Small supernumerary marker chromosomes (sSMC) - why do they break, where they break and how to distinguish harmful from harmless sSMC?

Dissertation

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This work is dedicated…

to my parents

to my dear wife Shaymaa

to my lovely kids Mustafa and Mayar

to the spirit of my dearest friend Dr. Zaid A. AL-Hilli

(10.09.1974 - 26.03.2011)

Abbreviations

aCGH	Array-based comparative genomic hybridization
AS	Angelman syndrome
BAC	Bacterial Artificial Chromosome
CCK	Color changing karyotyping
cenM-FISH	Centromere specific multicolor FISH
CES	Cat eye syndrome
CGH	Comparative genomic hybridization
CNV	Copy number variant(s)
COBRA	Combined binary ratio labeling
C-UBCA	Centromere-near - unbalanced chromosomal abnormalities
DAPI	4',-6-Diamidino-2-phenylindol-dihydrochlorid
der	Derivative chromosome
dn	de novo
DNA	Deoxyribonucleic Acid
ESAC	Extra structurally abnormal chromosome
FISH	Fluorescence in situ hybridization
HCM-FISH	Heterochromatin-directed M-FISH
i	Isochromosome
idic	Isodicentric chromosome
inv dup	Inverted duplicated chromosomes
ISCN	International System for Human Cytogenetic Nomenclature
Kb	Kilobase pairs
mar	Marker chromosome
Mb	Megabase pairs
SNP	Single nucleotide polymorphism
MCB	Multicolor banding
mFISH	multicolor FISH
M-FISH	Multiplex FISH
min	Centric minute chromosomes
NCBI	National Center for Biotechnology Information
NORs	Nucleolus-Organizing Regions
OMIM	Online Mendelian Inheritance in Man
p	Short arm of chromosome
PAC	Phage artificial chromosome
PCL-FISH	Pericentric-ladder-FISH
PCR	Polymerase chain reaction
PKS	Pallister-Killian syndrome
PWS	Prader-Willi syndrome
q	Long arm of chromosome

r	Ring chromosomes
rDNA	Ribosomal Deoxyribonucleic Acid
RNA	Ribonucleic Acid
rRNA	Ribosomal ribonucleic acid
SKY	Spectral karyotyping
SMC	Supernumerary marker chromosome
SNRPN	Small nuclear ribonucleoprotein-associated protein N
SRC	Supernumerary ring chromosome
sSMC	Small supernumerary marker chromosomes
subcenM-FISH	Subcentromere specific multicolor FISH
t	Translocation
UBCA	Unbalanced chromosomal abnormalities
UCSC	University of California, Santa Cruz
UPD	Uniparental disomy
WCP	Whole-chromosome painting
YAC	Yeast artificial chromosome

Contents

Dedication..... I

Abbreviations..... II-III

Contents..... IV-V

Summary..... 1

Zusammenfassung..... 2

1. Introduction..... 3

1.1. Cytogenetics – how to characterize chromosomes..... 3

1.1.1. Classical and banding cytogenetics..... 3

1.1.2. Molecular cytogenetics..... 4

1.1.3. Comparative Genomic Hybridization (CGH) and aCGH..... 6

1.1.3.1. Microdissection and aCGH..... 7

1.2. Small supernumerary marker chromosomes (sSMC)..... 8

1.2.1. Definition and nomenclature..... 8

1.2.2. Characterization..... 9

1.2.3. Formation of sSMC..... 11

1.2.3.1. Mixtures of Different sSMC Shapes..... 13

1.2.4. Epidemiology of sSMC in genetics disorders..... 14

1.2.4.1. Clinical consequences of sSMC..... 14

1.3. Aim of study/ Questions worked on..... 15

2. Results..... 16

2.1. Basic papers of thesis..... 16

2.2. Article-1..... 18

2.3. Article-2..... 23

2.4. Article-3..... 28

2.5. Article-4..... 34

2.6. Article-5..... 45

2.7. Article-6..... 53

2.8. Article-7..... 55

2.9. Article-8..... 62

2.10. Article-9..... 69

2.11. Article-10..... 75

2.12. Article-11..... 79

3. Discussion…….. 84
3.1. Development of probe sets for detection of euchromatic presence in sSMC…….. 84
3.2. sSMC and localization of chromosomal breakpoints…….. 87
3.3. Mosaicism in association with sSMC…….. 88
3.4. sSMC and genotype-phenotype correlation……. 90
4. **Conclusions and Outlook**……..……... 93
5. Bibliography ……. 94
6. Appendix ……... 105
6.1. List of own publications ……. 105
6.2. Curriculum Vitae……. 108
6.3. Acknowledgements……. 111
6.4. Ehrenwörtliche Erklärung…… 112

Summary:

Small supernumerary marker chromosomes (sSMC) are defined as additional centric chromosome fragments too small to be identified or characterized unambiguously by banding cytogenetics alone. Even though certain sSMC were associated with specific clinical pictures and syndromes, for most of the sSMC only first steps towards genotype-phenotype correlations were achieved. Therefore sSMC are still a problem in clinical cytogenetics and can be harmful due to different mechanisms like induction of genomic imbalances and/or uniparental disomy of the sSMC's sister chromosomes. This study had the aim to provide new insights into the questions (i) if and why sSMC include specific breakpoints and (ii) how to distinguish harmful from harmless sSMC. Thus, several approaches for better sSMC characterization (HCM-FISH) and/or, characterization of sSMC breakpoints were developed (PCL-FISH; 1MB sets spanning the transitions of dosage-sensitive and dosage-insensitive pericentric regions) and established. sSMC breakpoints were characterized in detail using these new approaches, but also by microdissection based array-comparative genomic hybridization. First hints were obtained that breakpoints involved in sSMC formation are situated preferentially in gene- poor regions of the pericentric regions. Concerning genotype-phenotype correlation of sSMC the present study further identified one new "complex sSMC" associated syndrome: the der(13 or 21)t(13 or 21;18) syndrome, which is associated with a mild clinical phenotype irrespective of partial trisomy 18p. Finally, influence of mosaicism on sSMC-related phenotypes was studied in detail.

In conclusion, the present study provided important new data for genotype-phenotype correlation and biological understanding of sSMC.

Zusammenfassung:

Kleine überzählige Marker-Chromosomen (sSMC) sind definiert als zusätzliche zentrische Chromosomenfragmente, die zu klein sind, als allein mittels Zytogenetik identifiziert oder eindeutig charakterisiert werden zu können. Auch wenn bestimmte sSMC schon mit spezifischen Krankheitsbilder und Syndromen assoziiert werden konnten, wurden für die meisten der sSMC bisher nur erste Schritte bezüglich Genotyp-Phänotyp-Korrelationen erreicht. Daher sind sSMC immer noch ein Problem in der klinischen Zytogenetik und können sich ungünstig auf den Phänotyp auswirken durch verschiedene Mechanismen, wie die Induktion von genomischen Imbalancen und/oder einer uniparentalen Disomie der Schwesterchromosomen des sSMC. Die hier vorliegende Studie hatte das Ziel, neue Einsichten in die Fragen (i) ob und warum sSMC spezifische Bruchpunkte haben, und (ii) wie schädliche von harmlosen sSMC zu unterscheiden sind. Daher wurden mehrere Ansätze für eine bessere sSMC-Charakterisierung (HCM –FISH) und/oder die Charakterisierung von sSMC Bruchpunkten entwickelt (PCL–FISH; 1MB Sondensets welche die Übergänge der dosisempfindlichen und der dosisunempfindlichen perizentrischen Regionen unterscheiden). sSMC Bruchpunkte wurden mit diesen neuen Ansätzen, aber auch im Detail durch mikrodissektion-basierende-array-komparative genomische Hybridisierung charakterisiert. Erste Hinweise wurden erhalten, dass die an der Bildung der sSMC-Bruchpunkte beteiligten Regionen bevorzugt in genarmen Bereichen des Perizentromers liegen. Bezüglich der Genotyp-Phänotyp-Korrelation von sSMC wurde in der vorliegenden Studie ein neues mit einem "komplexen sSMC" verbundenes Syndrom definiert: das der(13 oder 21)t(13 oder 21;18)-Syndrom, welches mit einem milden klinischen Phänotyp aber einer partiellen Trisomie 18p verbunden ist. Schließlich wurde der Einfluss von zellulären Mosaiken in sSMC-Syndromen auf den Phänotyp im Detail untersucht.

Insgesamt liefert die vorliegende Studie wichtige neue Daten für Genotyp-Phänotyp-Korrelation und das biologische Verständnis von sSMC.

1. Introduction

1.1. Cytogenetics – how to characterize chromosomes

Chromosomes are the factors that distinguish one species from another and that enable the transmission of genetic information from one generation to the next; the study of chromosomes and cell division are referred to as *Cytogenetics* (Turnpenny and Ellard 2007). Cytogenetic studies allow analyzing the chromosomal behavior in the organization and transmission of genetic information, variability mechanisms and evolutionary pathways, besides contributing to the genetic improvement of domestic species (Lacadena 1996) and its essential role in clinical genetics. Therefore, cytogenetics is mainly focused on structure and chemical/genetic organization of chromosomes, linking two formerly unrelated sciences, cytology and genetics (Griffiths et al. 1996).

1.1.1. Classical and banding cytogenetics

The essential step for the emergence of modern human cytogenetics approach was when Tjio and Levan (1956) correctly concluded that the normal human somatic cell contains 46 chromosomes (Tjio and Levan 1956) which was confirmed by examining meiotic chromosomes (Ford and Hamerton 1956). In 1959, Lejeune and colleagues found the trisomy for chromosome 21 as the underlying cause of the Langdon Down syndrome (Lejeune et al. 1959), and after that Nowell and Hungerford, in 1960 identified a minute chromosome in the peripheral blood of patient with chronic granulocytic leukaemia which was called later Philadelphia 1 chromosome (Nowell and Hungerford 1960). In 1961 Ilberry and coworkers provided the first description of a *small supernumerary marker chromosome (**sSMC; see also 1.2**)* when reporting a boy with epicanthic fold and protuberant tongue and a karyotype 47,XY,+mar/46,XY (Ilberry et al. 1961). Later, Ellis and colleagues (1962) reported an aberrant small acrocentric chromosome (Ellis et al. 1962), and Froland and colleagues (1963), described a boy with several congenital defects with a karyotype 47,XY,+mar (Froland et al. 1963). This altogether opened broad prospects to clinical cytogenetic studies and the literatures showed the relation between numerical and morphological chromosomal aberrations and disease in man (Luthardt and Keitges 2001).

Classical cytogenetic staining approaches can provide information regarding the structure of an sSMC (Rooney and Czepulkowski 1986). The size and shape is often more clearly observed in solid-stained preparations, since chromosome banding approaches like G-banding (Claussen et al. 2002) may suggest a particular chromosomal origin (Seabright 1971), such as in case of tetrasomy 12p (Graf and Schwartz 2002). So-called chromosomal satellites including the nucleolus-organizing regions (NORs) may be present at one or both ends of an

sSMC and can be visualized either by silver staining or observation of satellite association between the marker and other acrocentric chromosomes (Thangavelu et al. 1994). Centromeric heterochromatin can be highlighted by C-banding (Gardner et al. 2012). If a marker chromosome has two centromeres, one may be inactived, either in all or in a proportion of cells, as recent studies of our group showed (Ewers et al. 2010). DistamycinA/DAPI staining identifies the heterochromatin of chromosomes 1, 9, 15, and 16 and of the Y chromosome. In case an sSMC has chromosomal satellites and, in addition, a distamycinA/DAPI-staining region, then an origin from chromosome 15 is likely, even though this could not always be substantiated (Wisniewski et al. 1979, Callen 1991).

In 1971/1972 a system of nomenclature was proposed for banded human chromosomes and chromosome abnormalities and was based on the patterns observed in different cells stained with either of the chromosome banding techniques (Mitelman 1995). This international system for human cytogenetic nomenclature (ISCN) is still in place and actualized regularly (Shaffer et al. 2013). By standard banding techniques karyotypes a pattern of ~500 bands can be achieved. G-bands made it possible for a detailed analysis of each chromosome to be carried out, which led to improved definitions of different chromosomal aberrations and the discovery of new cytogenetic syndromes in clinical pathology. Thus, nowadays, it is still the starting point and gold standard of all cytogenetic techniques (Garcia-Sagredo 2008).

1.1.2. Molecular cytogenetics

The staining patterns produced on chromosomes by banding procedures are sometimes ambiguous, and the resolution is limited by the optical characteristics of microscopes and the complex manner in which DNA is packaged into chromosomes (Li and Pinkel 2006). But further characterization of particular rearrangements requires additional techniques. Among them, fluorescence in situ hybridization (FISH) becomes increasingly important in the characterization of both constitutional and acquired chromosomal abnormalities (Gerdes et al. 1997). In 1969, Gall and Pardue described the hybridization of radioactively labeled rRNA to tissue squashes allowing the in situ visualization of the complementary sequences, the rDNA, within cells (Gall and Pardue 1969) and then, in 1986, Pinkel and coworkers (1986) and Cremer and coworkers (1986) reported FISH using non-radioactively labeled probes. Since then, FISH has been further developed and widely used for the detection of DNA or RNA sequences (Pinkel et al.1986, Cremer et al. 1986). In situ hybridization is based on the specific base pairing of two complementary nucleic acid sequences, the probe and the target sequences. Hybridized probes are detected via fluorochromes using epifluorescence

microscopy, via colorimetric enzyme assays by transmission light microscopy, or via metallic compounds in the electron microscope (Joos et al. 1994).

The aim of the FISH technique is to characterize either imbalances, i.e gains or losses of chromosome material, or specific breakpoints with or without imbalance (Kjeldsen and Kølvraa 2002). A number of different types of probes for FISH can be distinguished on the basis of the complexities of probe or target sequences: the alphoid and satellite probes detecting repeat-targets; individual probes such as plasmid-, cosmid (35-55 kb)-, bacterial artificial chromosomes (BACs)-, yeast artificial chromosomes (YACs) or P1 filamentous phage artificial chromosomes (PACs) - clones detecting single copy sequences – nowadays mainly used BACs; or composite probes are generated using PCR with sequence-specific primers, allowing a specific painting of individual chromosomes or chromosomal regions (Pinkel et al. 1988, Speicher 2005). Following the sequencing of the human genome, large-insert clones that have been mapped and sequenced, and can be used as probes, are now readily available for almost any genomic region. Probes can be selected easily using internet-browsers such as Ensembl Cytoview, NCBI Map-Viewer or the UCSC genome browser (Speicher and Carter 2005). This relatively new field of molecular cytogenetics, which makes use of a variety of nucleic acid sequences as probes to cellular DNA targets has helped to bridge the gap between molecular genetics and classical cytogenetic analyses (Teixeira 2002).

Nederlof and coworkers reported in 1989 the first multicolor FISH (three-color FISH) experiments (Nederlof et al. 1989). For multicolor karyotyping with painting probes several approaches were developed, including multiplex FISH (M-FISH), spectral karyotyping (SKY), color changing karyotyping (CCK), and combined binary ratio labeling (COBRA) (Liehr and Claussen 2002a,b). Molecular cytogenetics has provided new tools to characterize aberrant karyotypes more precisely (Haddad et al. 1998) and became important component of molecular diagnostics, particularly for diagnosing congenital syndromes in which the underlying genetic defect is unknown (Speicher and Carter 2005).

1.1.3. Comparative Genomic Hybridization (CGH) and array-based CGH (aCGH)

Comparative genomic hybridization (CGH) is a molecular cytogenetic technique that allows comprehensive analysis of the entire genome. CGH permits the rapid detection and mapping of DNA sequence copy number differences between a normal and an abnormal genome (Kallioniemi et al. 1992). It has wide potential in application to basic research and clinical practice, particularly in areas such as tumour genetics. Indeed, because DNA copy number modifications are of pathogenic importance in cancer, CGH was initially developed for cancer research (Lapierre and Tachdjian 2002, Tachdjian 2009). In CGH, two DNA samples are differentially labelled, for example, with the test labelled in green and the reference in red (Fig.1.1). 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. The ratios of test to reference fluorescence along the chromosomes are quantified using digital image analysis. Gains and amplifications in the test DNA are identified as chromosomal regions with increased fluorescence ratios, whereas losses and deletions result in a reduced ratio (Speicher and Carter 2005, Kallioniemi 2008).

