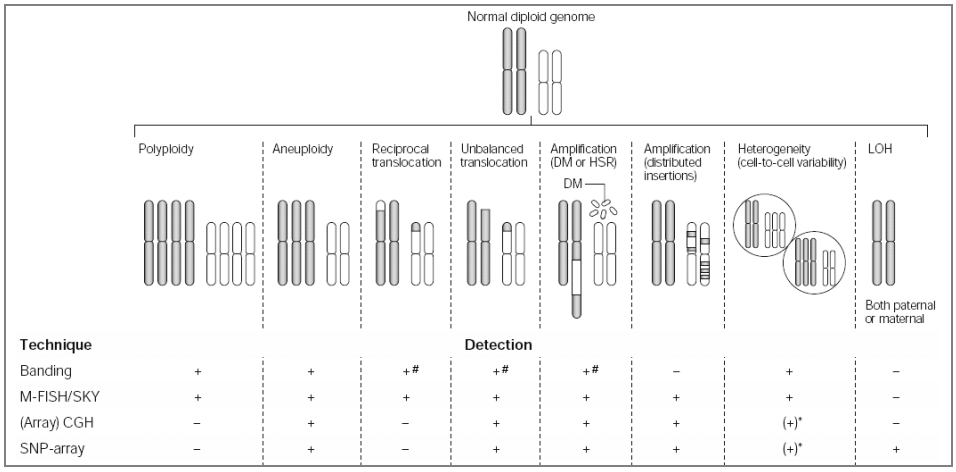


Figure 1.19. - Diagram showing the differences in resolution between conventional karyotyping and different array CGH platforms. Conventional karyotype allows a resolution of no more than 5 Mb. The resolution with array CGH depends on the platform used.

The advantage of array analysis is that it simultaneously assays discrete *loci* in a high-throughput manner at a resolution never before possible with conventional cytogenetic techniques. The high-resolution analysis afforded by microarrays can identify several pathogenic abnormalities. The recent use of microarray-based comparative array CGH has accelerated the identification of novel cytogenetic abnormalities. However, microarray analysis can also uncover gains and losses in regions of the genome that have unclear clinical significance. Some of these are apparently benign copy number variants (CNVs), but others may have clinical

relevance that remains unknown [Shaffer et al., 2007]. Like CGH, array CGH does not identify balanced chromosome rearrangements and complete ploidy.

Selecting the correct molecular cytogenetics strategy for the study of a particular disease or chromosomal imbalance is a main issue. Indeed, different chromosomal abnormalities require different cytogenetic methods for their identification. As summarized in figure 1.20., depending on the chromosomal abnormality the different cytogenetic techniques can have different sensibilities. For instance, array-CGH is not a suitable technique for the detection of polyploidy and balanced translocations, inversions or insertions. On the other hand, it has a high resolution for detecting submicroscopic deletions and duplications/amplifications.



Technique	Detection							
Banding	+	+	+#	+#	+#	–	+	–
M-FISH/SKY	+	+	+	+	+	+	+	–
(Array) CGH	–	+	–	+	+	+	(+)*	–
SNP-array	–	+	–	+	+	+	(+)*	+

Fig. 1.20. - Comparison of cytogenetic techniques for identifying chromosomal abnormalities. Various chromosomal aberrations that might be present in clinical samples are shown, with the ability of different cytogenetic techniques to detect them. A '+' indicates that an approach is suited for identifying the chromosomal rearrangement, a '–' indicates that the aberration would be missed. M-FISH, multiplex fluorescence *in situ* hybridization; SKY, spectral karyotyping; LOH, loss of heterozigoty. *Indicates that several experiments are needed [adapted from Speicher and Carter, 2005].
#rearrangements > 5Mb.

1.2. SMALL SUPERNUMERARY MARKER CHROMOSOMES (sSMCs)

1.2.1. Definition and Nomenclature of sSMCs

According to the ISCN published in 2009, a *marker chromosome* (mar) is a structurally abnormal chromosome that cannot be unambiguously identified or characterized by conventional banding cytogenetics [Shaffer et al., 2009]. A marker chromosome comprises a mixed collection of structurally rearranged chromosome regions. Numerous terms have been used in the literature to described markers, including *supernumerary marker chromosomes* (SMC), *extra structurally abnormal chromosomes* (ESAC), *supernumerary ring chromosomes* (SRC), *accessory chromosomes* (AC/ACH), *small accessory chromosome* (SAC), *extra* or *additional marker chromosome*, *supernumerary* or *extra microchromosome*, *additional or metacentric chromosome fragment* or *small bisatelited additional chromosome* [revised by Liehr et al., 2004]. Certain markers chromosomes are large enough to be identified by G banding and have a well-established phenotype. Examples include iso(12p), associated with Pallister-Killian syndrome (PKS) [Shinzel, 1991]; iso(18p), associated with mild-moderate mental retardation and a characteristic facial appearance [Callen et al., 1990]; or iso(9p)-syndrome, associated with mental retardation and craniofacial dysmorphisms. The other markers, smaller, are usually identified only by molecular cytogenetics. Some of these smaller supernumerary markers are also associated with well established syndromes, like cat-eye syndrome and the presence of an extra small marker derived from chromosome 22.

Crolla proposed a definition of *small supernumerary marker chromosomes* (sSMCs) as “small structurally abnormal chromosomes that occur in addition to the 46 chromosomes” [Crolla et al., 1997]. Liehr suggested a “more cytogenetic” definition, defining sSMCs “as structurally abnormal chromosomes that cannot be identified or characterized unambiguously by conventional banding cytogenetics alone, and are (in general) equal in size or smaller than a chromosome 20 of the same metaphase spread” [Liehr et al., 2004]. In contrast, a supernumerary marker chromosome larger than chromosome 20 usually can be identifiable based on chromosome banding. The

definition of small sSMC versus larger sSMC is not functional, but cytogenetic, because sSMC and larger sSMC can have the same karyotypic evolution [Liehr et al., 2004].

sSMC can either be present in addition to [Liehr et al., 2009]:

1) an otherwise normal karyotype (e.g.: 47,XX,+mar);
2) a numerically abnormal karyotype (e.g. Down syndrome – 48,XY,+21,+mar);
3) a structurally abnormal but balanced karyotype (e.g. 46,XX,del(mar-region),+mar).

sSMCs are a morphologically heterogeneous group of structural abnormal chromosomes that can appear in different shapes: inverted duplicated chromosomes (inv dup), centric minute chromosomes (min) and ring chromosomes (r) (Fig. 1.21.). The subgroup of inv dup is composed of different types of sSMC, including dicentric chromosomes.

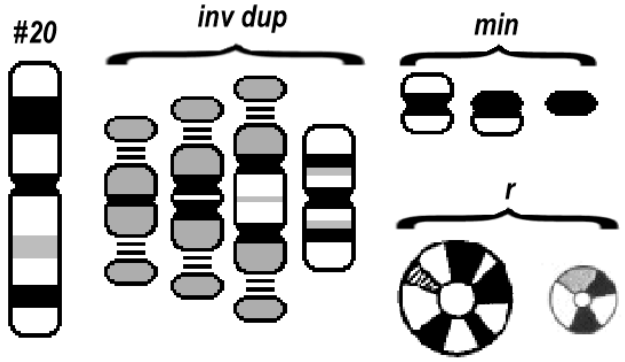


Figure 1.21.– Different shapes of sSMC. Small supernumerary marker chromosomes (sSMC) are defined as structurally abnormal chromosomes that cannot be identified or characterized unambiguously by conventional banding cytogenetics alone, and are (in general) equal in size or smaller than a chromosome 20 of the same metaphase spread. Different forms of sSMC can appear: inverted duplication chromosomes (inv dup), centric minute chromosomes (min) or ring chromosomes (r) [from Liehr et al., 2004].

Taking into account the morphology variety, the term “markers” seems to be more comprehensive, even after their identification by molecular cytogenetics [Liehr, 2009]. One problem not resolved in the new ISCN 2009 [Shaffer et al., 2009] is the difficulty encountered when a definition of subpopulation of small supernumerary marker chromosomes (sSMCs) is to be formulated. Additionally, no uniform regulations are available concerning the order of sSMC cell lines in the karyotype formula and the

description of the sSMCs main shapes (centric minute, inverted duplication and ring). When the "marker" chromosome becomes a well-characterized sSMC its shape should be indicated. However, according to the ISCN the only possibility of doing that correctly is using the abbreviation "der", for derivative. Liehr pinpointed this nomenclature problem in a recent letter to editor of the Journal of Histochemistry and Cytochemistry [Liehr, 2009]. According to the definition of derivative chromosomes (der) proposed in the 2009 ISCN, a "der" is a structurally rearranged chromosome generated either by a rearrangement involving two or more chromosomes or by multiple aberrations within a single chromosome. On the other hand, sSMCs are very specific and clearly definable according to their shapes. The only possibility according to the ISCN 2009 is to designate ring chromosomes as "r", being the term "der" used for the other shapes of sSMCs. The term "inv dup" is considered outdated and the replacement of this designation is suggested - for example *inv dup(15)(q11.2)* replaced by *idic(15;15)(q11.2;q11.2)*. However, this replacement could be confusing; in the way that "inv dup" is a widely accepted term.

Taking into account these considerations, Liehr proposed that the abbreviations "min" for centric minute-shaped sSMCs, "inv dup" for inverted duplicated sSMCs and "r" for ring-shaped sSMCs should continue to be applied and the use of "der" should be avoided in the subgroup of centric minute-shaped sSMCs. In table I, a comparison of the nomenclature proposed by ISCN 2009 and Liehr, in different sSMC cases, is described, showing that the system proposed by this author is more simple and informative [Liehr, 2009] (Table 1.I.). Furthermore and taking into account the order of the different cell lines in an sSMC mosaic case, this author purposes to sort the clones first by their modal chromosome number, second according to the number of cells containing the marker, and finally by the size of the imbalance they cause. Therefore, the normal cell line (46,XX or 46,XY) would be always referred at last[2].

[2] Considering the following example of a mosaic case with 4 different cell lines: 1) a normal complement in 4 analysed cells; 2) a cell line with a supernumerary marker chromosome (mar1) present in 5 analysed cells; 3) a cell line with a different, bigger, supernumerary marker chromosome (mar2) present in 4 analysed cells; 4) a cell line with a different supernumerary marker chromosome (mar3), smaller than mar2, present in 4 analysed cells; the order of the different cell lines should be: 47,XX,+mar1[5]/47,XX,+mar2[4]/47,XX,+mar3[4]/46,XX[4].

Table 1.I. - Comparison of ISCN 2009 nomenclature with the one suggested by Liehr for sSMCs shaped in centric minute, inverted duplication, or ring structure [from Liehr, 2009].

Marker type	ISCN 2009 description	Suggested sSMC description
(Centric) minute	Example for non-acrocentric chromosome der(20)(:p11.21→ q11.21:) or del(20)del(p11.21)del(q11.21)	min(20)(:p11.21→q11.21:)
	Example for acrocentric chromosome del(13)(q11.1)	min(13)(pter→q11.1:)
Inverted duplication	i(12)(p10)	inv dup(12)(pter→q11::q11→pter) or inv dup(12)(q11)
	i(15;15)(q11.2;q11.2) or psu dic(15;15)(q11.2;q11.2)	inv dup(15)(q11.2)
	inv dup(10)(qter→q25::q25→qter)	inv dup(10)(qter→q25::q25→qter)
Ring	r(20)(::p11.21→q11.21::)	r(20)(::p11.21→q11.21::)
	psu dic r(20;20)(::p11.21→q11.21::p11.21→q11.21::) or dic r(20;20)(::p11.21→q11.21::p11.21→q11.21::)	r(20;20)(::p11.21→q11.21::p11.21→q11.21::)

1.2.2. Relevance and risk of sSMCs

The first cases in the literature describing the presence of an sSMC appeared in the 60's. Indeed, in 1961, Ilberry and collaborators described a boy with epicanthic fold and protuberant tongue who had a karyotype 47,XY,+mar[53]/46,XY[16] *de novo* [Ilberry et al., 1961]. Ellis and colleagues published, in 1962, a case with an aberrant small acrocentric chromosome and Froland et al., in 1963, described a boy with several congenital defects with a karyotype 47,XY,+mar [Ellis et al., 1962; Froland et al., 1963]. Only several years latter, a correlation between several sSMCs cases and their possible phenotypic effects was described, when Buckton and collaborators showed that the ascertainment of sSMCs among individuals in psychiatric institutions was 3.27/1000 compared to their frequency of only 0.24/1000 among consecutive newborns [Buckton et al., 1985; Crolla et al., 2005].

Several attempts have been made to correlate specific marker chromosomes with a clinical picture, resulting in the description of few new syndromes like i(18p)-syndrome, the Pallister-Killian syndrome i(12p) (OMIM 601803) and the der(22) and cat-eye syndromes (CES) (OMIM 115470) [Crolla, 1998].

The risk for phenotypic abnormalities associated with an sSMC depends on several factors, including inheritance, mode of ascertainment, chromosomal origin, and the morphology, content, and structure of the marker [Graf et al., 2006]. Phenotypes associated with an sSMC are variable, from normal to severely affected. It has been shown that, depending on the chromosomal region of an sSMC, differences can be

observed and expected in clinical outcome. Indeed, this observation is confirmed by different reports of small trisomies in connection with the presence of an sSMC and specific clinical features [Crolla, 1998; Stankiewicz et al., 2000; D'Amato Sizonenko et al., 2002; Starke et al., 2003; Sarri et al., 2006; Baldwin et al., 2008]. However, the majority of markers published in the literature have not been fully characterized and in order to correlate an sSMC with a clinical outcome is essential to know it chromosomal origin and content.

sSMCs can be detected postnatally or prenatally, the last being a major problem for cytogenetic investigation and genetic counselling, due to time availability and, essentially, due to the difficulty in the majority of cases in establishing a robust genotype/phenotype correlation. Occasionally, sSMCs lead to spontaneous abortions. The majority of sSMCs do not grossly interfere with fetal development, because they contain little or no euchromatin.

Some markers are inherited directly from a phenotypically normal parent. In such cases, there are often no phenotypic consequences for individuals who inherit the marker, conferring the presence of a familial sSMC a low risk. However, there are exceptions to this, such as when there are imprinting effects from uniparental disomy (UPD), or low level, tissue specific mosaicism for the marker chromosome in a parent without phenotypic manifestations [Graf et al., 2006].

The risk of an abnormal phenotype in *de novo* prenatal cases with an sSMC is given as approximetly 13% [Warburton, 1991]. However, and taking in consideration the origin of the sSMC, the risk of an abnormal phenotype associated with a *de novo* acrocentric sSMC (excluding those derived from chromosome 15) is 7% (if sSMC is from 13, 14, 21 or 22) and 28% (for non-acrocentric autosomes) [Crolla, 1998]. Graf and colleagues estimated a 26% (28/108) risk for phenotypic abnormality for any non-sex chromosome *de novo* supernumerary marker chromosome [Graf et al., 2006]. It was suggested that if high resolution ultrasound studies are normal, this risk reduces to 18% [Graf et al., 2006]. As a result of the high risks and many uncertainties regarding the clinical outcome, many pregnancies with *de novo* sSMC are terminated.

Furthermore, the detection of mosaicism or uniparental disomy (UPD) of the parental chromosomes homologous to the sSMC confers more complexity to the case management.

1.2.3. Mechanisms of sSMCs formation

Different mechanisms of sSMC formation including trisomic rescue, monosomic rescue, post fertilization errors and gamete complementation have been proposed [Bartels et al., 2003; Liehr et al., 2004]. Another mechanism, that could explain the existence of multiple sSMCs of different origin, is the formation of sSMC from transfection of chromosomes into the zygote derived from one or more superfluous haploid pronuclei that would normally be degraded by deoxyribonucleases or other means [Daniel and Malafiej, 2003].

sSMCs can assume different shapes: inverted duplication (inv dup) chromosomes; centric minute chromosomes (min); neocentric chromosomes; ring chromosomes (SRC), and complex rearranged sSMCs. The mechanism of formation of the sSMC could be different according to the shape of the marker.

Inv dup supernumerary chromosomes

The most frequent mechanism proposed for the formation of inv dup sSMCs derived from acrocentric chromosomes is a U-type exchange resulting from crossover mistakes of chromatids of two homologous chromosomes during meiosis (Fig. 1.22.) [Schreck et al., 1977; Liehr et al., 2004]. A U-type exchange is also proposed for the formation of iso-chromosomes of non-acrocentric chromosomes, with a break within the centromeric DNA [Liehr, 2010 – sSMC homepage]. Besides U-type exchange, other theories have arise concerning the formation of dicentric iso-chromosomes including: a non-sister chromatid exchange followed by a second-division non-disjunction; errors in replication, caused by parental inversion heterozygosity; translocation exchange; and mitotic errors originating from ligation error in a replication fork during S-phase, followed by a new replication [Murmann et al., 2009].

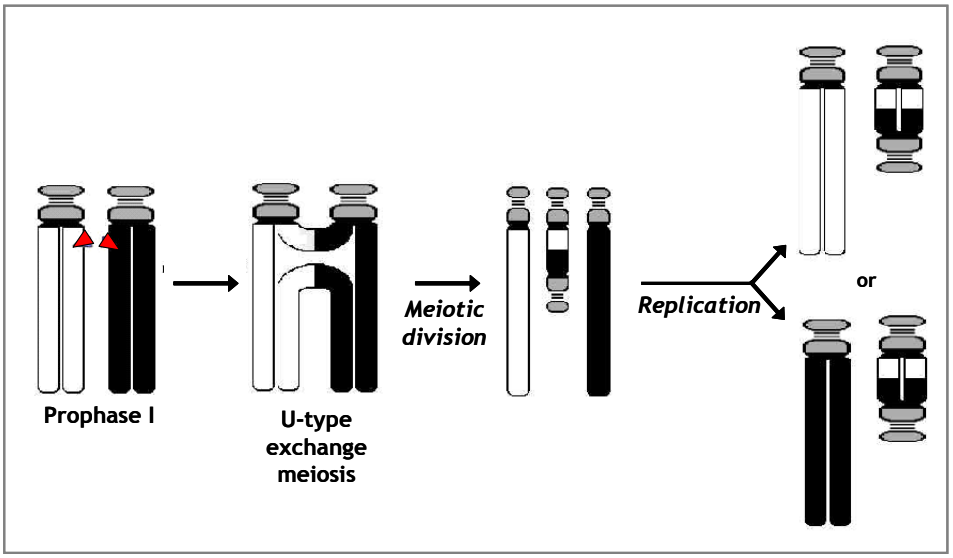


Figure 1.22. –Frequent mechanism proposed for the formation of inverted duplication sSMCs. A U-type exchange derived from crossover mistakes of the two homologous during the pachytene of prophase I [adapted from Liehr et al., 2004].

Small neocentric supernumerary chromosomes

Neocentric chromosomes do not contain detectable alpha-satellite DNA. They are called analphoid markers, carrying newly derived-centromeres (neocentromeres) that are formed within interstitial chromosomal sites that have not previously been known to express centromeres [Choo, 1997].

The development of the majority of neocentric sSMCs is based on a U-type exchange, between two chromatid of two different chromosomes (inter-chromosomal), and most of the neocentric sSMC are small iso-chromosomes (Fig. 1.23.). The acentric fragment created during a U-type exchange in these cases is included into a gamete, a neo-centromere is activated and the new "chromosome" is distributed throughout further cell cycles. This theory is supported by the fact that the frequency of inv dup(15) chromosomes is similar to that observed in neocentric chromosomes 15 among other acrocentric derived chromosomes of the corresponding group [Liehr et al., 2006]. However, Murmann et al. (2009) have proven that in contrary to the model shown in Fig. 1.23., in most neocentric inv dup-chromosomes the U-type exchange appears not intra- but inter-chromosomal, between two chromatids of the same chromosome (Fig. 1.24).

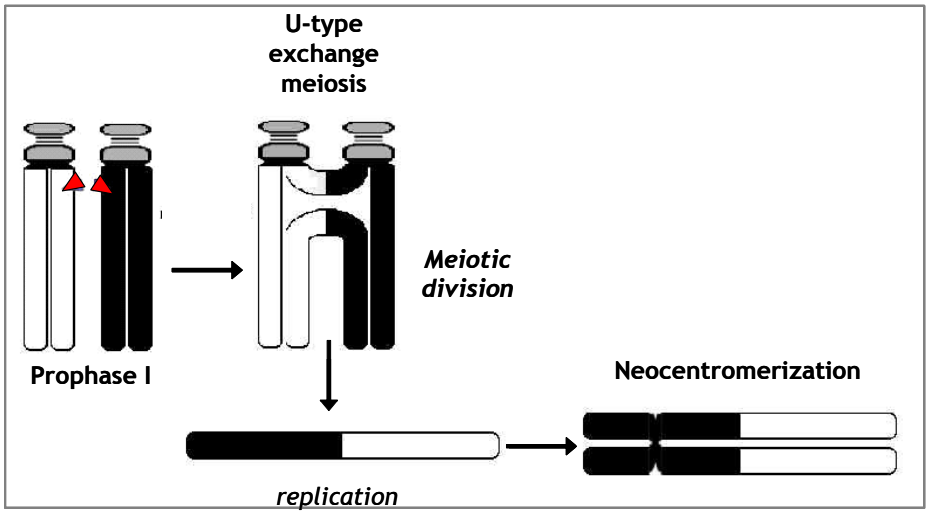


Figure 1.23. – Formation of a neocentromere supernumerary chromosome. An acentric inverted duplication chromosome is formed following a U-type exchange in meiosis I that forms a neocentromere, preventing it lost in subsequent cell divisions [adapted from Liehr et al., 2004].

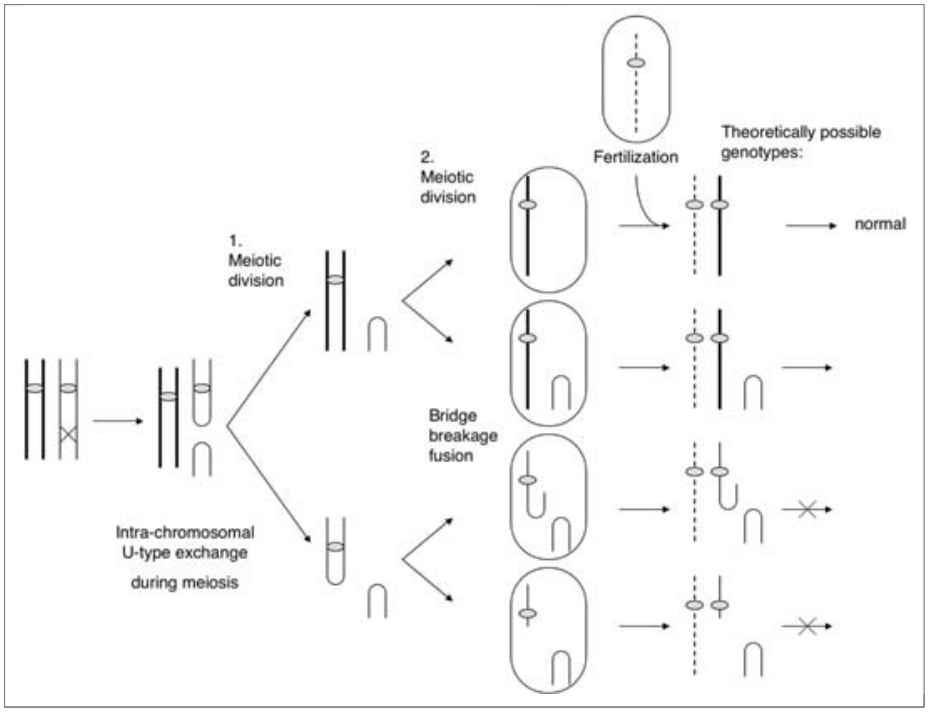


Figure 1.24. – U-type exchange could also occur between two chromatids of the same chromosome (intra-chromosomal). During meiosis crossing over leads to the formation of one acentric inv dup marker with two arms and one dicentric derivative. After meiosis I and the meiosis II division, respectively, the intra-chromosomal dicentrics break when both their centromeres are pulled apart resulting in a deleted derivative and an inv dup del derivative [adapted from Murmann et al., 2009].

There are some cases reported in the literature of sSMCs with a ring chromosome conformation and associated with the presence of a neocentrome [Slater et al., 1999; Spiegel et al., 2003, Mascarenhas et al., 2008]. The mechanism of formation in these cases should follow the one described for ring chromosomes (see below) with a part of one chromosome, without containing the centromere, is excised and a neocentromere is formed.

Small supernumerary ring chromosomes

In cases which the morphology of the sSMC can be determined, almost 50% appear to be ring chromosomes [Liehr et al., 2006]. Several explanations for formation of non-neocentric small supernumerary ring chromosomes (sSRC) are available in the literature. Two major models are proposed:

I) a sSRC can be formed after a break within the centromere ("centromere misdivision"), along with a break either in *p* arm or in *q* arm. This is the case where ring formation is in connection with an inverted duplication due to an U-shape reunion between broken sister chromatids [Michalski et al., 1993] (Fig. 1.25.A) or when a SRC is associated with a deletion of a part of the chromosome. In contrast to neocentric sSMC, parts of the centromere are included, leaving two centric chromosome fragments, one of which forms a small ring (Fig. 1.25.C);

II) a sSRC formation requires the occurrence of one break in the *p* arm and a second break in the *q* arm, and the two broken ends of a centric fragment fusing together to form a ring. The resulting SRC contains DNA from both p and q arms. sSRC formation can start with a centric minute chromosome (Fig. 1.25.B) or the formation of a sSRC could be associated with complex chromosomal rearrangements [Stankiewicz et al., 2001; Stavropoulou et al., 1998]. A dicentric iso-chromosome could be formed primarily, followed by an excision of one centromere, with the excised fragment subsequently forming a ring chromosome (Fig. 1.25.D).

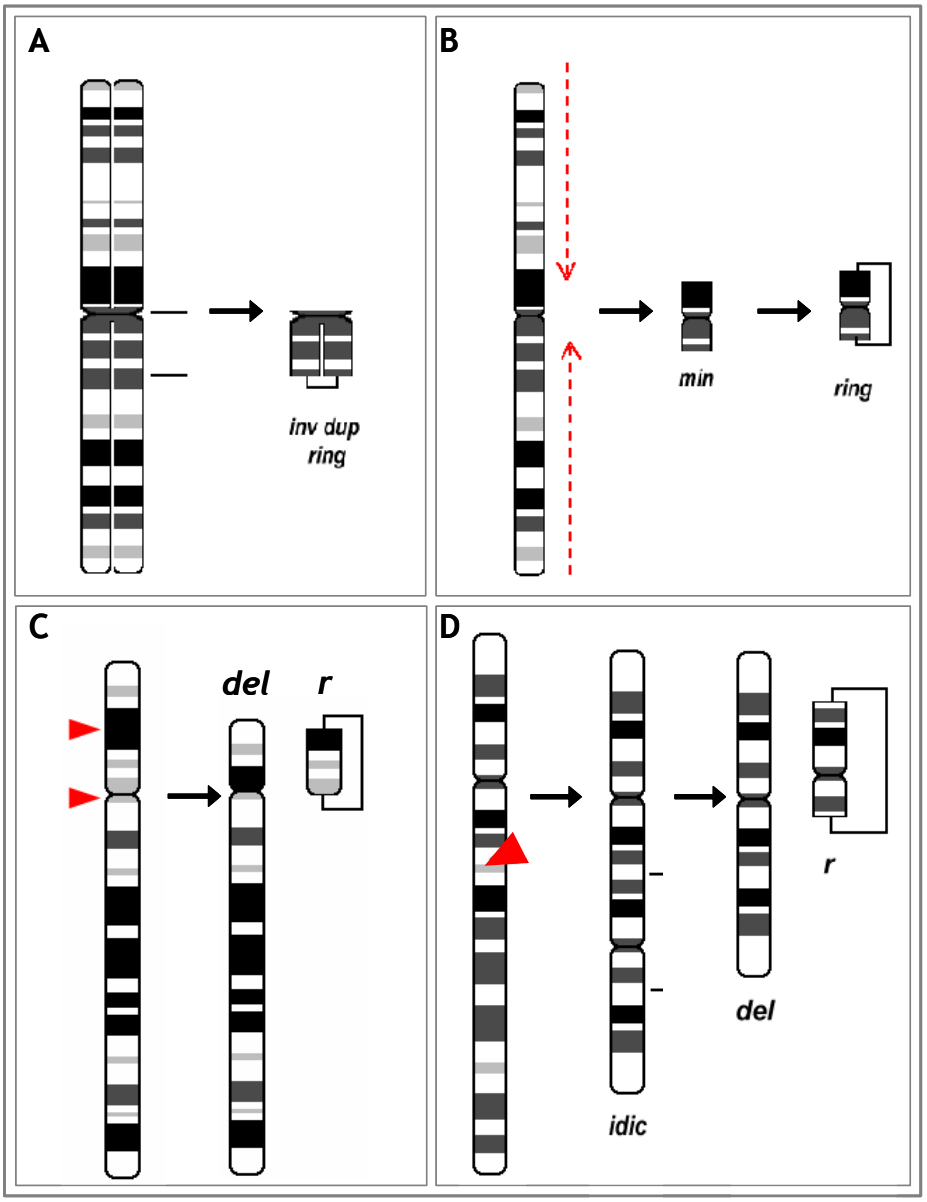


Figure 1.25. – Formation of sSRC. A) Formation of a sSRC after a U-shape reunion**; B)** Formation of a sSRC after a centric minute chromosome ; **C)** Formation of a sSRC in connection with a deletion; **D)** Formation of a sSRC associated with complex rearrangements, with a primary dicentric iso-chromosome formation, being one of the centromeres excised [adapted from Liehr, 2010 – sSMC homepage]**.**

Recently, Baldwin and co-workers proposed that small-ring formation with a "centromere misdivision", might be a predominant mechanism of origin [Baldwin et al., 2008]. These authors supported this theory by the existence of several deletion cases

reported in the literature associated with a complementary sSRC and of a cryptic deletion case reported in their paper, associated with a complementary sSRC. This mechanism of breakage within the centromere creating a pericentromeric deletion and a complementary ring chromosome was first described by Barbara McClintock, in 1938, using maize [McClintock, 1939].

Taking this into account, when in the presence of a familial SRC in a child with an abnormal phenotype and with a phenotypically normal parent (carrier of a SRC), this mechanism should be considered, given the possibility of cryptic pericentromeric deletions and a balanced deletion and ring state in a normal parent and other relatives [Baldwin et al. , 2008].

It is postulated that sSRC do not have telomeres [Ning et al., 1999]. However, the presence of telomeric and subtelomeric sequences at the fusion points of ring chromosomes was demonstrated for larger ring chromosomes [Pezzolo et al., 1993].

Complex rearranged supernumerary marker chromosomes

There are some cases reported in the literature of sSMCs with no consecutive chromosomal material, having complex intrachromosomal rearrangements [Trifonov et al., 2008]. There are also cases reported in the literature with more than one sSMC derived from 2 different chromosomes [Liehr et al., 2006], from 3 chromosomes [Huang et al., 2006], from 4 chromosomes [Tsuchiya et al., 2008]; from 5 chromosomes [Beverstock et al., 2003]; from 6 chromosomes [Vermeesch et al., 1999] and from 7 different chromosomes [Chen et al., 2006].

The formation of these complex rearranged sSMC could arise from a superfluous haploid pronucleus in which there is incomplete digestion of some chromosomes, and transfection of these leftover pieces into the zygote [Daniel and Malafiej, 2003].

1.2.4. Frequency and distribution of sSMCs

An important issue when studying the impact of an sSMC case is the frequency of sSMC in prenatal and in postnatal cases in different human sub-populations. Liehr and Weise addressed this question, reviewing 132 datasets derived from the literature and

from own data [Liehr and Weise, 2007] (Table I.2). The range of frequency of sSMC carriers in the different datasets analysed varied between 0.028% and 0.15%. These variations were mainly caused by the size of the analysed population and the bias caused by single studies. In this study 1 288 693 cytogenetically studied cases detecting 980 sSMCs were assembled, being the i(18p), der(22), i(2p) and inv dup(22) also counted as sSMC. According to this study, 70% of the reported sSMC are *de novo*, being the other 30% familial cases.

The frequency of sSMC in newborn cases is 0.044%, taking into account several reports of consecutive newborn children, without further selection criteria [Liehr and Weise, 2007]. In prenatal cases this rate is higher, being 0.075% in 688 030 cases provided by >240 laboratories. This detection rate was independent if chorion *villi* samples (CVS) or amniotic fluid cells were studied. Liehr and Weise evidenced a strong positive correlation (0.204 %) of sSMC presence and ultrasound abnormalities, but Pallister-Killian-, cat-eye-, der(22)- and iso(18p)-syndrome chromosomes were included and are usually correlated with malformations.

Table 1.II. – Frequency of sSMCs in human subpopulations.

Subpopulation	sSMCs Frequency
Prenatal cases	0.075%
Newborn cases	0.044%
Mentally retarded patients	0.288%
Sub fertile people	0.125% [0.165% in male; 0.022% female]

[adapted from Liehr and Weise, 2007]

The higher rate of cases with sSMC in prenatal compared to newborn could be explained by the bias caused by the maternal age effect in prenatal studies. Additionally, prenatal diagnostics is often performed when a suspicion of foetal pathology exists, increasing this bias, and foetus with sSMC may result in miscarriage and will not be accounted in newborn cases [Blennow et al., 1994]. Warburton reported a frequency of 0.043% of *de novo* sSMCs in prenatal studies [Warburton, 1991]. The discrepancy of this value could be attributed to the fact that only *de novo* cases were reported (the familial ones were excluded) and that the sSMCs accounted did not

include extra chromosomes like iso(18p), iso(9p), Pallister-Killian-, cat-eye- and der(22)-syndrome chromosomes.

The sSMC frequency rate in mentally retarded patients is 0.288% taking into account 26 studies with 2000 sSMCs carriers in 69 332 patients. The sSMC rate is ~7 times higher in developmentally retarded patients than in the normal population [Liehr and Weise, 2007].