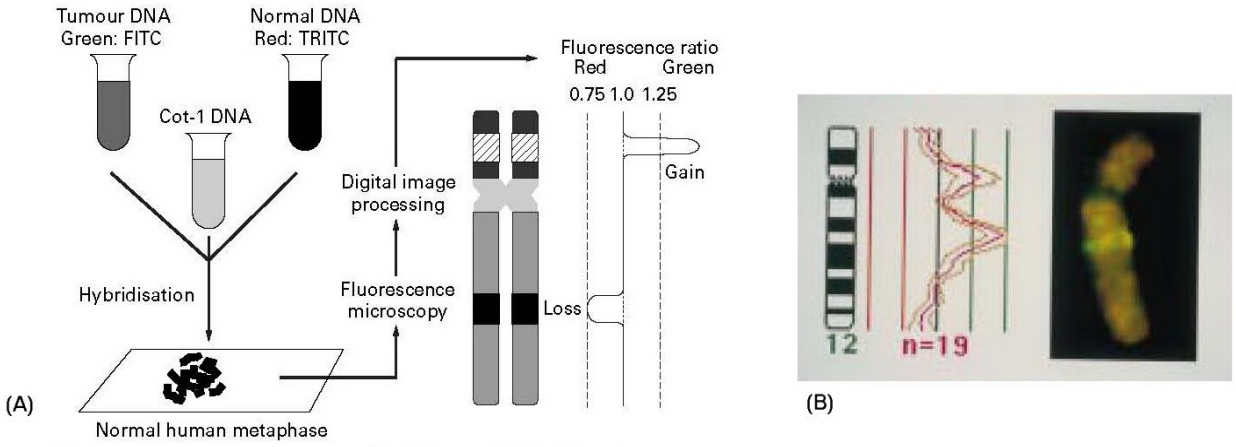

Figure 1.1. Principle of comparative genomic hybridization (CGH) as described by Weiss and coworkers (1999). **(A)** Schematic overview of the CGH technique. Tumor and reference DNA are labelled with a green and red fluorochrome, respectively, and hybridized to normal metaphase spreads. Images of the fluorescent signals are captured and the green to red signal ratios are quantified digitally for each chromosomal locus along the chromosomal axis. **(B)** High level gain on the long arm of chromosome 12. The clear green band shows the high level gain or amplification on the long arm of chromosome 12 (chromosomal band 12q15).

In analyzing the results of CGH, several limitations must also be taken into account. CGH can spot sequence copy number changes only if more than 50 % of the cells analyzed contain a chromosomal gain or loss. CGH is also impaired in its ability to identify balanced chromosomal abnormalities for which there are no copy number changes, such as those found in balanced translocations, inversions and intragenetic rearrangements (Tachdjian et al. 2008). Genetic changes are detected and mapped on chromosomes when the size of the chromosomal region affected is at least 10–12 Mb (Bentz et al. 1998).

Subsequently array-based CGH (aCGH) was established, an approach where arrays of genomic sequences replaced the metaphase chromosomes as hybridization targets by large numbers of mapped clones that are spotted onto a standard glass slide greatly increasing the resolution of screening for genomic copy number gains and losses (Solinas-Toldo et al. 1997, Pinkel et al. 1998). This solved many of the technical difficulties and problems caused by working with cytogenetic chromosome preparations. The main advantage of aCGH is the ability to perform copy number analyses with much higher resolution than was ever possible using chromosomal CGH (Davies et al. 2005, Pinkel and Albertson 2005, Lockwood et al. 2006).

Two major groups of microarray-based platforms are currently used in clinical cytogenetics: microarray-based comparative genomic hybridization (aCGH), and single nucleotide polymorphism (SNP) genotyping-based arrays (Li and Andersson 2009). In aCGH, the most apparent besides those already present in CGH includes the challenge of interpreting copy number variants (CNVs) of unknown significance and distinguishing disease-causing CNVs from normal CNV polymorphisms (Li and Andersson 2009, Bishop 2010).

1.1.3.1. Microdissection and aCGH

In 1981 Scalenghe and colleagues were the first to develop the chromosome microdissection and microcloning technique (Scalenghe et al. 1981). Then, it was extended to human chromosomes (Bates et al. 1986, Lüdecke et al. 1989, Senger et al. 1990). Microdissection can be used to isolate derivative chromosomes form a balanced translocation or marker chromosomes present in low mosaic. This DNA can be amplified and used in aCGH, thus overcoming parts of its above mentioned limitations (1.1.3). For sSMC this approach was applied by others (Shaw et al. 2004) and our group in single case studies (Liehr et al. 2006a, Backx et al. 2007).

1.2. Small supernumerary marker chromosomes (sSMC)

1.2.1. Definition and nomenclature

Small supernumerary marker chromosomes (sSMC) were first described in 1961 by Ilberry and coworkers and, today, it is known that sSMCs are present in approximately 3.0 million carriers worldwide in a population of 7 billion human beings (Liehr et al. 2004a, Liehr 2014a). sSMC can be defined as "small structurally abnormal chromosomes that occur in addition to the normal 46 chromosomes" (Crolla et al. 1997), and according to the definition of the ISCN 2013, *a marker chromosome* (**mar**) is a structurally abnormal chromosome that cannot be unambiguously identified or characterized by conventional banding cytogenetics (Shaffer et al. 2013). Numerous terms have been used in the literature to described sSMC in the last few decades. The three best known are supernumerary marker chromosome (**SMC**) which does not distinguish between larger and smaller SMCs, extra structurally abnormal chromosome (**ESAC**), and supernumerary ring chromosome (**SRC**). In addition, other designations summarized elsewhere were used (Liehr 2012, Liehr et al. 2004a). The chromosomal origin of some sSMCs has been identified and associated with known syndromes, such as isochromosome 12p [i(12p), OMIM #601803] Pallister-Killian syndrome (PKS), isochromosome 18p [i(18pS), OMIM #614290] syndrome, Emanuel syndrome (ES) or supernumerary-derivative chromosome 22 [der(22)t(11;22)(q23;q11.2), OMIM #609029] syndrome, and inverted duplication 22 [inv dup(22q), OMIM #115470] cat eye syndrome (CES) (Ballif et al. 2007). Liehr and colleagues reviewed, sSMCs are a morphologically heterogeneous group of structurally abnormal chromosomes: different types of inverted duplicated chromosomes (**inv**), centric minute chromosomes (**min**) and ring chromosomes (**r**) can be detected (Fig. 1.2), and they suggest for the first time a cytogenetic one as follows: *sSMC are 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. 2004a). In contrast, a SMC larger than chromosome 20 usually can be identified based on chromosome banding. The definition of small SMC versus large(r) SMC is a cytogenetic, but not functional, because sSMC and larger SMC can have the same modes of karyotypic evolution. sSMC can be present additionally (1) in a karyotype of 46 normal chromosomes, (2) in a numerically abnormal karyotype (e.g. Turner or Down syndrome) or (3) in a structurally abnormal but balanced karyotype (e.g. Robertsonian translocation) or ring chromosome formation (Liehr et al. 2004a, Liehr et al. 2009a, Liehr 2012).

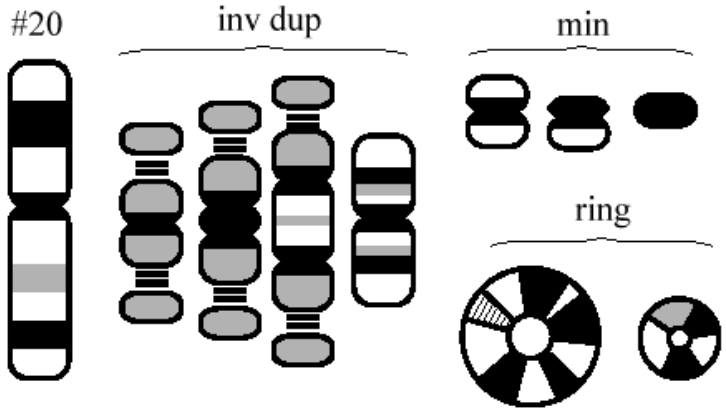

Figure.1.2. Different shapes of Small supernumerary marker chromosomes (sSMC). sSMCs can form three basic types of shapes: ring-structure (r), inverted duplication (inv dup), and centric minute (min) (Liehr et al. 2004a).

1.2.2. Characterization

Detection of an sSMC is nearly always unexpected by the clinician and more or less an accidental result in cytogenetics. The origin of sSMC is almost impossible to establish by routine cytogenetics alone, whereas fluorescence in situ hybridisation (FISH) methods are highly suited for this (Starke et al. 2003a). A variety of molecular cytogenetic techniques that provide more comprehensive analysis in a single or a few experiments have been described for sSMC characterization. M-FISH, multicolor banding (MCB), whole-chromosome painting (WCP), locus-specific FISH, centromere specific multicolor FISH (cenM-FISH), subcentromere specific multicolor FISH (subcenM-FISH), microdissection coupled with reverse painting and FISH approaches may all provide identification of the chromosome of origin of SMCs (Nietzel et al. 2001, Brecevic et al. 2006, Pietrzak et al. 2007). Even if M-FISH is readily available, this technique can result in ambiguous classification or misclassification of sSMCs, particularly if they are small. In addition, these multicolor FISH techniques cannot precisely determine the chromosome regions or breakpoints involved (Tsuchiya et al. 2008). Usually, sSMC larger than chromosome 20 can be identified based on chromosome-banding. Additionally, C-banding, silver staining of NOR or Q-banding were used for sSMC characterization (Gersen and Keagle 2005). WCP-FISH approaches are well-suited for the determination of the chromosomal origin of marker or derivative chromosomes providing that they are larger than 17p, whereas if they are smaller, WCP-FISH is, in general, non-informative (Haddad et al. 1998, Starke et al. 1999). Also, it is possible characterization sSMC with a euchromatic content of approximately half of the short arm of chromosome 17p

or more by the MCB technique is possible (Weise et al. 2002, Starke et al. 2003a). sSMCs have also been successfully characterized by glass needle-based chromosome microdissection and reverse chromosome painting (Starke et al. 2001). This approach is suited for all types of sSMCs, including neocentromeric ones (Liehr et al. 2007). Although a comprehensive characterization seems to be available by microdissection and reverse painting, it is restricted as it provides on information regarding the orientation of eventual present chromosomal fragments or the copy number of specific subregions, and no reliable information on the presence or absence of centromere-near euchromatic content (Liehr et al. 2009a). aCGH is an efficient and sensitive technique for detecting genome-wide copy number alterations at high resolution (Shaffer et al. 2007). aCGH can now provide accurate characterization of SMCs in terms of chromosomal origin, gene content, and other concomitant imbalances elsewhere in the genome (Reddy et al. 2013). However, most successfully characterized sSMCs were larger than 17p. Furthermore, centromeric and/or heterochromatic regions are problematic. CGH has the advantage that it provides informative results on the euchromatic region(s) involved in a sSMC, these regions must be larger than approximately 5-10 Mb to be visible in CGH and can be overcome that, in principle, by application of aCGH, where much higher resolutions can be achieved (Tsuchiya et al. 2008, Liehr et al. 2009a). Although aCGH using chips that provide comprehensive genome coverage may become the technology of choice for initial characterization of SMCs, G-banded and FISH analyses are still indispensable for determining the structure and level of mosaicism of these chromosomes. G-banded analysis may also be useful for detecting low level mosaic SMCs that could potentially be missed by array CGH (Tsuchiya et al. 2008). sSMC can be best characterized for their chromosomal origin by using centromeric probes. Nietzel and colleagues proposed the centromere-specific multicolor FISH (cenM-FISH), as fast and easy method for sSMC characterization (Nietzel et al. 2001). This approach overcomes the limitations of all the previously mentioned methods concerning the informational value of the centromeric regions (Liehr et al. 2009a).

Several probe sets were suggested as approaches to detect the presence of euchromatic on an sSMC. Besides FISH banding, approaches such as multicolor banding (Liehr et al. 2006b), and subcentromeric multicolor-FISH (subcenM-FISH), a probe set comprising of 43 bacterial or yeast artificial chromosome clones located in proximal regions of each human chromosome (Starke et al. 2003a) were suggested. Still the approaches available at the beginning of the present work were not ideal yet for sSMC-characterization.

As reported previously by (Chudoba et al. 1999, von Eggeling et al. 2002, Liehr et al. 2009a) and others (reviewed in: Kotzot 2002a), they recommended that, 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). However, most sSMCs have yet to be accurately characterized (Liehr et al. 2004b) because of variations in euchromatic DNA content, different degrees of mosaicism, UPD of the chromosomes homologous to the sSMC, and technical limitations of fluorescence in situ hybridization (FISH) and G-banding that do not allow for accurate detection of sSMCs at high resolution (Starke et al. 2003a). This has resulted in a lack of genotype/phenotype correlation for most sSMCs.

1.2.3. Formation of sSMC

Different mechanisms of sSMC formation including trisomic rescue, monosomic rescue, post fertilization errors and gamete complementation have been proposed in the literature (Bartels et al. 2003, Liehr et al. 2004a). Later, a new mechanism was proposed, that could provides a possible explanation for the formation of multiple sSMC of different origin, in which sSMC originated 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). Modes of sSMC-formation, which were not topic of this work, are summarized in Fig. 1.3.

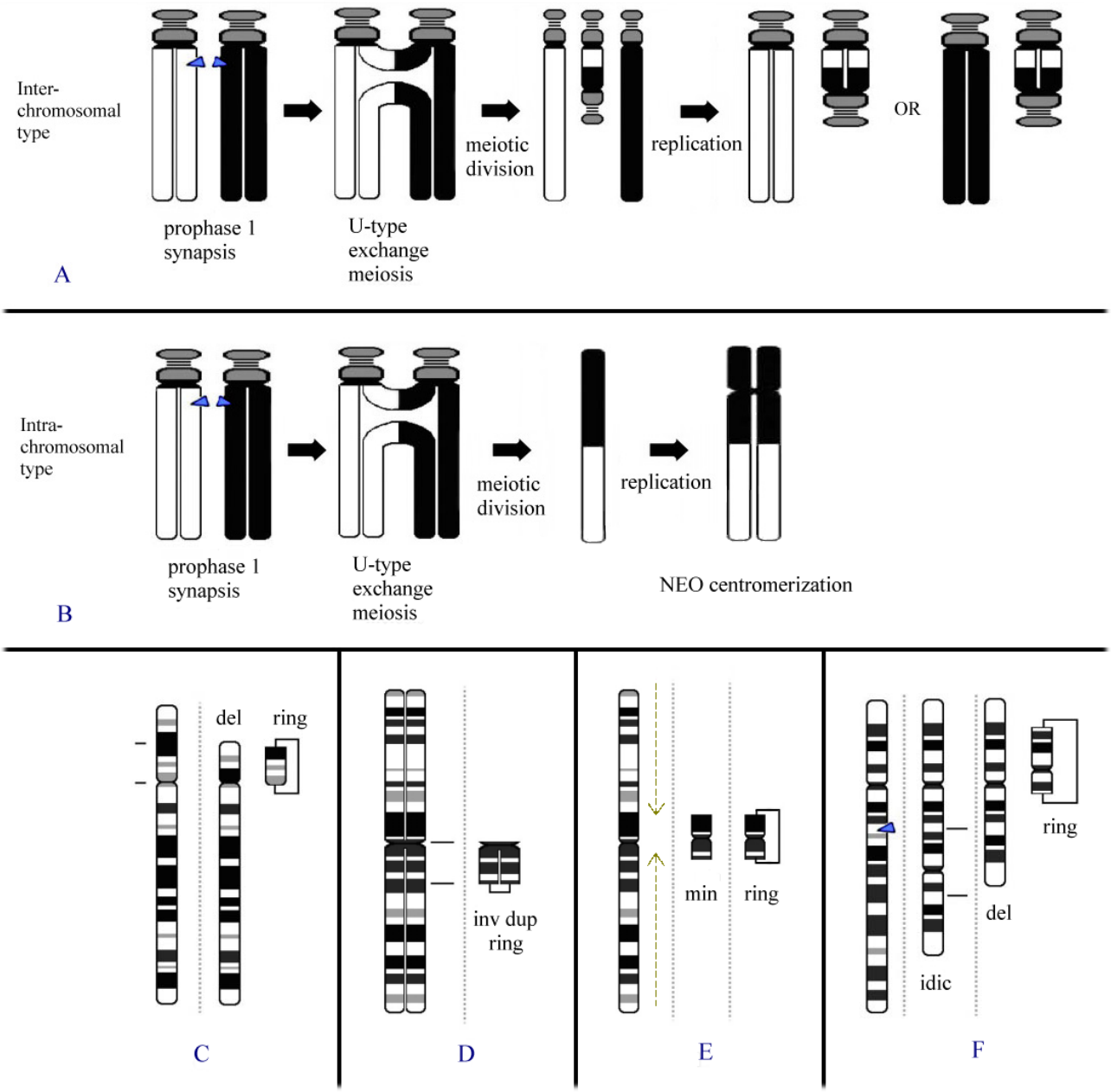

Figure 1.3. Different modes of sSMC formation: Development of an acrocentric inverted duplication chromosome (**A**) for non-acrocentric iso-chromosomes the same U-type exchange during meiosis is thought to be the most likely explanation for sSMC formation, as well. (**B**) The evolution of a neocentric chromosome in connection with a U-type exchange is depicted. Ring chromosome formation can be (**C**) due to an interstitial deletion, (**D**) in connection with a U-shape reunion between broken sister chromatids leading to an inverted duplicated ring or **(E)** evolve from a minute chromosome. The latter is postulated to evolve by degradation of a whole chromosome, which is indicated by the red arrows in the left part in E **(F)** arise connected with a complex chromosomal rearrangement leading to an inverted duplication prior to the formation of a ring (Liehr et al. 2004a).

1.2.3.1. Mixtures of different sSMC shapes

Recently Liehr and collaborators observed that one, two, or all three sSMC shapes (centric minute, ring, inverted duplication) can be present in a single patient with karyotype 47,XN,+mar (Liehr et al. 2006c). When previously unexpectedly different sSMC shapes are present, this condition is called cryptic mosaicism. Several patients with extremely active karyotypic evolution have been reported with up to ten different sSMC variants of the same derivative chromosome in their peripheral blood cells (Liehr et al. 2006c, Liehr 2009). Figure 1.4 reviews examples of how different shapes of sSMC can change to other ones. Presently, it can just be stated that this flexibility in sSMC shape exists; there are as yet no ideas on the mechanism of ring formation from a minute-shaped sSMC, for ring doubling, ring opening, and formation of inverted-duplication-shaped sSMC from centric minute-shaped sSMC, or for reduction of sSMC size and subsequent stabilizing of the sSMC again (Liehr 2012).

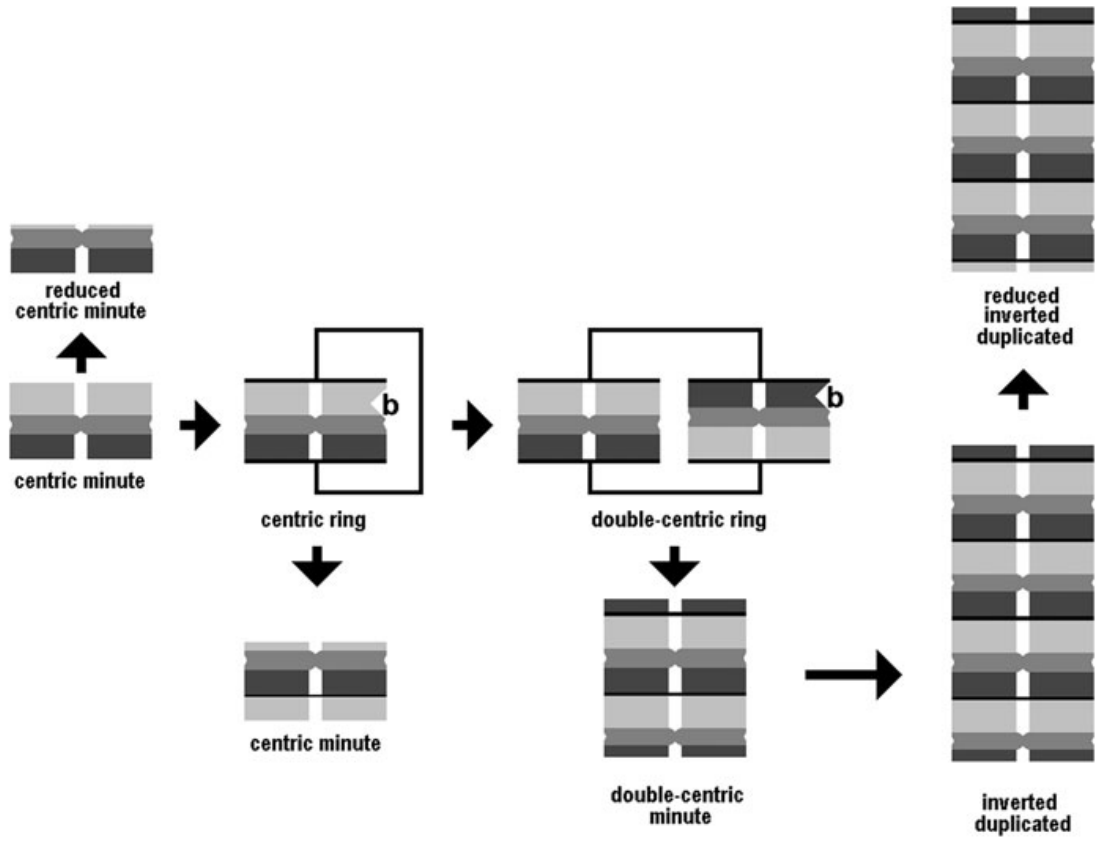

Figure 1.4. Multiple shapes of sSMC can evolve during the lifetime of an sSMC carrier. In the schematically given example according to the case reported in Liehr (2009), it is postulated that the starting point is a centric minute-shaped sSMC. This can undergo ring formation (***short horizontal arrows***), reduction in size (***vertical arrows to top***), ring opening (***vertical arrows to bottom***), and inverted duplication (***long horizontal arrow***) [Liehr 2012].

1.2.4. Epidemiology of sSMC in genetics disorders:

For several reasons sSMC are still a problem in clinical cytogenetics: (**i**) they are too small to be characterized for their chromosomal origin by traditional banding techniques and require molecular (cytogenetic) techniques for their identification (Liehr and Weise 2007); (**ii**) most of the sSMCs have not been correlated with clinical syndromes, even though progress was achieved, recently (Liehr et al. 2006c, Liehr 2014a); (**iii**) sSMC can be harmful due to different mechanisms like induction of genomic imbalance and/or UPD of the sSMC's sister chromosomes (Liehr et al. 2004b); (**iv**) also sSMC can be found just by chance and cannot be correlated with the clinical problems of a patient (Liehr 2010); finally (**v**) the percentage in which an sSMC is present (mosaicism) can, but must not have an influence on the clinical outcome (Liehr et al. 2004b, 2006c, Liehr 2014a). Thus, to understand the epidemiology of sSMC comprehensive studies of sSMC need to be done.

1.2.4.1. Clinical consequences of sSMC

In approximately 30% of SMC carriers an abnormal phenotype is observed. The clinical outcome of an sSMC is difficult to predict as they can have different phenotypic consequences because of (**1**) differences in euchromatic DNA-content, (**2**) different degrees of mosaicism, and/or (**3**) UPD of the chromosomes homologous to the SMC (Starke et al. 2003a). Also the risk for phenotypic abnormalities associated with a marker chromosome depends on several factors, including inheritance, mode of ascertainment, chromosomal origin, and the morphology, content, and structure of the marker (Graf et al. 2006). Thus, the main problem is de novo sSMC detected prenatally, which are not characterized in detail. It has been shown that most couples decide in such cases against the child, even though there is a 2:1 chance that the developing child would be normal.

Certain marker chromosomes are consistently identifiable by G-banding and have a well-established phenotype. Examples include i(12p), associated with PKS and i(18p), which cause both mild–moderate mental retardation and a characteristic facial appearance (Callen et al. 1990), and for chromosome 15-derived marker chromosomes, often seen as isodicentric 15q. FISH analysis allows discrimination between large markers that contain the *SNRPN* locus and thus are tetrasomic for the Prader–Willi syndrome (PWS) or Angelman syndrome (AS) critical region and those small markers that do not contain *SNRPN* (Crolla et al. 1995, Huang et al. 1997, Eggermann et al. 2002, Baldwin 2008).

1.3. Aims of the present study/ Questions worked on

The long term objective of the present project is get new insights in the regulation of gene-expression within the pericentric region of the human genome. There was some evidence at the begin of this study that there are dosage sensitive and insensitive regions around the human centromeres. This evidence was supported by own studies and thus the main focus of my studies were the following questions.

1) How to characterize sSMC quickly and comprehensively?
2) How to distinguish sSMC straight forward between benign and harmful?
3) Where are the borders of dosage-sensitive pericentric regions?

Or as summarized in the title of this thesis here should be studied **why sSMC break, where they break and how to distinguish harmful from harmless sSMC.**

To answer these questions FISH-probe sets were established, ~400 new sSMC cases were studied during the present work and microdissection based aCGH was systematically applied in 80 sSMC cases.

2.1. Basic papers of thesis

1. Liehr T, Weise A, **Hamid AB**, Fan X, Klein E, Aust N, Othman MAK, Mrasek K, Kosyakova N. **Multicolor FISH methods in current clinical diagnostics**. Expert Rev Mol Diagn, 2013;13(3):251–255.

2. Guilherme RS, Dutra ARN, Perez ABA, Takeno SS, Oliveira MM, Kulikowski LD, Klein E, **Hamid AB**, Liehr T, Melaragno MI. **First report of a small supernumerary der(8;14) marker chromosome**. Cytogenet Genome Res, 2013; 139:284-288.

3. Liehr T, Cirkovic S, Lalic T, Guc-Scekic M, de Almeida C, Weimer J, Iourov I, Melaragno M I, Guilherme RS, Stefanou E-GG, Aktas D, Kreskowski K, Klein E, Ziegler M, Kosyakova N, Volleth M, **Hamid AB**. **Complex small supernumerary marker chromosomes – an update**. Molecular Cytogenetics, 2013; 6:46

4. **Hamid AB**, Kreskowski K, Weise A, Kosayakova N, Mrasek K, Voigt M, Guilherme RS, Wagner R, Hardekopf D, Pekova S, Karamysheva T, Liehr T, Klein E. **How to narrow down chromosomal breakpoints in small and large derivative chromosomes – a new probe set**. J Appl Genet , 2012;53(3):259-269.

5. Liehr T, Karamysheva T, Merkas M, Brecevic L, **Hamid AB**, Ewers E, Mrasek K, Kosyakova N, Weise A. **Somatic mosaicism in cases with small supernumerary marker chromosomes.** Curr Genomics, 2010;11:432-439.

6. **Hamid AB**, Liehr T. **Pericentromeric BAC-probe set - thoughts about considering genedosage insensitive regions**. Mol Cytogenet 2013; 6:45/comments.

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11. Papoulidis I, Manolakos E, **Hamid AB**, Klein E, Kosyakova N, Kordaß U, Kunz J, Siomou E, Kontodiou M, Tzimina M, Nicolaides P, Liehr T, Petersen MB. **Tetrasomy 9p mosaicism associated with a normal phenotype in two cases**. Cytogenet Genome Res, 2012;136:237–241.

The main points of the present study - as highlighted in discussion - part are:

1) better sSMC characterization approaches,

2) characterization of chromosomal breakpoints involved in sSMC formation,

3) on mosaicism in sSMC, and overall,

4) on a refinement of the genotype-phenotype correlation in sSMC.

Those four points are also covered in the papers mentioned above as listed in the Table below:

Table.2.1. Articles of the present Ph.D. work is based on.

Article No.	probe sets	involved breakpoints	mosaicism	genotype/ phenotype
1	+	-	-	-
2	+	+	-	+
3	+	+	+	+
4	+	+	+	+
5	+	-	+	-
6	+	+	-	+
7	+	+	-	+
8	+	-	-	+
9	-	-	+	-
10	-	-	-	+
11	-	-	+	+

2.2. Article .1

Liehr T, Weise A, **Hamid AB**, Fan X, Klein E, Aust N, Othman MAK, Mrasek K, Kosyakova N. **Multicolor FISH methods in current clinical diagnostics**. Expert Rev Mol Diagn, 2013; 13(3): 251–255.

THEMED ARTICLE I Genetic & Genomics Applications Review

EXPERT REVIEWS

Multicolor FISH methods in current clinical diagnostics

Expert Rev. Mol. Diagn. 13(3), 251–255 (2013)

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Multicolor FISH (mFISH) assays are currently indispensable for a precise description of derivative chromosomes. Routine application of such techniques on human chromosomes started in 1996 with the simultaneous use of all 24 human whole-chromosome painting probes in multiplex-FISH and spectral karyotyping. Since then, multiple approaches for chromosomal differentiation based on multicolor-FISH (MFISH) assays have been developed. Predominantly, they are applied to characterize marker or derivative chromosomes identified in conventional banding analysis. Since the introduction of array-based comparative genomic hybridization (aCGH), mFISH is also applied to verify and further delineate aCGH-detected aberrations. For the latter, it is important to consider the fact that aCGH cannot detect or characterize balanced rearrangements, which are important to be resolved in detail in infertility diagnostics. In addition, mFISH is necessary to distinguish different imbalanced situations detectable in aCGH; small supernumerary marker chromosomes have to be differentiated from insertions or unbalanced translocations. This review presents an overview on the available mFISH methods and their applications in pre- and post-natal clinical genetics.

Keywords: derivative chromosomes • marker chromosomes • multicolor fluorescence *in situ* hybridization • postnatal diagnostics • prenatal diagnostics

Human genetics is a discipline, which includes pre- and post-natal counseling of patients and families. A genetic basis can be considered in individuals suffering from infertility and/or repeated abortions, or any kind of acquired or inherited syndrome [1]. At present, genetic counselors have a multitude of technical possibilities, some highly sophisticated, for the genetic analysis of an individual. Approaches such as next-generation sequencing of a whole genome has gained importance and has been helpful on many occasions [1,2].

Besides genetic counseling, another key element of human genetics is still the well-established approach of cytogenetics, including molecular cytogenetics [1]. In many western countries (e.g., Germany), insurance companies request, where appropriate, banding cytogenetics as the starting test for a genetic analysis. Thus, up to 40% of individuals in search of advice are still studied cytogenetically, and a subset of them are further analyzed by molecular cytogenetics [Schreyer I, Pers. Comm.]. Additionally, in most countries (except for North America and western Europe) cytogenetics is still the gold standard for any genetic analysis, with molecular cytogenetics becoming available over the last decade.

After the introduction of array-comparative genomic hybridization (aCGH), cytogenetics/molecular cytogenetics were considered to be outdated by some researchers [3,4]. However, it is common knowledge that aCGH results can only be correctly interpreted if cytogenetics is performed in parallel; in addition, abnormal aCGH results need to be confirmed by a second method, such as molecular cytogenetics [5,6].

Molecular cytogenetics

In banding cytogenetics – today often incorrectly called 'classical cytogenetics' (classical cytogenetics is Giemsa or Orcein staining without any banding) – only chromosome morphology combined with a black and white banding pattern is evaluated. Thus, only changes within the normal banding pattern, size variations in a chromosomal band or the chromosome itself, and changes to the centromere index, can be detected [7]. To overcome these limitations, FISH approaches were introduced in the 1980s,

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and the new field of 'molecular cytogenetics' was launched. For more information on FISH, readers are directed to [8], as such a discussion will not be covered in this review.

One-, two- and three-color FISH experiments are standard in every laboratory around the world performing molecular cytogenetics. Multicolor FISH (mFISH) is defined as the simultaneous use of at least three different ligands or fluorochromes for the specific labeling of DNA – excluding the counterstain [9]. Due to this definition, the first successful mFISH experiments were performed in 1989 [10]. The first mFISH probe sets were put together 7 years later in 1996 [11,12]. In the following review, the available mFISH probe sets for humans are summarized and their applications in pre- and post-natal diagnostics are highlighted.

mFISH probe sets

Whole-chromosome painting-based mFISH probe sets

Between 1996 and 2000, simultaneous staining of each of the 24 human chromosomes in different colors using whole-chromosome painting (WCP) probes was described repeatedly as multiplex-FISH (M-FISH) [11], spectral karyotyping (SKY) [12], mFISH, combined binary ratio labeling–FISH or 24-color FISH (reviewed in [9]). Between four and seven fluorescence dyes were used either for combinatorial labeling and/or ratio labeling (combinatorial labeling: three up to seven fluorochromes are combined; each flourochrome combination is only used once. Ratio labeling: a maximum of three different fluorochromes are combined and mixed for each chromosome in different ratios). It was also shown that additional probes can be added to the basic 24-color FISH probe set (summarized in [9]). Today, the WCP-based mFISH probe sets are the most frequently applied probe sets in diagnostics; they are mostly designated as M-FISH or SKY probe sets [101].

mFISH probe sets for FISH-banding

The definition of FISH-banding probe sets is "…any kind of FISH technique which provides the possibility to simultaneously characterize several chromosomal subregions smaller than a chromosome arm – excluding the short arms of the acrocentric chromosomes; FISH-banding methods fitting that definition may have quite different characteristics, but share the ability to produce a DNA-specific chromosomal banding…" [13].

The most often applied and also commercially available mFISH probe set for FISH banding is the high-resolution multicolor-banding (MCB) or m-banding technique [101]. It is based on overlapping microdissection libraries (partial chromosome paints [PCPs]) producing fluorescence profiles along the human chromosomes, which was first described using the example of chromosome 5 in 1999 [14]. MCB/m-banding allows for differentiation of chromosome region-specific areas at the band and sub-band level at a resolution of 550 bands per haploid karyotype. In addition, the simultaneous use of all MCB PCPs in one hybridization step for the characterization of complex karyotypes is possible [15]. For the MCB probe set, a molecular definition of all underlying microdissection libraries was performed, which converted MCB into a DNA sequence-anchored probe set [16].

Besides these, there were many other mFISH-banding probe sets, which either were never finished for all human chromosomes or are no longer (commercially) available, such as cross-species color banding (Rx-FISH) or the Harlequin-FISH probe set [17]; spectral color banding [18] ; or interspersed PCR-based M-FISH [19]. There are also many probe sets leading to chromosome bar codes with different resolutions and applications (for a more detailed review, see [9]).

Centromeric probe-based mFISH probe sets

Some mFISH probe sets are based on repetitive centromeric satellite probes. Such mFISH probe sets are extremely important in daily diagnostics, as combinations of different probes can principally be chosen freely according to the individual case and question [20]. There is also an mFISH probe set that allows the simultaneous characterization of all human centromeres in one step, the centromere-specific mFISH [21]. This probe set is especially useful for the characterization of the chromosomal origin of small supernumerary marker chromosomes (sSMC) [22,102].