In patients with fertility problems, and considering 41 studies with 30 510 patients, the overall rate is 0.125%, but with a higher frequency in male (0.165%) comparing with female (0.022%). Patients with fertility problems have a 2.9 times enhanced risk for an sSMC compared to the general population [Liehr and Weise, 2007]. It has been shown that the frequency of chromosome abnormalities in infertile male is approximately five times higher than that found among the normal male population [Chandley et al., 1975]. The majority of chromosome abnormalities appeared to be exerting their effect on male fertility through disturbance of spermatogenesis. Chromosomopathies account for approximately 2% of all men who attend infertility clinics, rising to 15% among those with azoospermia [Chandley, 1998].

An interesting estimation could be done – taking into account the estimated world population of 6 823 000 000 people and a 0.044% rate of sSMC in newborns, that will be at present ~$3.0x10^6$ living sSMC carriers [U.S. Census Bureau, 2010; Liehr and Weise, 2007].

1.2.5. Mosaicism and sSMCs

Mosaicism refers to the presence of genetically different cells in a multicellular organism that have been derived from a single fertilized egg. Many different mechanisms and types of genetic changes can lead to mosaicism, such as differences in the number and configuration of chromosomes (loss, gain, translocation, deletion), and various types of mutations of a single gene [Hall, 2005].

Chromosome instability is a hallmark of tumorigenesis but recently it was shown that is also common during early embryogenesis [Vanneste et al., 2009]. It is characterized by an elevated rate of gains or losses of complete chromosomes or segments of

chromosomes per cell cycle, resulting in cell-to-cell variability [Geigl et al., 2008]. Vanneste and collaborators revealed a high frequency of chromosome instability in cleavage-sate embryos involving complex patterns of segmental chromosomal imbalances and mosaicism for whole chromosomes and uniparental isodisomies [Vanneste et al., 2009].

The clinical impact of genetic mosaicism may be negligible, minor or dramatic. It has been known for a long time that chromosomal mosaicism can be seen in some of the more common chromosomal disorders, like Down syndrome or Turner syndrome. In general, it has been observed that the more normal cells that are present, the milder the effect on the phenotype could be. However, this is not a straightforward observation. The impact of the type of cell lines (normal or abnormal) present in different tissues is also very important to evaluate the severity of phenotypes, associated with mosaicism.

Mosaicism associated with sSMCs is a well-known fact. As previously discussed (section 1.2.3.) the different mechanisms proposed for sSMC formation, inluding trisomic rescue or monosomic rescue and post fertilization errors, would frequently lead to mosaicism. Additionally, sSMCs may tend to rearrange and/or reduce size during karyotype evolution. These phenomena could lead to the formation of different cell lines, for example:

a) in ring chromosomes, due to the characteristic instability of a ring structure at mitosis after sister chromatid exchange events, double or even more sSRC could be found [Starke et al., 2003; Carreira et al., 2007]
b) the decrease of cells with sSMC during lifetime [Hoo et al., 1974] or even the disappearance of the sSMC in the most frequent studied tissues during lifetime could occur (e.g. peripheral blood) [Fitzgerald and Mercer, 1980];
c) the formation of different variants and a highly complex mosaic arising from centric minute or ring chromosomes that are degraded differently in different cells/tissues [Liehr et al, 2004].

Fickelscher and collaborators characterized the levels of mosaicism of a prenatally ascertained case with a *de novo* sSMC derived from, chromosome 1. A molecular

cytogenetic study of eleven tissues of this foetus was performed showing that the presence of the sSMC varied between 13 and 62% within the different tissues. This finding is something common in sSMC carriers and could explain why up to the present no clinical correlations for sSMC mosaicism and clinical outcome in the corresponding carriers could be established [Fickelscher et al., 2007].

The retrospective study made by Warburton of 377,357 reported amniocenteses in a 10 years-period revealed that, of the 123 cases with sSMC, 53% were in mosaic and that mosaicism in phenotypically normal sSMC carriers was ~52.3% and in phenotypically abnormal sSMC carriers was ~56.3% [Warburton, 1991]. These numbers are approximate to the results of Daniel and Malafiej report – 61.9% mosaicism in phenotypically normal carriers and 56.6% in abnormal carriers, when reviewing supernumerary small ring marker in the literature [Daniel and Malafiej, 2003].

There are also cases showing that similar grades of mosaicism in two generations are associated with differences in clinical outcome [Tan-Sindhunata et al., 2000] or showing major differences in mosaicism percentage without phenotypic consequences [Anderlid et al., 2001]. In conclusion, the risk associated with the presence of an sSMC is difficult to ascertain in the presence of mosaicism.

With the use of molecular cytogenetic approaches and array techniques, it has been reported the presence of previously undetectable cryptic mosaicism associated with sSMC cases [Starke et al., 2003; Liehr et al., 2004; Tsuchiya et al., 2008; Liehr, 2009].

1.2.6. Uniparental Disomy and sSMCs

Uniparental disomy (UPD) is the inheritance of both homologues of one chromosome from only one parent, either the father or the mother (instead of inheriting one from the father and other from the mother) [Schinzel and Baumer, 2005]. If the parent passes on two copies of the same chromosome (non-disjunction in meiosis II) it is considered an isodisomy. But if the parent passes on one copy of each homolog (non-disjunction in meiosis I) it is called a heterodisomy (Fig. 1.26.). Additionally, UPD can occur for an entire chromosome or only in a chromosomal segment (interstitial or telomeric) together with biparental inheritance of the rest of the pair of chromosomes and a normal karyotype, in this case being called “segmental UPD”.

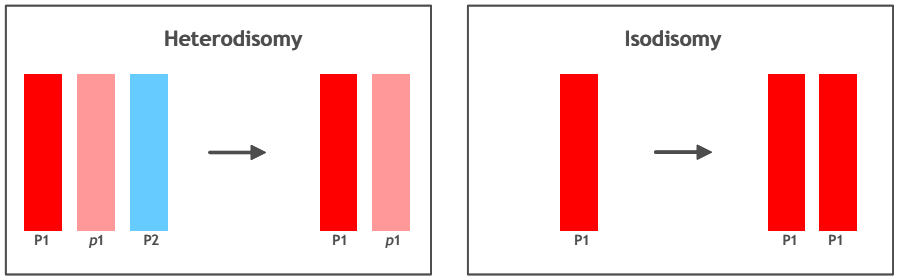


Figure 1.26. – Schematic representation showing the formation of an uniparental heterodisomy, usually after a trisomic “rescue” event and of an uniparental isodisomy, that can occur after pos-fertilization errors or by the fecundation of a nulissomic gamete followed by a postzygotic reduplication for aneuploidy “rescue”. P1 and *p*1 represent both homologs of progenitor 1 and P2 one of the homolog from progenitor 2.

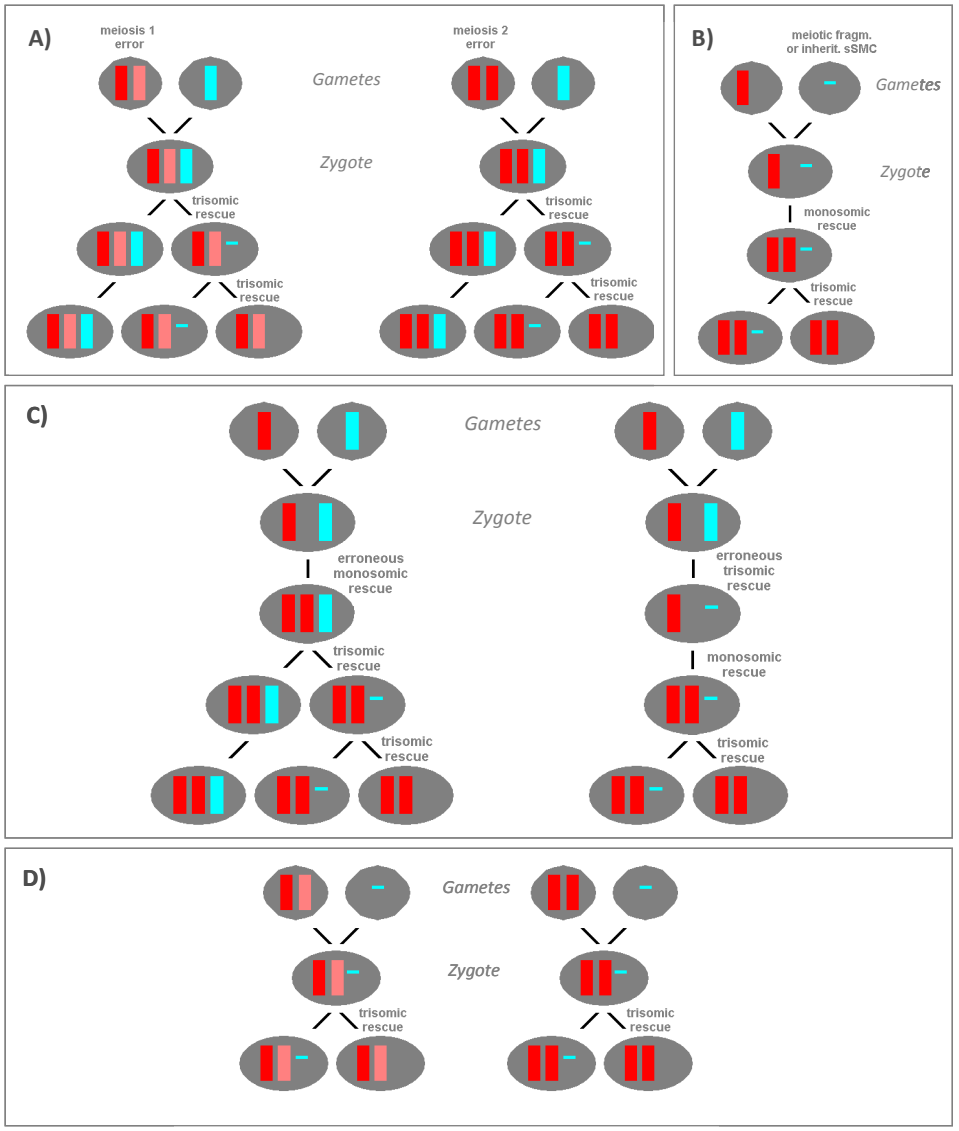


Figure 1.27.- Mechanisms of formation of UPD associated with an sSMC. (A) Functional trisomy rescue; **(B)** Postzygotic reduplication by fertilisation of a normal gamete by a gamete carrying a sSMC followed by somatic reduplication of the normal homologue; **(C)** Post-fertilisation errors by either mitotic non-disjunction followed by reduction of the single homologue or vice versa; **(D)** Complementation by fertilisation of a disomic gamete by a gamete carrying an sSMC.

There are several mechanisms proposed for the formation of UPD, but the bases are always two events, either two meiotic, or one meiotic and one mitotic, or two mitotic. Indeed, the coexistence of UPD with an sSMC could be explained by four different mechanisms (fig 1.27.) [Kotzot, 2002]:

A) Functional trisomy rescue: The zygote is trisomic (caused by an error in meiosis I or in meiosis II) and, either at random or by an active mechanism, parts of the single parental homologue are lost through one or two breakage events in an early embryonic cell division (fig 1.27.A). The sSMC is formed following two breakage events that can originate a sSMC with heterochromatic material (just near the centromere) or with also euchromatic regions (breakage more or less distal to the centromere).

B) Postzygotic reduplication when a normal gamete is fertilised with a gamete carrying a sSMC (a meiotic fragment or an inherited sSMC) (fig. 1.27.B). UPD will appear because a duplication of the normal homologue in a zygote which has inherited a sSMC in place of the normal corresponding chromosome occurs, "rescuing" an aneuploidy (fig 1.27.B). This mechanism seems to be less frequent.

C) Post-fertilization errors by either nondisjunction in an early mitosis and subsequent reduction of the monosomic homologue or by an inverse sequence (fig. 1.27.C). This subclass is the most complex and hard to demonstrate.

D) Fertilisation of a disomic gamete by a gamete with a sSMC formed before or during meiosis ("complementation") (fig. 1.27.D).

The major problems associated with UPD are trisomy mosaicism on the placenta or the foetus, genomic imprinting, homozygosity of autosomal recessive mutations or the combination of all these [Kotzot, 1999]. After identification of the origin of the sSMC, its normal sister chromosomes should be tested for their parental origin to exclude a possible uniparental disomy (UPD), particularly in chromosomes for which genomic imprinting is known. UPD is an example of the plasticity of the human genome, which for most chromosomes can tolerate the lack of biparental inheritance. In contrast a minority of genes expressed irregularly as a result of UPD will lead to severely harmful phenotypes [Kotzot, 2008]. Predicting the phenotypic effects of UPD is, therefore, complex. UPD phenomena in chromosomes 6, 7, 11, 14, 15 and 20 has shown in some

cases abnormalities in affected individuals [Chudoba et al., 1999; Shaffer et al., 2001; Kotzot, 2002].

The proportion of maternal *versus* paternal, meiotic *versus* mitotic nondisjunction in UPD parallels that of trisomy: predominance of maternal origin and increased mean maternal age. Thus, the vast majority of maternal UPD is heterodisomy, mostly stemming from maternal meiosis I nondisjunction. Mean maternal age in maternal UPD equals that usually found in maternal trisomy, i.e. an elevation of about 6 years over euploid controls. Paternal UPD, in contrast to maternal, is almost always isodisomy [Schinzel and Baumer, 2005].

UPD can be tested by molecular approaches, such as microsatellite analysis or methylation-specific polymerase chain reaction, or more recently with SNPs array analysis [Salafsky et al., 2001; Nietzel et al., 2003; Starke et al., 2003; Conlin et al., 2010].

Although UPD is often considered an event to be characterized exclusively by molecular genetic or epigenetic approaches, it seems that at least one third of UPD cases emerge in connection with or due to a chromosomal rearrangement [Liehr, 2010]. Thus, additional cytogenetic characterization of UPD cases is essential.

1.2.7. Parental age and sSMCs

Hook and Cross reported a significant maternal age effect associated with *de novo* sSMCs in a study with 75000 prenatal cases assessed by conventional cytogenetics [Hook and Cross, 1987]. In 2005, using FISH for ascertain chromosomal origin of 131 cases of sSMCs, Crolla and collaborators notice no overall maternal age effect associated with sSMCs [Crolla et al., 2005]. However, in this study, *de novo* sSMCs derived from chromosome 15 were associated with a significantly increased maternal age. Previous studies demonstrated the existence of an exponential increase with maternal age associated with maternal meiosis I nondisjunction involving chromosome 15 and with uniparental disomy for chromosome 15 [Robinson et al., 1996; Robinson et al., 1997]. The maternal age effect associated with *de novo* sSMC(15) in humans would suggest a common origin for both trisomy 15 following nondisjuction at meiosis I with

subsequent complete trisomy rescue leading to UPD(15) or incomplete trisomy rescue resulting in the formation of an sSMC(15) [Crolla et al., 2005].

Bartsch and collaborators reported a higher frequency of cat eye syndrome (CES) chromosomes in a prenatal retrospective study, that could be indicative of a maternal age effect in sSMC(22). In this study a CES chromosome was found in *de novo* cases (5/43 273 prenatal cases) in pregnant women referred because of advance maternal age [Bartsch et al., 2005]. Indeed, inverted duplications of chromosomes 15 and 22 share similar origins because they both may arise in female meiosis from errors of recombination at sites of low copy repeats (LCRs).

Apart from sSMCs derived from chromosome 15 and 22, the lack of comparable data concerning sSMCs from the other acrocentric and non acrocentric chromosomes does not allow taking a conclusion about the maternal age effect of *de novo* sSMCs. However, it seems that familial sSMCs are predominantly inherited via the maternal line [Liehr, 2006].

1.3. Aim of the work

Taking into account the following considerations:

1 - sSMCs can be detected postnatally or prenatally, the last being a major problem for cytogenetic investigation and genetic counselling, due to time availability and, essentially, due to the difficulty in the majority of cases in establishing a robust genotype/phenotype correlation;

2 - More precise knowledge of the size of the partial trisomy segment(s) and the gene content of the SMC would greatly improve the ability to predict phenotype and prognosis;

3 - The great majority of sSMCs reported in the literature lack of detailed characterization;

4 – The presence of a sSMC is often accompanied by mosaicism;

the main goal of this work was the ascertainment of the genetic content of a set of cases with sSMCs derived from different chromosomes, comparing them with similar cases reported in the literature with trisomies involving the same regions, in order to establish a genotype/phenotype correlation. A more precise knowledge of the size of the partial trisomy segment and the gene content of an sSMC would greatly improve the ability to predict phenotype, contributing to a more informed prenatal counseling or prognosis.

For this main achievement we aimed to:

a) Characterize the shape of the sSMCs by conventional banding and multicolour FISH, namely recurring to subcentromeric FISH or multicolour banding, in order to evaluate the sSMC structure and the presence of pericentromeric genes in the different marker chromosomes;

b) Evaluate the array CGH technique, with previous microdissection and amplification of the SMCs, for the ascertainment of the genetic content of the marker. If validated, this technique could have great value when the levels of mosaicism are low.

c) Evaluate, whenever possible, the presence of uniparental disomy (UPD);

d) Establish EBV-transformed linfoblastoid cell lines for all the sSMCs cases.

The final aim of this study is to propose guidelines for the management of sSMCs in a diagnostic cytogenetic laboratory, taking into consideration several issues like, for example: technical availability and presence/absence of mosaicism.

1.4. References

WEB RESOURCES
DECIPHER, https://decipher.sanger.ac.uk/

Ensembl, http://ensemble.org/

OMIM, http://www.ncbi.nim.nih.go/site/entrez?db=OMIM

PubMed, http://www.ncbi.nlm.nih.gov/pubmed/

Small supernumerary marker chromosomes database, Liehr T, www.med.uni-jena.de/fish/sSMC/00START.htm

U.S. Census Bureau, http://www.census.gov/ipc/www/popclockworld.html

UCSC genome browser, http://genome.ucsc.edu/

http://commons.wikimedia.org/wiki/Main_Page

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2. MATERIAL AND METHODS

2.1. Cell culture and harvesting

A good visualization of human chromosomes in somatic cells requires that dividing cells are studied during prometaphase. Large numbers of prometaphase cells can be obtained by growing cells in culture and by adding agents that inactivate spindle fiber formation, such as colcemid, to cell cultures during periods of active growth[5]. While the number of cells found in metaphase will increase as the length of exposure to colcemid increases, chromosome condensation also progresses with time. The optimal length of exposure to colcemid will be determined by the rate of cell division and the degree of condensation that is desired. Many cell types undergo growth and division spontaneously, but some cell types, such as peripheral lymphocytes, need to be stimulated into mitotic activity by the addition of a mitogen, like phytohemaglutinin, at the time cell cultures are initiated.

Cytogenetic procedures are optimized when all of the cells in culture are synchronized in their mitotic cycle. This is achieved by adding chemical agents, like thymidine, that block progression into S phase to an actively growing culture. The harvesting procedure, after culture, involves centrifugation of cell suspensions into a cell pellet, treatment with a hypotonic salt solution, fixation of the suspended cell pellet, and dropping of the cells onto glass slides [Moore and Best, 2001]. Cell cultures for this study were obtained from peripheral blood and amniocytes.

2.1.1. Peripheral blood cell culture

Peripheral blood culture was performed according to standard protocols, by inoculating sodium heparin collected whole blood in PB-MAX (Gibco), containing L-glutamin and phytohemaglutinin (PHA). PHA stimulates T lymphocytes to blast-like cells. After 48 to 72 h methotrexate (MTX)[6] was added (final concentration of 5 µM) in

[5]Colcemid and Colchicine are antimitotic drugs that bind to free tubulin and inhibit microtubule assembly [Fitzgerald, 1976]. These drugs bind to β-tubulin near the intradimer interface where they stabilize a "curved" tubulin conformation that inhibits microtubule elongation and destabilizes the polymer [Yin et al., 2007].

[6]Methotrexate is a folate antagonist that prevents thymidine syntesis. Depletion of thymidine pool prevents the cells from completing replication, and cells accumulate in the S-phase of the cell cycle. Subsequent addition of thymidine releases a synchronized wave of cells to complete replication and proceed through G2 and into mitosis [Bangs and Donlon, 2005].

order to block DNA replication. After 17h incubation at 37ºC, thymidine was added (final concentration of 0.05 μM) to synchronize cells. After 5 h cultures were subjected to colcemid treatment for 20 minutes (final concentration of 0.1 μg/ml), and the cells were then ressuspended in 0.4% KCl with buffered hypotonic solution (JL006A, GGS, Genial Genetic Solutions). The KCL hypotonic buffer was incubated for 25 minutes at 37ºC. A volume of 5 ml of methanol:acetic acid fixative solution (3:1) was added to the cell pellet under agitation and 3 more fixative washes were subsequently performed. Cell pellet(s) were stored at -20ºC in fixative solution. The best cell suspension, with high metaphase index and of very good quality, was chosen for subsequent studies [Haines et al., 1995; Bangs and Donlon, 2005].

2.2.1. Amniocytes cell culture

Amniocytes cell culture was performed using standard procedures [Haines et al., 1995; Meron, 2001] with modifications]. Briefly amniotic fluid was cultured with 5 ml of complete medium (Ham's F 10 medium supplemented with 2mM L-Glutamine, 10% Fetal Bovine Serum (FBS) and Penicillin/Streptomycin) and 3 ml of Amniomax (GIBCO^{TM}) or AmnioMed (EuroClone^{TM}). After 5-7 days in culture the proliferation of the culture was assessed in a daily basis. When a sufficient growth was detected cells were prepared for manipulation. Following a 3 hour treatment with colcemid (1 μg of KaryoMAX, GIBCO^{TM}) at 37ºC, cells were washed and tripsinized (0.05% trypsin with EDTA, without Ca or Mg). Hypotonic shock was done for 20 minutes with 0.4% KCl solution. Cells were fixed consecutively with 3 solutions: methanol/acetic acid solution (6:1); methanol/acetic acid solution (3:1) and methanol/acetic acid solution (1:1).

2.2. Chromosomes spreading

Cell suspensions in fixative were used to prepare metaphases for G-banding, C-banding, NOR-banding or FISH. When preparing slides with chromosome spreads one should be aware that it is not an exact science, although careful attention to a number of variables certainly increases the chance of successful results.

Cells in fixative solution were diluted at the appropriate density to produce a reasonable number of well-spread metaphases in each microscopic field. About 12-14 µl of cell suspension were distributed on several spots on a slide (pre-wet slide, when needed). A drop of new fixative solution was added to each area of spreading when needed. The slide was placed face up into a hot metal plate (37ºC) for evaporation of the fixative solution. The degree of spreading could be adjusted using different temperatures on the heated plate, with higher temperatures increasing chromosome spreading. For difficult-to-spread cells, after the surface became grainy, the slide was passed briefly through water vapours, and then some droplets of fixative were placed on the slide [Haines et al., 1995; Bangs and Donlon, 2005]. The rate at which the fixative evaporates is critical to the final dispersion of the chromosomes on the slide. Thus, humidity and temperature over the surface of the drying slide can be manipulated to produce optimal chromosome preparations. The ideal metaphase spread has all 46 chromosomes dispersed in the same optical field under the microscope (1000x magnification), with minimal overlapping chromosomes.

Aging of the slides was done at room temperature during 2-3 days or at 65ºC overnight. After dehydration of the slides, with ethanol (70%, 2min; 95%, 2min; 100%, 2 min) FISH was performed.

For multicolour FISH procedures, after aging, the slides were subjected to pepsin pre-treatment using 0.005% pepsin in 0.1 N HCl. This was followed by rinsing in PBS and a 10 min post-fix treatment with 4% formaldehyde for 5 min at room temperature. The post-fix solution was rinsed with water and the slides were dehydrated with ethanol series (70%, 95%, 100%).

2.3. Banding Techniques

2.3.1. G-Banding technique

Giemsa banding (G-Banding) produces a series of light and dark bands along the lengths of the chromosomes, allowing the identification of chromosomes and chromosomes regions. Bands that are dark with G-Banding generally correspond to late-replicating regions of the genome. Giemsa is specific for the phosphate groups of

DNA and attaches itself to regions of DNA where there are high amounts of adenine-thymine. These bands tend to contain relatively few active genes (Fig. 2.1.). G-Banding is produced by pre-treatment of chromosomes with trypsin before staining with Giemsa.

Briefly, aged slides of prometaphase chromosomes were incubated with trypsin (0.06g/100ml to 0.12g100 ml) for about 8 to 20 s, to obtain a partial digestion of the chromosomes. The concentration and time of trypsinization depend of the slides quality and cell types. Slides were washed with 5% FCS in phosphate buffer (pH 6.8) and incubated with 4% -10% Giemsa staining solution in phosphate buffer (pH 6.8) for 4 minutes. After a short wash (2x) with phosphate buffer, slides were ready to be observed at bright-field microscope [Schreck and Distèche, 2001]. Karyotypes were mounted and analysed using 1000x amplification and a Cytovision software.

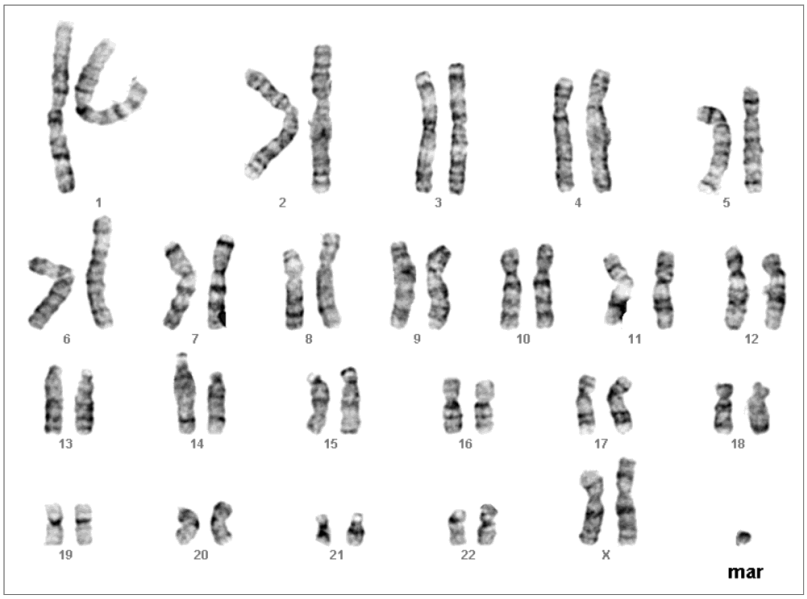

Figure 2.1. – Karyogram obtain after G-banding showing a small supernumerary marker chromosome – 47,XX,+mar. [photo from *Laboratório de Citogenética da Faculdade de Medicina da Universidade de Coimbra*].

2.3.2. C-Banding technique

Noncoding constitutive heterochromatin, such as the repetitive DNA surrounding the centromeres of all of the chromosomes, replicates later in the cell cycle than other

chromatin and exhibits special characteristics of stability under extreme conditions of heat and chemical exposure. This property of tightly condensed heterochromatin can be exploited to produce a unique banding pattern (C-Banding) in which the constitutive heterochromatin stains darkly and all other chromatin remains pale. C-Banding is produced by treatment of chromatin with acidic and then basic solutions followed by staining with Giemsa [Moore and Best, 2001].

After a treatment with HCl 1N for 10 min, at room temperature the slides were rinsed with water and let dry (air dry). Slides were immersed in a Coplin jar containing freshly prepared 5% $Ba(OH)_2$ solution (in SSC) at 50^{o}C . They were incubated during 30 s to 1 min at room temperature. After washing thoroughly with water, the slides were immersed in a SSC solution during 45 to 60 min, in a 60^{o}C water bath. The staining was done incubating the slides with 2% Giemsa Solution during 30 to 40 min. C-banded chromosomes were visualized with a bright-field microscope [Schreck and Distèche, 2001].

2.3.3. NOR-Banding technique

This staining technique uses silver nitrate that stains the active ribosomal DNA-containing Nucleolar Organizer Regions (NOR) located near the ends of the short arms of human acrocentric chromosomes 13, 14, 15, 21, and 22.

Slides (not-aged) were incubated with a 50% silver nitrate solution (in bi-distilled water) in a wet-chamber and incubated overnight at 37ºC (the slides are removed from heat when appear golden brown). The slides were rinse in water and air-dried. NOR-banded chromosomes were observed with a bright-field microscope [Schreck and Distèche, 2001].

2.4. Extraction of Bacterial Artificial Chromosomes (BAC)

The used Bacterial artificial (BAC) DNA was isolated from *Escherichia coli* cultures, Bacterial cultures were grown overnight, at 37^{o}C and 120 rpm, with the appropriate antibiotic in LB medium (Bacto Yeast extract 5g, Bacto trypton 10g, NaCl 10g, H_20 up to 1 liter). For isolation of the plasmid DNA, a QIAprep Miniprep (Quiagen®) kit was used, following manufacter's instructions.

2.5. Labelling of DNA

The DNA can be labelled for a direct detection, using dUTPs directly coupled to fluorochromes, or for an indirect detection. In this case, the probe is labelled with a hapten that itself is not fluorescent. The most common haptens used are biotin (that reacts with streptavidin that is bind to a fluorochrome) or digoxigenin (that can be detected by an antibody coupled with a fluorochrome). Whereas Fluoresecence *in situ* hybrydization (FISH) is faster with directly labelled probes, indirect labelling offers the advantage of signal amplification by using several layers of antibodies, and might therefore produce a signal that is brighter compared with background levels. For labelling DNA, hapten- or fluorescent labelled dUTPs are generally incorporated into the probe by either nick-translation or DOP-PCR (Fig. 2.2.) [Speicher and Carter, 2005].

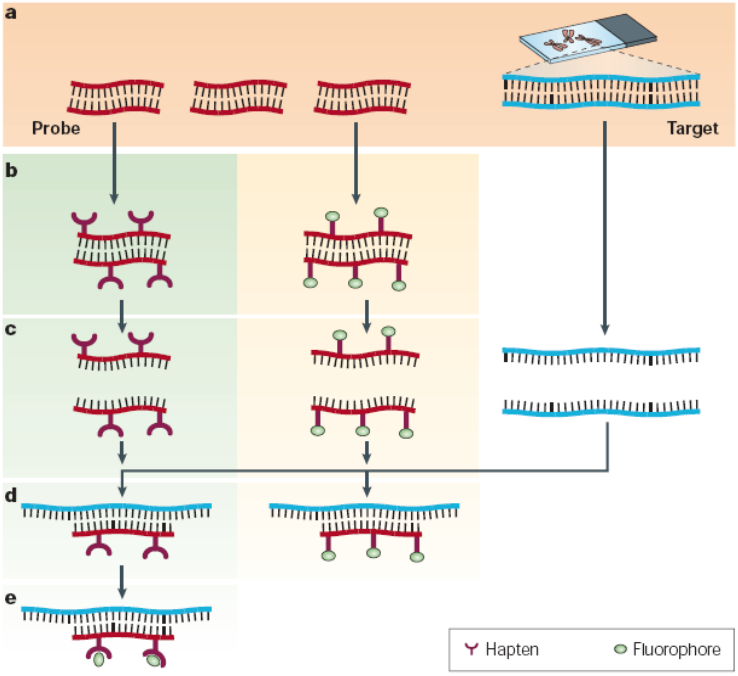

Figure 2.2. - Before hybridization (**a)**, the DNA probe is labelled by various means such as nick translation or DOP-PCR. Two labelling strategies **(b**) are commonly used — indirect labelling (left panel) and direct labelling (right panel). For indirect labelling, probes are labelled with modified nucleotides that contain an hapten, whereas direct labelling uses the incorporation of nucleotides that have been directly modified to contain a fluorophore. The labelled probe and the target DNA are denatured to yield single stranded DNA **(c)** and then they are combined **(d)**, which allows the annealing of complementary DNA sequences. If the probe has been labelled indirectly, an extra step is required for visualization of the non-fluorescent hapten that uses an enzymatic or immunological detection system (e) [adapted from Speicher and Carter, 2005].

During nick-translation the double stranded probe is "nicked" by DNAse and partially digested. At the same time, the strand is filled up by a polymerase that incorporates dNTPs and also labelled dUTP offered in the labelling reaction. Nick-translation is usually used for labelling of cloned DNA. However, PCR can also be used to label these probes with degenerated primers (DOP-PCR). Usually DOP-PCR is used to generate and label complex probes such as paints from flow sorted chromosomes or microdissection. DOP-PCR has the advantage that the entire probe is also being amplified.

BAC probes were amplified by degenerated oligonucleotide polymerase chain reaction[7] (DOP-PCR; 50 µl; 30 cycles). Each probe was labelled separately by using secondary DOP-PCR (20 µl volume; 20 cycles) either with Spectrum Green or TexasRed. Finally, the probe was dissolved in 30 µl hybridization solution [Senger et al., 1998; Nietzel et al., 2001; Starke et al., 2003]. The probes were derived from CHORI BACPAC Resources (Oakland, USA), Dr M Rocchi (*Universita degli Studi di Bari*, Italy) or Dr. Nigel Carter (Sanger Centre, Cambridge, UK).

2.6. Microdissection and Reverse Painting

Eight copies of the marker chromosome were dissected on a slide with a metaphase chromosome spread, using glass micro-needles controlled by a micro-manipulator under an inverted microscope. The dissected material was amplified with DOP-PCR and PCR products were labeled by nick-translation with biotin-14-dATP. Subsequently, this probe was used for reverse painting on patient metaphases (Fig. 2.3.) [Trifonov et al., 2003].

[7] The DOP Primer used was: 5'CCG ACT CGA GNN NNN NAT GTG G3'

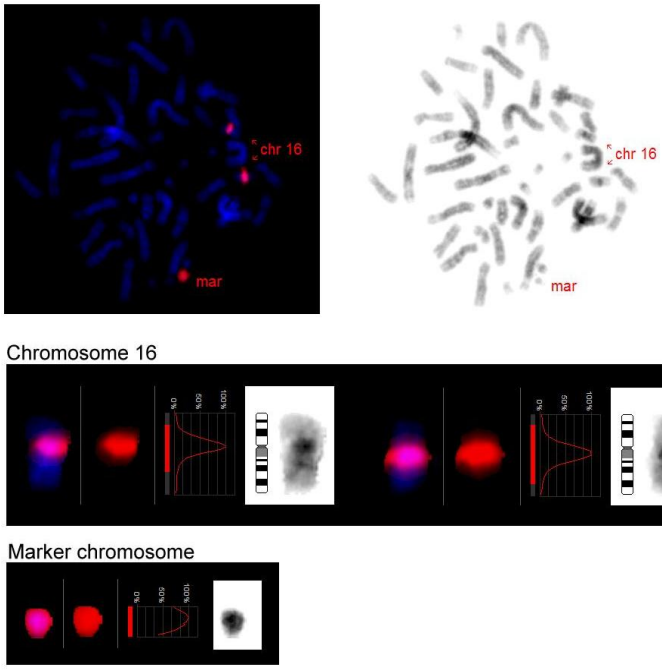

Figure 2.3. – Identification of the origin of a supernumerary marker chromosome derived from chromosome 16 using a reverse FISH approach. After microdissection and amplification of the marker, a probe was done. The hybridization of this probe with a metaphase plate of the same patient identified the normal 16 chromosomes and the marker.

2.7. Fluorescence *In situ* Hybridization (FISH)

FISH was performed using several probes including single probes (centromeric; subtelomeric, *locus* specific; BAC; and all-telomere); painting probes (whole chromosome painting – wcp, and partial chromosome painting – pcp) or multicolor probes (subcentromere specific probes - subcenM-FISH; multicolour banding probes – MCB) for different chromosomes, depending on the sSMCs cases. FISH was also done after marker chromosome microdissection, with reverse FISH (see section 2.5). For all FISH experiments standard procedures were used, depending on the probe. Centromeric- , locus-specific-, subtelomeric-, all-telomere-, and painting- probes were commercial probes and were hybridized according to manufacter's instructions. When using subcenM-FISH, MCB, or BAC probes, hybridization, post-washing, signal detection, and image acquisition were performed as previously reported [Chudoba et al. 1999; Liehr et al. 2002].

Hybridization

In general the hybridization step was done using dehydrated pre-treated slides (with a 15 min treatment in 2xSSC solution at 37ºC). When using commercial probes, slides and probes were denaturated together at 74ºC to 80ºC, during 2 to 3 min (depending on the probes used). The hybridization was done overnight in a wet-chamber at 37ºC.

For subcenM-FISH, MCB, and BAC, probes were denaturated using a thermocycler with the following condition: 75ºC (5 min), 4ºC (2 min) and 37ºC (30 min). The slide (pepsin pretreated slide) was denaturated with 70% formamide (in 2SSC) at 72ºC for 3 min, followed by immersion in 70% ice-cold ethanol, 95% ethanol and 100% ethanol at room temperature (3 min each). Hybridization was done in a wet-chamber at 37ºC over-night.

Post hybridization wash

When using commercial probes, with direct labelled probes, slides were washed in washing buffer at 70ºC, during 30 to 60 s (depending on the probe). After rinsing with PBS, slides were mounted using antifade solution, to prevent rapid fading of the fluorescence signal, with DAPI.

For subcenM-FISH, MCB, and BAC-FISH[8], slides were washed 3 times with a solution of 50% formamide at 42ºC during 5 min and 3 times with 2xSCC at 42ºC during 5 min. A final wash, with 4xSSCT at room temperature for 2 min was done. A blocking buffer (powder milk) was then used for 15 min at 37ºC. Each hybridization area of the slide was incubated with 100 µl antibody solution for 40 min at 37ºC (e.g., 5 µg/ml streptavidin-Cy5 in blocking buffer). The slides were washed with 4xSSCT (5 min) at room temperature and, after rinsing with water, were dehydrated with a series of ethanol (75%, 90%, 100 - 3 min each). The slides were mounted using antifade solution and using DAPI as a counter stain.

[8] In these FISH approaches, besides having direct labelled probes with fluorochromes, probes conjugated with biotin or digoxin are also used. An additional step is needed with binding of streptavidin or anti-digoxin (immunobinding), respectively, conjugated with a fluorochrome.

Visualization

For multicolor FISH approaches, like subcenM-FISH and MCB, the analysis was done using a Zeiss Axioplan fluorescence microscope with MetaSystems (Isis) software. For the other FISH experiences a Nikon fluorescence microscope coupled with Cytovision software was used.

SubcenM-FISH

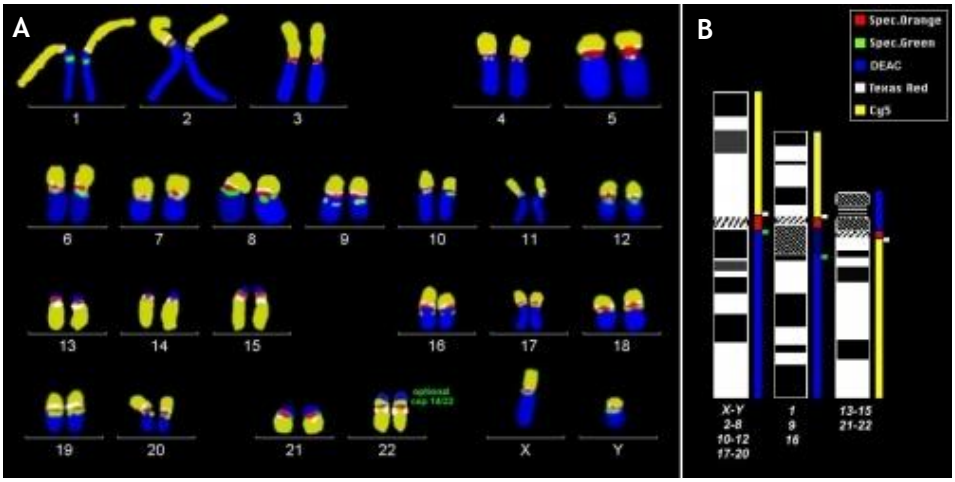

Figure 2.4. – A) SubcenM-FISH pseudo-color pattern for all 24 human chromosomes. Only one chromosome at a time can be stained; the chromosome figures were assembled from 24 different experiments B) Labelling scheme of the subcentromere specific multicolour FISH (subcenM-FISH) probe set. In the non-acrocentric chromosomes de p-arm is labelled with cyanine 5 (Cy5) and the q-arm with diethylaminocoumarin (DEAC). The centromere-near probe in the long arm is labelled with SpectrumGreen and in the short arm with Texas Red. In the acrocentric chromosomes, the long arm is stained with Cy5, the short arm with DEAC, and the centromere-near probe in the long arm with Texas Red. The centromeres are labelled with Spectrum Orange [adapted from Starke et al., 2003].

The proximal probes included in the subcenM-FISH set are chosen under the prerequisite that they were the most proximal probes presently mapped in the ENSEMBL database. Bacterial artificial (BAC) probes were amplified by DOP-PCR. For each chromosome a subcenM-Fish probe set is prepared. The set consists of the most proximally available single-copy probes, microdissection-derived partial chromosome (pcp) probes specific for the long and the short arm, and a centromere specific probe. For the acrocentric chromosomes, no proximal single copy probes and no chromosome specific pcp probes of the short arms are available. For all the acrocentric

chromosomes set, a probe that stains all p-arms of the acrocentric chromosomes (midi54) is used (Fig 2.4.) [Starke et al., 2003].

MCB-FISH

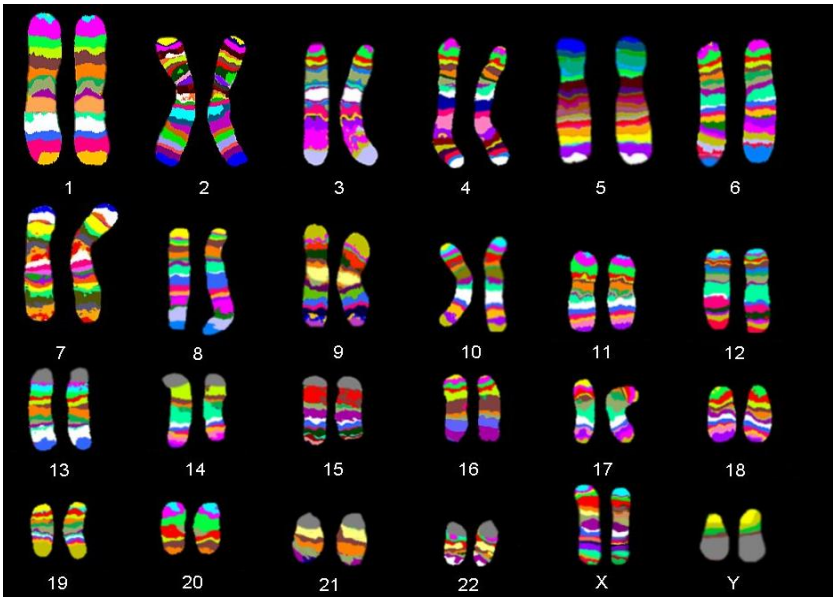

Figure 2.5. – Multicolor banding (MCB) pseudocolor pattern for all 24 human chromosomes on ~366 band level. Only one up to three chromosomes can be simultaneously stained by MCB. The chromosome figures were assembled from different experiments.

Between 3 and 12 microdissection libraries were combined per chromosome (Fig 2.5.; Fig. 2.6.). Three to five different fluorochromes were used to label the partial chromosome painting (pcp) probes: SpectrumOrange, SpectrumGreen, TexasRed, Cy5 (Cy5 coupled to streptavidin; detection of biotinylated probes), and diethylaminocoumarin (DEAC). The probe Midi 54 for the acrocentric chromosomes was added specifically to cover the corresponding short arms of these acrocentric chromosomes [Mrasek et al., 2001; Weise et al., 2008].

Recently, Weise and colleagues characterized the library of MCB probes with an array CGH approach. The possibilities of the MCB technique to characterize chromosomal breakpoints in one FISH experiment are now complemented by the feature of being anchored within the human DNA sequence at the BAC level (Fig. 2.7.) [Weise et al., 2008].

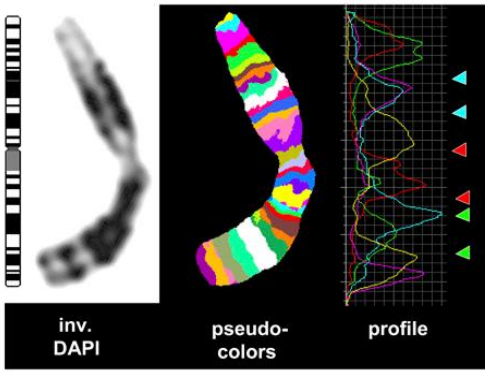

Figure 2.6. - Label scheme for chromosome 1 in MCB: From left to right: ideogram, inverted DAPI, pseudocolors, fluorochrome profiles where arrows indicate boundaries of the three new libraries of MCB 1 [adapted from Weise et al., 2008].

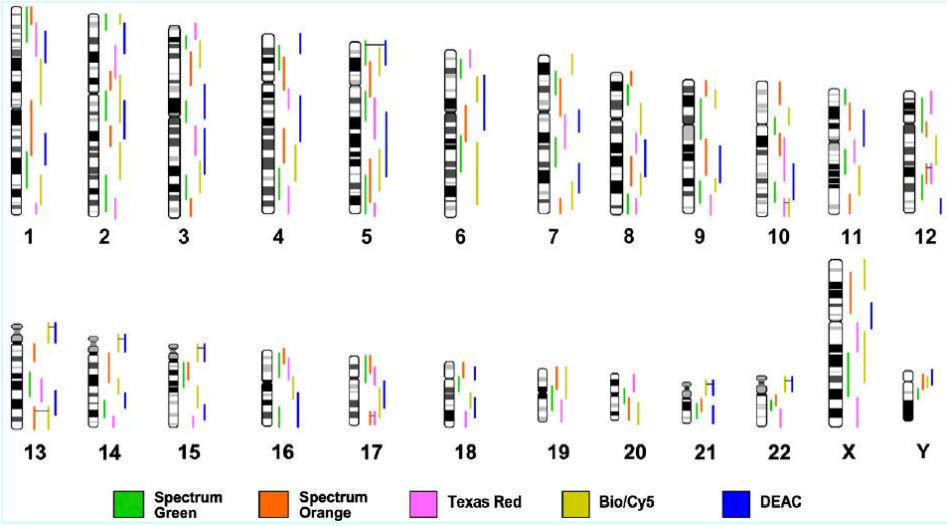

Figure 2.7. - Graphical overview and labelling scheme for all 169 array-mapped MCB libraries. Double labeling of a single MCB library is indicated with two vertical color lines connected by a horizontal black line. The fluorochrome color key is at the bottom of the legend [adapted from Weise et al., 2008].

2.8. Microsatellite analysis for Uniparental Disomy analysis

For uniparental disomy (UPD) analysis, genomic DNA was extracted from the peripheral blood lymphocytes of both parents, when possible, and the patient by using standard procedures. Twenty-five cycles of PCR were performed with labelled primers responsive to infrared (IRD800; MWG-Biotech, Germany). Denatured samples were

loaded onto a 6% denaturing polyacrylamide gel, separated electrophoretically on a Licor DNA 4000 sequencer (LI-COR, Nebraska), and detected with an infrared laser diode [Starke et al, 2003].

2.9. Array CGH – array painting

DOP PCR-amplified microdissected DNA from the marker chromosome was labeled by a random prime labeling system (Bioprime DNA Labeling System, Invitrogen, Carlsbad, CA) using Cy5 labeled dCTP's (Amersham Pharmacia Biotech, Piscataway, New Jersey) as described [Backx et al., 2008].

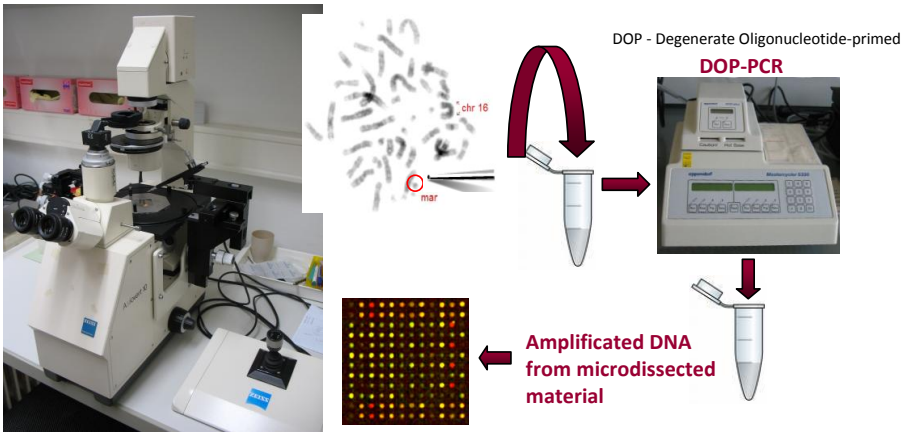

Figure 2.8. – Schemetic representation of the methodology used for array-painting. After microdissection of the sSMCs, the DNA was amplified by DOP-PCR. The DNA was labelled and hybridized on a microarray slide.

Except for some small modifications, probe preparation, and pre-blocking of the slide were performed as described [Fiegler et al., 2003]. To hybridize a spotting area of 24x24 mm, the probe was dissolved in 20 µl of hybridization solution. The hybridization was allowed to take place for 2 nights under a coverslip in a humid chamber saturated with 20% formamide and 2x SSC. Post hybridization washes were performed [Vermeesch et al., 2005]. A full tiling BAC array was used, with a resolution of 100-150 Kb (VIB MicroArrays Facility - www.microarrays.be). The array was scanned at 635nm using a GenePix4000B scanner (Axon Instruments) and image analysis was done using GenePix Pro 6.0. Spot intensities were corrected for the local background [Melo et al., 2009].

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3. RESULTS AND DISCUSSION

Chapter III

This chapter is subdivided in 10 sections (3.1 to 3.10), according to the chromosome derivative, as follows:

3.1. – sSMC derived from chromosome 1

3.2. – sSMCs derived from chromosome 2

3.3. – sSMC derived from chromosome 5

3.4. – sSMC derived from chromosome 11

3.5. – sSMC derived from chromosome 13

3.6. – sSMCs derived from chromosome 15

3.7. – sSMCs derived from chromosome 16

3.8. – sSMC derived from chromosome 17

3.9. – sSMCs derived from chromosome 18

3.10– sSMCs derived from chromosome 22

For a better sistematization in each section the description of the cases, results obtained, review of the literature for each subtype of sSMCs are described and discussed.

In section 3.2., 3.3., 3.4., 3.5., 3.7., 3.8., and 3.9., the text is extracted from the correspondent articles (published or submitted), as described in the list of publications included at the end of Chapter IV (page 212). In order to avoid repetition, material and methods subsection (included in the articles) was omitted, having Chapter II (Materials and Methods) the description of the materials and methods used. A general discussion of the results presented in this chapter is done in chapter IV followed by concluding remarks.

3.1. *sSMC from chromosome 1*

A case of a small supernumerary ring chromosome derived from chromosome 1

3.1.1. Introduction

Genetic counselling of patients with small supernumerary ring chromosomes (sSRCs) can be difficult, especially in prenatal testing, due to the complexity in establishing a karyotype-phenotype correlation. In cases which the morphology of the sSMC can be determined, almost 50% appear to be ring chromosomes [Liehr et al., 2006]. Mosaicism is quite common, resulting either from postzygotic mutation or from mitotic instability resulting in loss of the ring during cell division [Chen et al., 1995]. Clinical outcome of sSRCs is highly variable depending on their origin, size, euchromatin content, co-occurrence of uniparental disomy, and presence of mosaicism [Bernardini et al., 2007]. SRCs account for approximately 10% of the prenatal sSMC-positive cases and 60% of them are associated with abnormal phenotype [Blennow et al., 1994; Davidsson et al., 2008]. sSRC derived from chromosome 1 are among the most common aneuploid small marker chromosomes, excluding the ones derived from chromosomes 15 and 22 [Liehr et al., 2004]. Over two-thirds of cases carrying a sSMC derived from chromosome 1 are associated with clinical abnormalities [Liehr et al., 2010].

We report on a new case of mosaic small supernumerary ring derived from chromosome 1 in a female patient with a mild phenotype. The sSRC(1) was characterized by conventional cytogenetics, reverse FISH, subcenM-FISH and array CGH after microdissection.

3.1.2. Results

Case Report and Cytogenetic Studies

A young girl was referred to genetic clinic with learning difficulties, microcephaly, microtia, tooth problems, scoliosis and vesico-uretral bilateral reflux with normochromic normocytic anemia. She had an inguinal right and left hernia corrected with surgery. Cytogenetic analysis, by G-Banding, revealed a *de novo* supernumerary marker chromosome in 8 of the 22 analysed cells (mos 47,XX,+mar[8]/46,XX[14]). At eleven years old she was clinically reassessed revealing a normal growth (height - P50;

weight - P50-75; cephalic perimeter - P10-P25) and adequate learning capacities. She presented vesico-uretral reflux with nephropathy. Scoliosis and anaemia were not confirmed. Blood was collected from the patient in order to perform additional cytogenetic studies.

G-banding analysis revealed the presence of 3 different cell lines: one normal, other with a ring and the third with a double ring, being the karyotype mos 47,XX,+r[6]/47,XX,dic r[2]/46,XX[24] (Fig. 3.1.1.).

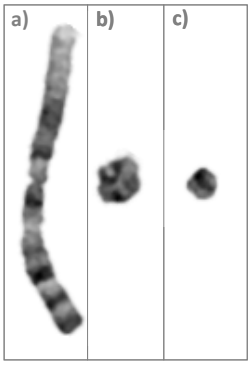

Figure 3.1.1. - G-banding pattern of one normal chromosome 1 (a) and of the two rings found in two different cell lines (b, c).

C-banding induced by barium hydroxide and Giemsa confirm the presence of the 3 cell lines, having the double ring and the ring C-banding positive regions interpreted as centromeric (Fig. 3.1.2.).

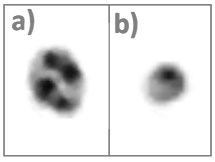

Figure 3.1.2. - C-banding pattern of the two sSRCs found in two different cell lines, showing a double ring with two centromeres (a) and a ring with one centromere (b).

Molecular Cytogenetics Analysis

The markers chromosomes were microdissected, labelled, and hybridized in a metaphase plate of the patient for reverse FISH, showing to be derived from chromosome 1 (Fig. 3.1.3.). SubcenM-FISH specific for chromosome 1 was made, the

karyotype being: mos 47,XX,+mar.ish r(1)(::p12->q12::)[5]/47,XX,+mar.ish r(1;1)(::p12->q12::p12->q12::)[2]/46,XX[24].

The microdissected DNA of the sSMCs of the patient was hybridized, after labelling, in a full tiling BAC array specific for chromosome 1, with a resolution of 100-150 Kb (VIB MicroArrays Facility - www.microarrays.be), having material of approximately 36.24 Mb (between 112.16 to 148.4 Mb), redefining the breakpoints between 1p13.2→1q21.2 region.

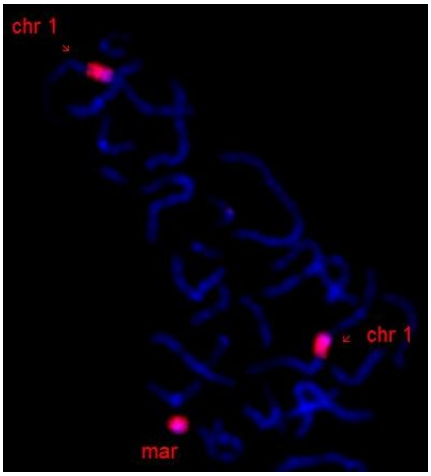

Figure 3.1.3. - Reverse FISH image showing the origin of the marker as derived from chromosome 1. The marker was microdissected, amplified, labelled and hybridized in a patient metaphase.

3.1.3. Discussion

We present a case of a female mosaic carrier of a small supernumerary ring derived from chromosome 1 in ~25% of her cells. The child presented a mild phenotype when clinically evaluated at 11 years old. The genetic content of the sSRC encompasses a ~36 Mb pericentromeric region rich in genes, both in p arm and in q arm (1p13.2→1q21.2) (Fig. 3.1.4.). The region encompasses 136 genes, 17 of them in Online Mendelian Inheritance in Man (OMIM)-morbid map. Of these 17, 14 are located in the 1p-arm, and the other 3 in 1q-arm. One example of an OMIM morbid gene located in this region (located at 1p between 120.45-120.61 Mb) is NOTCH2, a very highly conserved and important gene for cell differentiation, that seems to be involved in tooth development [Mitsiadis et al., 2003]. In about 94% of patients with Alagille syndrome

(ALGS; OMIM 118450), an autosomal dominant multisystem disorder, a mutation in the gene encoding the NOTCH signalling pathway ligand Jagged-1 is present. ALGS was also associated with mutations in NOTCH2 in 2 different families, having all the affected individuals renal manifestations [McDaniell et al., 2006]. Our proband presented tooth problems and nephropaty.

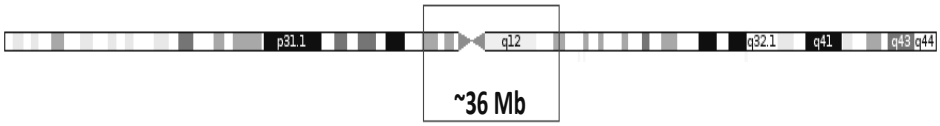

Figure 3.1.4. – Schematic representation of chromosome 1 with the ~36 Mb region involved in the sSMC(1) (square) [adapted from Ensembl].

However, it is difficult to establish a genotype/phenotype correlation in a sSMC carrier. It is essential to have several cases reported and very well characterized, both clinically and molecularly. The majority of the cases described in the literature with a sSMC(1) lack detailed molecular characterization or clinical details. Liehr established a sSMC database to overcome this problem [Liehr T, 2010 - www.med.uni-jena.de/fish/sSMC/00START.htm]. However, of the 46 cases with a sSMC(1) and clinical findings in his database, only 2 are nonmosaic, turning genotype/phenotype correlation more difficult.

Taking into account the cases described in sSMC(1) collection, including our case, [Liehr T, 2010 - www.med.uni-jena.de/fish/sSMC/00START.htm] (Table 3.1.I) the most common clinical symptoms of centromere-near proximal imbalances are, for 1p proximal region, development delay, mental retardation, microcephaly, dysmorphic face, finger and toe malformation and hypotonia. The symptoms more associated with 1q pericentromeric imbalances are dysmorphic face, growth retardation, heart defect, and mental retardation. Kidney problems and microcephaly were described in 11% of the cases. However, more cases should be reported, especially with a good cytogenetic characterization, to strength these observations. Taking into account the data from table 3.1.I., our patient showed features associated with imbalances in both 1p proximal (microcephaly in early childhood) and 1q proximal (growth retardation and kidney problems). With increasing of age, the proband revealed a milder phenotype.

Table 3.1.I. - Clinical symptoms of centromere-near proximal imbalances in 1p and 1q-proximal region, according to the sSMC(1) collection database [www.med.uni-jena.de/fish/sSMC/00START.htm]. The frequency of symtoms is indicated, taking into account the 46 cases with a sSMC(1) associated with clinical findings, as described in the sSMC(1) database.

Symptoms	1p - proximal	1q - proximal
ataxia	8 %	0 %
autism	25 %	0 %
brain malformations	8 %	11 %
developmental delay	83 %	33 %
dysmorphic face	83 %	89 %
finger or toe/foot malformations	42 %	22 %
genital abnormalities	0 %	11 %
growth retardation	17 %	55 %
heart defect	17 %	44 %
hypotonia	25 %	0 %
joint problems	8 %	11 %
kidney problems/ malformations	8 %	11 %
mental retardation	58 %	33 %
microcephaly	58 %	11 %
overgrowth	17 %	11 %
seizures	0 %	0 %
vision impaired	8 %	0 %

[adapted from Liehr T, 2010 - www.med.uni-jena.de/fish/sSMC/00START.htm]

Tönnies and co-workers, when reporting a child with a r(1) chromosome in about 14% of lymphocytes and 9% of buccal cells, stated that it would be important to verify the proportion of the mosaic cell line in different tissues to better understand the likely correlation between aneuploidy and clinical phenotype [Tönnies et al., 2003]. Fickelscher et al. verified the levels of mosaicism in different tissues in a prenatally ascertained sSMC(1). Post-mortem studies revealed that sSMC presence varied between 13 and 62% within different tissues [Fickelscher et al., 2007]. However, although analysis of different tissues can be important to better characterize mosaic patients, this kind of study does not seem to contribute significantly to the genetic counselling [Bernardini et al., 2007]. Indeed, the level of mosaicism in a patient with a SRC not always correlates with phenotypic features. For this purpose, it appears more appropriate the molecular characterisation of ring structures. It is also possible that the factors determining phenotypic severity in a mosaic carrier of a SRC are, rather

than the level of mosaicism, the cell lineage/type in which malsegregation occurs [Davidsson et al., 2008]. It is also important to re-evaluate the carriers of these sSMC at several ages, in order to establish the contribution of abnormal cell lines in their development.

Using high resolution array-based applications, it may be possible to delineate well-defined subgroups of patients with sSMC(1) and other sSMC in the future. This definition is essential for establishing a strong genotype/phenotype correlation, ultimately allowing a more informed genetic counselling, particularly in prenatal diagnosis.

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3.2. *sSMCs from chromosome 2*

Two new cases of *de novo* small supernumerary marker chromosomes (sSMC) detected at prenatal diagnosis [*]

[*] The work presented in this section was published in:

Jardim A, **Melo JB**, Matoso E, Pires LM, Ramos L, Carreira IM (2007) *Prenatal Diagnosis* 27: 380-381.

3.2.1. Introduction

Small supernumerary marker chromosomes (sSMC) can be defined as structurally abnormal chromosomes that cannot be identified or characterized unambiguously by conventional banding cytogenetics alone, and are (in general) equal in size or smaller than chromosome 20 of the same metaphase spread [Liehr et al., 2004]. sSMC are relatively uncommon in the general population. They have been detected with a frequency of 0.076% at prenatal diagnosis and at a much higher frequency of 0.426% in mentally retarded patients [Liehr et al., 2004].

The phenotypes associated with the presence of a *de novo* sSMC vary from normal to severely abnormal. In general, the risk associated with an abnormal phenotype in prenatally detected *de novo* cases with sSMC is 13% [Warburton, 1991], while for sSMC *de novo* derived from non-acrocentric chromosomes the risk is estimated to be approximately 28% [Crolla, 1998]. The phenotypic consequences of particular sSMC are difficult to predict because of differences in euchromatic DNA content, different degrees of mosaicism and/or uniparental disomy (UDP) of the parental chromosomes homologous to the sSMC [Starke et al., 2003].

The origin of sSMC is impossible to determine by routine cytogenetics alone but fluorescence *in situ* hybridization (FISH) enables the identification of its chromosomal origin. However, because of the several possibilities of different phenotypes attributable to the chromosomal origin of the sSMC, their characterization and also an adequate long-term follow-up are necessary [Starke et al., 2003; Liehr et al., 2006]. In cases with apparently normal phenotypes, the characterization of sSMC can provide important information about regions that are phenotypically silent in the presence of gene dosage imbalances [Sumption and Barber, 2001; Starke et al., 2003; Barber, 2005; Liehr et al., 2006]. In this report, we describe two new cases of *de novo* sSMC detected at

prenatal diagnosis derived from chromosome 2, with apparently normal phenotypes ascertained during the neonatal period and until the age of 2 years.

3.2.2. Results

Case A

The first case is the prenatal diagnosis of a 39-year-old pregnant woman who underwent an amniocentesis at 17 weeks' gestation due to advanced maternal age and also positive serum screening. Routine cytogenetics with GTG-banding (G-bands obtained by Trypsin and Giemsa) (Fig. 3.2.1.A) revealed the presence of sSMC in 15 of the 70 metaphases analysed. The sSMC were CBG positive (C-bands induced by barium hydroxide and Giemsa) (Fig 3.2.1.B) and NOR negative (silver staining of the nucleolus organizer regions). Chromosomal analysis of both parents' lymphocytes revealed normal karyotypes. FISH with the I-Multiprobe System (Cytocell) (complete set of alphasatellite/satellite III probes for the 24 chromosomes) (Figure 3.2.1.C) enabled the identification of the chromosome 2 origin of the sSMC. The sSMC showed hybridization with the centromeric probe D2Z2 and the karyotype of the fetus was mos47,XX,+mar.ishder(2)(D2Z2+)[15]/46,XX[55]. Ultrasound analysis did not reveal any abnormalities in the fetus. After counselling, the parents decided to carry on with the pregnancy, and the presence of the sSMC was confirmed in lymphocytes after birth although with different frequencies of the two cell lines (50% of metaphases analysed had the sSMC).

Case B

In the second case, amniocentesis of a 38-year-old pregnant woman was performed at 16 weeks' gestation because of advanced maternal age. The GTG-banding (Fig. 3.2.1.D) study revealed the presence of sSMC in 14 of 30 metaphase spreads analysed. The sSMC were CBG positive (Fig. 3.2.1.E) and NOR negative. The parents' karyotypes were normal. The origin of this sSMC was identified to be derived from chromosome 2 using the OctoChrome System (Cytocell) (includes whole chromosome painting probes for the 24 chromosomes) (Fig. 3.2.1.F). The sSMC hybridized with the wcp (whole

chromosome paint) for chromosome 2 and the karyotype of the fetus was mos47,XY,+mar.ishder(2)(wcp2+)[14]/46,XY[16]. Ecographic evaluation of the fetus was normal. After genetic counselling, the parents decided to continue the pregnancy, and postnatal chromosomal analysis confirmed the presence of the sSMC in 73% of the blood lymphocytes.

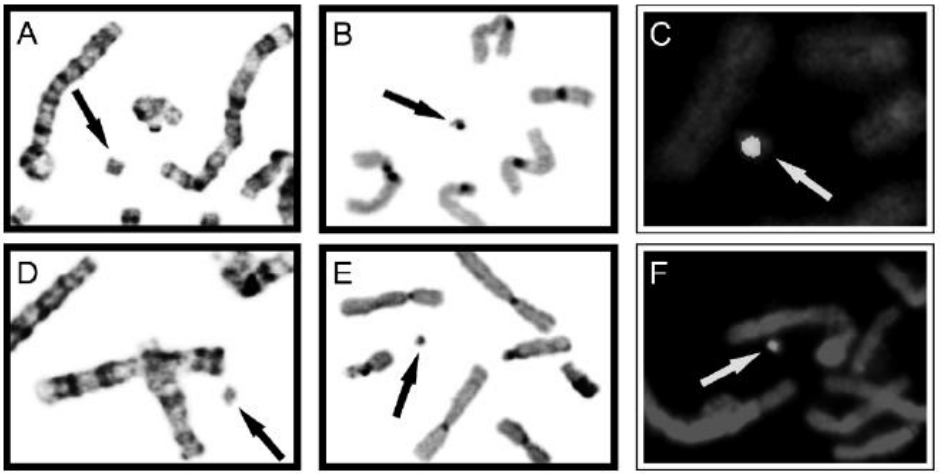

Figure 3.2.1. - (A) GTG, (B) CBG and **(C)** FISH with centromeric probe D2Z2 for the sSMC (indicated by arrow) in case 1; **(D)** GTG, **(E)** CBG and **(F)** FISH with wcp for chromosome 2 for the sSMC (indicated by arrow) in case 2.