Locus-specific probe-based mFISH probe sets

mFISH probe sets based on locus-specific probes can be created by every laboratory and many are commercially available [103–108]. Some of the abovementioned chromosome bar codes were based on such locus-specific probes [9]. At present, mainly bacterial artificial chromosome (BAC) probes are used, as the necessary BACs can easily be tracked in genome browsers [109–111] and are offered commercially [103]. One of the most imaginative mFISH probe sets developed during the last few years is the one that enables a type of single cell-directed microsatellite analysis; the so-called parental origin determination FISH (pod-FISH) approach, detecting copy number variant regions in the human genome on a single-cell level [23].

mFISH-probe sets based on combinations of a variety of probes

Finally, it is also possible to combine WCP, PCP, BAC or centromeric probes in one probe set. Recent examples are: the subcentromere-specific mFISH [24], which can specifically characterize the centromere near euchromatic material; the heterochromatin-M-FISH [25], which is specific for all larger heterochromatic regions in the human karyotype; or the 9het-mix [26], which enables subdifferentiation of chromosome 9 heteromorphisms in the human population.

Diagnostic applications of mFISH probe sets

mFISH probe sets are applied in pre- and post-natal clinical genetics (see below), tumor cytogenetics [9,13,101] and various research fields [9,13,101]. Here, the authors focus on their use in clinical genetics; that is, molecular cytogenetics performed on amnion, chorion, blood and, rarely, fibroblast cells. In all these tissues, it is possible to not only analyze the gain or loss of chromosomes or chromosomal segments in metaphase, but also in interphase. Structural rearrangements are normally studied on metaphase chromosomes in clinical genetics.

mFISH-probe sets applied in the interphase

Interphase mFISH diagnostics normally use the abovementioned combination of centromeric and/or locus-specific probes; PCPs and WCPs can be applied in interphase research, but are not suited for routine applications. The most commonly used diagnostic probe set is the AneuVision® (Vysis Inc., IL, USA) probe set [106] or comparable ones [104,106], suited to detect the most frequent numerical chromosomal aberrations of the human fetus in the second trimester [27]. Preimplantation diagnostics are performed with the aim of detecting up to 70% of the most frequent numerical chromosome aberrations responsible for spontaneous abortions [9].

All of the following mentioned applications are performed on metaphase chromosome preparations. Molecular cytogenetics is normally performed as a secondary diagnostic test; thus below, the primary test is listed as an entry criteria for mFISH.

mFISH used after a cytogenetic normal result

Banding cytogenetics in mentally retarded patients or prenatal cases with specific sonographic signs quite often give normal results. Still, the clinical signs may be indicative for some syndromes to be excluded by FISH, namely microdeletion or microduplication syndromes [28]. Therefore, the clinician needs to provide a suspected diagnosis and then the FISH probes for the corresponding microdeletion, or microduplication syndrome may be applied. In most cases, these probes are applied in two-color FISH experiments [104–106]; however, there was a suggestion for a simultaneous screening for Prader–Willi/Angelman (15q11.13), Williams–Beuren (7q11.23), Smith–Magenis (17p11.2) and DiGeorge/velocardiofacial (22q11.2) syndromes in one so-called 'multiFISH' assay [29]. In addition, there were mFISH probe sets with locus-specific probes for the subtelomeric regions, which were successfully applied to detect genetic imbalances in up to 6% of patients with iodiopathic mental retardation [9]. However, in many instances such probe sets are now successfully replaced by real-time PCR, multiplex ligation-dependent probe amplification or aCGH settings [30].

mFISH used after an abnormal cytogenetic result

A cytogenetically abnormal result, which needs further mFISH testing, can include mosaics [31], larger derivative chromosomes (balanced and unbalanced) [32] and/or the presence of an sSMC [22,102].

M-FISH or SKY probe sets will only be used in cases of complex chromosomal rearrangements [32] or if a derivative chromosome contains additional material of completely unknown origin [33]. As soon as the origin of the involved chromosomes is known, the chromosomal breakpoints are of interest and can be determined by mFISH-banding and/or locus-specific probes [9,13,101]. However, WCP-based mFISH-probe sets and mFISH-banding probe sets are not helpful for the characterization of sSMC or of heterochromatic variants.

Cytogenetically visible heterochromatic variants can be best characterized by the recently reported heterochromatin M-FISH probe set [25] or subsets of them [26,33].

sSMC, excluding neocentric ones [102], can be best characterized for their origin by centromere-specific M-FISH [21,22,24,102]. Subcentromere-specific M-FISH [24] is a straightforward approach for defining their euchromatic content, which might further be delineated by the pericentric ladder FISH probe set [34]. The latter enables a breakpoint analysis on a 10-Mb resolution.

mFISH used after an abnormal aCGH result

Since aCGH is applied for the characterization of subchromosomal imbalanced rearrangements [3–5], this is another starting point for the application of molecular cytogenetics. Here, individual combinations of locus-specific (BAC) probes are used to prove or contradict a gain or loss suggested after aCGH [5,35].

aCGH is not necessarily fully informative with regards to the number of copies gained in the patient; for example, a threefold gain of 18p detected in aCGH may be a hint of an intrachromosomal duplication or a derivative chromosome t(autosome;18)(?;p10) of the corresponding region in all cells of the patient. However, it can also be a hint on a mosaic karyotype 47,XN,+i(18)(p10)(50%)/46,XN(50%). FISH and mFISH applications following detection of an abnormal aCGH result were already repeatedly published [34–36] and are routine in clinical genetic diagnostics.

Conclusion

At present, mFISH methods are well established in clinical diagnostics. Apart from their longstanding role in refining and confirming cytogenetic results, mFISH approaches have gained additional importance in the verification of aCGH results. This underlines the truth that every approach has advantages and disadvantages: the conventional approach-banding cytogenetics has a lower resolution but provides a highly informative '*in situ*' view on the human genome; aCGH, however, results in a higher resolution but gives a result more distant from the *in vivo* situation and can only detect imbalanced rearrangements. Both approaches are connected by molecular cytogenetics, and a comprehensive view on a pre- or post-natal clinical case is most often only possible after applying several of the currently available approaches, including mFISH, in a majority of them.

Expert commentary

Molecular cytogenetics, especially mFISH, is still a progressive field. New mFISH probe sets are being developed up to the present date [25,34]. Otherwise, mFISH is necessary to confirm and refine diagnostic findings of cytogenetics and aCGH. Therefore, the method is the connecting approach for banding cytogenetics and molecular genetics.

Five-year view

The field of molecular cytogenetics/mFISH is an important tool to define and visualize chromosomal changes detectable in pre- and postnatal diagnostics. According to the fact that mFISH gained importance during the last years rather than lose it, in 5 years from now, it will be at least as significant as diagnostics are now. It can be expected that even findings seen in next-generation sequencing are necessary to be confirmed by mFISH in future [37].

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Key issues

- Human genetics is a discipline that includes pre- and post-natal counseling of patients and families, (molecular) cytogenetics and molecular genetics.
- In molecular cytogenetics, multiple multicolor FISH (mFISH) approaches are now available.
- mFISH is performed based on whole or partial chromosome painting, centromeric or locus-specific DNA probes.
- Since 1996, new mFISH probe sets have been established every year, and this development is still ongoing.
- mFISH can be applied during interphase and metaphase.
- mFISH assays are indispensable for a precise description of derivative chromosomes identified in banding cytogenetics.
- Small supernumerary marker chromosomes can still be best analyzed by mFISH.
- In the last few years, mFISH has become an important instrument for array-comparitive genomic hybridization confirmation.

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2.3. Article .2

Guilherme RS, Dutra ARN, Perez ABA, Takeno SS, Oliveira MM, Kulikowski LD, Klein E, **Hamid AB**, Liehr T, Melaragno MI. **First report of a small supernumerary der(8;14) marker chromosome**. Cytogenet Genome Res, 2013; 139:284-288.

Short Report

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First Report of a Small Supernumerary der(8;14) Marker Chromosome

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Key Words
Duplication 14q · FISH · SNP array · sSMC

Abstract
Small supernumerary marker chromosomes (sSMC) are structurally abnormal chromosomes, generally equal in size or smaller than a chromosome 20 of the same metaphase spread. Most of them are unexpectedly detected in routine karyotype analyses, and it is usually not easy to correlate them with a specific clinical picture. A small group of sSMCs is derived from more than one chromosome, called complex sSMCs. Here, we report on a patient with a de novo complex sSMC, derived from chromosomes 8 and 14. Banding karyotype analysis, multiplex ligation-dependent probe amplification (MLPA), single nucleotide polymorphism (SNP)-based array, and fluorescence in situ hybridization (FISH) were performed to investigate its origin. Array and FISH analyses revealed a der(14)t(8;14)(p23.2;q22.1)dn. The propositus presents some clinical features commonly found in patients with partial duplication or triplication of 8p and 14q. This is the first report describing a patient with a congenital der(14)t(8;14)(p23.2;q22.1)dn sSMC.

Small supernumerary marker chromosomes (sSMC) are 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]. As a rule, sSMCs are detected unexpectedly in routine karyotype analyses and are not easily correlated with a specific clinical picture [Liehr et al., 2004, 2010]. sSMCs are reported in 0.043% of newborn infants, 0.077% of prenatal diagnosis cases, 0.433% of patients with intellectual disability, and 0.171% of subfertile persons [Liehr and Weise, 2007]. sSMCs have been observed to be derived from any of the 24 human chromosomes, but most of them originate from chromosome 15, followed by chromosome 22 (http://www.fish.uniklinikum-jena.de/sSMC.html). One of the smallest sSMC subgroups is constituted by the so-called complex sSMCs, which contain chromosomal material of more than one chromosome [Trifonov et al., 2008]. Vetro et al. [2012] also described 4 patients with sSMCs originated by an un-

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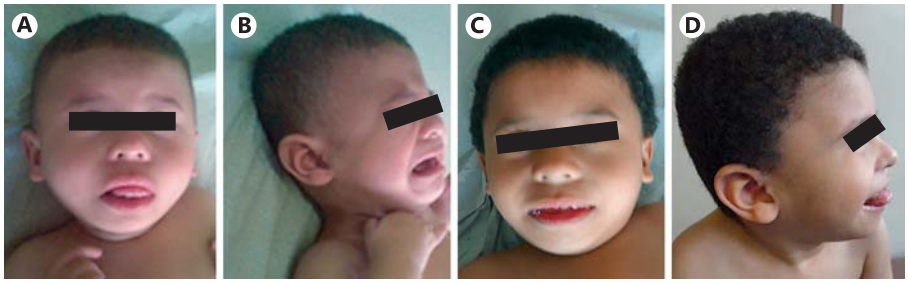

Fig. 1. Front and lateral view of the patient at ages 1 year and 9 months (**A**, **B**) and 2 years and 9 months (**C**, **D**).

expected mechanism. Among the comprehensively characterized der(14) sSMCs, the vast majority consists of inv/dup(14), min(14) and r(14) chromosomes [Liehr, 2012]. Only few sSMC(14) cases different from these have been reported, including dic(14;15), der(14)t(3;14), der(14)t(5;14), der(14)t(14;16), der(14)t(14;17), and der(14)t(3;14) chromosomes. With regard to sSMC(8), most of them comprise proximal centromere-near region imbalances, including minute and ring chromosomes or isochromosomes 8p, almost all in mosaic with normal cell lines. Inv/dup(8) chromosomes with neocentromeres have also been reported. Here, we report the first complex sSMC derived from chromosomes 14 and 8, characterized as der(14)t(8;14)(p23.2;q22.1)dn.

Clinical Report

The patient (fig. 1A–D), a male, was born to healthy and nonconsanguineous parents with 2 healthy older sons. At birth, the mother was 44 and the father 42 years old. The patient was born preterm (at 36.3 weeks) by vaginal delivery after an uneventful pregnancy. His birth weight was 2,550 g (P25), length 47 cm (P50), head circumference 32 cm (P50), and the APGAR scores were 7/9. The mother presented arterial hypertension during the pregnancy and bleeding in the third trimester. As a newborn, the patient presented feeding difficulties. A skull MRI revealed mild, not-hypertensive ventricular dilation, accentuation of tanks and cortical grooves. The patient further developed seizures since 2 months of age and had pneumonia. When he was 1 year and 9 months old, his weight was 7,885 g (<P3), length 76 cm (<P3) and head circumference 44 cm (<P3). His neuropsychomotor development was delayed (he could sit only with support), and he presented microsomia, brachycephaly, round face, blue sclera, upslanting oblique palpebral fissures, depressed nasal bridge, posteriorly placed low-set ears, high palate, short neck, downturned corners of the mouth, thin lips, large mouth with tongue protrusion, bilateral testicular retraction, and clinodactyly of the 5th fingers. At the age of 2 years and 9 months, he had similar anthropometric values (<P3), was unable to stand up without help, could not walk, and was followed-up by an audiologist, a physiotherapist, a neurologist, and an ophthalmologist. He had recurrent bronchopneumonia and seizures controlled by valproic acid.

Materials and Methods

Cytogenetic and Molecular Study

Chromosome analysis was performed on 72-hour lymphocyte cultures according to standard procedures. Fluorescent in situ hybridization (FISH) was carried out using a centromeric probe for chromosome 14/22 (Kreatech Diagnostics, Amsterdam, The Netherlands), a homemade probe specific for all acrocentric short arms (midi54, described in Starke et al. [2002]) and subtelomeric probes for chromosomes 8p and 14q (Vysis FISH technology from Abbott, North Chicago, Ill., USA). DNA was isolated from peripheral blood using a Gentra Puregene kit (Qiagen Sciences Inc., Germantown, Md., USA). A multiplex ligation-dependent probe amplification (MLPA) assay was performed using the subtelomeric SALSA MLPA kit P070 (MRC-Holland, Amsterdam, The Netherlands) as previously described by Christofolini et al. [2010]. Samples from the patient and his parents were genotyped using the Affymetrix Genome-Wide Human SNP Nsp/Sty 6.0 array (Affymetrix Inc., Santa Clara, Calif., USA) as previously described by Guilherme et al. [2010].

Results

G-banding revealed an sSMC in all 50 cells analyzed (fig. 2A). The parents' karyotypes were normal. FISH analysis showed an sSMC with a subtelomeric signal for the subtelomeric probe 8p and short arm 14p through midi54 probe, which confirmed the array findings (fig. 2B, C). MLPA results were analyzed by GeneMarker software and revealed an increased number of copies of the FBXO25 and ADPRTL2 probes, located in the subtelomeric 8p and proximal 14q region, respectively (results not shown). The array data were analyzed using annotation UCSC Genome Browser (http://genome.ucsc.edu/) on Human February 2009 (GRCh37/hg19) Assembly and showed duplication of 8p23.3p23.2 and 14q11.2q22.1 (fig. 2D, E). An ideogram illustrates how the sSMC was formed (fig. 2F). Thus, the patient presents a complex sSMC(14), derived from a translocation between chromosomes 8 and 14. The resulting karyotype can be described as 47,XY,+der(14)t(8;14)(p23.2;q22.1)dn.arr

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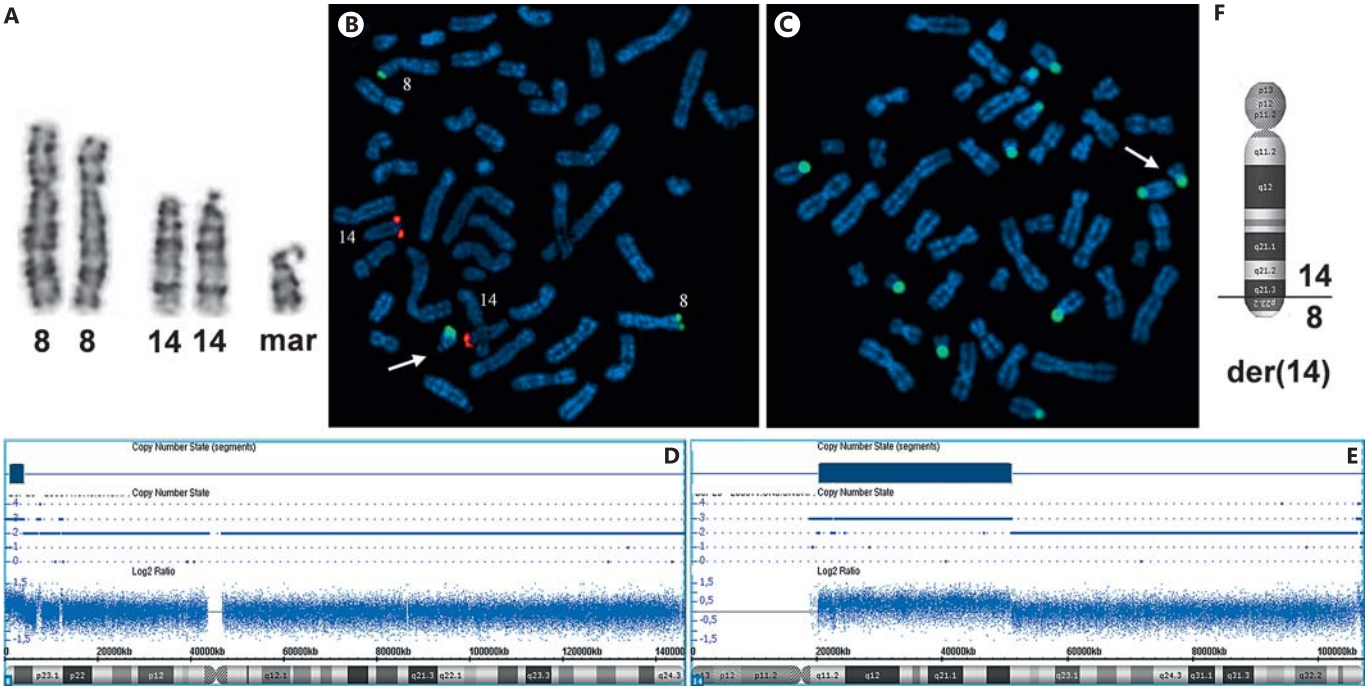

Fig. 2. Cytogenetic and molecular results for the sSMC(14). **A** Partial G-banded karyotype showing chromosome pairs 8 and 14 and the sSMC(14). **B** FISH with subtelomeric probes for chromosomes 8p (green) and 14q (red). **C** FISH using a probe specific for all acrocentric short arms (midi54), showing the presence of p-arm material on all acrocentric chromosomes and the sSMC. **D**, **E** Array results showing chromosomes 8 (left) and 14 (right) and their duplicated regions (blue bars). **F** Ideogram of the sSMC(14) as a der(14)t(8;14)(p23.2;q22.1).