3.2.3. Discussion

Chromosome 2 is rarely involved in the formation of marker chromosomes [Crolla, 1998; Ostroverkhova et al., 1999]. Only 11 other cases have been reported in the literature [Plattner et al., 1993; Daniel et al., 1994; Ostroverkhova et al., 1999; Villa et al., 2001; Giardino et al., 2002; Lasan Trcic et al., 2003; Starke et al., 2003; Guanciali-Franchi et al., 2004; Mrasek et al., 2005; Liehr et al., 2006] and of these only 2 at prenatal diagnosis [Villa et al., 2001; Mrasek et al., 2005; Liehr et al., 2006]. Only 8 of the 11 cases were characterized in detail for their chromosomal content, and analysis of the data seems to indicate a correlation between the centromere-near sequences of 2p11.2 and the presence of clinical abnormalities, and the absence of clinical symptoms with presence of proximal sequences of 2q11.2. [Starke et al., 2003; Mrasek et al., 2005; Liehr et al.,

2006]. Small partial trisomies of material derived distally from 2q11.2 are associated with clinical abnormalities [Giardino et al., 2002; Mrasek et al., 2005; Liehr et al., 2006]. The normal phenotype and development of both children up to the age of 2 years leads us to suspect that both cases are probably proximal trisomies of 2q. Nevertheless, a thorough characterization of both sSMC reported is still necessary and will be pursued. As soon as the parents give their consent, we intend to determine the euchromatic content of the sSMC by using microdissection and also bacterial artificial chromosomes (BACs) of the pericentromeric region of chromosome 2. The complete characterization of both sSMC as well as the continued follow-up of the children will take us one step further towards a more comprehensive genotype–phenotype correlation and thus ensure a more informed genetic counselling in the future.

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3.3. *sSMC from chromosome 5*

Characterization of sSMC derived from chromosome 5: Towards a Genotype/Phenotype correlation [*]

[*]The work presented in this section was partially published in:

Melo JB, Santos HL, Backx L, Vermeesch JR, Kosyakova N, Weise A, Ewers E, Liehr T, Carreira IM (2011) Chromosome 5 derived small supernumerary marker chromosome: towards a genotype/phenotype correlation of proximal chromosome 5 imbalances. *Journal of Applied Genetics (in press).*

3.3.1. Introduction

Small supernumerary marker chromosomes (sSMC) are defined as structurally abnormal chromosomes that cannot be identified or characterized unambiguously by conventional banding cytogenetics alone and are generally equal in size or smaller than a chromosome 20 of the same metaphase spread [Liehr et al., 2004]. Clinical outcome of sSMC is highly variable depending on their origin, size, euchromatin content, eventual co-occurrence of uniparental disomy, and prevalence of aneuploidy in mosaic cases. 70% of non-acrocentric sSMC do not have phenotypic consequence, while the remaining 30% have different clinical manifestations [Liehr et al., 2006]. The heterogeneous group of sSMC presents serious genetic counseling problems, especially if they are *de novo*. It is very important to characterize the content and the structure of the sSMC, in order to establish an adequate genotype-phenotype correlation. Most sSMC are derived from short arms and pericentric regions of the acrocentic chromosomes, while the occurrence of an additional derivative chromosome 5 is very rare.

In the present study we report features of a patient with a sSMC derived from chromosome 5 - sSMC(5), which was characterized by GTG-banding, fluorescence *in-situ* hybridization (FISH) and full-tiling resolution array CGH after sSMC microdissection. We also retrospectively compare and review all cases with sSMC from chromosome 5 previously reported and cases with chromosomal imbalances involving a trisomy with a genetic content overlapping our case. The use of molecular cytogenetics, especially array CGH approaches, is crucial for an efficient genotype/phenotype correlation.

3.3.2. Results

Case Report

The patient is the third female child of healthy non-consanguineous parents, with two normal sisters. The delivery was uneventful, after a pregnancy with reduced fetal movements, with a birth weight of 2,750 g, no evidence for anoxia and she started crying soon after birth. Hypotonia was observed since birth. She revealed moderate psychomotor delay, started walking late and had speech difficulties, with the first sentence only at 4 years old. She presented mild facial dysmorphisms, namely mild hypertelorism, high and wide nasal bridge, a prominent and bulbous nose, thin upper lip, dental anomalies and slight protrusion of the chin. The patient revealed learning difficulties throughout her life but has succeeded finishing a professional course, although she can't be persistent in a job. She is now 35 years old and has a healthy son with a normal karyotype.

Cytogenetics and Molecular Cytogenetics

Lymphocyte culture was established from peripheral blood and GTG-banding analysis was performed revealing a female karyotype with an sSMC in 70% of the 20 analyzed metaphases. The marker chromosome was very small and appeared to have a ring shape (Fig 3.3.1A). Karyotypes of both parents were normal (data not showed).

Centromeric-FISH, using the probe SE 1/5/19 (Poseidon Kreatech Diagnostics, The Netherlands) revealed that the sSMC was derived from chromosomes 1, 5 or 19 (data not showed). Reverse FISH confirmed the origin of the marker and suggested the involvement of the euchromatic region of 5q in the sSMC (Fig 3.3.1B). SubcenM-FISH specific for chromosome 5 confirmed the ring-shape of the marker and demonstrated that the sSMC possessed euchromatic material from 5q, the karyotype being mos 47,XX,+r(5)(::p11.1→q11.2?::)[70%]/46,XX[30%] (Fig 3.3.1.C). With array CGH, after microdissection of the sSMC, the breakpoints of the marker were redefined. Thus, the sSMC led to a partial trisomy with approximately 15 Mb length, between 46.15~49.56 MB (5p11.1) and 61.25~61.33Mb (5q12.1) on chromosome 5 (Fig 3.3.2).

UPD was excluded in this patient for chromosome 5, with the following markers being informative: D5S2505, GATA145D09, D5S1457, D5S1501, D5S1456, D5S211, D5S1453 (data not shown).

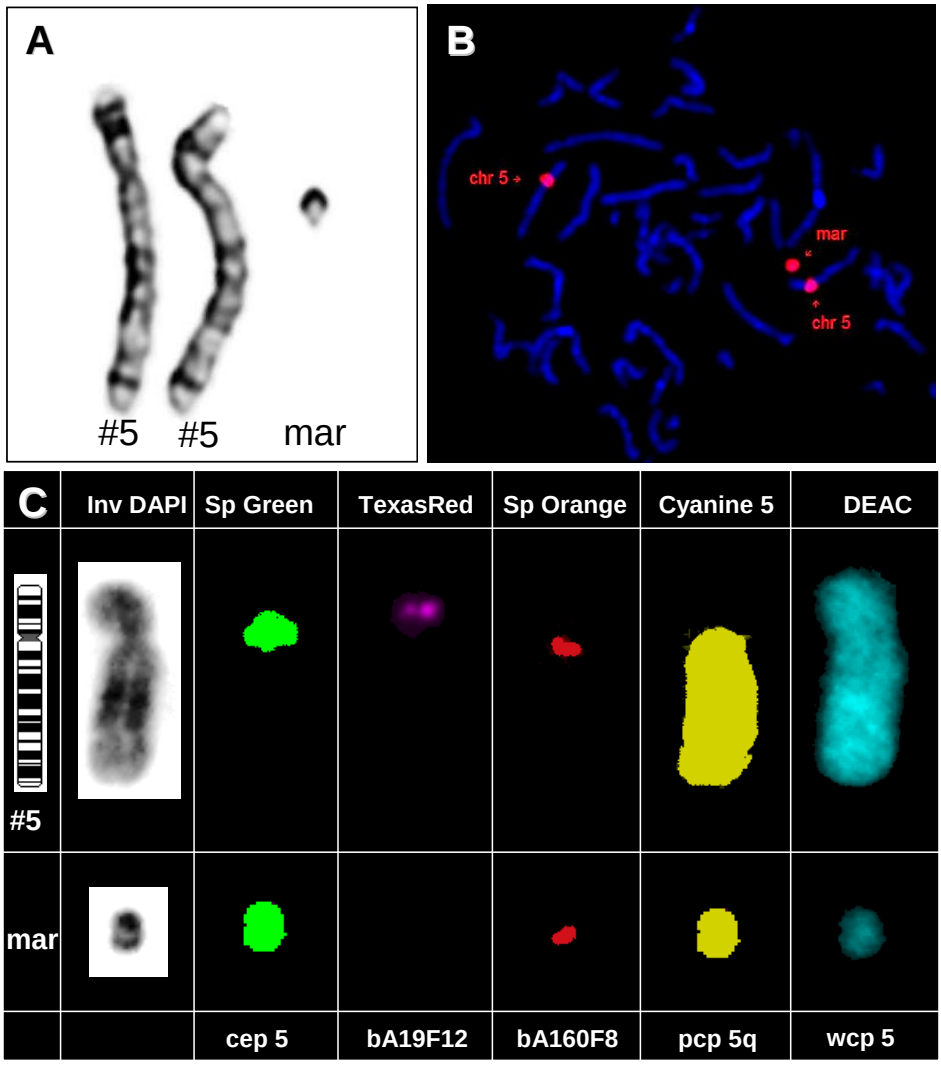

Figure 3.3.1. - G-banding and FISH results. The sSMC(5) is signed as mar. **A.** GTG banded images of both normal chromosomes 5 and of the sSMC(5). **B.** Reverse FISH image confirming the origin of the sSMC and the extent of the chromosome 5 involved in the marker. The sSMC was microdissected and the DNA was amplified, labeled and hybridized in a patient metaphase. **C.** FISH image applying the subcentromeric probes for chromosome 5, revealing that the centromere-near region in p-arm (bA19F12, in purple) is absent in the marker, the centromere-near region in q-arm (bA160F8, in red) is present in the marker, being the sSMC mainly derived from q-arm (in yellow). For space reasons only one of the two normal chromosomes 5 is shown.

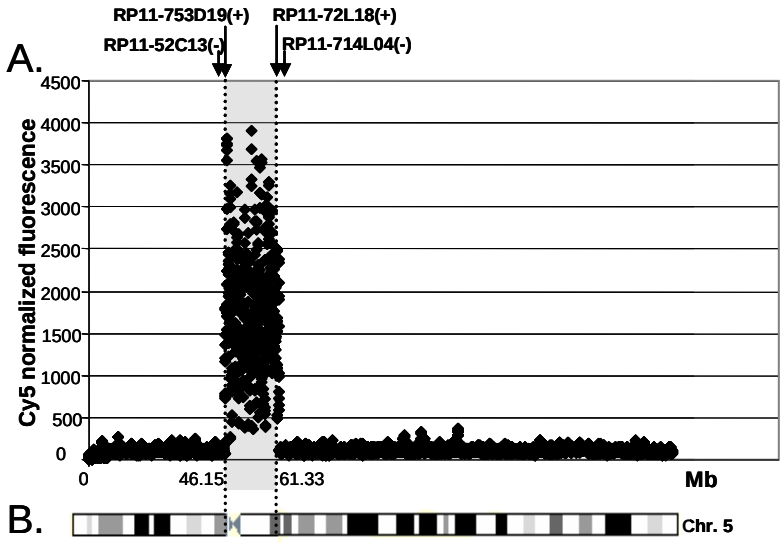

Figure 3.3.2 – A. Array CGH result after microdissection and amplification of the sSMC(5). **A.** The plot shows a gain for region 5q11 to 5q12.1, between 46.15~49.56 MB and 61.25~61.33 Mb, with the flanking BAC clones, RP11-52C13 (absent in the sSMC), RP11-753D19 (present in the sSMC), RP11-72L18 (present in the sSMC), RP11-714L4 (absent in the sSMC). **B.** Ideogram of chromosome 5 showing the region involved in the sSMC, corresponding to the flanking BAC clones identified in 3A.

Literature review

To the best of our knowledge, there are 21 cases with a sSMC derived from chromosome 5 reported in the literature. Beside these reports, 14 additional cases are reported in sSMC homepage [Liehr 2010, sSMC homepage], i.e. 1.2% of the sSMC cases included in this data base. However not all are correlated with a clinical outcome.

Taking into account the cases reported in the literature, in three of these cases clinical information is not available and/or the marker chromosomes were poorly characterized, being therefore excluded of a possible genotype/phenotype correlation [Masuno et al.,1999, Heng et al., 2003 – case 12; Karaman et al., 2006 – case18]. Four of the reported cases with a sSMC(5) do not present clinical findings [Starke et al., 2003 – case 11; Leite et al., 2006; Baldwin et al., 2008 – case 10; Manvelyan et al., 2008 – case 9, Fig 3.3. – i) to iv)]. The other fourteen reported sSMC(5) cases present significant clinical findings (Fig 3.3.3 – v) to xvii)), including one with a neocentromere (Fig 3.3.3.v) [Schuffenhauer et al., 1996; Avansino et al., 1999; Stankiewicz et al., 2000 – case 1; Anderlid et al., 2001 - case G; Fritz et al., 2001; D'Amato Sizonenko et al., 2002 – case 1 and case 2; Brecevic et al., 2006 -

case 5; Huang et al., 2006- cases 53 and 54; Liehr et al., 2006a – case 5-6; Sarri et al., 2006; Baldwin et al., 2008 – case 9; Polityko et al., 2008,]. Additionally to these cases of sSMC(5), there is one with the presence of two sSMC with euchromatin from two different chromosomes (chromosomes 5 and 6) [Liehr et al., 2006b].

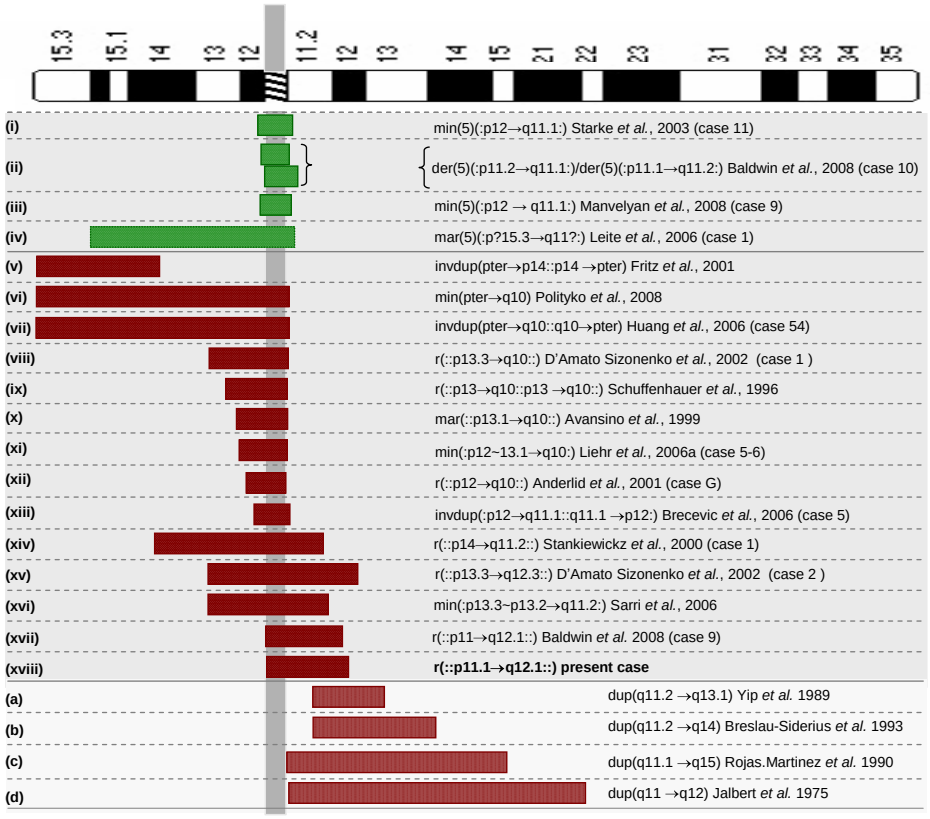

Figure 3.3.3. – Representation of the approximate breakpoints/genetic content of the reported sSMC(5) cases with or without clinical symptoms and with a sufficient cytogenetic characterization and of the cases with a duplication or insertion involving trisomy of proximal 5q arm. The sSMC(5) cases are designated from i) to xviii) and the duplications/insertions cases are designated from a) to d). Green squares correspond to reported sSMC(5) without clinical symptoms and red squares to reported sSMC(5) or partial trisomy 5q associated with clinical findings. Breakpoints' representation is approximated.

Of the sSMC (5) so far reported, approximately 50% have a ring shape, the others being inverted duplications or minutes. The great majority of sSMC(5) described are mainly derived from 5p arm. The approximately breakpoints/genetic content of the sSMC(5) documented with or without clinical findings are shown in figure 3.3.3. The

characterization of the sSMC(5) summarized in figure 3.3.3 were mainly performed by FISH, using in some cases multicolor FISH approaches like subcenM-FISH and multicolor banding, or using other FISH probes specific for chromosome 5. The present case is the first reported sSMC(5) characterized by array CGH, allowing a more precise information about the genetic content of the marker. The features of the patients with a trissomic region, associated with the presence of a sSMC with a molecular cytogenetic content overlapping our case, are described in Table 3.3.I [Stankiewicz et al., 2000 – case 1; D'Amato Sizonenko et al., 2002 –case 2; Sarri et al., 2006; Baldwin et al., 2008 – case 9].

Besides the cases with sSMC(5), there are reports in the literature describing probands with a proximal 5q trisomic region located close to the centromere and overlapping the region of our sSMC(5): 1) a boy with a *de novo* duplication involving 5q11.2→q14 region [Breslau-Siderius et al., 1993]; 2) a case with a *de novo* duplication 5q11.1→q15 [Rojas-Martinez et al., 1990]; 3) a boy with a trisomy 5q11.2→q13.1 as a result of a paternal balanced insertion in the long arm of chromosome 20 [Yip et al., 1989] and 4) a girl with a trisomy 5q11→q22 as a result of a maternal balanced insertion at chromosome 1 [Jalbert et al., 1975]. The features of these patients are also included in Table 3.3.I and represented in figure 3.3.3. (a) to d)), as the genetic content involved in the trisomy overlaps our case.

3.3.3. Discussion

We have reported a mosaic case with a sSMC derived from chromosome 5 with approximately 15 Mb length characterized by multicolor FISH and array CGH, after microdissection. The proband karyotype was initially established as mos 47,XX,+r(5)(::p11.1 →q11.2?::)[70%]/46,XX[30%]. With array CGH, after microdissection of the sSMC, the breakpoints of the marker were redefined, leading to a partial trisomy between 46.15~49.56 MB (5p11.1) and 61.25~61.335Mb (5q12.1) on chromosome 5. We reviewed all reported cases with a sSMC(5) and with a trisomy involving the region contained in the marker chromosome.

sSMC(5) involving regions just near the centromere, apparently without euchromatin involvement, do not present clinical manifestations (Fig 3.3.3, i) to iii)). The other reported sSMC(5), with involvement of euchromatic regions in p-arm and/or q-arm, are associated with clinical manifestations. The exception seems to be the case of Leite and collaborators [Leite et al., 2006], which reported a sSMC(5) involving a long extension of p-arm (p?15.3→q11?) but with no apparent pathological phenotype (Fig 3.3.3, iv). This could be associated to the fact that the sSMC is present in mosaic (< 40% of cord cells with the marker). The characterization of this marker was done using a cross-species color banding FISH approach with gibbon whole chromosome painting probes, homologous to human chromosomes, leading to an unclear characterization of the sSMC. It would be interesting to evaluate this case using another molecular cytogenetic approach, in order to confirm the genetic content of the marker.

The majority of the sSMC(5) reported involved partial trisomy of 5p arm, being the sSMC(5) with 5q material rarer (Fig 3.3.3.). A critical region has been proposed for chromosome 5 p-arm in 5p13, being trisomy including this region associated with psychomotor delay and a characteristic facies following a pregnancy complicated by polyhydramnios [Avansino et al., 1999; D'Amato-Sizonenko et al., 2002]. The dysmorphic features include macrocephaly, epicanthic folds with apparent hypertelorism, enlarged anterior fontanelle, midfacial hypoplasia with a small nose, micrortrognathia, short neck, low-set dysplastic ears with preauricular skin tag, arachnodactyly and talipes equinovarus. Psychomotor retardation may be accompanied by a seizural disorder [D'Amato-Sizonenko et al., 2002].

sSMC(5) involving euchromatin in q arm are rare and a genotype/phenotype correlation is difficult to ascertain due to heterogeneity of the size and content of the sSMC. Of the 4 reported cases with a sSMC(5) and with a molecular cytogenetic content overlapping our case (described in Table 3.3.I and represented in Fig 3.3.3. – xiv to xvii), 3 sSMC(5) cases (Fig 3.3.3. - xiv to xvi) involve 5p arm euchromatic content, namely the critical region proposed for 5p13, and euchromatic material from 5q arm [Stankiewicz et al., 2000 – case 1; D'Amato Sizonenko et al., 2002 –case 2; Sarri et al., 2006]. The dysmorphic features of these cases are concordant in some extent with the ones

described for trisomy involving 5p13 [Avansino et al., 1999; D'Amato-Sizonenko et al., 2002]. Interestingly, the report of Stankiewicz and colleagues [Stankiewicz et al., 2000 – case 1] (Fig 3.3.3.- xiii) describe besides the dysmorphic features and mental development slightly delayed, speech difficulties, with beginning of an indistinctly speech at 4 years old (Table 3.3.I.). The observation of learning difficulties and speech delay was also seen in our proband. The case reported by Baldwin and co-workers [Baldwin et al., 2008 – case 9] (Fig 3.3.3.- xvii) revealed also development delay, but no speech difficulty was reported. However, this proband was clinically evaluated at 1 year old and it would be interesting to reassess him later in life to confirm a possible speech difficulty. There are in the literature some cases with a duplication involving proximal 5q arm [reviewed by Douyard et al., 2006], 4 of them having a cytogenetic content overlapping our case (Figure 3.3.3 – a to d; Table 3.3.I) [Jalbert et al., 1975; Yip et al., 1989; Rojas-Martinez et al., 1990; Breslau-Siderius et al., 1993] and 3 of these cases reported speech difficulties, with speech delay [Yip et al., 1989] (Fig 3.3.3. – a) or even absence of speech [Jalbert et al., 1975; Rojas-Martinez et al., 1990] (Fig 3.3.3.- c and d). It was not possible to assess this characteristic in the other remaining case, due to the age of examination (8 months) (Breslau-Siderius et al., 1993] (Fig 3.3.3. – b). It seems that cases with trisomy involving 5q11.2 to 5q12.1 are associated with speech delay and cases with more distal duplications of 5q arm are related with absence of speech. Douyard and collaborators, when reviewing proximal 5q duplications cases, already noted this observation, and our report could reinforce this correlation [Douyard et al., 2006]. This evidence could suggest a possible critical region associated with speech deficiency in proximal 5q arm. Nonetheless, in order to strengthen this association, it would be important to have more sSMC(5) or partial 5 trisomies documented, even if they are associated with normal phenotypes.

Hypotonia and development delay are also a common feature of the cases reviewed involving a 5q proximal arm trisomy. The dysmorphic features observed in all cases are broad, but in most of the reported cases, including our patient, was described a prominent nose with a wide nasal bridge.

Mosaicism, a very common phenomenon in sSMC cases, is a very important factor regarding the heterogeneity of clinical manifestations. It would be expected that patients with a low-grade of mosaicism would display a milder phenotype, but this is not confirmed by the literature. Depending on the specific tissues with the sSMC, namely if cerebral tissue is involved, the impact of the marker size could be overcome. Imprinting phenomena could also influence the clinical impact of the sSMC, and it would be interesting to evaluate this in all sSMC cases.

A genotype-phenotype correlation is very difficult to ascertain mainly due to breakpoint distribution heterogeneity and to different levels and distribution of mosaicism. A more precise knowledge of the size of the partial trisomy segment and the gene content of a sSMC would greatly improve the ability to predict phenotype, contributing to a more informed prenatal counseling and prognosis. Indeed, this kind of studies gives detailed information that would allow data sequence mining for disease-related genes, where dosage gene increase could have an important role. Multicolor FISH approaches, recurring to subcenFISH and/or multicolor banding allow a good characterization of the heterochromatic/euchromatic content of a sSMC. Currently, with the use of array CGH, it is possible to identify the specific gene content of sSMCs. However this technique could present some problems: 1) when the sSMC is present in a low percentage of cells, it could be difficult to have significant results; 2) depending on the coverage of the pericentromeric region on the array it could be difficult to assess the content just near the centromere. In such cases the use of array CGH after the microdissection and amplification of the sSMC coupled with an array with high resolution in pericentromeric regions, namely a BAC full-tilling array, could overcame these problems. In conclusion, it is very important to characterize thoroughly the genetic content of all sSMC and report them, in order to establish a more precise genotype-phenotype correlation.

Table 3.3.I – Clinical and cytogenetic features of cases with an sSMC(5) or with a duplication, involving a trisomy in proximal 5q arm.

Report	Features		
Stankiewickz *et al.* (case 1) **Fig 3.3.3.– xiv)**	**Karyotype**	**Trisomic region**	**Method**
	mos47,XX,+r(5)[57%]/46,XX[43%]	5p14→q11.2	FISH: alphoid repeat, telomeric probes, WCP, locus specific probes, reverse painting
	Clinical Symptoms		
	At birth: Born after uncomplicated pregnancy. At 4y: speech indistinctly with 4y; mental development delayed. At 27y: Baker's cysts; bilateral talipes valgus and genu valgum; small facial DF - epicanthic folds, high arched palate, hypertelorism, left strabismus, upward slanting palpebral fissures, synophrys, prominent nose with wide nasal bridge, micrognathia, low set posteriorly rotated dysplastic ears, low frontal and posterior hairline. At 30 y: mildly mentally retarded.		
D'Amato-Sizonenko *et al.* (case 2) **Fig 3.3.3.– xv)**	**Karyotype**	**Trisomic region**	**Method**
	mos47,XY, +r(5)[81%]/46,XY[19%]	5p13.3→q12.3	FISH: CEP, WCP, PCP, YACs probes
	Clinical Symptoms		
	Pregnancy: Polyhydramnions. At birth: Birth weight at 75th centile; OFC >90th centile; length at 50th centile; Apgar scores 6/7/-; macrocephaly; large anterior fontanel; upslanting palpebral fissures; wide nasal bridge; abnormal ears; preauricular pits; bilateral palmar crease; cardiac murmur; anterior anus; bilateral talipes equinovarus; hypotonia, VSD; hydrocephalus; bilateral inguinal herniae. At 9m: facial eczema; dolichomacrocephaly; epicanthic folds; upslanting palpebral fissures; hypertelorism; broad bridge of nose; microretrognathia, mildly low-set ears; bell-shape thorax; normal renal scan; normal brain and spine MRI; normal ophthalmological and hearing examination. At 1y: weight and length < 3rd centile; OFC >90th centile with large anterior fontanel; feeding problems; same DF; respiratory problems; unable to sit; development progress – alert, babbling, grabbing and transferring objects, smiling socially.		
Sarri *et al.* **Fig 3.3.3.– xvi)**	**Karyotype**	**Trisomic region**	**Method**
	mos47,XX,+mar(5)[70%]/46,XX[30%]	5p13.3~p13.2→q11.2	M-FISH: subcenM-FISH, MCB
	Clinical Symptoms		
	Karyotyped initially because of atrial septal defect, ventricular septal defect and odd facies; walked at 18 m; moderate learning difficulties but attends normal school; mother describes her as "slow" and "heavy" when she has tasks to perform; At 9y 8m: Weight > 97th centile; length 92th centile; OFC >97th centile; ears 55th centile; mild dolichocephaly; tongue with a vertical furrow; thick thorax with 85 cm circumference (> 97th centile), imd 18.3 cm (90th pc); 2 supernumerary nipples, tapering fingers; palms 85th centile; middle finger > 97th centile, thumb distally placed, foot length 97th centile; clino/camptodactyly of toes IV and V; DF: low set ears with preauricular pits, broad eyebrows, hypertelorism, down slanted palpebral fissures, heavy epicanthus;, high nasal bridge. She began to gain weight from ~ 7 y. Ophthalmologic investigation was normal.		

Baldwin *et al.* (case 9)

Fig 3.3.3.– xvii)

Karyotype	Trisomic region	Method
47,+r(5)[25%]/46[75%]	p11→q12.1	subcenM-FISH with 3 BACs

Clinical Symptoms

Pregnancy: Intrauterine growth retardation and small for gestational age.
At 55d (neonatal intensive care unit): apnea; poor feeder; elevated triglycerides; normal head ultrasound; mild DF.
At 1y: developmental delay; atrial septal defect; hemangioma; mild DF.

Yip *et al.*

Fig 3.3.3.– a

Karyotype	Trisomic region	Method
46,XY,der(20)t(20;5)(q11.2;q11.2q13.1)	q11.2→q13.1	Conventional cytogenetcis

Clinical Symptoms

Birth weight 2800g
At 2y 9m: global development delay with speech delay; slight clinodacyly of the 4th and 5th digits; urinary tract defect; DF: prominent forehead, wide-flat nose, upslating palpebral fissures, upper set ears with lower helices protruding, hypertelorism.

Breslau-Siderius *et al.*

Fig 3.3.3.– b

Karyotype	Trisomic region	Method
46,XY,dup(5)(q11.2→q14)	q11.2→q14	FISH

Clinical Symptoms

At 8m: development delay; growth retardation; congenital heart defects – atrial septal defect and valvular pulmonic stenosis; hypotonia; craniostenosis; DF: prominent forehead, large nose bridge, epicanthal folds, low set, posteriorly rotated ears, ear tags.

Rojas-Martinez *et al.*

Fig 3.3.3.– c

Karyotype	Trisomic region	Method
46,XY,dup(5)(q11.2→q14)	q11.1→q15	n.a.

Clinical Symptoms

At 3.5y: development delay; absence of speech; growth retardation; mild hypertonia; microcephaly; DF: prominent nose, downslating palpebral fissures, epichantic folds, low set dysplastic ears.

	Karyotype	Trisomic region	Method
Jalbert *et al.*	46,XX,rec(1;5)ins (1;5)(q32;q11q22)	q11→q22	Conventional cytogenetics
Fig 3.3.3.– d	**Clinical Symptoms**		
	At 3y7m: development delay, no growth retardation; absent speech; ventricular septal defect; hypotonia; microcephaly; thin hands and tapering fingers; eye sight and hearing normal; DF: low set, protruding ears, epicanthal folds, bulbous and prominent nose, prominent forehead, thick upper lip; teeth abnormally set		
Present case	**Karyotype**	**Trisomic region**	**Method**
	mos47,XX,+r(5)[70%]/46,XX[30%]	p10→q12.1 (46,15~49.46Mb→ 61.25~61.33 Mb)	FISH: CEP, reverse painting M-FISH: subcenM-FISH Array CGH
Fig 3.3.3. – xviii)	**Clinical Symptoms**		
	At birth: Normal delivery, weight 2750g. Childhood: Hypotonic; psychomotor delay; speech delay (first sentence at 4y); learning difficulties; DF –hypertelorism, high nasal bridge with a bulbous prominent nose, thin upper lip, dental anomalies, slight protrusion of the chin. Adulthood: Normal height (160 cm) and weight (53 Kg); OFC 54 cm; Mildly mentally retarded; Maintains DF described features; Healthy son with normal karyotype.		

Adapted from http://www.med.uni-jena.de/fish/sSMC/05.htm and Douyard *et al.*, 2006. n.a. – data not available; d, days; y, years; m, months; FISH, fluorescence *in situ* hybridization; DF, dysmorphic features; CEP, centromeric probe; WCP, whole chromosome painting; PCP, partial chromosome painting; YAC, yeast artificial chromosome; OFC, head circumference; BAC, bacterial artificial chromosome; MCB – multicolour banding; M-FISH – multicolor FISH.

3.3.4. References

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3.4. *sSMC from chromosome 11*

Unbalanced chromosome abnormality in the pericentromeric region 11q: mother and child with distinct phenotypes with a small supernumerary marker chromosome derived from chromosome 11 [*]

[*] The work presented in this section is under submission:

Melo JB, Jardim A, Soares G, Backx L, Vermeesch JR, Kosyakova N, Weise A, Liehr T, Carreira IM. Unbalanced chromosome abnormality in the pericentromeric region 11q: mother and child with distinct phenotypes with a small supernumerary marker chromosome derived from chromosome 11. Submitted to *American Journal of Medical Genetics A*.

3.4.1. Introduction

Unbalanced chromosomal abnormalities (UBCAs) usually involve several megabases of DNA, and the great majority are ascertained because of phenotypic or reproductive effects that bring patients to medical attention. The more severely affected an individual is, the more likely he will be investigated; creating an ascertainment bias that does not reflect the full range of phenotypes that may be associated with imbalance of a particular chromosomal segment [Barber, 2005]. Small supernumerary marker chromosomes (sSMC) are small structurally abnormal chromosomes that occur in addition to the 46 chromosomes and cannot be identified or characterized unambiguously by conventional banding cytogenetics alone and are generally equal in size or smaller than a chromosome 20 of the same metaphase spread (Liehr et al., 2004). The risk for phenotypic abnormalities associated with an sSMC depends on several factors, including pattern of inheritance, levels of mosaicism, mode of ascertainment, chromosomal origin, uniparental disomy, and the morphology, content, and structure of the marker [Graf et al., 2006].

We report a case of mother and son with the identical sSMC derived from chromosome 11 (sSMC(11)) with distinct phenotypes.

3.4.2. Results

Case Report

The 13 years old boy presents facial dysmorphisms, macrocephaly, strabism, ptosis, mild mental retardation / developmental delay (global IQ on WISC-III evaluation at the

age of 15 years: 56). The 36 years old mother, besides a congenital cardiopathy – atrial septum defect (corrected with surgery), is phenotypically normal.

Cytogenetics and Molecular Cytogenetics

The sSMC was characterized comprehensively for its genetic content by G-banding, molecular cytogenetics using reverse FISH and subcentromere-specific multicolor fluorescence *in situ* hybridization (subcenM-FISH) [Starke et al., 2003], and array Comparative Genomic Hybridization (Array CGH) after microdissection and amplification of the sSMC-specific DNA [Melo et al., 2009]. G-banding showed that the sSMC was present in 83% of the 30 analysed metaphase in the boy and in 70% in the mother (Fig 3.4.1). The origin of the marker was ascertained by reverse FISH, as derived from chromosome 11 (Fig 3.4.2).

The karyotype, after characterization of the marker by G-banding and subcenM-FISH, was in the mother –

47,XX,+min(11)(:p11.12~11.2->q12:)[7]/47,XX,+r(11)(::p11.12~11.2->q12::)[2]/46,XX[4];

and in the son –

47,XY,+r(11)(::p11.12~11.2->q12::)[6]/47,XY,+r(11;11)(::p11.12~11.2->q12::p11.12~11.2->q12::)[3]/47,XY,+min(11)(:p11.12~11.2->q12:)[4]/46,XY[3] (Fig. 3.4.3.A).

The microdissected DNA of the sSMCs of the son was hybridized in a full tiling BAC array specific for chromosome 11, with a resolution of 100-150 Kb (VIB MicroArrays Facility - www.microarrays.be), having material of approximately 14.55 Mb size extension from 11q (between 50.47 Mb to 65.02 Mb) (Fig. 3.4.3 B and C).

Uniparental Disomy analysis

Uniparental Disomy (UPD) for chromosome 11 was excluded in the boy, with the following markers being informative: D11S1984, D11S2362, D11S1999, D11S1981, D11S1392, D11S2000, D11S1986, D11S1998, D11S4464, D11S912, and D11S968.

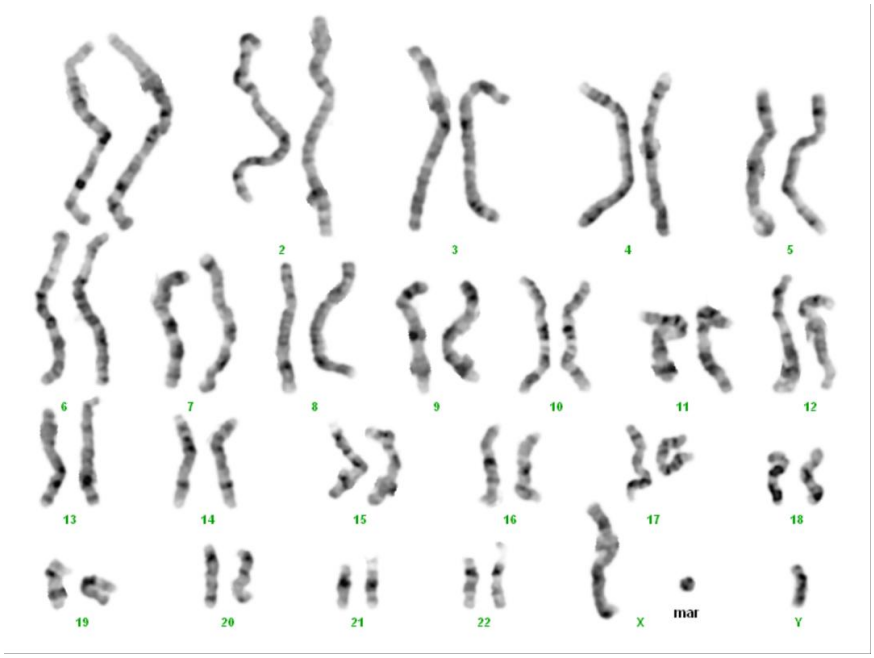

Figure 3.4.1 – Karyogram of the child showing a 46,XY with a small supernumerary marker chromosome that was encountered in 25 of the 30 analyzed cells.

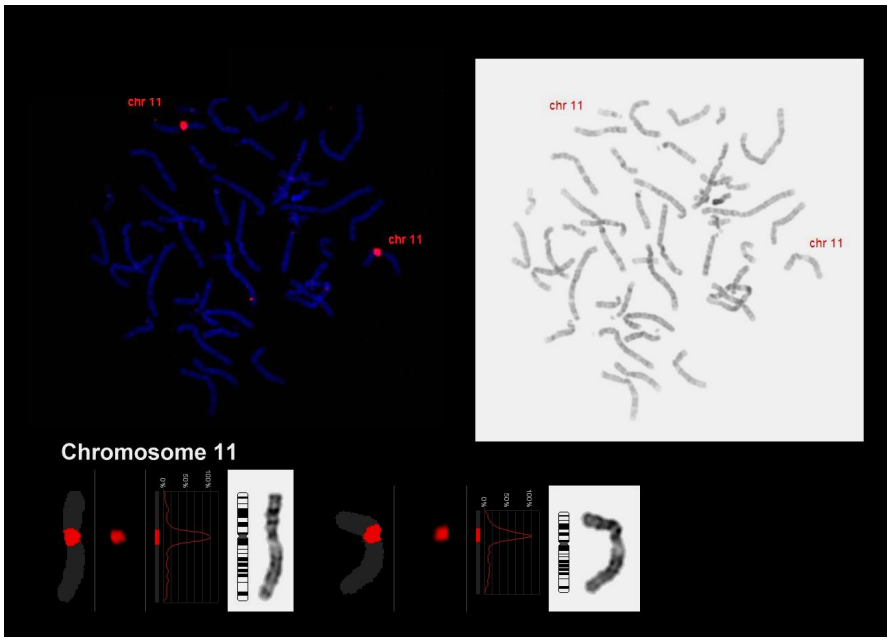

Figure 3.4.2 – Reverse FISH after microdissection and amplification of the the sSMC. The amplificated material was labelled and hybridized in a nomal metaphase plate. FISH image shows that the origin of the marker is the pericentromeric region of chromosome 11.

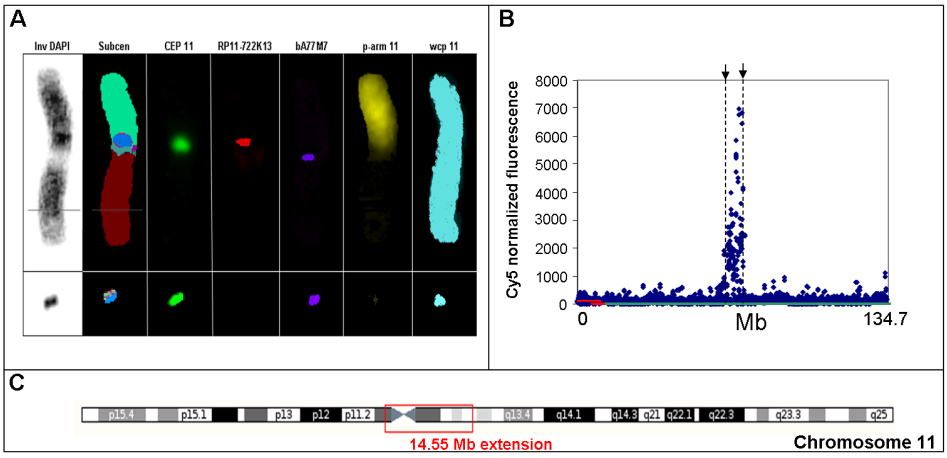

Figure 3.4.3 – A. SubcenM-FISH for chromosome 11, revealing that the centromere-near region in p-arm (RP11- 722K13, in red) is absent and the centromere-near region in q-arm (bA77M7, in purple) is present in the marker, being the sSMC derived from q-arm. The p-arm is absent in the marker (in yellow). The centromeric probe – CEP11 is identified in green and whole chromosome painting of 11 in blue (for space reasons only one of the two normal chromosomes 11 is shown). **B.** Array CGH result after microdissection and amplification of the sSMC(11). The plot shows a gain for region 11p11.12 to 11q13.1, between 50,47 Mb to 65,02Mb **C.** Ideogram of chromosome 11 showing the region (red square) involved in the sSMC, corresponding to the flanking BAC clones identified in 1.B.

3.4.3. Discussion

Some sSMCs are inherited directly from a phenotypically normal parent. In such cases, there are often no phenotypic consequences for individuals who inherit the marker. However, there are exceptions to this. Although the sSMC reported here is maternally derived, the mother has a normal phenotype and the son an abnormal one. The difference in the phenotypes of mother and child could be due to both the different degree and distribution of mosaicism and to the different forms of the sSMC encountered.

The variability of phenotypes among relatives observed in transmitted UCBAs can also be due to other causes, like chromosomal non-penetrance, uniparental disomy or undetected differences at the molecular level, in this or other locci. The normal outcome for a cytogenetically visible UBCA, deletion or duplication, could be due to several reasons [Barber et al., 2005], like for example:

1) Low gene content;

2) Absence of dosage sensitive loci: many genes are not dosage sensitive, and imbalances involving a limited number of genes may not include genes that are dosage sensitive.

3) Functional redundancy: deletions or duplications of genes that have additional or related copies outside an imbalanced segment may have no detectable effect on the phenotype. Metabolic pathways can substitute for a pathway affected by mutation or functional complementation can arise from duplicate genes. It has also been suggested that deletions involving gene clusters may be better buffered because of the remaining cluster of related genes on the normal homologue.

4) Allelic exclusion: specific alleles have allele-specific levels of expression – a high expressing allele could compensate for a deleted locus and a low expressing allele for a duplicated gene in a given individual, but unlikely that these would be coinherited over several generations of the same family.

Barber reviewed 130 families with directly transmitted, cytogenetically visible unbalanced chromosome abnormalities (UBCAs), and noticed that in 30 families (23%), the affected proband had the same UBCA as other phenotypically normal family members. There are more female than male transmitting carriers of UBCAs, suggesting that unbalanced chromosome complements may have a more deleterious effect on male than female meiosis, as has previously been suggested for balanced translocation and ring chromosome carriers [Barber, 2005].

sSMCs derived from chromosome 11 (sSMC) are rare and, so far, it is not yet totally clear which regions of chromosome 11 are critical and have clinical consequences. In the literature there are 5 cases reported of sSMC(11) associated with an abnormal phenotype [Neill et al., 2010; Baldwin et al., 2008; Rauch et al., 1992; Daniel and Malafiej, 2003; Sanz et al., 2005] and 3 cases without phenotypical consequences [Mannens et al., 1991; Haaf et al., 1992; Bartsch et al., 2005]. In the majority of these cases the characterization of the genetic content of the markers is not exhaustive.

There is a difficulty in establishing a genotype–phenotype correlation in a ring carrier. Rings often appear as mosaics because of sister chromatid exchange within a ring in

mitotic crossing-over events, which generates aneuploid cells with increased mortality [Carreira et al., 2007].

It is essential to report all cases of sSMC, associated or not with clinical findings, with a good molecular cytogenetic content characterization to have a robust genotype/phenotype correlation. This case highlights the problem of correlating familial sSMCs encountered in a phenotypic normal parent with the expected phenotypic outcome in a child with the same imbalance. This is a major problem, especially in prenatal diagnosis.

3.4.4. References

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3.5. *sSMC from chromosome 13*

First prenatally detected small supernumerary neocentromeric derivative chromosome 13 resulting in a non-mosaic partial tetrasomy 13q [*]

[*] The work presented in this section was published in:

Mascarenhas A, Matoso E, Tönnies H, Gerlach A, Julião MJ, **Melo JB,** Carreira IM (2008) *Cytogenetic and Genome Research* 121: 293-7.

3.5.1. Introduction

Human centromeres are defined cytogenetically by a primary constriction of the chromosomes and are required for proper chromosome segregation in both meiosis and mitosis. They are characterized by tandemly repeated alpha satellite DNA and heterochromatin [Choo, 1997; Warburton, 2004]. A class of mitotically stable human derivative chromosomes containing fully functional centromeres, so called neocentromeres lacking alpha satellite DNA has been described. Despite the complete absence of normal centromeric alpha-satellite DNA, human neocentromeres are able to form a primary constriction and assemble a functional kinetochore, suggesting that centromere formation is not strictly dependent on primary DNA sequence [Karpen and Allshire, 1997; Amor and Choo, 2002]. An analysis of the distribution of neocentromeres suggests clustering in chromosomal hotspot regions 15q25, 3q, and 13q [Amor and Choo, 2002; Warburton, 2004]. Neocentromeres often result in partial tri- or tetrasomy, because their formation confers mitotic stability to acentric chromosome fragments that would normally be lost [Levy et al., 2000]. The most common class of neocentromeric chromosomes are supernumerary inverted duplications of the distal segments of a chromosome [Choo, 1997; Warburton et al., 2000]. Small supernumerary marker chromosomes (sSMC) are defined as structurally abnormal chromosomes that cannot be identified or characterized unambiguously by conventional banding cytogenetics alone, and are generally equal in size or smaller than a chromosome 20 of the same metaphase spread [Liehr et al., 2004].

We present a case of a sSMC without centromeric alpha satellite DNA, which is an inverted duplication of distal 13q31, found in a prenatal diagnosis with ultrasound malformations, of a 34 year-old pregnant woman. Fifteen supernumerary

neocentromeric marker chromosomes originating from the distal region of chromosome 13q have been reported, supporting the idea that this chromosome may have an increased propensity for neocentromere formation (For review: http://www.med.uni-jena.de/fish/sSMC). However, only ten of these cases are sSMC, according to the definition proposed by Liehr et al. (2004) (see Table3.5.I.). Prenatal diagnosis of tetrasomy for distal 13q, mosaic or nonmosaic, is very uncommon [Knegt et al., 2003]. To the best of our knowledge, this is the first case reported prenatally of a supernumerary neocentromeric chromosome from distal 13q.

3.5.2. Results

A 34 year old woman was referred to prenatal cytogenetic diagnosis due to foetal abnormalities identified on ultrasound at 23 weeks of pregnancy. There was oligohydramnios, a large cisterna magna, ventriculomegaly, enlarged and hyperechogenic kidneys and club left foot.

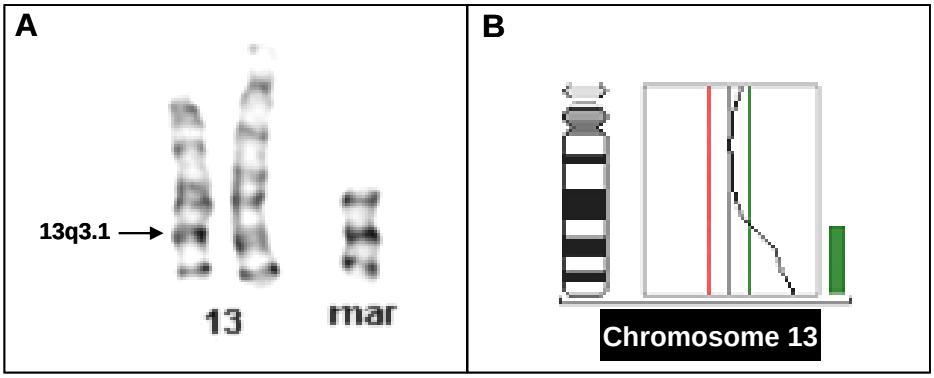

Figure 3.5.1. – A. GTG banded images of normal chromosomes 13 and the marker chromosome. **B.** CGH profile for chromosome 13. The gain of the chromosome region 13q31→qter is shown.

Studies on both the culture amniotic fluid and foetal blood cells revealed a *de novo* small supernumerary marker chromosome, in all 50 analysed metaphases. This marker showed a symmetrical G-banding pattern (Fig 3.5.1.A). CBG-staining revealed no pericentromeric heterochromatin and Ag-NOR staining showed no NOR on the marker (data not shown). FISH studies, using whole chromosome painting, centromeric and subtelomeric probes, revealed this marker to be 13 in origin (Fig 3.5.2.A) and an inverted duplication of the distal portion of chromosome 13q (Fig 3.5.2.B), with no

detectable alpha satellite DNA (Fig 3.5.2.C-D). The neocentromeric constriction was apparently at band 13q31 (Fig 3.5.1.A). Comparative genomic hybridization confirmed the origin and the chromosomal region involved in the partial tetrasomy (Fig 3.5.1.B). The presence of a functional neocentromere at band 13q31 on this marker chromosome was confirmed by immunofluorescence with antibodies to CENP-C (Fig 3.5.2.E).

The karyotype of the foetus was 47,XX,+mar.ish inv dup(13)(qter→q31::q31→neo→qter)(wcp13+,D13Z1/D21Z1–,D13S327++), resulting in a tetrasomy of the distal portion of chromosome 13q.

After genetic counselling, parents decided for pregnancy termination. After the induced abortion the foetal observation with post-mortem autopsy identified the presence of short nose and neck, hypothelorism (inner canthal distance 1 cm), short palpebral fissures, low set ears and thymic hypoplasia. It also confirmed the presence of club left foot and renal dysplasia. The central nervous system study was unable to confirm the presence of the large cisterna magna and ventriculomegaly.

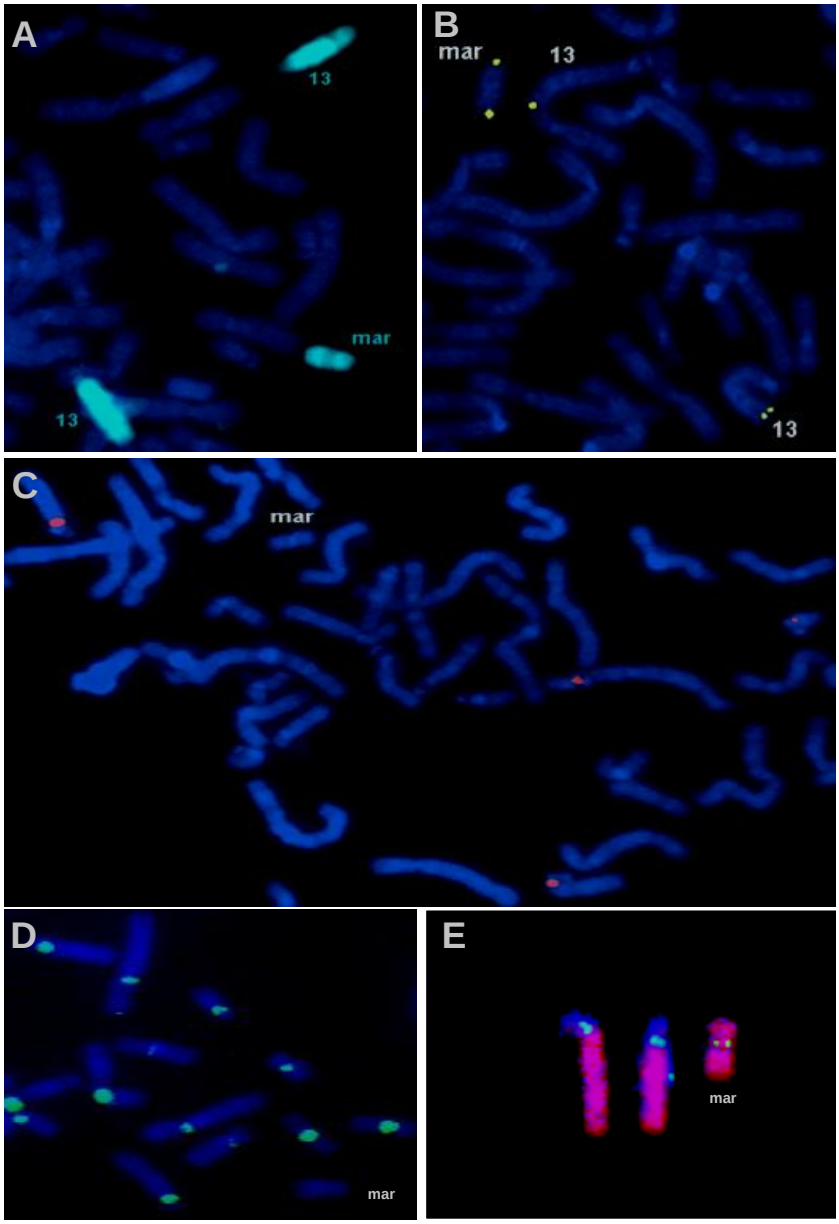

Figure 3.5.2. - FISH using different probes. The supernumerary marker chromosome is signed as *mar*. **A**) Whole chromosome painting 13 identifying the supernumerary marker having chromosome 13 origin. **B)** Subtelomeric 13q probe showing the inversion duplication of the derivative. **C)** CEP 13/21 probe showing the absence of a normal alphoid centromere in the derivative. **D)** All centromeric probe reinforcing the absence of any alphoid centromere in the supernumerary derivative chromosome 13. E. FISH with wcp13 (red) and immunofluorescence with antibodies to CENP-C (green) that marks all active centromeres, including the neocentromere in the derivative chromosome 13.

Table 3.5.I - Summary of the eleven cases reported with sSMC derived from chromosome 13 with neocentromeres [adapted from Liehr et al., 2007].

Case	Rearrangement	Location of neocentromere	Mosaicism	Prenatal/ Postnatal	Clinical Efects	Reference sSMC homepage[1]
1	r(13)(::q21.31→q22.2::)	13q21	47,XX,del(13)(q21.32q22.2), +mar[100%]	postnatal	none	13-N-p21.31/1-1
2	inv dup(13)(qter→q31: :q31→qter)	13q32	47,XX,+mar[54%]/46,XX[46%]	postnatal	severe	13-N-qt31/1-1
3	inv dup(13)(qter→q31: :q31→qter)	13q31	47,XY,+mar[60%]/46,XY[40%]	postnatal	severe	13-N-qt31/1-2
4	inv dup(13)(qter→q31: :q31→qter)	13q31	47,XX,+mar[13%]/46,XX[87%]	postnatal	severe	13-N-qt31/1-3
5	inv dup(13)(qter→q22.3~31.1: :q22.3~31.1→qter)	13q31	47,XY,+mar[100%]	postnatal	severe	13-N-qt31/1-5
6	inv dup(13)(qter→q31: :q31→qter)	13q31	47,XX,+mar[100%]	prenatal	severe	This Report
7	r(13)(::q31.1q32.3::)	13q32/31	47,XY,del(13)(q31.1q32.3),+r(13)(::q31.1q32.3::)[50%]/ 46,XY,del(13)(q31.1q32.3)[50%]	postnatal	moderate	13-N-q31.1/1-1
8	inv dup(13)(qter→q32: :q32→qter)	13q32	47,XX,+mar[98%]/46,XX[2%] (sSMC in fibroblasts 8%)	postnatal	severe	13-N-qt32/1-1
9	inv dup(13)(qter→q32: :q32→qter)	13q32	48,XX,+marx2[15%]/47,XX,+mar[75%] 46,XX[10%] at birth	postnatal	severe	13-N-qt32/1-2
10	inv dup(13)(qter→q32: :q32→qter)	13q32	47,XY,+mar[100%] (sSMC in fibroblasts 100%)	postnatal	severe	13-N-qt32/1-3
11	inv dup(13)(qter→q32: :q32→qter)x2	13q32	48,XY,+marx2[26%]/46,XY[74%] at age of 7y	postnatal	severe	13-N-qt32/1-4

[1] http://www.med.uni-jena.de/fish/sSMC

3.5.3. Discussion

We report the first case of a prenatal diagnosis that showed a small supernumerary marker chromosome consisting of an inverted duplication of the distal portion of chromosome 13q, containing no detectable alpha satellite DNA. The neocentromeric constriction was located at band 13q31. The presence of a functional neocentromere was proved by immunofluorescence studies using antibodies against a centromere protein - CENP-C, that is known to be important for centromere function, namely for the assembly of the kinetochore and in the correct segregation of sister chromatids [Politi et al., 2002]. CENP-C is associated with active neocentromeres that apparently do not contain repetitive sequences typical of conventional centromeres [Amor et al., 2004].

The high number of observed 13q neocentromeres cases may be related with the viability of chromosome 13 trisomies. However in other chromosomes, like 18, 21 and X, that are well tolerated in trisomy, neocentromerization is a rare event: there is one case reported for chromosome 21 [Barbi et al., 2000], one for chromosome X [Kaiser-Rogers et al., 1995] and one reported neocentromere for chromosome 18 [Rauch et al., 1992]. These facts suggest that 13q has a higher propensity for formation of neocentromeric derivatives, but the reason for it remains to be elucidated. At least four distinct regions in 13q have been shown to contain neocentromeres: 13q21 (five cases), 13q31 (three cases), 13q31/32 (one case) and 13q32 (seven cases) [For review: http://www.med.uni-jena.de/fish/sSMC], which could suggest that these bands are "hotspot" regions for neocentromere formation. A study by Alonso and coworkers has analysed the DNA sequence of three chromosome 13 neocentromeres at 13q32, using CENP-A chromatin immunoprecipitation and genomic microarray analysis. They showed that there is no evidence of any overlap between the three neocentromeres, indicating, therefore, that there is no DNA sequence defined as a neocentromere hotspot in this region [Alonso et al., 2003]. Interesting also is that the sequence analysis of the DNA isolated from several independent neocentromeres revealed no common sequence, with the exception of an increase AT richness, a shared characteristic with

alpha satellite DNA sequences of human centromeres [Lo et al., 2001; Satinover et al., 2001; Li et al., 2002].

The most frequent features seen in patients with partial trisomy or tetrasomy of chromosome 13q are microphthalmia, ear abnormalities, hypotelorism, facial dysmorphisms, urogenital defects and severe learning difficulties [Schinzel, 2001]. The phenotype of the case in this report also showed hypotelorism, low set ears, facial dysmorphisms and renal dysplasia. Regarding the size of the partial tetrasomy only three cases are cytogenetically comparable to our case (case 2; 3 and 4 – Table 3.5.I). However, these cases which involve a tetrasomy of 13q31-qter, two with the neocentromere location like ours at 13q31 (case 3 and 4 – Table 3.5.I) and one with the neocentromere at 13q32 (case 2 – Table 3.5.I), are different from the present case not only because they were mosaic but they were also postnatally diagnosed. Therefore the clinical comparison with our case can only be extrapolated. There are some clinical features that can be correlated in all of these cases, namely the cranio/facial abnormalities and ear malformations. Since this is the first case reported prenatally, it is important to notice the relevance regarding the ultrasound malformations, specially the ones involving the head (large cisterna magna, ventriculomegalia and the hypotelorism). The severity of the foetus phenotype could be explained by the full tetrasomy in the two tissues studied, in contraposition to the mosaic status of the previous reported postnatal cases involving the same region.

The complexity and variability of the phenotypes seen in patients with sSMC of chromosome 13 do not support a genotype/phenotype correlation [reviewed by Liehr et al., 2007]. Furthermore, the wide range of mosaicism and the chromosome imbalances seen in all these cases must account to some extent for the wide variability of the observed phenotypes. Moreover, the presence of the neocentromere could lead to chromatin changes, influencing gene expression over an extended chromosomal region [Levy et al., 2000].

In conclusion, there are, to the best of our knowledge, only three more cases described with sSMC with the location of the neocentromere in band 13q31 (case 3, 4 and 5 – Table 3.5.I), as in our case. However, this is the first one with a neocentromere

in 13q31 reported prenatally and that was not a mosaic. The origin and mechanisms involved in neocentromeric marker chromosomes are interesting subjects that deserve further investigation. Every sSMC should be characterized by cytogenetic and molecular methods, in order to define the euchromatic content and to prove the physical neocentromere position, providing further information for genetic counselling and future genotype/phenotype correlations.

3.5.4. References

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3.6. - *sSMCs from chromosome 15*

Refined molecular cytogenetic characterization of the breakpoints of small supernumerary marker chromosomes derived from chromosome 15 [*]

[*] The work presented in this section is under submission:

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3.6.1. Introduction

Many rearrangements may occur in the imprinted chromosome region 15q11->q14, which is known for its instability due to the presence of repeated DNA elements [Battaglia, 2008]. This region harbours six common sites that mediate chromosomal rearrangements. These duplicons or breakpoints (BPs) consist of large segments with low copy repeats referred to as BP1–BP5 located in 15q11->q13, whereas BP6 is within 15q14 [Kleefstra et al., 2010] (Fig. 3.6.1.). The rearrangements include deletions associated either with Angelman syndrome (OMIM 105830) or with Prader-Willi syndrome (OMIM 176270), according to parental origin [Lalande, 1996], translocations, inversions and supernumerary marker chromosomes formed by the inverted duplication of proximal chromosome 15. Interstitial duplications, triplications and balanced reciprocal translocations are much less frequent [Battaglia, 2008].

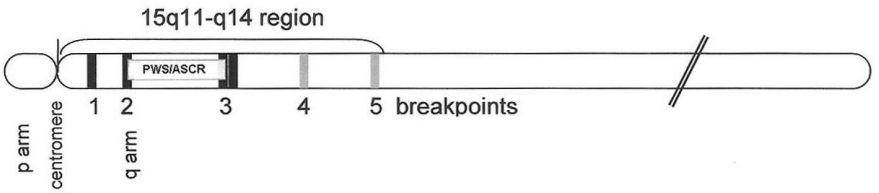

Figure 3.6.1. Schematic representation of chromosome 15, showing the five recurrent breakpoints (BP), and the ~4 Mb segment (15q11-q14) that encompasses the Prader-Willi/Angelman syndrome critical region (PWS/ASCR) [from Battaglia, 2008].

Supernumerary marker chromosomes (SMC) originating from chromosome 15 are the most common SMCs. They encompass clinically irrelevant SMC(15)s containing only heterochromatin and 15p material, and clinically relevant SMC(15)s that consist of both eu- and heterochromatic 15q material [Kleefstra et al., 2010]. The two most

proximal breakpoints (BP1 and BP2) are implicated in the origins of clinically insignificant SMCs [Huang et al., 1997]. The three distal breakpoints give rise to larger SMCs [Roberts et al., 2003; Makoff and Flomen, 2007]. So far, BP6 has not been reported to be involved in the formation of SMCs [Kleefstra et al., 2010]. The critical ~4 Mb region, responsible for PWS/AS and the duplication chromosome 15 syndromes, lies between BP2 and BP3.

As with others SMCs, a SMC(15) can be monocentric or dicentric. The dicentric SMC(15)s are the most common and may have originated from recombination between two homologs or between the two chromatids of one chromosome 15. Such a dicentric SMC(15) consists of two centromeres, with one inactivated, two bisatellited telomeres, and two inverted copies of the proximal end of the q arm [Kleefstra et al., 2010]. These dicentric SMC(15)s are also referred to as "pseudodicentric chromosome 15" or "inv dup(15)" [Roberts et al., 2003]. Two cytogenetic types of inv dup (15) markers chromosomes have been identified, with different phenotypic consequences [Battaglia, 2008; Crolla et al., 1995; Huang et al., 1997; Roberts et al., 2003]:

a) small SMC(15)s, which are metacentric or submetacentric chromosomes without euchromatic material, not containing the Prader-Willi/Angelman syndrome critical region (PWS/ASCR), and that can be familial or *de novo*. Most children with this aberration show a normal phenotype, although exceptions have been reported [Hou and Wang, 1998]. They have been associated with male infertility [Eggermann et al., 2002; Morel et al., 2004];

b) large SMC(15)s, with the PWS/ASCR, between BP2 and BP3, being associated with an abnormal phenotype - the idic(15) syndrome [Battaglia, 2008]. On the basis of size, these clinically relevant SMC(15)s can be subdivided into type A,''large'' asymmetric and type B, ''small'' symmetric SMC(15)s. Type B SMC(15)s contain the triplicated 15pter to BP3 (located at 26.5 Mb) region, while type A SMC(15)s consist of 15pter->BP4(28.5Mb)::BP5(30.5Mb)->15pter [Kleefstra et al., 2010].

The inv dup(15) or idic(15) (inverted duplication of proximal chromosome 15 or isodicentric 15 chromosome) syndrome is characterized by a distinct neurobehavioral phenotype including moderate to profound developmental delay/intellectual

disability, absent or very poor speech, hypotonia, epilepsy, and an autism spectrum disorder [Robinson et al., 1993; Crolla et al., 1995; Battaglia, 2008; Battaglia et al., 2010]. The combination of severe epilepsy and the particular behaviour disorder renders a more distinctive clinical picture that distinguishes idic(15) syndrome from other conditions involving severe neurodevelopmental disability [Battaglia et al., 2010]. Incidence at birth is estimated to be 1/30 000 with a sex ratio of almost 1 [Schinzel and Niedrist, 2001; Battaglia et al., 2010].

Genotype/phenotype studies have not been able, to date, to show any correlation between type and size of the idic(15), and the degree of severity of the clinical spectrum. We describe 6 new cases with SMC(15)s that were characterized by molecular cytogenetics and array CGH techniques, for the evaluation of the exact genetic content.

3.6.2. Results

We report 6 new cases of patients with a SMC(15) (cases I, II, III, IV, V and VI) (Table 3.6.I.).

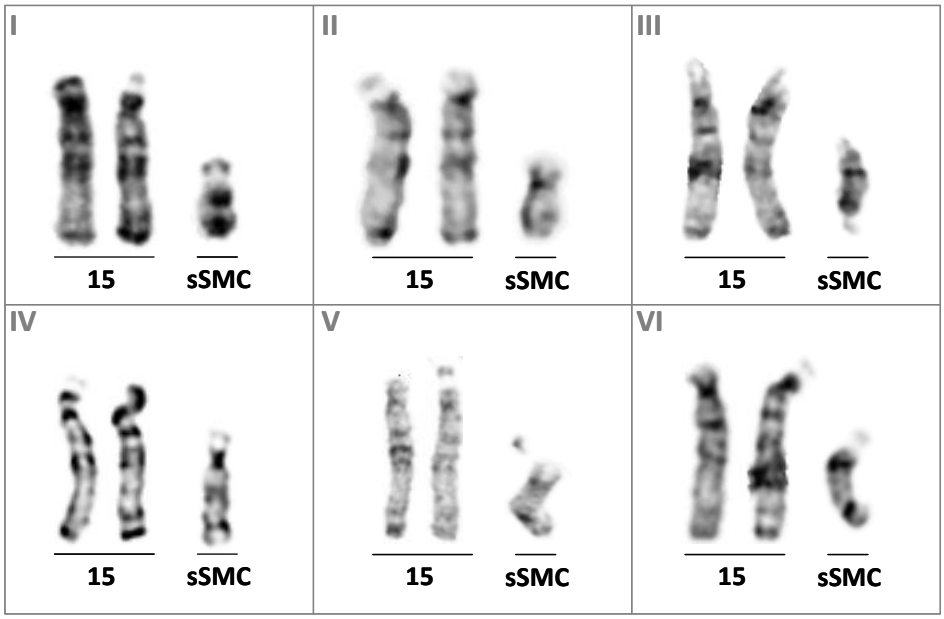

Figure 3.6.2. GTG banding of normal chromosomes 15 and the marker chromosome in 6 cases (I to VI).

Case I

The sSMC(15) was ascertained prenatally in a case referred for advanced maternal age and confirmed in lymphocytes after birth. A normal child was born (Table 3.9.I). After G-Banding, the karyotype was mos 47,XX,+mar[27]/46,XX[3] (Fig. 3.6.2.). Karyotypes of both parents were normal. Centromeric FISH identified the origin of the marker as being derived from chromosome 15. SubcenM-FISH and Multicolour Banding (MCB) analysis revealed the sSMC as an inv dup(15)(q11.2).