8p23.3p23.2(46,385–4,301,995)×3,14q11.2q22.1 (19,002,111–51,106,110)×3. The parent's array results were normal, and the comparison of the child's and his parents' SNP genotypes revealed that the duplicated chromosome 8 and 14 material was of maternal origin.

Discussion

Here, we report a rare case with a complex sSMC derived from chromosomes 8 and 14, resulting in a 4.3-Mb duplication of the 8pterp23.2 and a 51-Mb duplication of the region 14q11.2q22.1. We did not find any directly comparable case in the literature on patients with congenital abnormalities. However, there are 4 more complex sSMCs derived from chromosome 14 (cases 14-U-12, 14-U-17, 14-U-18, and 14-U-23) and 3 complex sSMCs involving the 8pter region, which are derived from chromosomes 13, 18 and 22 (cases 13-U-8, 18-U-10 and 22-U-43), described by Liehr [2012]. The breakpoints in chromosomes 14 are reported as being located at q13, q12, q11.2, and q21.1. None of the previously reported cases were studied by array. Thus, the observed breakpoints might be the same, at least in a subset of these cases. For the 8pter region, recently 2 low-copy repeats were identified which promote translocations of this chromosomal region [Giorda et al., 2007]. According to Mapview (http://www.ncbi.nlm.nih.gov/mapview), the region 8p23.3p23.2, which is duplicated in our patient's sSMC, presents only a few genes, including 6 OMIM genes (http://www.ncbi.nlm.nih.gov/projects/mapview/maps.cgi). The duplicated 14q region contains several genes, including 159 OMIM genes, among which the *NPAS3* (neuronal PAS domain protein 3) gene that may regulate genes involved in neurogenesis, the *FOXG1* (forkhead box G1) and *AKAP6* (A kinase (PRKA) anchor protein 6) genes which may play a role in brain development, and the *PNP* (purine nucleoside phosphorylase), *CZMB* (granzyme B), *CLEC14A* (C-type lectin domain family 14, member A), and *IL25* (interleukin 25) genes which are important for normal immune response and could be responsible for some clinical features observed. Partial duplications of 8p are quite often reported in the clinical genetics literature. The 8p duplication syndrome

comprises profound mental retardation, cardiac malformation, agenesis of the corpus callosum, neonatal hypotonia, feeding difficulties, and other minor anomalies [Brooks et al., 1998]. Patients with duplication of region 8p22p23.3 have been reported to be clinically normal or to have mild mental retardation with no dysmorphic features [Brooks et al., 1998; Engelen et al., 2000], suggesting that the genes located in this region may play only a minor role in the neurobehavioral and physical development. We propose that the clinical features presented by our patient result mainly from trisomy 14q. The common characteristics reported in patients with partial 14q duplication or triplication found in our patient are: intellectual disability, seizures, delayed neuropsychomotor development, brachycephaly, low-set ears, high palate, macrostomia, and tongue protrusion [Lemire and Cardwell, 1999; Schinzel, 2001; Lee-Jones et al., 2004; Eggermann et al., 2005]. The partial 14q duplication presents with a variable phenotype, depending on the breakpoints location. Most of the patients with 14q12 microduplication including the *FOXG1* gene described in the literature suffered from seizures or infantile spasms [Yeung et al., 2009; Brunetti-Pierri et al., 2011; Tohyama et al., 2011], similar to our patient. This microduplication is found more often in males and is frequently associated with West Syndrome [Allou et al., 2012]. Recently, *FOXG1* was reported to be a dose-sensitive gene whose duplication could result in severe epilepsy, infantile spasms and developmental delay [Yeung et al., 2009; Brunetti-Pierri et al., 2011], while its deletion could cause seizures, but not infantile spasms [Tohyama et al., 2011]. The clinical phenotype of our patient may be the result of the additional genes present in the duplicated region and not only of the *FOXG1* gene, which represents the most interesting candidate to explain his abnormal neurodevelopment. The sSMC originated from maternal chromosomes 8 and 14, probably due to errors in meiosis that could be related to the mother's age. Region 14q22.1 also presents segmental duplications. The sSMC derived from chromosomes 8 and 14 produces partial upd(14) and upd(8). Considering that no imprinted genes are reported in chromosome 8, only in the distal part of chromosome 14 [Kotzot, 2004], we concluded that the patient's phenotype was not influenced by imprinting, since the imprinted region is not present in his sSMC. To our best knowledge, this is the first report describing a patient with a congenital sSMC(14) derived from an 8p/14q translocation who presents a clinical phenotype commonly found in patients with partial 8p and 14q duplication or triplication. In addition, the repeated involvement of 8pter in chromosomal translocations was confirmed once more.

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2.4. Article .3

Liehr T, Cirkovic S, Lalic T, Guc-Scekic M, de Almeida C, Weimer J, Iourov I, Melaragno MI, Guilherme RS, Stefanou E-GG, Aktas D, Kreskowski K, Klein E, Ziegler M, Kosyakova N, Volleth M, **Hamid AB**. **Complex small supernumerary marker chromosomes – an update**. Molecular Cytogenetics, 2013; 6:46

Liehr et al. Molecular Cytogenetics 2013, 6:46
http://www.molecularcytogenetics.org/content/6/1/46

MOLECULAR CYTOGENETICS

RESEARCH Open Access

Complex small supernumerary marker chromosomes – an update

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Abstract

Background: Complex small supernumerary marker chromosomes (sSMC) constitute one of the smallest subgroups of sSMC in general. Complex sSMC consist of chromosomal material derived from more than one chromosome; the best known representative of this group is the derivative chromosome 22 {der(22)t(11;22)} or Emanuel syndrome. In 2008 we speculated that complex sSMC could be part of an underestimated entity.

Results: Here, the overall yet reported 412 complex sSMC are summarized. They constitute 8.4% of all yet in detail characterized sSMC cases. The majority of the complex sSMC is contributed by patients suffering from Emanuel syndrome (82%). Besides there are a der(22)t(8;22)(q24.1;q11.1) and a der(13)t(13;18)(q11;p11.21) or der(21)t(18;21)(p11.21;q11.1) = der(13 or 21)t(13 or 21;18) syndrome. The latter two represent another 2.6% and 2.2% of the complex sSMC-cases, respectively. The large majority of complex sSMC has a centric minute shape and derives from an acrocentric chromosome. Nonetheless, complex sSMC can involve material from each chromosomal origin. Most complex sSMC are inherited form a balanced translocation in one parent and are non-mosaic. Interestingly, there are hot spots for the chromosomal breakpoints involved.

Conclusions: Complex sSMC need to be considered in diagnostics, especially in non-mosaic, centric minute shaped sSMC. As yet three complex-sSMC-associated syndromes are identified. As recurrent breakpoints in the complex sSMC were characterized, it is to be expected that more syndromes are identified in this subgroup of sSMC. Overall, complex sSMC emphasize once more the importance of detailed cytogenetic analyses, especially in patients with idiopathic mental retardation.

Keywords: Complex small supernumerary marker chromosomes (sSMC), Genotype-phenotype correlation, Mosaicism, SSMC shape, Emanuel syndrome

Background

Small supernumerary marker chromosomes (sSMC) are structurally abnormal chromosomes that cannot be identified or characterized in detail by banding cytogenetics, are generally about the size of or smaller than a chromosome 20, and molecular cytogenetic techniques are necessary for their comprehensive characterization [1]. It is estimated that there are ~3 million of sSMC carriers in the human population of 7 billion individuals. Fortunately, only in 1/3 of the cases the sSMC is associated with clinical abnormalities [2]. Besides some specific syndromes, i.e. Pallister-Killian {= i(12p), OMIM #601803}, isochromosome 18p {i(18p), OMIM #614290}, cat-eye {i(22p ~ q), OMIM #115470}, idic(15) {no OMIM number} and Emanuel or derivative chromosome 22 {der(22)t(11;22), OMIM #609029} syndromes [2], for the remaining sSMC-cases only first steps towards genotype-phenotype correlations were achieved [2,3].

sSMC can present with different shapes (ring-, centric minute- and inverted duplication-shape), and consist in the majority of the cases of pericentric chromosomal material. Besides, sSMC can be derived from any part of the

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Liehr *et al. Molecular Cytogenetics* 2013, **6**:46
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human chromosomes and form neocentrics [2,4]. If they derived from the chromosomal ends, in most cases they lead to partial tetrasomies [2]; for one of those conditions also an OMIM entry was introduced recently (#614846 - tetrasomy 15q26 syndrome).

One of the smallest subgroup of sSMC is constituted by the so-called complex marker chromosomes [5]. 'Complex' are such sSMC which consist of chromosomal material derived from more than one chromosome [1]. Thus, besides the aforementioned large group of Emanuel or derivative chromosome 22 {der(22)t(11;22), OMIM #609029} syndrome cases, there was identified a second recurrent complex sSMC in 2010, designated as supernumerary der(22)t(8;22) syndrome {OMIM #613700} [6].

In 2008 we speculated that the then described 22 complex sSMC cases, excluding the der(22)t(11;22) cases, could be part of an underestimated entity [5]. Here the yet reported 412 complex sSMC cases are summarized based on the sSMC database (http://www.fish.uniklinikum-jena.de/sSMC.html, [3]) and analyzed for their chromosomal constitution, breakpoints and special features.

Results

The 412 complex sSMC available in literature constitute 8.4% of all yet in detail characterized sSMC cases. The majority of the complex sSMC cases is contributed by der(22)t(11;22)(q23;q11.2) cases, i.e. 339/412 cases (82%). Besides there are two additional types of complex sSMC which have been observed in more than 2 independent patients: the der(22)t(8;22)(q24.1;q11.1) and the der(13)t(13;18)(q11;p11.21) or der(21)t(18;21)(p11.21;q11.1) = der(13 or 21)t(13 or 21;18) (Figure 1A). Both represent another 2.6% and 2.2% of complex sSMC-cases (Figure 1B).

Concerning the shape, complex sSMC present in banding cytogenetics normally as centric minutes: this accounts for all Emanuel syndrome cases and 94% of the remainder ones. Only 2% each of the complex sSMC (excluding Emanuel syndrome cases) occur as inverted duplicated and ring shaped sSMC (Figure 1C). All complex sSMC, apart from one, derive from two chromosomes; only case 07-U-1 is reported to be constituted of three different chromosomes.

As summarized in Table 1, each of the human chromosomes, excluding chromosome 10, was involved in the formation of complex sSMC already. All apart from 14 complex sSMC are derivatives of acrocentric chromosomes. Of the non-acrocentric complex sSMC, derivatives of chromosome 18 were observed most often (3 times).

For 57 of the 73 complex sSMC (excluding Emanuel syndrome) parental studies were done. As depicted in Figure 1D 36% of those were de novo, the remainder

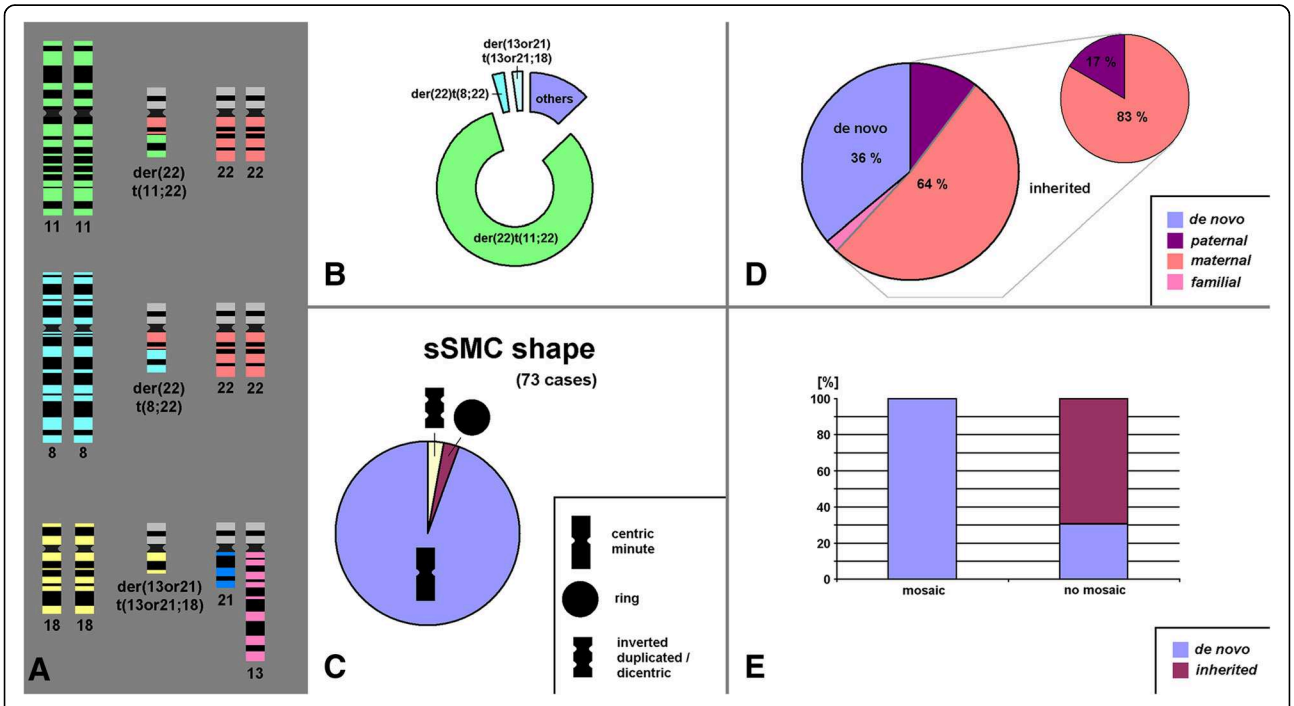

Figure 1 Complex sSMC: frequencies, shapes, origin and mosaicism. A) Schematic depictions of the three yet known complex sSMC leading to specific syndromic conditions: the Emanuel = der(22)t(11;22), the der(22)t(8;22) and the der(13 or 21)(13 or 21;18) syndrome. **B)** Frequency of the known three syndromes from **A)** and the other complex sSMC (others) depicted as a ring diagram. **C)** Distribution of the sSMC shapes among the reported complex sSMC cases excluding the cases with Emanuel syndrome. **D)** Distribution of de novo and inherited cases among complex sSMC excluding the cases with Emanuel syndrome. **E)** Complex sSMC tended to be mosaic only among the de novo cases.