The microdissected DNA of the sSMC was hybridized in a full tiling BAC array specific for chromosome 15, with a resolution of 100-150 Kb (VIB MicroArrays Facility - www.microarrays.be), having material of approximately 2.49 Mb size extension from 15q (between 18.47 Mb to 20.96 Mb). This region encompasses only one gene (BCL8) described in OMIM morbid map, which is associated with large - cell Lymphoma [Dyominet al., 1997].

Uniparental Disomy (UPD) for chromosome 15 was excluded, with the following markers being informative: D15S652, D15S816, and D15S642.

Case II

During the first years of life the patient presented hipotony, psychomotor development delay and strabismus. At 7 years old started having mioclonic crisis. The child was clinically reassessed at 14 years old, presenting severe psychomotor delay; daily convulsions; and frequent respiratory infections (Table 3.6.I). He is the second child of nonconsanguineos parents with no familiar history, being the older child normal.

G-banding revealed the presence of a supernumerary marker chromosome in all the analyzed cells, the karyotype of the boy being 47,XY,+mar (Fig. 3.6.2.). Karyotypes of both parents were normal. After MCB and subcenM-FISH analysis the sSMC characterization revealed an inv dup(15)(q13~14) (Fig. 3.6.3.) (Table 3.6.I.).

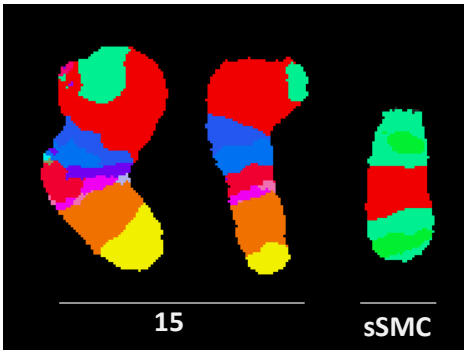

Figure 3.6.3. - Multicolour Banding (MCB) specific for chromosome 15, in the two normal chromosomes and in the sSMC of case II, revealing an inv dup(15)(q13~14).

The microdissected DNA of the sSMC was hybridized in a full tiling BAC array specific for chromosome 15, with the same resolution, having material of approximately 8.35 Mb size extension from 15q (between 18.47 Mb to 26.82 Mb). This region encompasses six genes described in OMIM morbid map [OMIM], like UBE3A and GABRB3, located in PW/ASCR [Kleefstra et al., 2010].

Case III

The patient was clinically assessed at 8 years old presenting severe psychomotor development delay; speech problems, no control of the sphincters, growth delay, autism and hyper-reactivity, and frontal lobe epilepsy (Table 3.6.I).

G-banding revealed the presence of a supernumerary marker chromosome in all the analyzed cells, the karyotype being 47,XY,+mar (Fig. 3.6.2.). The characterization of the sSMC by subcenM-FISH and MCB, revealed an inv dup(15)(q11.2). However, array CGH analysis identified the marker with material of approximately 8.35 Mb size extension from 15q (between 18.47 Mb to 26.82 Mb), larger than expected with MCB results and with the same genetic content as case II (Table 3.6.I.).

Uniparental Disomy (UPD) for chromosome 15 was excluded, with the following markers being informative: D15S659, D15S818, D15S655, D15S652, D15S816, D15S642, and D15S153.

Case IV

A female patient was clinically re-evaluated at 18 years old having psychomotor development delay; speech problems; behaviour alterations; aggressiveness; and agitation (Table 3.6.I.). The age of the mother at birth was 39 years old.

The karyotype revealed a *de novo* marker chromosome in mosaic (mos 47,XX,+mar[16]/46,XX[8]) (Fig. 3.6.2.). MCB and subcenM-FISH characterization defined the marker derived from chromosome 15, as an inv dup(15)(q13~14), with duplicated PW/ASCR. Array CGH analysis of the microdissected DNA of the marker revealed a marker with 10.47 Mb size extension, between 18.47 Mb and 28.94 Mb (Table 3.6.I.). This region encompasses more two genes described in OMIM morbid map [OMIM] comparing with cases II and III: OCA2 and HERC2, associated with skin/hair/eye pigmentation [Sturm, 2009].

Uniparental Disomy (UPD) for chromosome 15 was excluded, with the following markers being informative: D15S1507, D15S653, D15S642, and D15S153.

Case V

A female child with hypotonia; mental retardation; autism; and speech problems was assessed at 5 years old. The karyotype revealed a sSMC in all cells (47,XX,+mar), maternally derived. The origin of the marker was identified by centromeric FISH. The PW/AS critical region was identified by locus-specific FISH probes, the marker being an inv dup (with duplicated PW/AS critical region) (Fig. 3.6.4.).

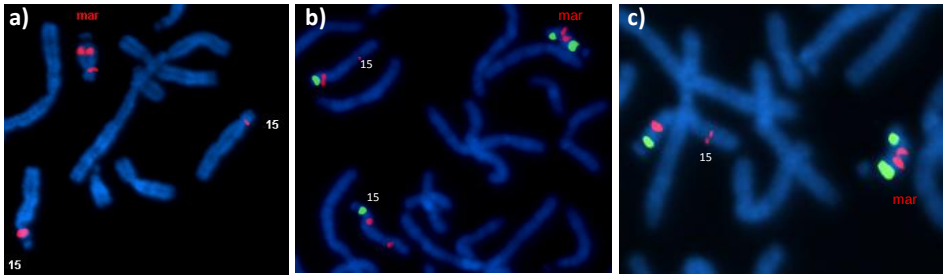

Figure 3.6.4. a) Centromeric FISH for chromosome 15 showing the origin of the sSMC in case V.; **b)** Locus-specific probes for chromosome 15 - SNRPN in red (PW/AS critical region) and D15Z1 in green; **c)** Locus-specific probes for chromosome 15 **–**D15S10 in red (PW/AS critical region) and D15Z1 in green.

Table 3.6.I. – Description of the sSMC(15)s cases characterized by molecular cytogenetics and array CGH, after microdissection of the marker.

C	Origin	Pre or Pos	UPD	Karyotype/ sSMC characterization M-FISH	Array result	OMIM*	Clinical presentation
I	*de novo*	PRE + POS	No UPD	47,XX,+mar[27]/46,XX[3] **sSMC characterization: ish. inv dup(15)(q11.2)**	sSMC size: 2.49 Mb 18.47→20.96 Mb 15q11.2→15q11.2	1	Normal child was born
II	*de novo*	POS	n.a.	47,XY,+mar **sSMC characterization: ish. inv dup(15)(q13~14)**	sSMC size: 8.35 Mb 18.47→ 26.82 Mb 15q11.2→15q13.1	6	14 years: Severe psychomotor delay; Convulsions – daily; Frequent respiratory infections
III	*de novo*	POS	No UPD	47,XY,+mar **sSMC characterization: ish. inv dup(15)(q11.2)**	sSMC size: 8.35 Mb 18.47→ 26.82 Mb 15q11.2→15q13.1	6	8 years: Severe psychomotor development delay; speech problems, no control of sphincters Growth delay Autism and hyper-reactivity Frontal lobe epilepsy
IV	*de novo*	POS	No UPD	47,XX,+mar[16]/46,XX[8] **sSMC characterization: ish. inv dup(15)(q13~14)**	sSMC size: 10.47 Mb 18.47→28.94 Mb 15q11.2→15q13.2	8	18 years: Psychomotor development delay; speech problems; behaviour alterations; aggressiveness; agitation
V	mat	POS	n.a.	47,XX,+mar **sSMC characterization: ish.inv dup(15)(q13~14)**	sSMC size: 12.38 Mb 18.47→30.85Mb 15q11.2→15q13.3	8	5 years: Mental retardation; Autism; Hypotonia; Speech problems
VI	*de novo*	POS	n.a.	47,XX,+mar **sSMC characterization: ish.inv dup(15)(q13~14)**	sSMC size: 12.38 Mb 18.47→30.85Mb 15q11.2→15q13.3	8	27 years: Low IQ; Learning disabilities (mother of V)

Mat – maternal; Pre – prenatal; Pos – postnatal; M-FISH – Multicolor Fluorescence *in situ* hybridization; Mb – Megabases; n.a. – not available; UPD – uniparental disomy; *-number of genes in OMIM morbid map; C- case.

SubcenM-FISH and MCB results identified the breakpoints of the inv dup, as inv dup(15)(q13~14) (Fig. 3.6.5.). Array CGH analysis, after microdissection of the marker, revealed a sSMC with 12.38 Mb extension, between 18.47Mb and 30.85Mb (Table 3.6.I.). This region encompasses the same number of genes (8) described in OMIM morbid map [OMIM] comparing with case IV.

Case VI

Patient VI is the mother of case V. She has the same sSMC(15) as the daughter, as characterized by MCB and SubcenM-FISH. At 27 years old she presents low IQ and learning disabilities (Table 3.9.I).

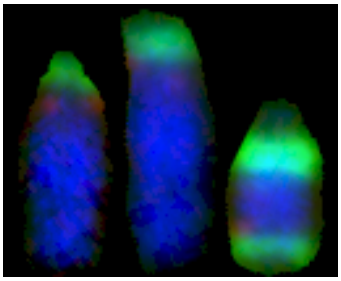

Figure 3.6.5. SubcenM-FISH specific for chromosome 15 in case V, with the two normal chromosomes 15 and the sSMC. The sSMC(15) is dicentric (centromere in green) with breakpoints between q13~q14.

3.6.3. Discussion

A wide range of structural rearrangements may occur in the 15q11->q13 region, including supernumerary marker chromosomes. In the present study, we characterized the molecular content of SMC(15), present in 6 different patients, correlating clinical features with SMC(15) sizes. One case is a small SMC(15)s, without PWS/ASCR (case I), and without clinical features and/or development delay. The other 5 cases are large SMC(15)s with PWS/ASCR (cases II, III, IV, V, and VI), all having clinical features associated. Of these large SMC(15)s described, two cases are from group B, having symmetrical pattern corresponding to BP3 (BP3:BP3) (case II and Case III). The other 3 cases have large SMC(15)s from group A, showing an asymmetrical pattern with distal margins at the known breakpoint BP4 (Fig. 3.6.6). As described in previous studies, all large SMC(15)s in this study had distal margins at the known breakpoints: at BP3 (BP3:BP3), or at BP4 and BP5 (BP4:BP5) [Wandstrat et al., 1998; Roberts et al., 2003; Wang et al., 2004; Sahoo et al., 2005; Makoff and Flomen, 2007, Kleefstra et al., 2010].

Large SMC(15)s have been associated with increased mean maternal age at conception, similar to other trisomies [Crolla et al., 1995; Dennis et al., 2006; Roberts et al., 2003; Valente et al., 2006; Battaglia et al., 2010, Kleefstra et al., 2010]. Indeed, nondisjunction is likely to play a role in the formation of all SMC(15)s. The fact that the majority of SMC(15) are nonmosaics suggests a meiotic origin of these markers and this is supported by the observations that all *de novo* SMC(15)s characterized molecularly have been shown to be maternal in origin [Crolla et al., 2005]. No paternally derived large SMC(15)s have been reported. The reasons for this remain unresolved. During spermatogenesis, cells carrying a SMC can be selected against [Cotter et al.,

2000; Eggermann et al., 2002]. Alternatively, large SMC(15)s could be lethal if paternally inherited, although paternally inherited interstitial triplications have been described [Ungaro et al., 2001] that have the same PWACR copy number as those in patients with large SMC(15).

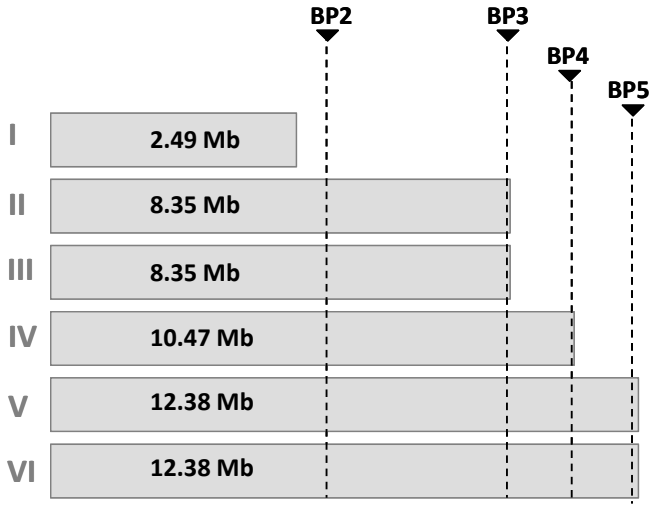

Figure 3.6.6. – Schematic representation of the 15q region involved in all the SMC(15)s described in this study. The extension of each marker is described.

The presence of a large SMC(15)s in all cells, with the PWS/ASCR, has been associated with phenotypic effects [Battaglia, 2008]. Previous reports on SMC(15) have shown that on average 80%-85% of SMC(15)s are present as nonmosaics [Leana-Cox et al., 1994; Mignon et al., 1996; Roberts et al., 2003; Crolla et al., 2005]. Mosaicism with a normal cell line has been described in a small subset of individuals [Roberts et al., 2003; Crolla et al., 2005; Loitzsch and Bartsch, 2006; Saitoh et al., 2007; Guanciali-Franchi et al., 2008]. In case IV of this study, the large SMC(15) is present in 66% of the analyzed cells (Table 3.6.I). It has been seen that the features of patients with mosaic large SMC(15) are in the phenotypic spectrum of large SMC(15)s, depending on the percentage and type of cell line which contain the SMC(15) [Dennis et al., 2006; Kleefstra et al., 2010]. To date, mosaicism for large SMC(15) (with PW/ASCR) with no phenotypic effects has been observed in very few cases [Roberts et al., 2003; Loitzsch and Bartsch, 2006; Guanciali-Franchi et al., 2008].

Numerous copies of region-specific repeats that lie within proximal 15q facilitate mispairing and unequal meiotic exchanges, which promote a relatively high frequency of rearrangements within this region. Gene expression in this critical region is regulated by an imprinting mechanism [Dittrich et al., 1996]. Only maternally inherited aberrations of chromosome 15q11-13 seem to be pathogenic, with the exception of one report [Mohandas et al., 1999]. Maternal genes, contained in this genomic region, could act in a dosage dependent manner their copy number being critical for normal brain development and function [Battaglia, 2008]. Indeed, several cases of partial hexasomy for chromosome 15q have been reported, that arose because of the presence of either two idic(15) chromosomes or a very large idic(15) chromosome, that have a clinical presentation more severe than typical cases of idic(15) [Huang and Bartley, 2003; Maggouta et al., 2003; Nietzel et al., 2003; Qumsiyeh et al., 2003; Mann et al., 2004]. It seems that whereas the size of the duplication chromosome does not seem to be directly related to the phenotype of patients with idic(15), the severity of the presentation of these hexasomic patients indicates that increasing dosage of the region has a negative effect on outcome [Mann et al., 2004].

In our cohort, patients with large SMC(15)s presented common features of idic(15) syndrome, like mental retardation or psychomotor development delay, epilepsy, or autistic-like behaviour, that is associated with high prevalence of behavioural problems. As pinpointed by Kleefstra and co-workers, these features could be explained by a dosage effect of various genes located in the 15q11.2->q13 region like: GABRB5, GABRB3, APBA2, CHRFAM7A, and SGNE1 that encode proteins present in nervous tissue; and NIPA1, CHRNA7, and UBE3A that show a possible association with neurologic and neuropsychiatric disorders [Kleefstra et al., 2010]. We did not identify significant differences in phenotype between patients with type A and type B large SMC(15)s. This observation is in concordance with other studies that reported phenotypic data of types A and B SMC(15) separately [Dennis et al., 2006; Kleefstra et al., 2010]. As expected, the patient with a small SMC(15), without PW/ASCR, presents a normal phenotype.

In one patient (case III) multicolour FISH analysis (with MCB and SubcenM-FISH) showed a different size of the SMC(15) compared to the assessment by array CGH analysis. Additionally, routine cytogenetic analysis was not an accurate method to determine the size of the SMC(15). A full characterization of SMC(15) by array CGH techniques is essential to establish with more precision breakpoints position and size of duplicated segments. Array CGH analysis, after microdissection of the marker, showed to be a powerful approach to detect the duplication and its extent.

3.6.4. References

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3.7. *sSMCs from chromosome 16*

Molecular cytogenetic characterization of two cases with *de novo* small mosaic supernumerary marker chromosomes derived from chromosome 16: Towards a genotype/phenotype correlation [*]

[*] The work presented in this section was published in:

Melo JB, Matoso E, Polityko A, Saraiva J, Backx L, Vermeesch JR, Kosyakova N, Ewers E, Liehr T, Carreira IM (2009) *Cytogenetic and Genome Research* 125:109-14.

3.7.1. Introduction

The chromosome 16 long arm (16q) is characterized by the presence of a block of heterochromatin in the pericentromeric region (16q11.1→q11.2). Cases with an sSMC consisting of only these centromeric and heterochromatin regions do not show clinical manifestations [Callen et al., 1990, 1991; Crolla, 1998; Paoloni-Giacobino et al., 1998; Sanz et al., 2000]. However, trisomy involving band 16q12 is usually associated with a clinical impact [Barber et al., 2006].

We present two cases with an sSMC derived from chromosome 16. These cases were characterized by molecular cytogenetics, namely with centromere specific FISH probes and subcenM-FISH. Case A was also characterized by reverse FISH and array painting, using a full-tiling BAC array specific for chromosome 16 (with resolution of 100--150 kb). The characterization of these sSMC demonstrated the involvement of 16q12 euchromatin in both markers.

3.7.2. Results

Case A:

A prenatal diagnosis due to advanced maternal age with no ultrasound anomalies was performed in the 16th week of gestation. Autopsy was done at 23 weeks, revealing a female fetus in the 50th percentile with signals of maceration and signals of acute anoxia. The fetus had an extra suprarenal gland near the left ovary and only slight facial dysmorphisms, namely discrete hypertelorism, large philtrum and asymmetrical implantation of the ears.

GTG-banded amniocytes were studied revealing a female karyotype with an sSMC in 24% of the analyzed metaphases. The marker chromosome was very small and

appeared to have a ring shape (Fig. 3.7.1.A). Karyotypes of both parents, performed in cultured lymphocytes, were normal (data not shown). The de novo sSMC was positive with C-banding, suggesting that the marker chromosome contained heterochromatin (Fig. 3.7.1.B).

Centromere FISH, using probe D16Z1–revealed that the sSMC was derived from chromosome 16 (data not shown). All-telomere probe was negative in the sSMC (Fig. 3.7.2.A). Reverse FISH confirmed the origin of the marker and suggested the involvement of the euchromatic region of 16q12 in the sSMC **(fig. 2B)**. SubcenM-FISH specific for chromosome 16 confirmed the ring-shape of the marker and demonstrated that the sSMC possessed euchromatic material from 16q, the karyotype being mos 47,XX,+r(16)(::p11.1→q12.1~q12.2::)[25%]/46,XX[75%] (Fig. 3.7.2.C). With array-CGH the breakpoints of the marker were redefined to 16p11.2 and 16q12.1. Thus, the sSMC led to a partial trisomy of 33.43 Mb to 47.02 Mb on chromosome 16 (Fig. 3.7.3.).

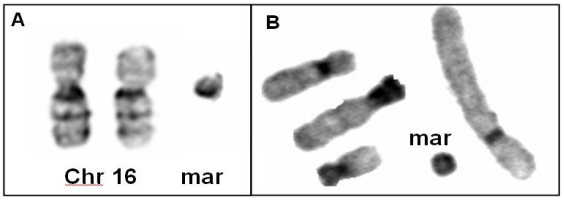

Figure 3.7.1. - A. GTG banded images of both normal chromosomes 16 and of the marker chromosome of case A. **B.** CBG banded images showing the marker chromosome of case A. The supernumerary marker chromosome is designated as *mar.*

Case B:

A cytogenetic study of a boy presenting congenital malformations, born at term, with birth weight 3200 g and length 50 cm, was done. At 13 years of age the weight was 47 kg, length 160.5 cm and head circumference 54 cm. He presented low-set dysplastic ears, hypoplastic testes and cryptorchidism. No further clinical details were available.

GTG-banding in metaphase spreads from lymphocytes revealed a male karyotype with a ring shape sSMC in 50% of the analyzed cells. Karyotypes of both parents were normal (data not shown). Centromere FISH revealed that the sSMC derived from chromosome 16 and subcenM-FISH specific for chromosome 16 demonstrated a

karyotype mos 47,XY,+r(16)(::p11.1→q12::)[7]/46,XY[7] (results not shown due to similarity of the figures).

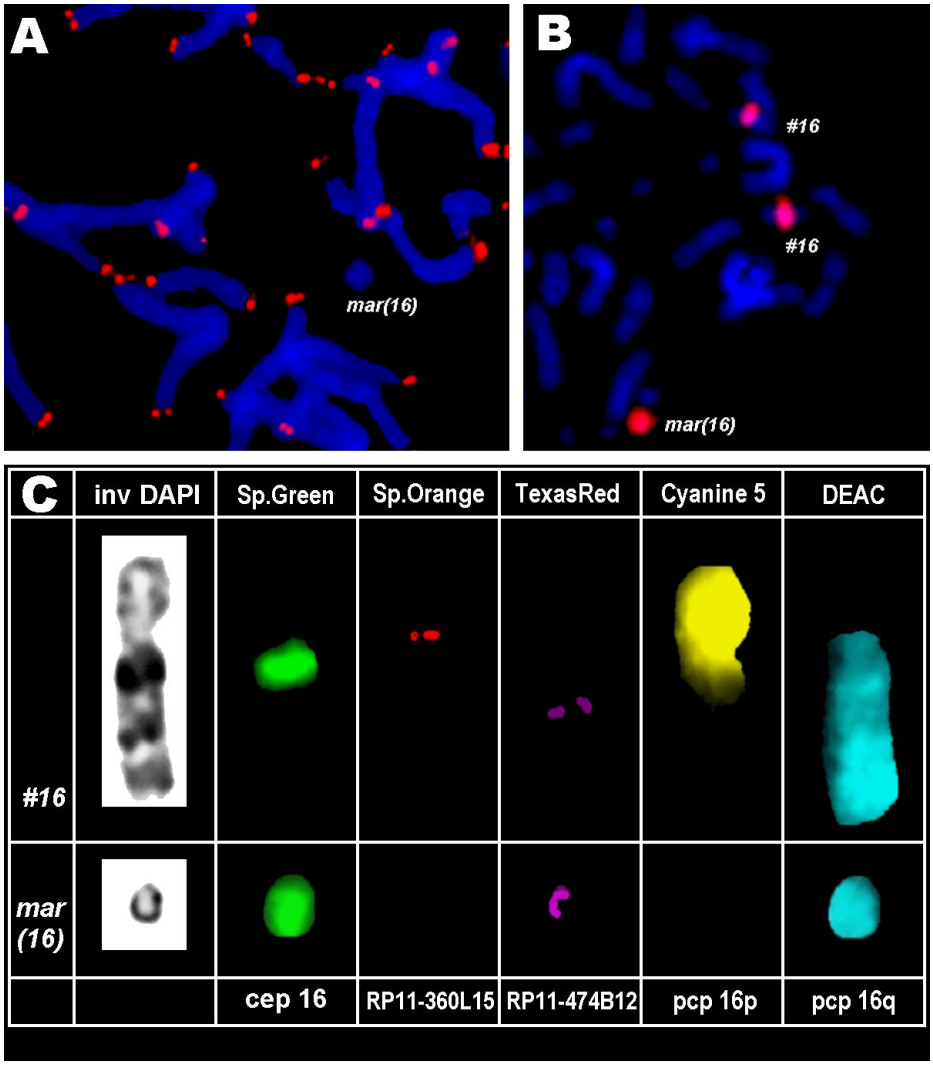

Figure 3.7.2. – FISH using different probes in cases A (subfigures A-C). The supernumerary marker chromosome is signed as *mar(16)*. **A.** Image of a hybridization of an all telomeric probe, showing an absence of signs in the small supernumerary marker in case A. **B.** Reverse FISH image confirming the origin of the sSMC and the extent of the chromosome 16 involved in the marker of case A. The sSMC was microdissected and the DNA was amplified, labeled and hybridized in a patient metaphase. **C.** FISH image applying the subcentromeric probes for chromosome 16, revealing in case A that the centromere-near region in p-arm (RP11-360L15; in red) is absent in the marker, the centromere-near region in q-arm (RP11-474-B12; in purple) is present in the marker, being the sSMC mainly derived from q-arm (in blue). For space reasons only one of the two normal chromosomes 16 is shown.

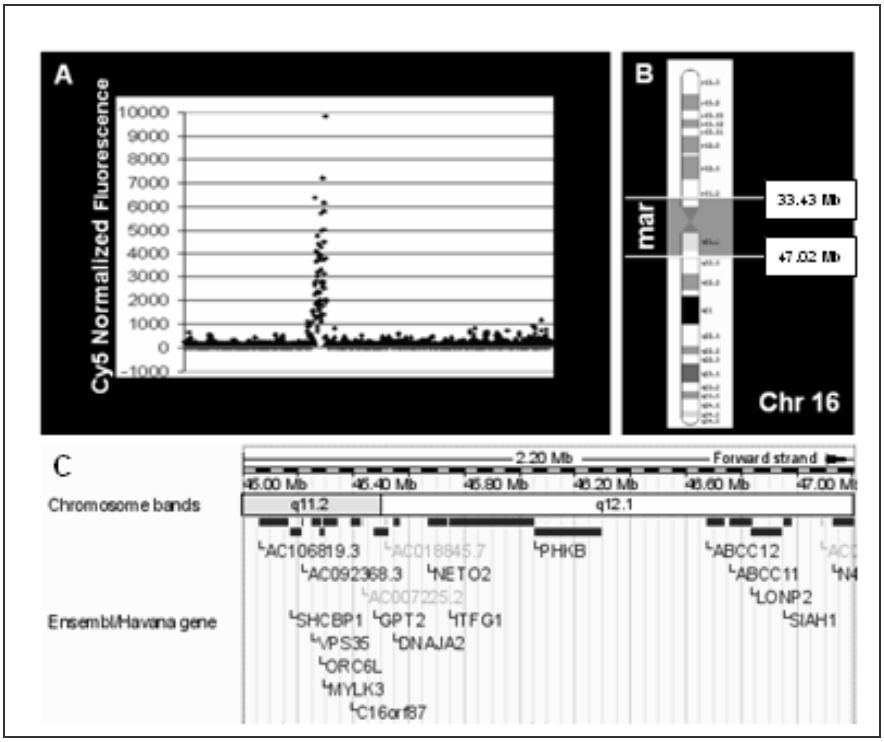

Fig. 3.7.3. – A. Array painting result of the marker chromosome in case A. **A.** The plot shows a gain for region 16p11.2 to 16q12.1 with breakpoints mapping at BAC clones RP11-258P17 and RP11-520B19**. B.** Ideogram of chromosome 16 showing the region involved in the sSMC, corresponding to the flanking BAC clones identified in 3A. C. Diagram from Ensembl (www.ensembl.org) showing the genes present in the region between 16q11.2 and 16q12.1, which is the euchromatic content of the sSMC from case A.

3.7.3. Discussion

If a structural chromosome rearrangement is diagnosed in a pregnancy it is crucial to determine the implications of this particular abnormality in order to counsel the parents in their decision for a suitable course of action [Gardner and Sutherland, 2004]. If neither a family history nor ultrasound fetal abnormalities exist and a chromosome aberration is detected, the prognosis for the fetus is largely deduced from the results of the cytogenetic analysis. Depending on the type of aberration, the risk for abnormalities and/or mental retardation ranges from almost nil in case of amplification of heterochromatic material to a high risk in the presence of genetically active euchromatin [Trimborn et al., 2006]. Indeed, heterochromatic C-band negative variants of 16q11.2 involving α-satellite DNA have been described in the literature

[Jalal et al., 1993]. The carriers show neither phenotypic abnormalities nor mental retardation. There are several reports describing the presence of an sSMC from chromosome 16 associated with no clinical manifestations [Callen et al., 1990, 1991; Crolla, 1998; Paoloni-Giacobino et al., 1998; Sanz et al., 2000; Hengstschlager et al., 2001; Bartsch et al., 2005, Pater et al., 2006, Karaman et al., 2006, Rodríguez et al., 2008]. In only a few of these cases the markers have been characterized extensively, however their content seems to be limited to the centromeric and heterochromatin regions. Trisomy involving band 16q12 is usually associated with clinical impact [reviewed by Barber et al., 2006]. It seems that individuals with duplications of this proximal 16q do not have characteristic facies but frequently have short stature, developmental and speech delay, learning difficulties and behavioral problems which range from mild to severe [Barber et al., 2006]. However, a recent report showed that not all euchromatic trisomies of 16q12 have a clinical repercussion [Rodríguez et al., 2008]. Indeed, the authors showed the presence of a partial trisomy of the long arm of chromosome 16, involving the heterochromatin block and the proximal euchromatin region at 16q12 in a healthy patient. This discrepancy could be attributed to the fact that the sSMC reported in this case was present in a mosaic form (~65% of the analyzed cells).

To the best of our knowledge there are 6 reported cases of sSMC derived from chromosome 16 with clinical findings as described in table 1 [Krauss et al., 1987; Liehr et al., 2006; Crolla et al., 1998; Aviv et al., 2005; Avela et al., 2007; Baldwin et al., 2008]. In only 3 of these cases (case 1, 2 and 6) the sSMC is characterized sufficiently. Case 1 is a postnatal case with a minute shaped centric marker with material from 16p11.1 to 16q11.2. Case 2 is a ring 16 with p10 to q13 material but associated with a deletion in chromosome 1. Case 6 is a postnatal case with an sSMC consisting of 16p11.2 to 16q11.2 characterized by array-CGH, but with a diagnosis of Beckwith-Wiedemann syndrome, associated with alterations in the methylation pattern of the 11p15 region. Therefore, it is difficult to assess genotype/phenotype correlations in carriers of sSMCs derived from chromosome 16. However, taking into account these reports and the two cases reported here, the presence of the proximal euchromatic region of 16q12 (between 45.5 and 47.02 Mb) in the sSMC seems to result in broad clinical

consequences (Table 3.6.I), although more cases of affected and unaffected individuals with sSMC from chromosome 16 should be reported in order to confirm this observation. In both cases reported here and in the sSMC described by Krauss and colleagues [Krauss et al., 1987] the patients have broad facial dysmorphism, and in common alterations related to ear implantation (Table 3.7.I).

Some protein-coding genes have been located in band 16q12, the region present in the sSMC of case A reported here (Fig 3.7.3.C). These include the E3 ubiquitin protein ligase gene *(SIAH1)*, the peroxisomal Lon protease homologue 2 gene *(LONP2)*, two multidrug resistance-associated proteins genes *(ABCC11* and *ABCC12)*, the beta subunit gene of phosphorylase kinase gene *(PHKB)* which is an enzyme that activates glycogen phosphorylases in muscle, liver, and other tissues, the gene for a precursor a T-cell immunomodulatory protein *(ITFG1)*, the gene for a brain specific transmembrane protein *(NETO2)*, the gene for a protein important for cell cycle progression restoration *(DNAJA2)*, the alanine aminotransferase-2 gene *(GPT2)*, a gene of the family of myosin light chain kinases *(MYLK3)*, a gene from ORC family *(ORC6L)*, a gene related with vacuolar protein sorting-associated proteins (VPS35) and a gene of the family of SHC-SH_2 domain binding proteins *(SHCBP1)* (www.ensembl.org).

In conclusion, molecular cytogenetic techniques, namely subcen-FISH and especially array CGH-based techniques, allow a more accurate characterization of the heterochromatic/euchromatic content of an sSMC, which is important for the establishment of genotype-phenotype correlation and for the improvement of prenatal counseling and patient follow-up.

Table 3.7.I - Description of previously reported cases with a sSMC derived from chromosome 16 with clinical findings and the two cases reported here.

Case	age	de novo/ inherited	Karyotype	clinical symptoms	Ref.
1	2 years	de novo	47,XY,+min(16)(:p11.1→q11.1 or q11.1→p11.1::p11.1→q11.2:) [6%]	normal at birth, edema at hands at 9m; little asymmetry of skull, no dysmorphic signs, slight penis anomaly	Liehr et al., 2006 *case 16-1*
2	23 years	n.a.	47,XX,del(16)(p10q13), +r(16)(::p10→q13::)[100%]	mild general hypotonia, Head circumference 51cm = very small; bow shaped mouth, abnormal posterior rotated ears; detected due to a malformed child without the mar but with the del(16) chromosome	Krauss et al., 1987
3	30 years	de novo	47,XY,+mar(16).ish(DZ16Z2+; D16Z3+, wcp16-) [100%]	mild mental handicap; psychosis; schizophrenia, normal appearance without abnormalities	Crolla et al., 1998 *case 21*
4	Pre natal	de novo	47,XX,+mar(16) [88%]	amniocentesis due to posterior fossa cyst with extension of the region of cerebellar vermis with splaying of cerebellar hemispheres suggesting Dandy-Walker complex in week 20 of gestation; termination of pregnancy in week 23; no autopsy, external examination showed normal female phenotype.	Aviv et al., 2005
5	Pos natal	de novo	47,XY,+mar(16).ish(DZ16Z2+; D16Z3+, wcp16-) [50%]	Patient born at term after normal pregnancy and delivery: 3150g, 51cm, Head circumference 35.5cm. facial dysmorphic; epicanthic folds, thin, distinct eyebrows, simian crease in both hands, short neck, ventricular septal defect and open foramen ovale, delayed development; up to 3y of age patient progressed at -2SD, weight +5% and Head circumference at -2.5 SD curve.	Avela et al., 2007
6	Pos natal	de novo	47,XX,+mar(16)(:p11.2→q11.2:) [85%] size p 0.7MB (array CGH)	macroglossia, dysmorphic features, height, weight and OFC ~90 centile; mild gross motor delay, asymmetric lower extremities; clinical features suggestive of Beckwith-Wiedemann syndrome (BWS)- testing for LIT1 methylation confirmed a diagnosis of BWS.	Baldwin et al., 2008
7	Pre natal	de novo	47,XX,+r(16)(::p11.2→q12.1::) [24%]	TOP, extra supra-renal, slight facial alterations, like discrete hypertelorism, large *philtrum* and asymmetrical implantation of the ears	Case A
8	13 years	de novo	47,XY,+r(16)(::p11.1→q12::) [50%]	congenital malformations, born at term, low-set dysplastic ears, hypoplastic testes, cryptorchidism	Case B

Adapted from http://www.med.uni-jena.de/fish/sSMC/00START.htm
n.a. – not available; SD – standard deviation; TOP = termination of pregnancy

3.7.4. References

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3.8. - *sSMC from chromosome 17*

Prenatal diagnosis of a small supernumerary marker chromosome 17 with a normal phenotypic outcome [*]

[*]The work presented in this section is under submission:

Melo JB, Jardim A, Saraiva J, Backx L Vermeesch JR, von Eggeling F, Kosyakova N, Liehr T, Carreira IM. Prenatal diagnosis of a small supernumerary marker chromosome 17 with a normal phenotypic outcome. Submitted to *Cytogenetic and Genome Research.*

3.8.1. Introduction

Small supernumerary marker chromosomes (sSMCs) are structurally abnormal chromosomes that cannot be identified or characterized unambiguously by classic cytogenetics analysis alone, and are (in general) equal in size or smaller than a chromosome 20 of the same metaphase spread [Liehr et al., 2004]. Phenotypes associated with a sSMC are variable, from normal to severely affected. It has been shown that, depending on the chromosomal region of a sSMC, differences can be observed and expected in the clinical outcome. Also other factors like uniparental disomy, mosaicism and mode of inheritance have to be considered. When detected prenatally, *de novo* sSMCs constitute a major problem for cytogenetic investigation and genetic counselling, due to time availability and, essentially, due to the difficulty, in the majority of cases, in establishing a robust genotype/phenotype correlation.