Liehr *et al. Molecular Cytogenetics* 2013, **6**:46
http://www.molecularcytogenetics.org/content/6/1/46

Table 1 Complex sSMC cases summarized from Liehr (2013), not including 339 der(22)t(11;22)(q23;q11.2) cases

Karyotype	Origin	Mosaic	Gender	Case acc. to Liehr (2013)
der(4)t(4;7)(q12;p22.1)	n.a.	-	F	04-U-10
der(4)t(4;9)(q12;p21.2)	mat	-	F	04-U-11
der(7)t(X;5;7)(p22.1;q35;p13q21)	dn	-	F	07-U-1
der(8;12)(8pter → 8q11.1::12q11.1 → 12pter)	dn	+	M	08-U-10
der(9)t(3;9)(p25;q21.1)	mat	-	F	09-U-22
r(11)t(11;20)(::11p11.1 → 11q12.1::20q13.1?2 → 20q13.32::)	dn	+	F	11-U-12
der(11)t(11;13)(q25;q14)	pat	-	M	11-U-13
der(12)t(4;12)(p16;q11)	mat	-	n.a.	12-U-6
der(13)t(1;13)(q32;q12)	n.a.	-	F	13-U-16
der(13)t(4;13)(q31.3;q13)	mat	-	F	13-U-14
der(13)t(8;13)(p23.1;q12.11)	mat	-	M	13-U-8
der(13 or 21;14)(q10;q10)	n.a.	+	F	13/21-O-q10/4-1
der(13 or 21;15)(q10;q10)	n.a.	-	F	13/21-O-q10/5-1
der(13 or 21)t(13 or 21;18)(q11;p11.2)	dn	-	F	13/21-U-8
der(acro)t(acro;18)(q11;p11.21)	dn	-	F	13/21-U-8d
der(13)t(13;18)(q11;p11.21) or der(21)t(18;21)(p11.21;q11.1)	n.a.	-	F	13/21-U-8a
der(13)t(13;18)(q11;p11.21) or der(21)t(18;21)(p11.21;q11.1)	dn	-	M	13/21-U-8b
der(13)t(13;18)(q11;p11.21) or der(21)t(18;21)(p11.21;q11.1)	n.a.	-	F	13/21-U-8c
der(13)t(13;18)(q11;p11.21) or der(21)t(18;21)(p11.21;q11.1)	n.a.	-	M	13/21-U-8e
der(13)t(13;18)(q11;p11.21) or der(21)t(18;21)(p11.21;q11.1)	dn	-	F	13/21-U-8f
der(13)t(13;18)(q11;p11.21) or der(21)t(18;21)(p11.21;q11.1)	dn	-	M	13/21-U-8g
der(13)t(13;18)(q11;p11.21) or der(21)t(18;21)(p11.21;q11.1)	dn	-	M	+21-U-35
der(14)t(3;14)	mat	n.a.	n.a.	14-U-11
der(14)t(3;14)(p24.1;q21.1)	mat	-	M	14-U-23
der(14)t(5;14)(q13;p13.3)	n.a.	-	F	14-U-12
der(14)t(8;14)(p23;q22)	n.a.	-	M	14-U-27
dic(14;15)(14pter- > 14q11.2::15q11.1- > 15pter)	dn	-	M	14-O-q11.2/1-1
der(14)t(14;16)(q12;q21)	n.a.	-	F	14-U-17
der(14)t(14;17)(q11.2;q25.3)	mat	-	M	14-U-18
der(14)t(14;19)(14pter → 14q11.1::19p13.12 → 19p13.2:)	dn	+	F	14-U-26
der(14 or 22)t(2;14 or 22)(p11.2;q11.1)	dn	+	F	14/22-U-19
der(15)t(15;?)(q24;?)	dn	-	F	15-CW-3
der(15)t(9;15)(p24;q11.2)	mat	-	M	15-O-q11.2/5-1
dic(15;22)(15q11.1;22q22.1)	dn	-	M	15-U-6
der(Y;15)	n.a.	-	F	15-CO-1
der(15)t(Y;15)(q12;q22)	dn	-	M	15-U-10
der(15)t(8;15)(p23.2;q21.3)	dn	-	M	15-U-208
der(15)t(9;15)(p12;q14)	mat	-	F	15-U-189
mar(15;16)	n.a.	n.a.	n.a.	15-U-160
der(15)t(15;16)(q13;p13.2)	mat	-	F	15-U-15
inv dup(13;15)(p11.2p11.2)	n.a.	+	F	15-U-161
der(15)t(15;16)(q13;q13)	mat	-	M	15-U-206
der(15)t(15;16)(q13;p13.2)	mat	-	F	15-U-207
der(15)t(15;17)(q12;q25.3)	mat	-	M	15-U-214
der(15)t(15;18)(q11.1;p11.1 ~ 11.21)	n.a.	-	M	15-U-205
der(17)t(17;acro)(q11;p11.2)	dn	-	M	17-W-p13.3/1-1

Liehr *et al. Molecular Cytogenetics* 2013, **6**:46
http://www.molecularcytogenetics.org/content/6/1/46

Table 1 Complex sSMC cases summarized from Liehr (2013), not including 339 der(22)t(11;22)(q23;q11.2) cases *(Continued)*

der(18)t(2;18)(p23.1;q11.1)	dn	+	F	18-U-24
der(18)t(8;18)(p23.2 ~ 23.1;q11.1)	n.a.	-	M	18-U-10
der(19)t(18;19)	n.a.	n.a.	F	19-U-15
der(18)t(18;21 or 22)	fam	n.a.	n.a.	18-CW-2
der(21)t(4;21)(q32.1;q21.2)	mat	-	F	21-U-15
der(21)t(7;21)(p21;q21.3)	mat	-	M	21-U-7
der(13/21;22)(13/21pter → 13/21q11::22q11.1 ~ 11.2 → 22q11.21 ~ 11.22: :22q11.21 ~ 11.22 → 22pter)	dn	-	F	22-Wces-5-101
der(22)t(6;22)(p22.1;q11.21)	?pat	-	F	22-U-53
der(22)t(8;22)(q24.1;q11.2)	pat	-	M	22-U-11
der(22)t(8;22)(q24.1;q11.1)	mat	-	M/F	22-U-11a1/a2
der(22)t(8;22)(q24.1;q11.1)	pat	-	M	22-U-11b
der(22)t(8;22)(q24.1;q11.1)	mat	-	M	22-U-11c
der(22)t(8;22)(q24.1;q11.1)	mat	-	M	22-U-11d
der(22)t(8;22)(q24.1;q11.1)	mat	-	M	22-U-11e
der(22)t(8;22)(q24.1;q11.1)	mat	-	M	22-U-11f
der(22)t(8;22)(q24.13;q11.21)	n.a.	-	M	22-U-11g
der(22)t(8;22)(q24.13;q11.21)	pat	-	F	22-U-11h
der(22)t(8;22)(q24.1;q11.2)	mat	-	M	22-U-11i
der(22)t(8;22)(q24.1;q11.2)	n.a.	-	M	22-U-11j
der(22)t(8;22)(p22;q11.21)	mat	-	M	22-U-43
der(22)t(9;22)(p13.1;q11)	mat	-	M	22-U-57
der(22)t(12;22)(p12;q11.2-12)	dn	-	M	22-U-18
der(22)t(12;22)(p13.3;q12)	mat	-	M	22-U-18a
der(22)t(14;22)(q31;q11)	mat	-	F	22-O-q11/3-1
der(22)t(17;22)(17pter → 17p10::22q10 → 22pter)	mat	-	M	22-U-6
der(22)t(17;22)(p13.3;q11.21)	pat	-	M	22-O-q11.21/3-1
der(22)t(19;22)(q13.42;q11.1)	n.a.	-	M	22-U-50
r(15)ins(15;5)(?;q35.5q35.3)der(18)(:p11.21 → q11.1:)der(18)(:p11.1 → q11.1:)	dn	+	M	mult 3-9

ones were inherited form a balanced translocation in one parent. The majority of the latter group (83%) was maternally derived. Interestingly, mosaic cases with karyotypes 47,XN,+mar/46,XN were only seen in de novo complex sSMC (Figure 1E). However, no balanced translocation t(13;18)(q11;p11.21) or t(18;21)(p11.21;q11.1) was seen yet in any of the corresponding nine cases.

In the 73 complex sSMC only 67 breakpoints were involved. 44/67 breakpoints were unique, the remainder observed two to 14 times (Table 2).

Finally, only seven of the 73 (~10%) complex sSMC-cases not leading to Emanuel syndrome (case numbers 13/21-O-q10/4-1, 13/21-O-q10/5-1, 14-O-q11.2/1-1, 15-O-q11.2/5-1, 15-CO-1, 22-O-q11/3-1, 22-O-q11.21/3-1) were not associated with clinical signs (Table 1). However, clinically affected carriers of a der(13 or 21)t(13 or 21;18) inherited the sSMC in parts by their mothers, which were considered to be clinically normal [3].

Discussion

In 2008 complex sSMC seamed to be something rather unusual, apart from the cases with Emanuel syndrome [5]. Since then ~4 times more complex sSMC were characterized and reported, thus enabling more detailed follow up analyses of our previous studies.

~40% (408 of 1,040) of all centric minute shaped sSMC are complex sSMC, including der(22)t(11;22) cases [3]; the latter needs to be kept in mind, if a minute shaped sSMC is detected in diagnostics. Moreover, if a centric minute shaped sSMC turns out to be NOR-positive at one end, thus being acrocentric derived, this means that there is a 70% chance that it is a complex sSMC: of the yet known 567 centric minute shaped sSMC 408 are complex [3]. Also, if a centric minute shaped sSMC is present in all cells of the carrier, this might be another hint for a complex sSMC. Centric minute shaped non-complex sSMC are mosaic in ~70% of the cases [3], while complex sSMC

Liehr et al. Molecular Cytogenetics 2013, 6:46
http://www.molecularcytogenetics.org/content/6/1/46

Table 2 Breakpoints present between two and fourteen times in 73 complex sSMC

Present X times	Breakpoint
	4q12
	4q31.3 ~ q32.1
	12q11
	13q11 ~ q11.2
	13q13 ~ q14
	14/22q10 ~ q11.1
	15q11.2 ~ 12
	15q21.3 ~ q22
	16p13.2
	17p10 ~ q11
	17q25.3
2	21q21.2 ~ 21.3
	5q35
	14q11.1 ~ 11.2
	15q13
3	22q11.21 ~ q12
4	15q10 ~ q11.1
5	8p22 ~ p23
9	22q11.1 ~ 11.21
10	22q10 ~ 22q11.1
11	8q24.1
	13/21q11
14	18p11.1 ~ 11.21

are mosaic in only ~10% of the cases. This indicates the importance of cytogenetic analyses, as only this kind of study enables to characterize the sSMC-shape and mosaicism reliably, and gives first hints on the possible complex nature of an sSMC.

In 2010 the der(22)t(8;22)(q24.1;q11.1) syndrome was reported. It was suggested that, like in Emanuel syndrome, a 3:1 meiotic non-disjunction is causative for the occurrence of the corresponding sSMC in the offspring of t(8;22)(q24.1;q11.1) carriers [6]. Besides in the present study it became obvious that there is at least one more syndrome present among the patients with complex sSMC – nine patients with a der(13 or 21)t(13 or 21;18) were reported yet. It is not known yet if it is always de novo or can also be due to a balanced t(13;18)(q11;p11.21) or t(18;21)(p11.21;q11.1) in one of the parents. However, in contrast to most other complex-sSMC associated syndromes symptoms are very variable, even though a complete trisomy 18p is induced [3].

64% of complex sSMC are due to parental balanced translocations, 36% are de novo. This is a much lower rate that seen in sSMC in general, with a de novo rate of 70% [2; 3]. Still, like in other sSMC the majority of them is maternally derived [2].

At present it seems, complex sSMC fall into two major groups: such with unique and such with (more) common breakpoints. The later group comprises at present 23 different breakpoints involved 2 to 14 times in one of the 73 complex sSMC. As reason for this preference several mechanisms are discussed, including palindrome mediated recurrent translocations [6], homologous recombination between olfactory receptor gene clusters [7] or an involvement of fragile sites in the formation of constitutional breakpoints [8].

While the formation of complex sSMC due to a parental balanced translocation is comprehensible, it is unclear how such sSMC are formed de novo. Mosaicism in the germ-cells of one parent may be a possible explanation. Also, as only de novo cases have been seen in mosaic yet (Figure 1E), postzygotic origin of de novo cases has also to be considered.

As complex sSMC comprise in most cases besides centromeric material also chromosomal parts from gene-rich subtelomeric regions, it is not surprising that in the majority of the cases the clinical consequences are adverse. The seven cases with complex sSMC and no clinical signs only comprised genomic regions without dosage-dependant genes or even only heterochromatin.

Conclusions

In conclusion, complex sSMC are with 8.4% (including Emanuel syndrome cases) or ~1.5% (excluding der(22)t(11;22) cases) an essential part of the reported sSMC cases. Their frequency was really underestimated in 2008. Especially in cases of clinical abnormal patients with a centric minute shaped sSMC present in 100% of the cells a complex sSMC should be considered.

Methods

Data was acquired from the freely available sSMC database (http://www.fish.uniklinikum-jena.de/sSMC.html, [3]). 412 sSMC cases were identified as being complex among the 4,913 sSMC cases summarized there. The 339 der(22)t(11;22)(q23;q11.2) cases were not further analyzed; in Table 1 only the details on chromosomal constitution, parental origin, mosaicism and gender for the remainder 73 complex sSMC cases are summarized. Data from Table 1 together with previous knowledge on non-complex sSMC are bases for the here reported and discussed results.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
SC, TaL, MG-S, CdA, JW, II, IMM, RSG, E-GGS, DA provided the case and/or did primary cytogenetic and parts of FISH-tests; KK, EK, MZ, NK, ABH and TL did detailed FISH studies. TL drafted the paper and all authors read and approved the final manuscript.

Liehr *et al. Molecular Cytogenetics* 2013, **6**:46
http://www.molecularcytogenetics.org/content/6/1/46

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2.5. Article .4

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HUMAN GENETICS • ORIGINAL PAPER

How to narrow down chromosomal breakpoints in small and large derivative chromosomes – a new probe set

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Abstract Here a new fluorescence in situ hybridization (FISH-) based probe set is presented and its possible applications are highlighted in 34 exemplary clinical cases. The so-called pericentric-ladder-FISH (PCL-FISH) probe set enables a characterization of chromosomal breakpoints especially in small supernumerary marker chromosomes (sSMC), but can also be applied successfully in large inborn or acquired derivative chromosomes. PCL-FISH was established as 24 different chromosome-specific probe sets and can be used in two- up multicolor-FISH approaches. PCL-FISH enables the determination of a chromosomal breakpoint with a resolution between 1 and ~10 megabasepairs and is based on locus-specific bacterial artificial chromosome (BAC) probes. Results obtained on 29 sSMC cases and five larger derivative chromosomes are presented and discussed. To confirm the reliability of PCL-FISH, eight of the 29 sSMC cases were studied by array-comparative genomic hybridization (aCGH); the used sSMC-specific DNA was obtained by glass-needle based microdissection and DOP-PCR-amplification. Overall, PCL-FISH leads to a better resolution than most FISH-banding approaches and is a good tool to narrow down chromosomal breakpoints.

Keywords Chromosomal breakpoints · Fluorescence in situ hybridization (FISH) · Pericentric-ladder-FISH (PCL-FISH) · Small supernumerary marker chromosomes (sSMC)

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Introduction

Chromosomal rearrangements detected in routine banding cytogenetics currently can be characterized easily by fluorescence in situ hybridization (FISH) and/or array-comparative genomic hybridization (aCGH) (Manolakos et al. 2010; Weimer et al. 2011). Obviously, aCGH provides higher resolutions; however, FISH still has several advantages over the array-based approaches (Tsuchiya et al. 2008; Manolakos et al. 2010). While aCGH is restricted to the analysis of unbalanced rearrangements, FISH can also do balanced ones. Also chromosomal aberrations present in low mosaic level can be characterized by FISH without problems (Iourov et al. 2008; van der Veken et al. 2010), as this approach is single cell directed.

The exact determination of breakpoints present in derivative chromosomes is one major goal of a cytogenetic analysis and therefore various FISH-probe sets have been developed in the last decades (Liehr 2012a). Besides the sets based on whole chromosome painting probes (multiplex-FISH=M-FISH (Speicher et al. 1996); spectral

J Appl Genetics (2012) 53:259–269

karyotyping=SKY (Schröck et al. 1996)) or all centromeric probes (centromere-specific M-FISH=cenM-FISH (Nietzel et al. 2001)), also various FISH-banding approaches (Liehr et al. 2002a) were introduced. One early idea on how to implement FISH-banding was chromosome-bar coding, using well mapped locus-specific probes (Lengauer et al. 1992). However, no such probe set was ever finished for routine use in human chromosomes (Liehr et al. 2006a).

Here we present a new FISH probe set based on 174 bacterial artificial chromosome (BAC) probes called pericentric-ladder-FISH (PCL-FISH). It enables a chromosome-specific characterization of breakpoints with a resolution between 1 and ~10 megabasepairs (Mb) in small and large inborn or acquired derivative chromosomes; it is directed mainly toward the pericentric regions, as it is primarily intended for characterization of small supernumerary marker chromosomes (sSMC) (Liehr et al. 2004 and 2006b). PCL-FISH was successfully applied in 29 cases with sSMC and in five patients with larger derivative chromosomes.

Material and methods

PCL-FISH probe set

The PCL-FISH probe set (Fig. 1) is based on 174 BAC probes (Table 1). As centromere-near "starting points" for each of the chromosome-arms served probes used in the previously published so-called subcentromere-specific multicolor- (subcenM-) FISH probes set (Liehr et al. 2006b). These probes are denominated Np1 or Nq1 in Table 1, whereby "N" stands for the corresponding chromosome number. Distal from the probes Np1 or Nq1 follow between two and six other BAC probes with an average distance of 10 Mb to each other. Those were selected from the published human DNA-sequence. The BAC-probes were either kindly provided from the Sanger Center, Cambridge, UK, or purchased via BAC/PAC Chori, Oakland, CA, USA.

Fifty to 100 ng of DNA per BAC-probe was in vitro amplified and labeled by degenerated oligonucleotide primed polymerase chain reaction (DOP-PCR) (Telenius et al. 1992). Amplification procedure followed a published scheme (Fig. 2A in Liehr et al. 2002b). Here we used only the fluorochromes SpectrumOrange (SO) and diethylaminocoumarin (DEAC) for labeling of the BAC-probes and combined them with the corresponding commercially available centromeric probe labeled with a green fluorochrome (Kreatech, Amsterdam, The Netherlands; see Fig. 1). Still, it would be no problem to also label the BACs with more and/ or combined fluorochromes to achieve additional colors and an individual identification of the probes. As in the present study PCL-FISH was exclusively used to narrow down chromosomal breakpoints in derivative chromosomes with known structures, a three-color FISH as shown in

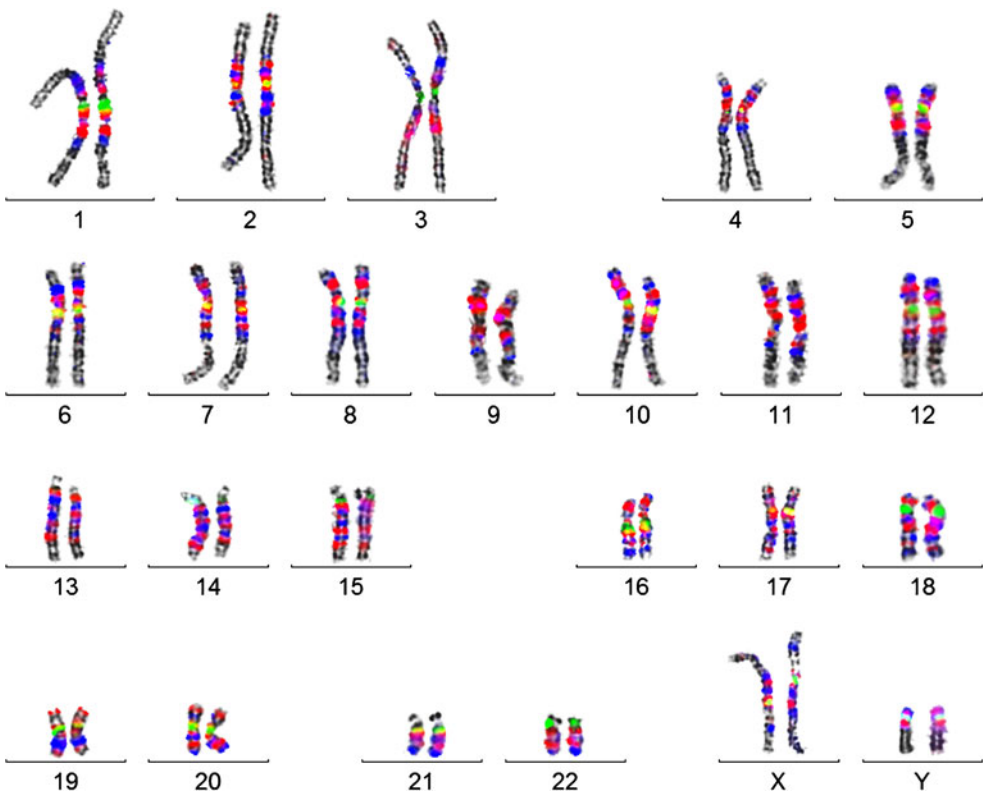

Fig. 1 Karyogram combined of two homologous for each chromosome-position labeled with the PCL-FISH probe set. The chromosome-pairs are taken from 24 different experiments and one metaphase, each, except for the Y-chromosomes, which are from two different metaphases. A cytogenetically normal female and a male were hybridized, each. Locus-specific probes are labeled in red and blue according to the scheme shown in Table 1; corresponding centromeric probes labeled in green were additionally applied

J Appl Genetics (2012) 53:259–269

Table 1 List of used BAC-probes, their official names, the abbreviation under which the probes are designated in Tables 2 and 3, the cytogenetic localization of the probes (cytoband), molecular position and distance to centromere, acc. to hg18

Abbreviation	BAC-probe	Cytoband	Position (hg18)	Distance to centromere [Mb]/label
1p4	RP11-324 C23	1p22.2	91,953,842-91,986,170	29.11/blue
1p3	RP11-260 K3	1p21.2	99,670,767-99,835,823	21.26/red
1p2	RP11-392B1	1p13.3	111,226,829-111,256,235	9.84/blue
1p1	RP11-27 K13	1p13.1	117,282,240-117,407,086	3.69/red
1q1	RP11-30I17	1q21.1	144,174,052-144,361,891	1.77/red
1q2	RP11-205 M9	1q21.3	152,393,706-152,511,165	10.00/blue
1q3	RP11-343 F16	1q23.3	162,274,665-162,447,403	19.87/red
1q4	RP11-415 M14	1q25.1 ~ 25.2	174,374,254-174,386,794	31.97/blue
2p4	RP11-440P5	2p16.1	60,553,094-60,688,102	30.31/blue
2p3	RP11-436 H22	2p13.3	70,583,656-70,774,208	20.23/red
2p2	RP11-303I4	2p12	80,657,637-80,808,678	10.19/blue
2p1	RP11-294I20	2p11.2	88,939,371-88,940,029	2.06/red
2q1	RP11-708D7	2q11.1	94,981,836-95,110,967	−0.59/red
2q2	RP11-332 H14	2q12.3	105,152,958-105,351,875	9.65/blue
2q3	RP11-115 F20	2q14.1	115,731,180-115,780,908	20.03/red
2q4	RP11-48 K7	2q14.3	127,180,319-127,253,883	31.48/blue
3p4	RP11-449E4	3p14	59,757,660-59,924,092	29.48/blue
3p3	RP11-152 N21	3p14.1	69,417,957-69,541,168	19.86/red
3p2	RP11-16 M12	3p12.3	78,395,761-78,564,998	10.84/blue
3p1	RP11-91A15	3p11.1	89,670,648-89,771,786	0.37/red
3q1	RP11-529P9	3q12.1	98,526,229-98,714,854	5.32/red
3q2	RP11-49I4	3q12.3	102,993,643-103,144,287	9.94/blue
3q3	RP11-572 C15	3q13.13 ~ 13.2	113766654-113980366	20.80/red
3q4	RP11-299 J3	3q21.1	123,488,724-123,632,323	30.43/blue
4p4	RP11-339D20	4p15.31	19,642,622-19,763,590	29.07/blue
4p3	RP11-417 M17	4p15.1	29,249,879-29,381,035	19.32/red
4p2	RP11-617D20	4p14	38,270,133-38,416,177	10.28/blue
4p1	RP11-793 H20	4p12	47,946,771-48,061,917	0.64/red
4q1	RP11-365 H22	4q11	52,354,875-52,530,859	−0.05/red
4q2	RP11-24I7	4q13.1	62,331,990-62,451,691	9.93/blue
4q3	RP11-499 N1	4q13.3	72,066,561-72,265,534	19.67/red
4q4	RP11-570 L13	4q21.23	85,690,662-85,871,431	33.29/blue
5p4	RP11-88 L18	5p15.1	17,465,420-17,636,603	28.27/blue
5p3	RP11-351 N6	5p14.1	26,370,550-26,539,899	19.26/red
5p2	RP11-7 M4	5p13.2	36,988,518-37,177,098	8.26/blue
5p1	RP11-301A5	5p13.1	40,982,971-41,157,849	4.81/red
5q1	RP11-269 M20	5q11.1	49,913,068-50,093,939	−0.59/red
5q2	RP11-103A15	5q12.1	60,540,925-60,617,076	10.04/blue
5q3	RP11-551B22	5q13.2	69,777,035-69,816,329	19.28/red
5q4	RP11-90A9	5q14.1	79,882,591-80,063,088	29.38/ blue
6p4	RP11-192 H11	6p22.1	29,962,436-30,073,097	28.33/blue
6p3	RP11-100B10	6p21.2	38,054,811-38,217,746	20.18/red
6p2	RP11-334 H12	6p12.3	48,824,692-48,956,050	9.44/blue
6p1	RP11-421P21	6p11.2	57,228,514-57,292,599	1.11/red
6q1	RP11-349P19	6q12	65,158,548-65,208,779	1.76/red
6q2	RP11-256 L9	6q13	73,180,924-73,217,550	9.78/blue
6q3	RP11-25O6	6q14.1	83,407,494-83,562,457	20.01/red
6q4	RP11-538A16	6q16.1	93,629,676-93,823,839	30.23/blue

J Appl Genetics (2012) 53:259–269

Table 1 (continued)

Abbreviation	BAC-probe	Cytoband	Position (hg18)	Distance to centromere [Mb]/label
7p4	RP4-781A18	7p15.2	27,976,171-28,166,812	29.23/blue
7p3	RP11-302 L6	7p14.2 ~ 14.1	37,482,196-37,597,749	19.80/red
7p2	RP11-651 K8	7p12.3	46,734,018-46,843,890	10.56/blue
7p1	RP11-10 F11	7p11.2	55,222,879-55,347,931	2.05/red
7q1	RP11-144 H20	7q11.21	61,606,122-61,791,403	0.51/red
7q2	RP11-53 M13	7q11.22	71,272,716-71,279,228	10.18/blue
7q3	RP11-448A3	7q21.11	81,263,387-81,349,902	20.16/red
7q4	RP11-313 N23	7q21.2	91,801,440-91,966,446	30.70/blue
8p4	RP11-366 J3	8p22	14,724,716-14,893,465	27.31/blue
8p3	RP11-115 K10	8p21.2	24,116,701-24,305,391	17.89/red
8p2	RP11-11 N9	8p12	32,909,252-33,028,630	9.71/blue
8p1	RP11-503E24	8p11.21	42,503,724-42,674,302	−0.47/red
8q1	RP11-197I11	8q11.23	53,135,835-53,175,019	5.04/red
8q2	RP11-430 H18	8q12.1	58,249,439-58,400,298	10.15/blue
8q3	RP11-409 C19	8q13.2	68,012,453-68,120,862	19.91/red
8q4	RP11-652 L3	8q21.11	78,197,078-78,348,048	30.10/blue
9p4	RP11-492A12	9p22.2	17,361,476-17,489,085	29.21/blue
9p3	RP11-438B23	9p21.2	27,866,315-28,042,166	18.66/red
9p2	RP11-61I3	9p13.2	37,607,753-37,776,264	8.92/blue
9p1	RP11-113O24	9p13.1	38,263,089-38,427,295	8.27/red
9q1	RP11-109D9	9q21.11	71,449,567-71,636,843	1.45/red
9q2	RP11-574 G7	9q21.2	79,668,774-79,884,072	9.67/blue
9q3	RP11-249 H20	9q21.33	89,375,412-89,446,315	19.38/red
9q4	RP11-535 C21	9q22.33	99,744,616-99,824,931	29.74/blue
10p4	RP11-575 N15	10p14	8,728,048-8,906,592	29.89/blue
10p3	RP11-51E20	10p12.31	20,784,567-20,938,614	17.86/red
10p2	RP11-350D11	10p11.23	30,675,157-30,865,135	7.93/blue
10p1	RP11-365P10	10p11.21	36,945,343-36,974,907	1.85/red
10q1	RP11-92P6	10q11.21	43,174,613-43,219,888	1.07/red
10q2	RP11-532 F4	10q11.23	52,151,487-52,328,351	10.05/blue
10q3	RP11-166B18	10q21.2	61,904,197-62,069,022	19.80/red
10q4	RP11-367 H5	10q22.1	71,504,053-71,672,097	29.40/blue
11p4	RP11-701I24	11p15	20,531,828.-20,709,763	30.69/blue
11p3	RP11-297A4	11p13	31,195,990-31,386,312	20.01/red
11p2	RP11-1 G18	11p12	41,077,222-41,230,282	10.17/blue
11p1	RP11-397 M16	11p11.2	48,260,247-48,436,072	2.96/red
11q1	RP11-644A8	11q12.1	56,558,447-56,613,460	0.16/red
11q2	RP11-157 K17	11q13.1	66,670,500-66,841,190	10.44/blue
11q3	RP11-263 C24	11q13.5	75,265,230-75,385,345	18.87/red
11q4	RP11-665E10	11q14.2	87,158,646-87,317,430	30.92/blue
12p4	RP11-298 J22	12p13.33	2,247,231-2,406,919	30.79/blue
12p3	RP11-161A14	12p13.1	13,255,212-13,430,711	19.77/red
12p2	RP11-12D15	12p12.1	22,250,749-22,369,579	10.83/blue
12p1	RP11-310I24	12p11.22	29,551,976-29,607,135	3.59/red
12q1	RP11-498B21	12q12	39,833,150-39,900,092	3.33/red
12q2	RP11-89 H19	12q13.11	46,571,184-46,627,459	10.07/blue
12q3	RP11-181 L23	12q13.3	56,118,000-56,288,135	19.62/red
12q4	RP11-542B15	12q15	66,138,644-66,250,773	29.64/blue
13q1	RP11-523 H24	13q12.11	19,137,338-19,306,540	0.74/red

J Appl Genetics (2012) 53:259–269

Table 1 (continued)

Abbreviation	BAC-probe	Cytoband	Position (hg18)	Distance to centromere [Mb]/label
13q2	RP11-904 N23	13q12.2 ~ 12.3	27,754,495-27,943,490	9.35/blue
13q3	RP11-50D16	13q13.3	38,421,313-38,577,271	17.32/red
13q4	RP11-278A16	13q14.2	48,237,412-48,368,518	29.84/blue
13q5	RP11-100 C24	13q21.1	56,650,462-56,729,961	38.25/red
13q6	RP11-521 L15	13q21.33	67,417,069-67,493,555	49.02/blue
13q7	RP11-80 N10	13q31.1	80,203,304-80,367,022	61.80/red
14q1	RP11-14 J7	14q11.2	20,057,964-20,172,932	0.96/red
14q2	RP11-125A5	14q12	28,581,578-28,768,137	9.48/blue
14q3	RP11-111A21	14q21.1	38,968,002-39,128,583	19.87/red
14q4	RP11-831 F12	14q22.1	49,199,101-49,342,562	30.10/blue
14q5	RP11-701B16	14q23.1	59,039,114-59,210,327	39.94/red
14q6	RP11-486O13	14q24.2	69,613,365-69,799,632	50.51/blue
14q7	RP11-242P2	14q31.1	79,100,266-79,261,442	60.00/red
15q1	RP11-26 F2	15q11.1	20,542,381-20,704,897	2.14/red
15q2	RP11-261B23	15q13.2	28,285,619-28,402,108	9.89/blue
15q3	RP11-380D11	15q15.1	39,589,023-39,754,407	21.19/red
15q4	RP11-416 K5	15q21.2	48,177,332-48,330,980	29.78/blue
15q5	RP11-219B17	15q22.2	58,672,146-58,821,992	40.27/red
15q6	RP11-96 C21	15q23	68,530,717-68,651,733	50.13/blue
15q7	RP11-210 M15	15q25.2	78,397,187-78,541,279	60.00/red
16p4	RP11-295D4	16p13.3	4,320,670-4,461,705	29.94/blue
16p3	RP11-114I21	16p13.11	15,601,001-15,767,810	18.63/red
16p2	CTD-2515A14	16p12.1	24,668,123-24,856,494	18.63/blue
16p1	RP11-408D2	16p11.2	32,206,388-33,203,783	9.73/red
16q1	RP11-474B12	16q12.1	45,880,869-46,027,419	0.53/red
16q2	RP11-250E14	16q13	55,932,916-56,094,253	10.59/blue
16q3	RP11-167P11	16q22.1	66,238,838-66,393,296	20.89/red
16q4	RP11-358 L22	16q23.1	76,606,529-76,754,301	31.25/blue
17p3	RP11-135 N5	17p13.3	2,316,192-2,492,178	19.61/blue
17p2	RP11-471 L13	17p12	11,940,105-12,065,839	9.58/red
17p1	RP11-299 G20	17p11.1	22,177,022-22,177,618	−0.02/red
17q1	RP11-229 K15	17q11.2	26,452,331-26,604,331	3.25/blue
17q2	RP11-678 G7	17q12	33,529,231-33,710,106	10.33/red
17q3	RP11-100E5	17q21	38,799,089-38,932,045	15.60/blue
17q4	RP11-502 F1	17q22.23	51,736,564-51,905,016	28.54/red
17q5	RP11-147 L13	17q24.2	63,619,435-63,836,065	40.42/blue
18p3	RP11-835E18	18p11.3	5,183,555-5,306,587	12.91/blue
18p2	RP11-419 J16	18p11.2	10,132,108-10,203,413	5.20/red
18p1	RP11-178 F10	18q11.2	20,259,694-20,371,249	2.96/red
18q1	RP11-317 G20	18q12.1	28,046,335-28,218,210	10.75/blue
18q2	RP11-89 M10	18q12.3	37,455,958-37,620,554	20.16/red
18q3	RP11-346 H17	18q21.1	48,239,496-48,386,192	30.94/blue
18q4	RP11-13 L22	18q21.33	58,418,051-58,578,530	41.12/red
18q5	RP11-45A1	18q22.3	67,916,649-68,041,626	50.62/blue
19p3	RP11-110A24	19p13.3	209,326-374,094	26.33/red
19p2	RP11-177 J4	19p13.2	10,430,651-10,606,709	16.09/blue
19p1	CTC-451A6	19p12	22,661,070-22,729,110	3.97/red
19q1	CTD-2043I16	19q12	33,298,064-33,399,253	3.10/red
19q2	RP11-1096 L2	19q13.31	48,118,601-48,308,257	17.92/blue