The finding of unbalanced chromosomal abnormalities (UBCAs) was reviewed and summarized from a total of 200 families [Barber, 2005; http://www.ngrl.org.uk/Wessex/collection/ubca_chart.htm]. UBCAs involve the addition (duplication) or removal (deletion) of one of the two copies of each chromosomal segment present in normal diploid individuals. UBCAs usually involve several megabases of DNA, and carriers are usually ascertained either through an abnormal phenotype or because of adverse reproductive effects. UBCAs have also been reported in families for “incidental” reasons such as prenatal diagnosis because of maternal age [Barber, 2005].

UBCAs without phenotypic effect are frequently not well characterized and so they could be more common than is apparent from the literature. We report a possible new chromosomal region involved in a cytogenetic UBCA, in a case of a sSMC detected

through prenatal diagnosis. The sSMC was characterized by molecular cytogenetics, namely with subcenM-FISH, and by array painting, using a full-tiling BAC array specific for chromosome17.

3.8.2. Results

Case report

A pregnant woman was referred for amniocentesis, at 20 weeks gestation, due to advance maternal age. The pregnancy was normal with no relevant familiar history. High resolution ultrasound did not show any abnormality in the female foetus.

She was born at 38 weeks gestation by normal delivery, with a birth weight of 2,900 kg and an Apgar score of 9/10/10. The clinical evaluation at birth was normal. She was clinically reassessed at 2 years of age, being a healthy child, with normal growth and development, and with no malformations nor dysmorphisms.

Cytogenetics and Molecular Cytogenetics

Routine cytogenetics with GTG-banding documented the presence of a centric minute-shaped sSMC (47,XX,+mar) in 83 % of the analysed metaphases. Whole chromosome painting (WCP) identified the origin of the marker as being from chromosome 17. The parents had normal karyotypes. The sSMC was later characterized comprehensively for their genetic content by molecular cytogenetics using subcentromere-specific multicolor fluorescence *in situ* hybridization (subcenM-FISH) [Starke et al., 2003], and by array Comparative Genomic Hybridization (Array CGH) after microdissection and amplification of the sSMC by degenerated oligonucleotide polymerase chain reaction [Melo et al., 2009]. SubcenM-FISH results confirmed the origin of the sSMC as being a der(17)(::p11.2->q11.1::) (Fig.3.8.A). The microdissected DNA of the marker was hybridized in a full tiling BAC array specific for chromosome 17, with a resolution of 100-150 Kb (VIB MicroArrays Facility - www.microarrays.be), having material of approximately 3.27 Mb size extension from 17p (between 19.32~19.74 Mb to 22.15~22.59 Mb) (Fig. 3.8.B and C).

Uniparental Disomy analysis

Uniparental Disomy (UPD) was excluded in this patient for chromosome 17, with the following markers being informative: D17S130; D17S520; D17S1293; D17S1795; D17S1290; D17S1301.

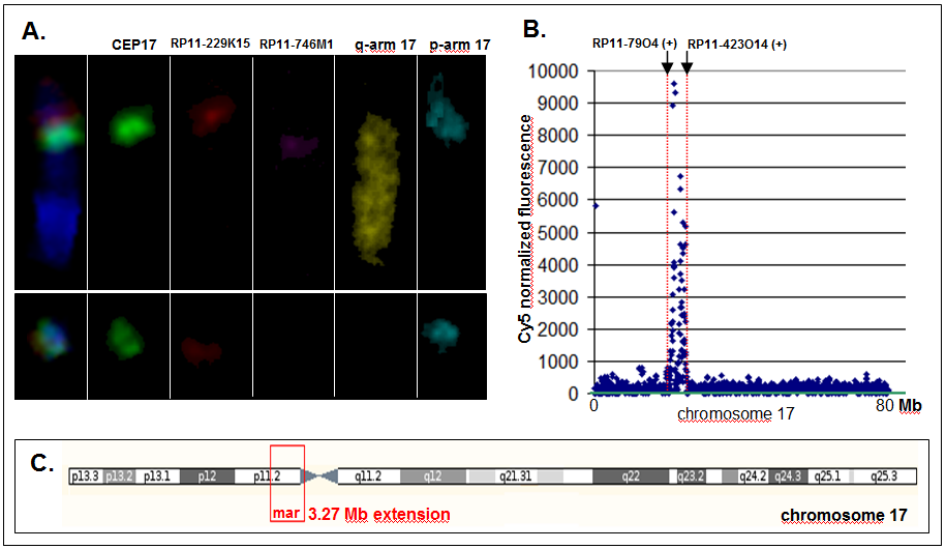

Figure 3.8. –A. SubcenM-FISH for chromosome 17, revealing that the centromere-near region in q-arm (RP11-229K15, in purple) is absent and the centromere-near region in p-arm (RP11-746M1, in red) is present in the marker, being the sSMC derived from p-arm (in blue). The centromeric probe – CEP17 is identified in green and partial chromosome painting of 17q in yellow (for space reasons only one of the two normal chromosomes 17 is shown). **B.** Array CGH result after microdissection and amplification of the sSMC(17). The plot shows a gain for region 17p11.2 to 17q11.1, between 19.32~19.74 Mb to 22.15~22.59 Mb with the flanking BAC clones present in the sSMC, RP11-79O4 (17p11.2) and RP11-423O14 (17q11.1). **C.** Ideogram of chromosome 17 showing the region (red square) involved in the sSMC, corresponding to the flanking BAC clones identified in 1.B.

3.6.3. Discussion

In the literature there are 15 cases reported of sSMC derived from chromosome 17, 3 of them being associated with a normal phenotype [Friedman et al., 1992; Starke et al. 2003 – case 25; Bartsch et al., 2005 – case 30] (Table 3.8.I) and the others associated with affected phenotypes [Shabtai et al., 1979; Brondum-Nielsen and Mikkelsen, 1995 – case 11; Morrison et al., 1997; Kozma et al., 1998; Kulharya et al., 1998; Stankiewicz et al., 2001; Dupont et al., 2003; Shaw et al., 2004 – patient 1934 and patient 2170; Yatsenko et al., 2005; Baldwin et al. 2008 – case 17; Lee et al., 2009; Manolakos et al., 2010].

Table 3.8.I – Cases described in the literature with a sSMC derived from chromosome 17 and without clinical findings

Case	sSMC shape and content	%Mosaicism*	sSMC Characterization
Bartsch et al., 2005 (case 3)	r(17)(::p1?1→q1?1::)	68%	FISH: centromeric probes, wcp 17
Friedman et al., 1992	min(17)(:p11.2→q10:)	89%	n.a.
Starke et al. 2003 (case 25)	min(17)(:p11.2→q11.1:)	66%	SubcenM-FISH
Present case	min(17)(::p11.2->q11.1::) 19.32~19.74-22.15~22.59 Mb	83%	SubcenM-FISH Array CGH

*%of metaphases with a sSMC; n.a. – not available information

Apart from the present report, in the literature there is only one comparable case with a similar imbalance in 17p and with a good molecular cytogenetic characterization [Starke et al., 2003]. Additional chromosomal material of proximal 17p11.2 apparently does not contribute to an abnormal phenotype. However, this observation should be confirmed with other cases, and the degree of mosaicism should be considered.

Unbalanced chromosomal abnormalities without phenotypic effect are frequently not published and therefore more common than is apparent from the literature. The data published by Barber and colleagues [Barber et al., 2005] are consistent with the idea that microscopic and submicroscopic imbalances of multiple evolutionarily conserved *loci* can be compatible with a normal phenotype. However, it should be taken into account that few of the children who were reportedly normal at term in cases ascertained at prenatal diagnosis for maternal age, have been followed up over a period of years by a medical geneticist.

In summary, the presence of a *de novo* sSMC in prenatal diagnosis presents a problem for genetic counselling. The time to investigate the genetic content of the marker is limited and the degree of mosaicism might influence the phenotype. It is important to report all cases of sSMC associated or not with clinical findings. A good molecular cytogenetic content characterization is essential for a robust genotype/phenotype correlation. A chromosomal imbalance of 17p11.2, involving a partial trisomy of 19.32~19.74 Mb to 22.15~22.59 Mb region, does not seem to be correlated with phenotypic effects. It is important to quickly and clearly characterize prenatal sSMCs, with molecular cytogenetic techniques such as SubcenM-FISH or array CGH. The knowledge of the exact genetic content of the marker, the determination of the levels

of mosaicism, and the exclusion of UPD will definitely help genetic counselling when a sSMC is encountered at prenatal diagnosis.

3.8.4. References

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3.9. *sSMCs from chromosome 18*

Three unusual but cytogenetically similar cases with up to five different cell lines involving structural and numerical abnormalities of chromosome 18 [*]

[*] The work presented in this section was published in:

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3.9.1. Introduction

The occurrence of structural abnormalities that involve chromosome 18 is relatively high [Baumer et al., 2002; Miller et al., 2003], being the most frequently ones deletions and ring formation. About 70 cases have been reported with ring chromosome 18 and most of them are female [Schinzel, 2001].The phenotypic variability observed in patients with r(18) depends primarily on the extension of the deleted chromosome segment [Stankiewicz et al., 2001]. In most cases patients that present r(18) show a phenotype like the 18q- which is highly variable, probably depending on the extent of the terminal or interstitial 18q deletions [Brkanac et al., 1998; Cody et al., 1999; Engelen et al., 2003]. Few cases share the phenotype like 18p- syndrome or, a combination of these two syndromes [Schinzel, 2001; Stankiewicz et al., 2001; Bird et al., 1997]. In general, it is thought that malformations in these patients result of terminal deletions of both arms of the chromosome [Vermeesch et al., 2002]. Rings often appear as mosaics and in some cases loss of the ring, double sized rings or multiple copies of the ring have been observed, as a consequence of structural instability of the ring during cell division [Miller et al., 2003; Baumer et al., 2002]. Mosaicism involving structural and numerical chromosome anomalies in a single individual is rare [Sutcliffe et al., 2001; Eiben et al., 1992]. We present two prenatal and two postnatal cases (twins) showing mosaicism for; rings, marker and monosomy 18. In these cases the application of several high resolution molecular cytogenetics techniques such as, FISH using centromeric probes, MCB and locus specific probes for chromosome 18 were important to increase the precision of the definition of the rearrangement itself, as to define more accurately the level of mosaicism, attained by the evaluation of a larger number of cells.

3.9.2. Results

Case Report

Case A

Prenatal diagnosis of a 34 year-old gravida was performed in the 14th week of gestation due to ultrasound malformations including holoprosencephaly (HPE) and hypotelorism (Figure 3.9.1.A). It was the first gestation of a non-consanguineous healthy couple. After cytogenetic evaluation of the amniocytes (results see below) the parents chose, after genetic counselling, to terminate the pregnancy in the 15th week of gestation. An autopsy was performed and the anatomopathologic study revealed a foetus with female-like gonads, holoprosencephaly (Figure 3.9.1.B), low set ears and without other internal or external malformations.

Case B

A prenatal diagnosis was performed in a 27 year-old pregnant woman in the 28th week of gestation due to abnormal ultrasound result of the heart, suggesting a vitium cordis, i.e. pulmonar stenosis with poststenotic dilatation and hypoplastic right ventricle. The pregnancy was terminated at the 34th gestational week. Autopsy revealed deep setting ears, large back of the head and flat arch of feet. Moreover, the presence of a valvulary pulmonar stenosis with poststenotic dilatation was confirmed and it was also detected a premature closure of ductus botalii, and an obstructive fetal uropathy with left sided ureterstenosis including dilatation of renal pelvis and urethra-dilatation plus fibrosis.

Cases C1 and C2

Monozygotic twins were born to healthy Caucasian, non-consanguineous parents. The mother, a 1st gravida, was 28 years old at delivery. After an uneventful pregnancy, the girls were delivered by caesarean section at 36 weeks of gestation. At birth, auxological data for the first twin were, weight 2.180g [<3rd centile], length 43cm [4th centile], head circumference [OFC] 31.5cm [20th centile], and for the second twin weight 1.460g, length 40cm, OFC 29.5cm [all <3rd centile]; Apgar scores were 9/10/10, respectively.

According to similar abnormal clinical features observed in both girls, like minimal dysmorphisms including a smooth philtrum, a thin upper lip, low set ears, wide spaced

nipples, and clinodactyly of the fifth finger, cytogenetic analysis from lymphocytes was done.

Developmental delay was first observed at 6 months. Independent walking was achieved at the age of 20 months. At the age of 4 they spoke short sentences of 3 – 4 words. At the age of 8 years they were able to read single words and it was expected that they will acquire adequate reading abilities. Both girls displayed growth retardation with all growth parameters developing below the 3rd centile. At the age of 8 4/12 years, height was 107.8cm (- 4,7 SDS) for the first twin and 103.7cm (-5,5 SDS) for the second twin. Ossification was delayed according to an age of 5 9/12 years and 5 years, respectively. Growth hormone and related factors (IGFI and IGFBP3) were measured in the lower normal range at several occasions. Ultrasound examination excluded major malformations of internal organs. MRI of the brain performed in the first twin at the age of 4 years showed an Arnold-Chiari-I malformation without hydrocephalus and delayed myelination. Seizures have not been observed. Major clinical problems were recurrent infections, bronchial hyper reactivity, dry and eczematic skin.

Cytogenetic Results

Case A

Cytogenetic analysis of G-banded chromosomes after the first passage from different cultures lines of amniotic fluid cells, using flask technique, revealed a mosaicism involving two cell lines: mos 46,XY,r(18)[36]/45,XY,-18[7] . Fluorescence *in situ* hybridization (FISH) was performed in order to clarify the structural rearrangement and the distribution of the different cell lines. Chromosome 18 centromeric probe D18Z1 showed signals on both, the normal chromosome 18 and the ring chromosomes. With this probe was also possible to identify a metaphase with a duplicated ring and another with a double ring. Hybridization with subtelomeric probes D18S552 (18p) and D18S1390 (18q), showed normal signals on the normal chromosome and deletion of both terminal regions in the ring chromosomes (data not

shown). With these FISH studies it was possible to identify two extra cell lines, so that the karyotype was:

mos 46,XY,r(18)[36]/45,XY,-18[7]/47,XY,+r(18)x2[1]/46,XY,dupr(18)[1] (Fig. 3.9.1.C) (Table 3.9.I).

After termination of the pregnancy a cytogenetic analysis and FISH studies were performed in the fibroblasts of the expelled foetus after the third passage. A new cell line with a small marker chromosome was detected, as well as three of the cell lines observed in the amniocytes. The line with two rings, observed in the amniocytes, was not found in the fibroblasts. Thus, result of the cytogenetic analysis in this tissue was: mos 46,XY,r(18)[89]/45,XY,-18[22]/46,XY,dupr(18)[2]/ 46,XY,mar(18)[4]. FISH with a centromere 18 specific probe was also performed to determine the origin of the marker chromosome, which was found to contain material from the pericentromeric region of chromosome 18 (Fig. 3.9.1.E).

Applying subtelomeric probes 18p and 18q (Figure 1D), subcenM-FISH (Figure 1E) and MCB (Fig. 3.9.1.F) analysis, only the most frequent variant of the derivative chromosome 18 could be detected. Thus, a final karyotype like the following can be suggested:

mos 46,XY,r(18)(::p11.1→::q22)/45,XY,-18/

46,XY,dupr(18)(::p11.1→q22::)/46,XY,del(18)(:p11.1→q12:) (Table 3.9.I).

Karyotypes of both parents were normal.

Case B

After G-banding the amniocytes, two cell lines were accounted for with the following karyotype: 46,XY,r(18)[90%]/45,XY,-18[10%].

MCB analyses revealed a complex karyotype with five cell lines: r(18)(::p11.2?2→q21.?2::)[12]/r(18;18)(::p11.2?2→q21.?2::p11.2?2→q21.?2::)[4]/r(18;18)(::p11.2?2→q21.?2::p11.2?2→q21.?2::)x2[1]/del(18)(:p11.21→q11.2:)[1]/ace(18)(:q11.2→q12.?2:)[1] (Fig. 3.9.2, Table 3.9.I). Karyotypes of both parents were normal.

Cases C1 and C2

Structurally abnormal female karyotypes with ring chromosomes 18 [karyotype 46,XX,r(18)(p11.32q22.3)de novo] were observed in both girls without evidence for

mosaicism. MCB, subcenM-FISH and application of subtelomeric probes in combination with centromeric probes were very useful because they allowed the identification of adicional cell lines and a variety of rearrangemnts revealing therefore differing complex karyotypes in each of the twins (see Table 3.8.I).

Parental karyotypes were normal. Two younger sisters of the twins are healthy.

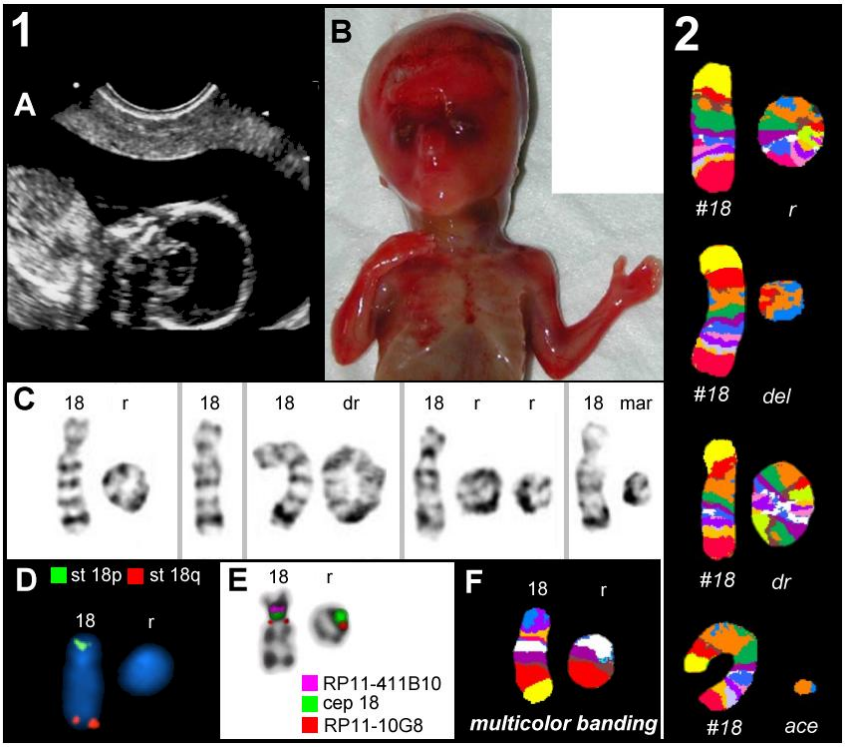

Figure 3.9.1. - A) Sonography revealed in case A a holoprosencephaly.**; B)** Frontal view of the autopsy of the fetus at 17 weeks, showing the holoprosencephaly; **C)** Partial G-banded karyotype showing the normal chromosome 18, the single ring (r), double ring (dr), two rings and the marker (mar) in case A; **D)** Commercially available FISH subtelomeric probes (Abbott/Vysis) for chromosome 18p (st 18p) and18q (st 18q) demonstrated that both corresponding regions are deleted in the ring chromosome of this case; **E)** FISH applying subcentromeric probes revealed that the centromere-near region in 18p11.21 was absent and the centromere-near region in 18q11.21 was present on the ring.; **F)** Multicolor banding (MCB) confirmed the aforementioned findings.

Figure 3.9.2 - MCB results obtained in case B, besides a normal chromosome 18 either a ring chromosome (r), a double ring (dr) a minute shaped chromosome (del) or an acentric fragment (ace) was present.

3.8.3. Discussion

We report here 4 cases, 2 prenatal and 2 postnatal, with complex karyotypic changes involving the formation of rings of chromosome 18.

Table 3.9.I - Karyotype after application of MCB, subtelomeric probes 18pter and 18qter in combination with the centromeric probe for chromosome 18 and subcenM-FISH in the four cases. As highly complex karyotypes were present in all cases slightly different results were obtained for the probe sets used, as listed here.

Case	Karyotype
Case A	
Amniocytes	
Routine cytogenetics	mos 46,XY,r(18)[36]/45,XY-18[7]
Results after MCB	mos 46,XY,r(18)(::p11.1→:q22)/45,XY,-18/46,XY,dupr(18) (::p11.1→q22::)/46,XY,del(18)(:p11.1→q12:)
Results after subtelomeric and centromeric probes	mos 46,XY,r(18)(::p11.1→:q22)[36]/45,XY,-18[7]/ 47,XY,-18, +r(18)(::p11.1→::q22)x2[1]/46,XY,dupr(18)(::p11.1→q22::) [1]
Fibroblasts	
Routine cytogenetics	mos 46,XY,r(18)[89]/45,XY-18[22]/46,XY,dupr(18)[2]/ 46,XY,mar(18)[4]
Results after subtelomeric and centromeric probes	mos 46,XY,r(18)(::p11.1→::q22)[89]/45,XY,-18[22]/ 46,XY,dupr(18)(::p11.1→q22::)[2]/46,XY,mar(18)
Case B	
Routine cytogenetics	46,XY,r(18)[90%]/45,XY,-18[10%]
Results after MCB	r(18)(::p11.2?2→q21.?2::)[12]/r(18;18)(::p11.2?2→q21.?2::p11.2?2→q21.?2::)[4]/r(18;18)(::p11.2?2→q21.?2::p11.2?2→q21.?2:)x2[1]/del(18)(:p11.21→q11.2)[1]/ace(18)(:q11.2→q12.?2)[1]
Case C1	
Routine cytogenetics	46,XX,r(18)(p11.32q22.3)
Results after MCB	46,XX,r(18)(::p11.32→q23::)[8]/46,XX,r(18)(::p11.32→q23::p11.32→q23::)[4]/45,XX,-18[2]
Results after subtelomeric and centromeric probes	46,XX,r(18)(::p11.32→q23::)[11]/46,XX,r(18)(::p11.32→q23::p11.32→q23::)[3]/45,XX,-18[1]/47,XX,r(18)(::p11.32→q23::p11.32→q23::), +del(18)(q?11.2)[1]/47,XX,-18, +del(18) (:p11.?1→q11.?1:)x2[1]/46,XX,min(18)(:p11.?1→q11.?1:)[1]
Result after subcenM-FISH	46,XX,r(18)(::p11.32→q23::)[6]/46,XX,r(18)(::p11.32→q23::p11.32→q23::)[4]
Case C2	
Routine cytogenetics	46,XX,r(18)(p11.32q22.3)
Result after MCB	46,XX,r(18)(::p11.32→q23::p11.32→q23::)[7]/46,XX,r(18)(::p11.32→q23::)[4]/45,XX,-18[3]/46,XX,r(18)(::p11.32→q22.1::)[1]/45,XX,-18,r(18)(::p11.32→q23::)[1]
Result after subtelomeric and centromeric probes	46,XX,r(18)(::p11.32→q23::)[7]/46,XX,r(18)(::p11.32→q23::p11.32→q23::)[3]/45,XX,-18[1]/45,XX,-18,r(18)(::p11.32→q23::)[1]46,XX,-18,r(18)(::p11.32→q23::)x2[1]/46,XX,del(18)(:p11.?1→q11.?1:)[1]
Result after subcenM-FISH	46,XX,r(18)(::p11.32→q23::)[3]/46,XX,r(18)(::p11.32→q23::p11.32→q23::)[3]/46,XX,-18x2, +r(18)(::p11.32->q23::p11.32→q23::)x2[3]/47,XX,del(18)(:p11.2→q11.2:)x2[1]

FISH, fluorescence in situ hybridization; MCB, multicolor banding. Because highly complex karyotypes were present in all cases, slightly different results were obtained for the probe sets used, as listed here.

There are reports of rings for all chromosomes, although the most frequent are those involving autosomes 13 and 18 [Mohamed et al., 2001]. It is suggested that chromosome 18 might carry an elevated number of certain repeated sequences susceptible to interchromosomal and intrachromosomal rearrangements [Pater et al., 2003]. The most frequently structural rearrangements that involve chromosome 18 are the deletions and the ring chromosomes.

One of the mechanisms of formation of a ring chromosome can be explained by breakage of the chromosome at both ends and the joining back of both extremities, with loss of terminal regions [Sigurdardottir et al., 1999]. Depending on the size of the deletion at each end of the chromosome, the phenotypic appearance ranges from normal fertile carriers in a minority of cases, to those with severe malformations. Incidences and types of congenital malformations are similar to those carrying the del(18)(q21-qter) [Schinzel, 2001].

There is a difficulty in establishing a genotype-phenotype correlation in a ring carrier. It is necessary to determinate primary deletions associated with ring chromosome formation and also secondary loss or gain of material that may occur due to instability of ring chromosomes. This can cause a dilemma in prenatal diagnosis [Tümer et al., 2004]. Rings often appear as mosaics due to sister chromatid exchange within a ring in mitotic crossing-over events, which generates aneuploid cells with increased mortality [Sigurdardottir et al., 1999; Vermeesch et al., 2002].

Patients with ring chromosome 18 usually share clinical features of 18q- syndrome, such as, hypotonia, poor coordination, microcephaly, hearing abnormalities, malformation of genitalia [Schinzel, 2001; Cody et al., 1999; Brkanac et al., 1998]. Less frequently these patients show characteristics in common with 18p- syndrome, such as, mild to moderate growth deficiency, microcephaly, ptosis and more occasionally holoprosencephaly (HPE) [Schinzel, 2001]. Case A shares features with both syndromes. Cytogenetic analysis revealed a male foetus and the anatomopathologic study reported a foetus with female-like gonads. This discrepancy may be related to malformation of genitalia, a clinical feature of 18q- syndrome. Low set ears is also a hearing abnormality associated with deletion of the long arm of chromosome 18. Holoprosencephaly (also found in the autopsy) is probably due to deletion of 18p [Muenke, 1989; Bird et al., 1997]. The genetic causes of HPE can be cytogenetic anomalies or monogenic syndromes [Croen et al., 1996; Olsen et al., 1997; Cohen 1989; Muenke, 1989]. The association of HPE and deletion of chromosome 18p was first described in a child with HPE and monosomy 18p due to an unbalanced translocation [Johnson and Bachman, 1976]. The region 18p11.3 is defined as one of the critical regions for HPE [Overhauser et al., 1995]. Despite the fact that all the cases reported here showed deletions on 18p11.3, HPE was only found in case A. This could mean that the HPE critical region was most probably not disrupted in the other 3 cases, ie. the chromosome breaks were more distal, towards the telomere. According to Strenge and Froster (2004), HPE only occurs in 10-20% of the cases showing the terminal deletion [Strenge and Froster, 2004].

The occurrence of a r(18) together with a duplication of a segment of chromosome 18 or with a small marker chromosome is rare [Madan et al., 1981; Stankiewicz et al., 2001]. In all the 4 cases reported here, a marker was detected in a small percentage of the cells observed from different cultures suggesting that it is not a culture artefact (Table 3.8.I). The most likely explanation for the mosaic occurrence of the small marker in such small proportion could be that its loss would not be associated with a decreased cell survival as it would be if a complete ring was lost [Sigurdardottir et al., 1999].

Cytogenetic analysis of case A revealed a mosaicism involving a total of five cell lines (four in the amniotic fluid and four in post mortem fibroblasts, with two lines that only appear each in one or the other tissue) showing the mitotic instability of the ring [Fig. 3.9.1., Table 3.9.I]. Mosaicism of monosomy 18 was considered as a culture artefact, due to mitotic loss of the ring [Schinzel, 2001; Fischer et al., 2001]. However, there are reasons that make us think otherwise and to agree with Yardin et al. (2001), two different cultures of the amniotic fluid in case A gave approximately the same level of mosaicim and the monosomic cell line was also present in fibroblasts [Yardin et al., 2001]. In both tissues the percentage of this cell line was similar (16% and 18% respectively). Also in all 4 cases, a cell line with -18 was observed [Table 3.9.I]. One could suggest that this cell line, may have started as a r(18) which, because of its mitotic instability and crossing-over events between the ring sister chromatids, led to its loss during cell division generating the monosomy. So, the monosomic cell line may be part of the biological mechanism associated with the propagation of the ring during cell division. These events are also responsible for loss or duplications of ring chromosomes.

The growth retardation observed in both twins (Cases C1 and C2) could be associated with the occurrence of sister chromatid exchange within the rings that generate aneuploid cells with double rings and consequently serious genetic imbalance. This process may lead to increase cell death that can led to a decreased number of viable cells at any given interval of development and subsequently to growth deficiencies in the carriers. Generally severe growth failure is seen more often in patients with larger

rings than among patients with smaller ones, because of the probability of the higher frequency of sister chromatid exchanges in the first ones [Kosztolányi, 1987].

In all the cases presented here a *de novo* r(18) was established, showing mitotic instability that seems to be an important tool for the investigation of the dynamics of ring chromosome mosaicism, through multiple cell divisions. Mosaicism in all four reported cases can be explained by post-zygotic errors during mitosis. The variability of the cell lines observed and the variability in structure of the ring chromosomes found highlights the importance of analyzing a large number of cells and the importance of using various cytogenetic techniques.

The use of molecular cytogenetics is also of great value in the establishment of the presence of either loss or gain of chromosomal material in the ring structure as it will help to elucidate the role played by that material in determining the phenotypes, facilitating the search for candidate genes on those regions.

3.9.4. References

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3.10. *sSMCs from chromosome 22*

Molecular cytogenetic characterization of two cases with a small supernumerary marker chromosome derived from chromosome 22

3.10.1. Introduction

Segmental duplications, or low-copy repeats (LCRs) of >95% sequence identity, cluster within different chromosome regions and constitute approximately 5% of the human genome [Bailey et al., 2002]. Low-copy repeats range in size from 10-250 kb [Stankiewicz and Lupski, 2002]. They are considered highly dynamic regions in the genome because they mediate meiotic unequal nonallelic homologous recombination events, resulting in altered gene dosage associated with human genomic disorders [Babcock et al., 2003]. The LCRs on chromosome 22q (LCR22) are a complex mosaic of genes and pseudogenes formed by duplication processes. The 22q11 region is involved in chromosomal rearrangements that lead to altered gene dosage, resulting in genomic disorders that are characterized by mental retardation and/or congenital malformations. Three of such disorders- cat-eye syndrome (CES, OMIM 115470), der(22) syndrome, and velocardiofacial syndrome/DiGeorge syndrome (VCFS/DGS, OMIM 192430/ MIM 601362) - are associated with four, three, and one dose, respectively, of parts of 22q11 (Fig. 3.10.1) [McDermid and Morrow, 2002]. The reason for such a multitude of rearrangements lies in the complicated inverted and direct orientation of sequences in the LCR22 [McDermid and Morrow, 2002].

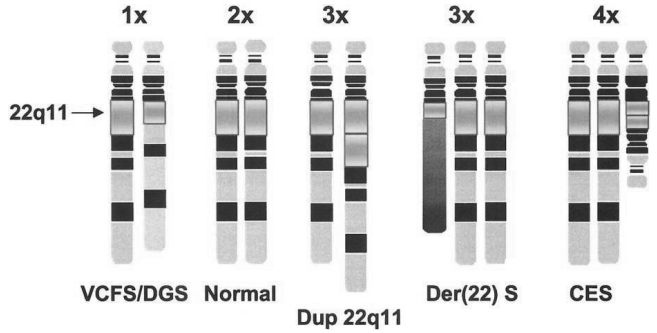

Figure 3.10.1. - Rearrangements on 22q11. VCFS/DGS is associated with interstitial deletions; Der(22) syndrome occurs in offspring of unaffected carriers of the constitutional t(11;22) translocation; patients with der(22) syndrome have a partial trisomy for 22pter-q11 and 11q23-qter. Patients with CES harbour a bissatellite supernumerary chromosome 22 that results in a partial tetrasomy [from McDermid and Morrow, 2002].

The phenotypes of partial trisomy 22q include the CES, the der(22) syndrome, the microduplication 22q11.2 syndrome, and the chromosome 22q duplication syndrome. Full trisomy 22 typically results in abortion, but partial 22q trisomy including 22q11.2 and/or parts of 22q12–q13 was reported with phenotypes that were compatible with survival [Bartsch et al., 2005]. The der(22) syndrome is more severe than CES and includes numerous additional signs that can be attributed to partial trisomy of 11q [Emanuel, 2008].

A very large subset of SMCs is derived from the human chromosome 22 and usually confers tri- or tetrasomy for the cat eye syndrome chromosomal region (the proximal 2Mb of chromosome 22q) and/or other segments of 22q [Emanuel, 2008]. Features of CES include ocular colobomata, anal atresia, congenital heart defects, renal malformations, craniofacial anomalies (e.g., preauricular skin tags and pits), male genital anomalies, skeletal defects, and borderline to moderate mental retardation [Schinzel et al., 1981].

A number of different SMCs containing the CES critical region (CESCR) have been described, including the typical bisatellite small and larger cat eye syndrome chromosomes and small ring-like SMCs(22) [Liehr et al., 1992; Mears et al., 1995; Crolla et al., 1997; McTaggart et al., 1998; Bartsch et al., 2005]. Owing to their small size and complex rearrangements, SMCs(22) have remained a diagnostic challenge [Bartsch et al., 2005]. McTaggart and co-workers classified the CES chromosomes into two types based on the location of the two breakpoints required to generate them within LCR22 region (Fig 3.10.2.) [McTaggart et al., 1998]:

- Type I - small CES chromosomes, that are symmetrical, with both breakpoints located within the proximal interval;
- Type II - Large CES chromosomes, which are either asymmetrical (type IIa), with one breakpoint located in each of the two intervals, or symmetrical (type IIb), with both breakpoints located in the distal interval.

Bartsch and colleagues, when reporting a set of SMC(22) with CESCR, suggested a type III CES chromosome defined by at least one breakpoint outside the LCRs22, larger than type II (Fig 3.10.2.).

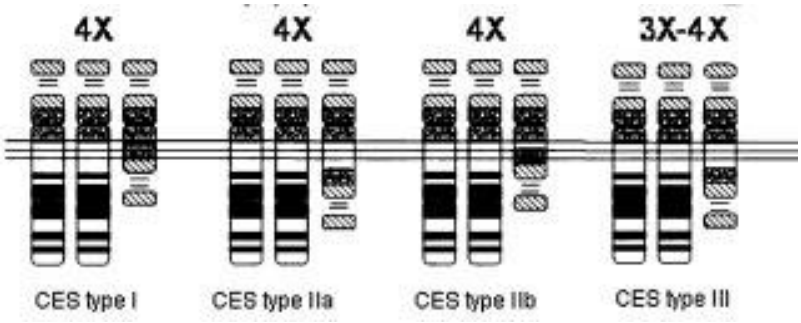

Figure 3.10.2. - Representation of the CES chromosomes: type I (frequent); symmetrical type IIa, rare; asymmetrical type IIb, rarer; and type III (exceptional), with association of trisomy (3x) and/or tetrasomy (4x) of the 22q11.1 region [adapted from Bélien et al., 2008].

We performed FISH studies on two patients with different sSMC(22). In one of the cases the marker was additionally studied by array CGH after microdissection. One of the patients presented the more frequent type of CES (Type I) and the other the exceptional type (Type III).

3.10.2. Results

Case I

A 6 years old boy with cat-eye syndrome presented congenital cardiopathy, psychomotor development delay, anal atresia, poor ponderal progression, bilateral iris *coloboma*, palate groove, lateral ventricles asymmetry, and small kidneys with deficient cortico-medullar differentiation (Table 3.10.I). Conventional cytogenetics revealed a karyotype of 47,XX,+mar, *de novo*, in all the analyzed cells. The SMC showed two AgNOR and two CBG positive regions, corresponding two a bisatellite and dicentric SMC (Fig. 3.10.3).

FISH using centromeric probes (D14Z1/D22Z1) revealed the origin of the marker as being derived from chromosome 14 or 22, and confirmed the presence of two centromeres. The SMC did not present a signal for D22S75, a probe located in the VCFS/DGS region (~17.5 Mb)(Fig. 3.10.4.).

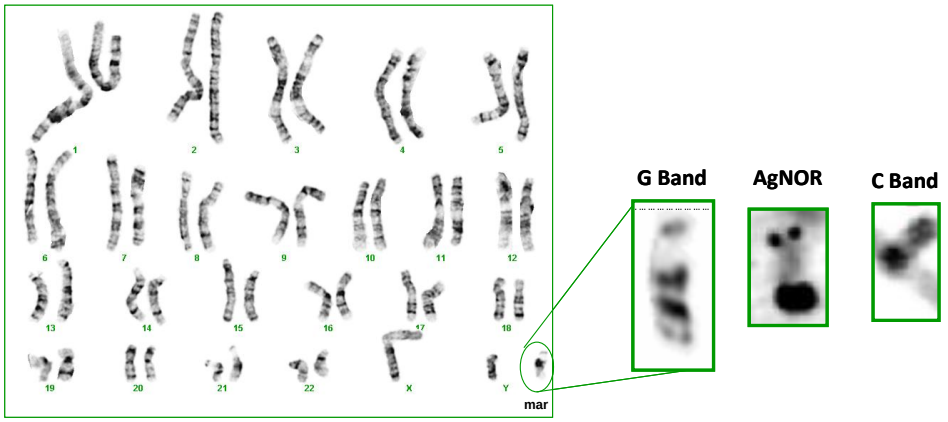

Figure 3.10.3. – Karyogram of Case I obtained by GTG-banding showing a 47,XY,+mar. The sSMC has two AgNOR positive regions and two CBG positive regions.

FISH using BAC clones confirmed the presence in the marker of two signals for RP11-172D7(~16.5 Mb) and for RP11-81B3 (~16.8 Mb) in 22q11.21, and two positive regions for rRNA regions (corresponding to acrocentric p-arms). The 22q11.22 region corresponding to RP11-1058B20 is not present in the sSMC (Fig. 3.10.5.).

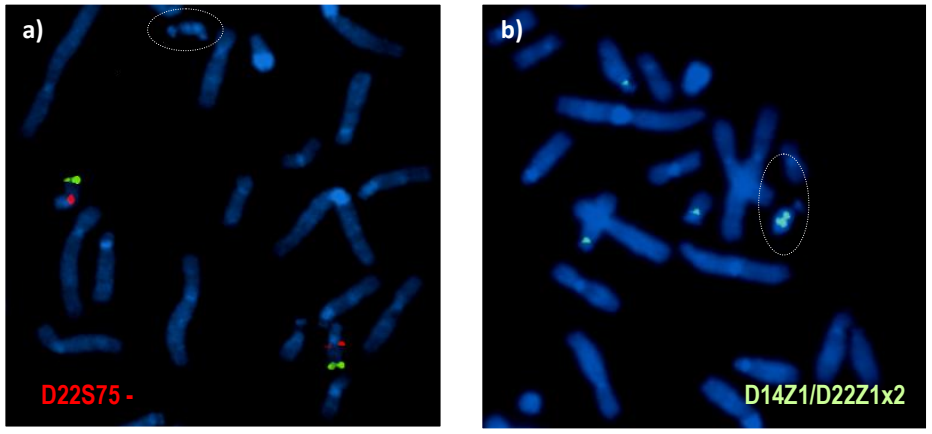

Figure 3.10.4. – a) Partial metaphase showing the two normal 22 chromosomes and the SMC (white circle) in Case I. The SMC does not present a signal for D22S75 (red), a probe located in the VCFS/DGS region; **b)** Partial metaphase showing the two normal 22 chromosomes and the SMC (white circle) with two signals for the centromeric probe specific for chromosomes 14/22 ( )

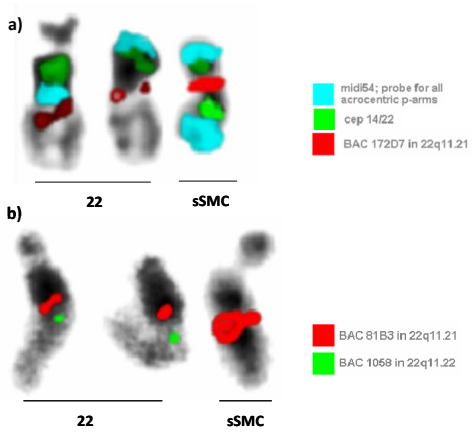

Figure 3.10.5. – Normal 22 chromosomes and the sSMC(22) showing the presence in Case I **a)** 2 centromeres (cep14/22 in green), two signals (2 signals merged in a bigger signal, when comparing to the signal in normal 22 chromosomes) for RP11-172D7 in 22q11.21 (red) and two regions corresponding to acrocentric arms (aqua); **b)** Two signals (2 signals merged in a bigger signal, when comparing to the signal in normal 22 chromosomes) in the marker for RP11-81B3 in 22q11.21 (red) and absence of signal for RP11-1058B20 in 22q11.22.

The microdissected DNA of the sSMC was hybridized in a full tiling BAC array specific for chromosome 22, with a resolution of 100-150 Kb (VIB MicroArrays Facility - www.microarrays.be), having material of approximately 2.8 Mb size extension from 22q (between 14.55 Mb to 17.35 Mb). (Table 3.10.I). The sSMC revealed to be an inv dup(22)(q11.21) (Table 3.10.I).

Case II

A sSMC present in mosaic was detected after prenatal diagnosis. G-banding of amniocytes culture revealed a karyotype 47,XY,+mar in 61% of the analyzed cells. The study after cordocentesis revealed a percentage of mosaicism of 56%. The presence of the marker was confirmed postnatally in peripheral blood in 37% of the prometaphases studied. The marker was characterized by CBG and AgNOR banding, revealing the presence of one centromere and 2 positive AgNOR regions (Fig. 3.10.6.). FISH studies by whole chromosome painting (wcp), revealed that the sSMC was derived from chromosome 22; presented two regions corresponding to the acrocentric arms (rRNA probes); and two positive regions for D22S75 probe (~17.5 Mb) (Fig. 3.10.7.).

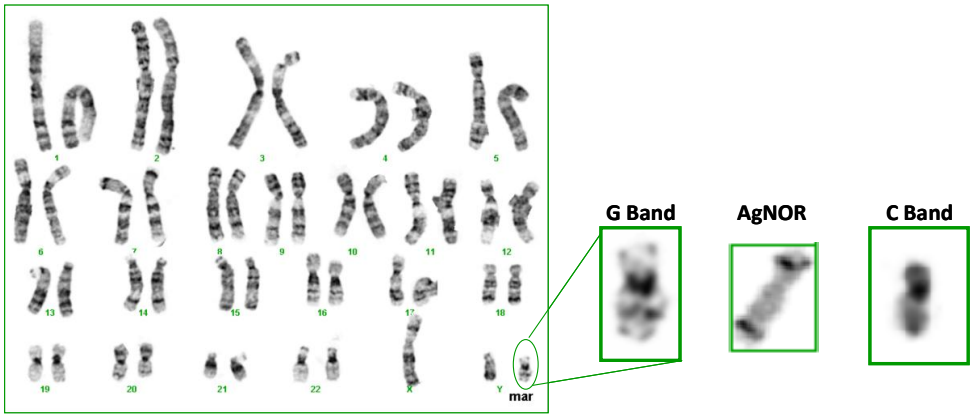

Figure 3.10.6. – Karyogram of Case II obtained by GTG-banding showing a 47,XY,+mar. The sSMC s two AgNOR positive regions and one C-band positive region.

The boy was clinically assessed after birth and revaluated at 5 years old. He presents a "Cat-eye like syndrome", with normal psychomotor development, normal growth development, normal echocardiography, normal renal and abdominal ecography, and bilateral preauricular skin pits (Table 3.10.I).

Additional FISH studies, using BAC clones, revealed the presence in the sSMC of two signals for RP11-172D7 in 22q11.21 (~16.5 Mb), two signals for RP11-1058B20 in 22q11.21 (~19.6 Mb), and apparently two signals for RP11-229C18 in 22q11.23 (~24 Mb), which appear merged (Fig. 3.10.8.). The sSMC revealed to be an inv dup(22)(q11.23~12.1) (Table 3.10.I).

Table 3.10.I. - Description of karyotype and clinical presentation of the sSMC(22)s cases characterized by molecular cytogenetics.

Case	Karyotype/ sSMC characterization M-FISH	Array CGH result	Clinical presentation
Case I	47,XY,+mar[100%] **sSMC characterization:** **ish.inv dup(22)(q11.21)**	sSMC size: 2.8 MB 14.55→ 17.35 MB 22q11.1→22q11.21	6 years: Cat-eye syndrome Congenital cardiopathy, psychomotor development delay, anal atresia, poor ponderal progression; bilateral iris *coloboma*; palate groove; lateral ventricles asymmetry; small kidneys with deficient cortico-medullar differentiation
Case II	47,XY,+mar[36%]/46,XY[64%] **sSMC characterization:** **ish.inv dup(22)(q11.23~12.1)**	n.a.	5 years: Cat-eye like syndrome; normal psychomotor development; normal growth development; normal ecocardiography; normal renal and abdominal ecography; Bilateral preauricular skin pits.

n.a. – not available

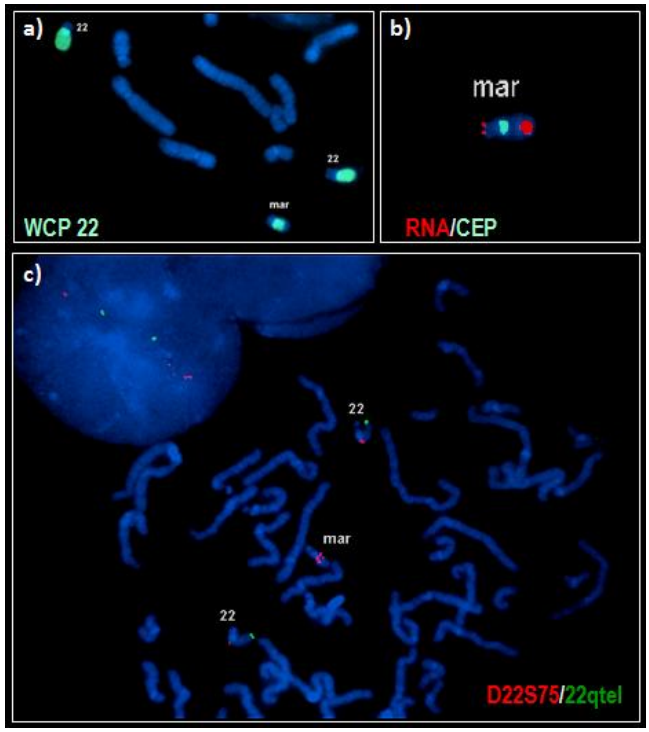

Figure 3.10.7. – FISH images in Case II. **a)** Partial metaphase showing the two normal 22 chromosomes and the marker painted with wcp specific for chromosome 22; **b)** The SMC has one centromere (aqua) and two rRNA positive regions (red); **c)** Partial metaphase showing the two normal 22 chromosomes and the sSMC with apparently two signals for D22S75, a probe located in the VCFS/DGS region.

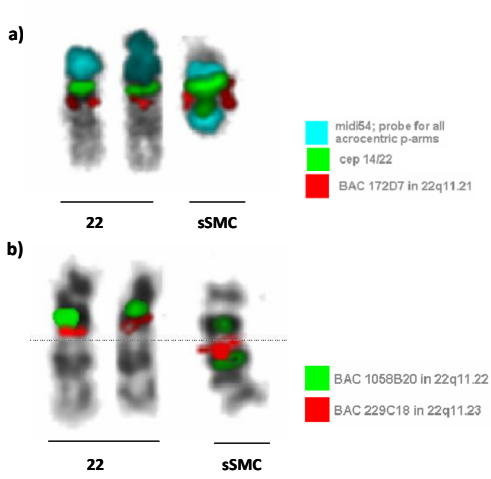

Figure 3.10.8. – Normal 22 chromosomes and the sSMC(22) in Case II showing the presence of: **a)** One centromere (cep14/22 in green), two signals for RP11-172D7 in 22q11.21 (red) and two regions corresponding to acrocentric arms (aqua); **b)** Two signals in the marker for RP11-1058B20 in 22q11.22 (green) and apparently two signals (2 signals merged in a bigger signal, when comparing to the signal in normal 22 chromosomes) for RP11-229C18 in 22q11.23.

3.10.3. Discussion

We report two cases with a sSMC(22), studied by molecular cytogenetics, using several BAC probes and, in one case, using array CGH after marker microdissection. Using a set of probes corresponding to different regions of 22q (Fig. 3.10.9.), and correlating with the data obtained by array CGH, we can conclude that the sSMC(22) from case I is a type I CES, the most common, and the sSMC(22) from case II corresponds to a type III CES chromosome, the rarest type of CES chromosomes.

Babcock and co-workers defined LCR22 localization, discovering that some LCR22s are large, such as LCR22-2 and LCR22-4, which are both 240 kb in size. LCR22-3a, situated between the two, contains an uncloned region within; thus, its size is unknown. Some LCR22s are quite small—less than 2 kb. LCR22s are composed of blocks or modules forming a complex pattern. Most of them appear to be partial duplicates of each other. The two greatest in size, LCR22-2 and LCR22-4, contain a large region of overall direct orientation [Shaikh et al., 2000]. Homologous recombination events between these two LCR22s mediate recurrent 22q11.2 rearrangements associated with VCFS/DGS and CES. Thus, these two intervals, due to their high degree of direct identity (97%-99%), are excellent substrates for unequal crossing-over events [Babcock et al., 2003].

Classical CES is characterized by the presence of a dicentric and bisatellite small inv dup (22) supernumerary marker chromosome. Thus, patients with CES usually have a partial tetrasomy of a region that spans chromosome 22p and part of 22q11.21 [McDermid and Morrow, 2002; Bartsch et al., 2005; Gentile et al., 2005, Bélien et al., 2008]. We can cytogenetically distinguish three types of CES chromosomes (Fig. 3.10.2.), which are correlated with breakpoints localization (Fig. 3.10.9). Type I CES chromosomes have a breakpoint between 16.688 - 17.690 Mb, corresponding to the LCR22-3a (17.248–17.433 Mb (previously named LCRA). Type II CES chromosome have their breakpoints between 19.466 - 21.847 Mb, matching the molecular positions of LCR-4 and LCR22-6. [Babcock et al., 2003; Bartsch et al., 2005; Gentile et al., 2005; Bélien et al., 2008]. Type III CES chromosome, is defined by at least one breakpoint outside the LCRs22 [Bartsch et al., 2005] (Fig. 3.10.9.).

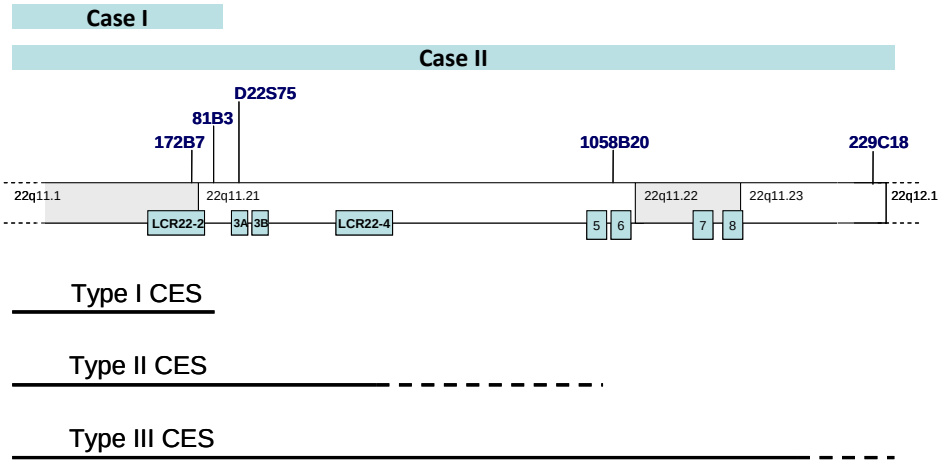

Figure 3.10.9. – Different types of CES according to LCR22 position and BAC clones position. In Case I of this report a type I CES is described with positive signal for 172B7 (~16.4 Mb) and 81B3 (~16.8 Mb) clones and negative for D22S75 (~17.5 Mb). In Case II a type III CES is present, with positive signal for RP11- 229C18 (24.18 Mb). LCR 22 position is indicated according to Babcock et al., 2003.

Bartsch and co-workers were the first to report a case with a type III CES chromosome. It was a case with a SMC present only in a small subset of cells (19% of amniocytes), with phenotype of the patient at the age of 6 months being normal. The sSMC in case II, that we report, is also present only in a subset of cells. Cytogenetic mosaicism significantly influences the clinical outcome of patients with a SMC. This could explain the mild phenotype observed, with a normal psychomotor development. Indeed, this SMC conferred trisomy of 22p11-q12.3 and would predict, if present in all cells, a severe phenotype. Recently, Karcaaltincaba and colleagues reported a non mosaic partial trisomy 22q with *de novo* duplication of chromosomal region 22q11.1-22q13 and a different phenotype from the reported cat eye syndromes, with absence of heart defects and presence of brain anomalies [Karcaaltincaba et al., 2010].

For now, a clear delineation of the phenotypes associated with rearrangements of 22q11.2 cannot be accomplished. CES phenotype is variable and it does not appear to correlate with the size of the marker [Bartsch et al., 2005; Bélien et al., 2008]. A careful clinical and molecular delineation of additional patients with these disorders is necessary to understand the relationship between genotype and phenotype.

3.10.4. References

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4. GENERAL DISCUSSION AND CONCLUDING REMARKS

About 3 million individuals of the whole human population would have one small supernumerary marker chromosome (sSMC) additionally to their normal chromosomal complement, i.e. 46 normal chromosomes and one sSMC (47,XX,+mar or 47,XY,+mar). These sSMCs can originate from each of the 24 human chromosomes and have different shapes. Marker chromosomes are of particular interest in clinical cytogenetics. They are more frequent in individuals with mental retardation than in the normal population. However, they are often found in only a small percentage of cells, making them difficult to detect and characterize in a diagnostic setting. About 30% of the carriers of a sSMC are clinically abnormal and 70% are clinically normal. The major problem in connection with sSMC appears when the diagnosis of the sSMC presence is made prenatally. A clear prediction about the outcome of the pregnancy is difficult to ascertain.

So, when a sSMC is encountered by classical cytogenetics in a prenatal or postnatal diagnosis, the main questions are:

- *What to do next? Should the marker be more extensively characterized?*
- *Which techniques should be used for its correct characterization?*
- *In prenatal diagnosis, is it possible to predict the outcome of a sSMC?*
- *How to interpret its characterization especially in the mosaic states?*

In this study we have characterized several cases, prenatal and postnatal, with a small Supernumerary Marker Chromosome (sSMC):

- One *de novo* case with a sSMC derived from chromosome 1, with a ring shape, present in mosaic associated with a mild phenotype (section 3.1.);

- Two *de novo* cases detected at prenatal diagnosis with a sSMC derived from chromosome 2, present in mosaic and with no apparent phenotype (section 3.2.);

- One *de novo* case with a sSMC derived from chromosome 5 in mosaic and with a ring shape, present in a female patient with learning difficulties and mild facial dysmorphisms (section 3.3.);

- A case of a sSMC derived from chromosome 11 present in mosaic, in a mother and a child with distinct phenotypes (section 3.4.);

- A case with a *de novo* small supernumerary neocentromeric chromosome derived from chromosome 13 ascertained prenatally (section 3.5.);
- Six postnatal cases of SMCs derived from chromosome 15 some associated with clinical findings others not (section 3.6.);
- Two cases of *de novo* sSMCs derived from chromosome 16, present in mosaic, associated with clinical findings (section 3.7.);
- A prenatal case with a sSMC derived from chromosome 17 with a normal phenotype (section 3.8.);
- Three cases with complex karyotype changes involving the formation of rings of chromosome 18, and with the presence of a marker in a small percentage of cells (section 3.9.);
- Two cases with a sSMC derived from chromosome 22 associated with cat-eye syndrome (section 3.10.).

In all these cases we tried to obtain the best possible clinical description of the patients or features, and to follow a specific series of methodologies in order to achieve the ultimate goal – a strong genotype/phenotype correlation.

Based on our results and experience, we propose approaches for a correct management of sSMC cases, which are dependent on a good and motivated clinical set up and on the techniques available at the laboratory (Fig. 4.1.). The management of a sSMC case depends primarily if it is ascertained pre or postnatally. When the case is prenatal, some of the techniques, like microdissection, are not appropriate for the diagnosis because they are time consuming, not compatible with the time for termination of pregnancy we currently have (24 weeks of gestation). However, they should be done later for a more precise characterization of the sSMC, as well as the good characterization of the foetuses by the Clinical Geneticist and Anatomopathologist, to establish a strong genotype/phenotype correlation.

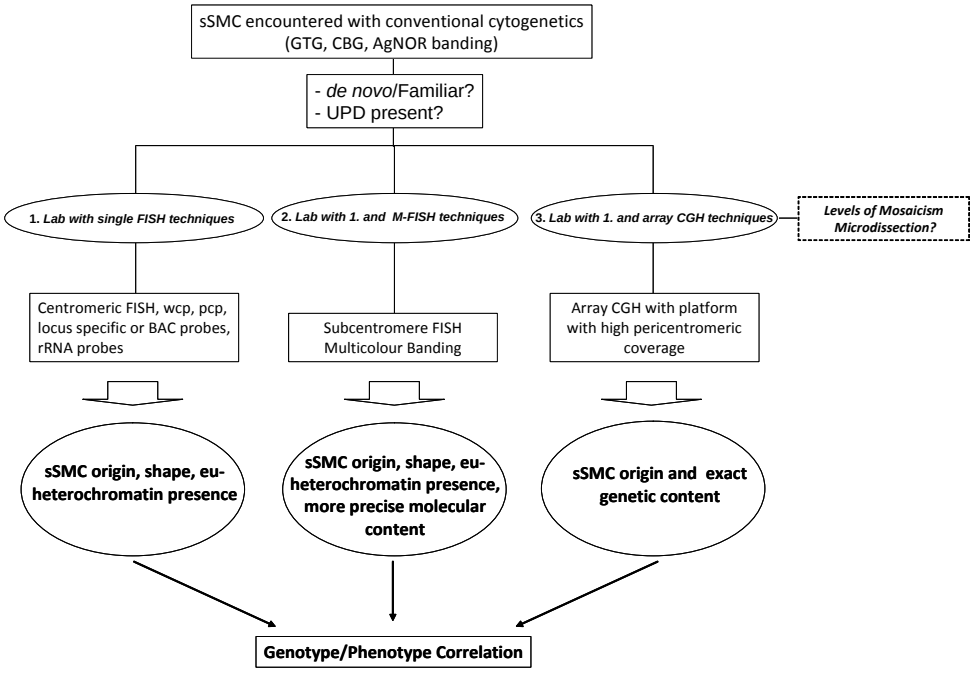

Figure 4.1. - Management of a sSMC encountered with conventional cytogenetics. The approach for its characterization depends on the techniques available and the genotype/phenotype correlation depends on a strong clinical set up.

Some main issues should be emphasized:

1) The follow-up of patients with a sSMC is crucial, either by recurring to autopsy data or by collecting data after birth. We have described cases where the phenotype becomes milder with age. It is a well know fact that when the sSMC appears in mosaic, the percentage of mosaicism could decrease over the years. Patients with a sSMC should be followed up, and laboratory testing as well as clinical details at different ages are crucial for establishing strong genotype/phenotype correlations.

2) The sSMC should be extensively characterized for its shape, origin and molecular content. The cases presented in this work were extensively characterized using conventional cytogenetics and molecular cytogenetics. We can conclude that molecular cytogenetic analysis is essential for the characterization of a sSMC. A variety of molecular cytogenetic techniques for SMC characterization can be used for a more comprehensive analysis in a single or a few experiments. Fluorescence *in situ*

hybridization (FISH) approaches, including centromeric FISH, whole-chromosome painting, and locus-specific probes, allow the characterization of the chromosomal origin of the sSMC. Other FISH strategies can be used, like subcentromere specific multicolor FISH (subcenM FISH) and multicolour banding (MCB), detecting the presence of euchromatin on a sSMC. These FISH techniques are feasible both in prenatal and in postnatal cases. The characterization by glass-needle-based chromosome microdissection and reverse painting could also be used, this approach being suited for all types of sSMC, including neocentromeric sSMC. Due to short time availability that usually is a problem in prenatal diagnosis, microdissection would be more suitable for postnatal diagnosis or for latter characterization of the sSMCs detected in prenatal cases.

3) As we can observe from our results, these multicolour FISH techniques can not precisely determine the chromosome regions or breakpoints involved. Microarray-based comparative genomic hybridization (array CGH) is a high-resolution and comprehensive method for detecting both genome-wide and chromosome-specific copy-number imbalance. The use of array CGH seems to be a good strategy for the characterization of the genetic content of sSMCs and allows the determination of the breakpoints and chromosomal regions present in the marker. Nevertheless, the characterization of centromeric and heterochomatic regions is problematic with this technique. Additionally, array CGH does not give information in cases with less than 30% mosaicism, leading to erroneous conclusions, being a major problem in sSMCs cases, which often appear in mosaic.

4) This study clearly demonstrates the utility of combining molecular cytogenetic analysis, namely with multicolour FISH approaches, and array CGH in the detection and characterization of marker chromosomes. Additionally, the use of combined microdissection and array CGH allows the determination of molecular breakpoints of the marker, even when the sSMC was present in mosaic. The use of conventional cytogenetic and M-FISH approaches permits the characterization of the sSMC shape.

5) Uniparental disomy should be analysed and excluded, especially when the sSMC is derived from chromosomes associated with genomic imprinting.

6) Biological material from the proband, and if possible from the parents, should be collected for future studies. For the majority of the cases described in this study an EBV-immortalized cell line was established.

In conclusion, for a good characterization of a sSMC several approaches should be taken, in order to ascertain not only the genetic content but also the shape of the sSMC. This information, about the shape and content, is crucial for moving towards a correct genotype/phenotype correlation of a sSMC and also for establishing possible mechanisms of formation for each marker, essential for risk assessment. The detailed molecular characterization of sSMCs will play a valuable role in the diagnosis and medical management of both pre- and postnatal cases in which marker chromosomes have been identified. Array CGH is a step in answering to the request of aetiological diagnosis in families with affected individuals. This technology is being installed in laboratories worldwide, including our own. Only with these combined approaches we can move forward for a better understanding of the impact of a sSMC on the phenotype, especially in prenatal cases.

List of publications in the field of Cytogenetic:

A) Book Chapters

Liehr T, Mrasek K, Kosyakova N, Mkrtchyan H, **Melo J**, Polityko A, Brecevic L Multiplex FISH and Spectral Karyotyping in Fluorescence In Situ Hybridization (FISH) – Application Guide. Springer-Verlag Berlin Heidelberg 2009 T. Liehr (ed.)
ISBN-10: 3540705805; ISBN-13: 978-3540705802.

B) Peer-reviewed articles:

Jardim A, **Melo JB**, Matoso E, Pires LM, Ramos L, Carreira IM (2007) Two new cases of de novo small supernumerary marker chromosomes (sSMC) detected at prenatal diagnosis. *Prenatal Diagnosis* 27: 380-381.

Carreira IM, Mascarenhas A, Matoso E, Couceiro AB, Ramos L, Dufke A, Mazauric M, Stressig R, Kosyakova N, **Melo JB**, Liehr T (2007) Three unusual but cytogenetically similar cases with up to five different cell lines involving structural and numerical abnormalities of chromosome 18. *Journal of Histochemistry and Cytochemistry* 55:1123-1128.

Mascarenhas A, Matoso E, Tönnies H, Gerlach A, Julião MJ, **Melo JB,** Carreira IM (2008) First prenatally detected small supernumerary neocentromeric derivative chromosome 13 resulting in a non-mosaic partial tetrasomy 13q. *Cytogenetic and Genome Research* 121: 293-7.

Melo JB, Matoso E, Polityko A, Saraiva J, Backx L, Vermeesch JR, Kosyakova N, Ewers E, Liehr T, Carreira IM (2009) Molecular cytogenetic characterization of two cases with de novo small mosaic supernumerary marker chromosomes derived from chromosome 16: Towards a genotype/phenotype correlation. *Cytogenetic and Genome Research* 125:109-14.

Carreira IM, **Melo JB**, Rodrigues C, Backx L, Vermeesch J, Weise A, Kosyakova N, Oliveira G, Matoso E (2009) Molecular cytogenetic characterisation of a mosaic add(12)(p13.3) with an inv dup(3)(q26.31 -> qter) detected in an autistic boy. *Molecular Cytogenetics* 4(2):16. doi: 10.1186/1755-8166-2-16.

Ferreira SI, Matoso E, Pinto M, Almeida J, Liehr T, **Melo JB**, Carreira IM (2010) X-chromosome terminal deletion in a female with premature ovarian failure: Haploinsufficiency of X-linked genes as a possible explanation. *Molecular Cytogenetics* 3:14. doi: 10.1186/1755-8166-3-14.

Melo JB, Santos HL, Backx L, Vermeesch JR, Kosyakova N, Weise A, Ewers E, Liehr T, Carreira IM (*in press*) Chromosome 5 derived small supernumerary marker chromosome: towards a genotype/phenotype correlation of proximal chromosome 5 imbalances. *Journal of Applied Genetics.*

Under submission:

Melo JB, Jardim A, Saraiva J, Backx L Vermeesch JR, von Eggeling F, Kosyakova N, Liehr T, Carreira IM. Prenatal diagnosis of a small supernumerary marker chromosome 17 with a normal phenotypic outcome. Submitted to *Cytogenetic and Genome Research.*

Melo JB, Jardim A, Soares G, Backx L, Vermeesch JR, Kosyakova N, Weise A, Liehr T, Carreira IM. Unbalanced chromosome abnormality in the pericentromeric region 11q: mother and child with distinct phenotypes with a small supernumerary marker chromosome derived from chromosome 11. Submitted to *American Journal of Medical Genetics A.*

Melo JB, Jardim A, Matoso E, Backx L, Vermeesch JR, Kosyakova N, Liehr T, Carreira IM. Molecular breakpoints definition of six supernumerary marker chromosomes derived from chromosome 15 by array techniques after microdissection. Submitted to *Journal Histochemistry and Cytochemistry.*