J Appl Genetics (2012) 53:259–269

Table 1 (continued)

Abbreviation	BAC-probe	Cytoband	Position (hg18)	Distance to centromere [Mb]/label
19q3	RP11-256B9	19q13.33 ~ 13.41	57,561,808-57,749,888	27.36/red
20p3	RP11-12 M19	20p13	2,748,056-2,851,325	22.85/red
20p2	RP5-822 J19	20p12.1	15,512,928-15,602,699	10.10/blue
20p1	RP11-96 L6	20p11.21	25,465,310-25,522,324	0.18/red
20q1	RP11-243 J16	20q11.21	29,756,779-29,925,538	1.36/red
20q2	RP11-101E14	20q12	37,981,424-38,106,380	9.58/blue
20q3	RP11-644 F19	20q13.1	46,715,027-46,898,782	18.32/red
20q4	RP11-429E11	20q13.33	59,655,701-59,786,565	31.26/blue
21q1	RP11-89 H21	21q11.2	14,850,742-15,000,742	1.65/blue
21q2	RP11-132 H24	21q21.2	24,509,021-24,633,021	11.31/red
21q3	RP11-410P24	21q22.11	32,849,566-33,019,511	19.65/red
21q4	RP11-88 N2	21q22.3	43,556,416-43,769,964	30.36/blue
22q1	RP11-172D7	22q11	16,239,476-16,239,639	−0.06/blue
22q2	CTA-125 H2	22q12	24,555,255-24,728,767	8.26/red
22q3	RP11-89D12	22q12.3	32,849,566-33,019,511	16.55/red
22q4	RP1-100 G10	22q13.32	43,556,416-43,769,964	27.26/blue
Xp4	RP11-430 F3	Xp21.3	27,828,710-28,012,235	28.59/blue
Xp3	RP11-492O8	Xp21.1	36,531,334-36,664,328	19.94/red
Xp2	RP11-14O9	Xp11.3	46,616,221-46,695,415	9.90/blue
Xp1	RP11-465B24	Xp11.21	56,467,529-56,573,161	0.03/red
Xq1	RP11-403E24	Xq11.1	63,222,525-63,351,189	−1.88/red
Xq2	RP13-36 G14	Xq13.2	73,120,826-73,206,160	8.02/blue
Xq3	RP11-496 J2	Xq21.2	84,741,613-84,850,590	19.64/red
Xq4	RP11-358 K18	Xq21.33	94,900,252-95,033,143	29.80/blue
Yp2	RP11-515 L2	Yp11.31	2,838,554-2,845,472	9.25/blue
Yp1	RP11-122 L9	Yp11.2	4,917,081-5,077,603	7.02/red
Yq1	RP11-235I1	Yq11.2	15,267,383-15,447,103	0.97/red

Fig. 2 was sufficient. The derivatives were characterized before by cenM-FISH (Nietzel et al. 2001), subcenM-FISH (Liehr et al. 2006b) or array proven multicolor banding (aMCB) (Liehr et al. 2002b; Weise et al. 2008) (results not shown).

Twenty metaphase spreads were analyzed, each, using a fluorescence microscope (Axioplan 2 mot, Zeiss) equipped with appropriate filter sets to discriminate between all three fluorochromes and the counterstain DAPI (Diaminophenylindol). Image capturing and processing were carried out using an isis mFISH imaging system (MetaSystems, Altlussheim, Germany).

Probe generation for and performing of array-comparative genomic hybridization (aCGH)

The sSMC of cases 1, 4–7, 9, 12, and 15 were microdissected, the DNA amplified by DOP-PCR (Telenius et al. 1992) and subsequently hybridized to a genome-wide array (Liehr et al. 2011). aCGH was done according to standard protocols using the 180 K chip of Agilent. Evaluation was done with the scanner provided by Agilent and the corresponding software.

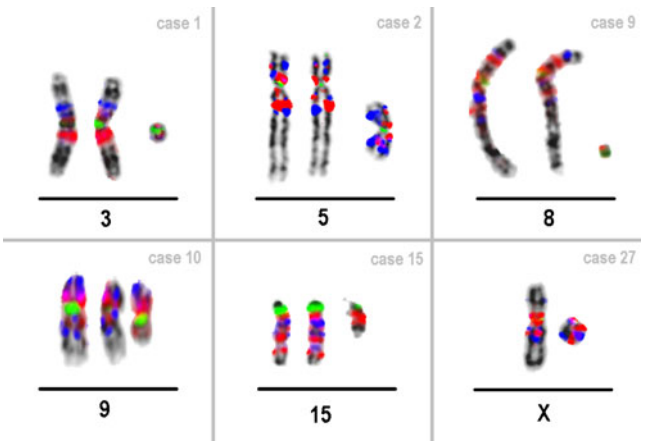

Fig. 2 Representative PCL-FISH results for cases 1, 2, 9, 10, 15 and 27 are depicted

J Appl Genetics (2012) 53:259–269

Table 2 sSMC-cases solved by PCL-FISH – clinical details are available in Liehr (2012b) – i.e., sSMC homepage; the karyotype after molecular cytogenetic characterization and array-CGH and the breakpoints are given corresponding to the used BAC probes in PCL-FISH, which are abbreviated acc. to Table 1 and the position acc. to hg 18. Also the percentage of the sSMC is given

Case	Case no. sSMC homepage	Karyotype array-CGH-data	sSMC present in %	Breakpoints p-arm / q-arm (Table 1) positions of breakpoints (hg 18)
1	03-O-p11.2/2-1	r(3)(::p11.2->q11.2::)	33	3p2 ~ 3p1 / 3q2 ~ 3q3
		74.67-104.78		78.56-89.67 / 103.14-113.77
2	05-W-iso/1-4	inv dup(5)(pter->q10::q10->pter)	33	- / 5cep ~ 5q1
		n.a.		- / 50.09-50.50
3	05-W-p13.2/2-1	min(5)(:p13.2->q11.1:)	80	5p2 / 5cep ~ 5q1
		n.a.		37.00-37.18 / 50.09-50.50
4	06-W-p11.2/1-1	min(6)(:p11.2->q11.1:)[55]/inv dup(6)(:q11.1->p11.2::p11.2->q11.1:)[5]	60	6p2 ~ 6p1 / 6cep ~ 6q1
		57.09-64.11		48.96-57.23 / 63.40-65.16
5	06-W-p12.3/1-1	r(6)(::p12.3->q12::)[2]/r(6)(::p12.3->q12::)x2[5]/min(6)(:p12.3->q12:)[9]/ min(6)(:p12.3->q12:)x2[2]/min(6)(:p12.3->q1?2::q1?2->p12.3:)[1]/r(6)(::p12.3->q12::),r(6;6)(::p12.3->q12::p12.3->q12::)[1]	86	6p2 ~ 6p1 / 6q1 ~ 6q2
		51.55-66.71		48.96-57.23 / 65.21-73.18
6	06-W-p11.1/2-1	min(6)(:p11.1->q13:)[5]/r(6)(::p11.1->q13::)[2]/r(6)(::p11.1->q13::p11.1->q13::)[2]/r(6)(::p11.1->q13::p11.1->q13::p11.1->q13::p11.1->q13::)[1]/ inv dup(6)(:p11.1->q13::p11.1->q13:)[10]	85	6p2 ~ 6p1 / 6q1 ~ 6q2
		58.40-65.20		48.96-57.23 / 65.21-73.18
7	07-W-p11.2/1-2	min(7)(:p11.2->q11.21:)[5]/r(7)(::p11.2->q11.21::)[2]/r(7;7)(::p11.2->q11.21::p11.2->q11.21::)[1]	81	7p2 ~ 7p1 / 7q1 ~ 7q2
		55.42-63.45		46.84-55.23 / 61.61-71.27
8	07-U-8	min(7)(:p11.2 ~ 11.1->q11.2:)	81	7p2 ~ 7p1 / 7q1 ~ 7q2
		n.a.		46.84-55.23 / 61.61-71.27
9	08-W-p11.21 ~ 11.22/1-1	r(8)(:p11.21 ~ 11.22->q11.1:)	100	8p2 ~ 8p1 / 8cep ~ 8q1
		42.50-49.50		33.03-42.50 / 48.10-53.14
10	09-W-pter/1-1	del(9)(q21.1)	100	- / 9q1 ~ 9q2
		n.a.		- / 71.64-79.67
11	09-W-iso/1-1	inv dup(9)(q12)	85	- / 9cep ~ 9q1
		n.a.		- / 70.00-71.45
12	11-U-15	min(11)(:p11.21->q13.1:)	70	11p1 ~ 11cep / 11q1 ~ 11q2
		49.85-64.40		48.44-51.40 / 56.61-66.67
13	12-W-p11.1/2-1	r(12)(::p11.1->q14::)[7]/r(12)(::p11.1->q11::)[2]	25	12p1 ~ 12cep / 12q2 ~ 12q312p1 ~ 12cep / 12cep ~ 12q1
		n.a.		29.61-33.20 / 46.63-56.12 29.61-33.20 / 36.50-39.83
14	15-W-q13/4-1	inv dup(15)(pter->q11.1::q13->pter)	100	- / 15cep ~ 15q1 / 15q1 ~ 15q2
		n.a.		- / 18.40-20.70 /20.70-28.29
15	15-CWw-148	inv dup(15)(q12 ~ 13)	100	- / 15q1 ~ 15q2
		0.00-26.01		- /20.70-28.29
16	15-W-q14/4-1	der(15)(:q14->q13::q14->p11.1::p11.1->q14::q13->q14:)	25	- / 15q2 ~ 15q3
		n.a.		- / 28.40-39.59
17	15-W-q13.2/1-3	inv dup(15)(q13.2)	100	- / 15q2 ~ 15q3
		n.a.		- / 28.40-39.59
18	18-Wi-143	inv dup(18)(q11.1)	100	- / 18cep
		n.a.		- / 15.40-17.30

J Appl Genetics (2012) 53:259–269

Table 2 (continued)

Case	Case no. sSMC homepage	Karyotype array-CGH-data	sSMC present in %	Breakpoints p-arm / q-arm (Table 1) positions of breakpoints (hg 18)
19	18-Wi-132	inv dup(18)(q11.1)	100	- / 18cep
		n.a.		- / 15.40-17.30
20	19-U-10	r(19)(::p13.1->q13.1::)[17]/r(19;19)(::p13.1->q13.1::p13.1->q13.1::)[4]	88	19p2 ~ 19p1 / 19q2 ~ 19q3
		n.a.		10.61-22.66 / 48.31-57.56
21	20-U-11	r(20)(::p11.1->q11.23::)[10]/ min(20)(:p11.1->q11.23:)[2]	90	20p1 ~ 20cep / 20q2 ~ 20q3
		n.a.		25.52-25.70 / 38.11-46.72
22	21-W-q11.2 ~ 21.1/1-2	del(21)(q11.2 ~ 21.1:)	25	- / 21q1 ~ 21q2
		n.a.		- / 15.00-24.51
23	22-Wces-5-86	inv dup(22)(q11.21)	100	- / 22q2
		n.a.		- / 24.56-24.73
24	22-Wces-5-94	inv dup(22)(q11.23 ~ 12.1)	37	- / 22q1 ~ 22q2
		n.a.		- / 24.73-32.85
25	22-Wces-5-130	inv dup(22)(q11.21)	100	- / 22q1 ~ 22q2
		n.a.		- / 24.73-32.85
26	plus21-U-39	min(22)(pter->q11.21)	73	- / 22q2
		n.a.		- / 24.56-24.73
27	minX-p11.1/6-1	min(X)(:p11.1->q22:)	15	Xp1-Xcep / Xq4 ~ Xtel
		n.a.		56.57-56.60 / 95.03-145.91
28	m-iY-p11.32/1-3	inv dup(Y)(p11.32)	20	Ypter ~ Yp2 / -
		n.a.		0.00-2.84 / -
29	m-rY-p11.3/2-4	r(Y)(::p11.3->q11.2::)[7]/r(Y)(::p11.3->q11.2::p11.3->q11.2::)[3]	53	Ypter ~ Yp2 / Yq1 ~ Yq2
		n.a.		0.00-2.84 / 15.45-22.59

Clinical cases

Overall, 33 clinical cases were studied by PCL-FISH (see Tables 2 and 3). For cases listed in Table 2 the clinical details are reported in Liehr (2012b). In general, the clinical indications were infertility or repeated abortions (like, e.g., also in cases A, C and D, Table 3), dysmorphic features and/or mental retardation (like in case B, Table 3) or prenatal detection of the aberration due to different reasons (like in case E, Table 3).

Results

sSMC and large derivative chromosomes listed in Tables 2 and 3 were characterized by established FISH-approaches and aCGH. Detailed results for these studies are not shown, but the karyotypes defined after their application for each of the cases are listed in Tables 2 and 3. Afterward for each case the appropriate chromosome-specific PCL-FISH-probe sets were applied. Representative PCL-FISH results for cases 1, 2, 9, 10, 15 and 27 are shown in Fig. 2.

In the present study it could be demonstrated that PCL-FISH can be used to characterize chromosomal breakpoints with a high accuracy on the single cell level (Tables 2 and 3). Results obtained by PCL-FISH were in complete concordance with aCGH results in the eight sSMC cases 1, 4–7, 9, 12, and 15 studied by both approaches.

Overall, most of the breakpoints could be narrowed down to ~10 Mb, as the raster of the PCL-FISH-probe set lets us expect. However, when chromosomal breakpoints were located between the centromere and the first proximal probe in the p- or q-arm, the characterized critical region could be smaller, i.e., between 0.03 and 5.04 Mb in size (cases 2–4, 9, 11–14, 18–19, 21, 27 and C). The same was true if the break was between the distal applied probe of a chromosome arm and its end, like in cases 28, 29, D and E (2.84 to 3.17 Mb); however, with respect to the chromosomal aberration studied it also could be larger than 10 Mb: in case 27 it was 50.88 Mb, however, as it was obvious that the sSMC was not much larger than the most distal probe of the PCL-FISH set the break must have appeared

J Appl Genetics (2012) 53:259–269

Table 3 Non-sSMC-cases solved by PCL-FISH; breakpoints are given in principle as in Table 2

case	karyotype	breakpoints p-arm / q-arm (Table 1) positions of breakpoints (hg 18)
A	46,XY,inv(10)(p11.1q21.3)	10p1 ~ 10cep / 10q1 ~ 10q2
		36.97-38.80 / 43.22-52.15
B	46,XY,der(14)(pter->q22.1 ~ 22.3::q11.2->qter)[46]/46,XY[4]	14q1 ~ 14q2 / 14q4 ~ 14q5
		20.17-28.58 / 49.34-59.04
C	46,inv(14)(p11.2q13.2 ~ 21.1)	14cep ~ 14q1 / 14q2 ~ 14q3
		19.10-20.06 / 28.77-38.97
D	47,XX,r(21)(::p11.2->q22.3::).+min(21)(:pter->p11.2:) or r(21)(::p13->p11.2::)[1]/46,XX,r(21)(::p11.2->q22.3::)[33]/46,XX,r(21;21)(::p11.2->q22.3::p11.2->q22.3::)[5]/46,XX[1]	- / 21q4 ~ 21qter
		- / 43.77-46.94
E	46,XN,del(21)(:p11.1 ~ 11.2->q22.3:)[7]/46,XN,r(21)(::p11.1 ~ 11.2->q22.3::)[4]/46,XN,der(21)(:q11.2->p11.1 ~ 11.2::p11.1 ~ 11.2->q22.3:)[8]/45,XN,-21[1]	- / 21q4 ~ 21qter
		- / 43.77-46.94

between positions 95.03 and ~110 of the X-chromosome here. Finally, it could also happen that the break appeared directly in the region spanned by one of the applied probes, like in cases 3, 23 and 26; then the break event could be characterized with an accuracy of 0.17 and 0.18 Mb in the studied cases.

In several of the sSMC cases there were so-called cryptic mosaics (Liehr et al. 2010), i.e., the cells with the sSMC split into different subclones, distinguishable only by FISH. In case 13 this was expressed as two sSMC derived from chromosome 12, both, with identical breakpoints in the short, but different ones in the long arm. As one of the sSMC of this case was completely heterochromatic the sSMC of this case would not be resolved comprehensively by aCGH alone.

Discussion

During the last decades numerous FISH-approaches were developed (Liehr 2012a). M-FISH/ SKY is able to characterize the origin and/or composition of larger euchromatic derivative chromosomes (Speicher et al. 1996; Schröck et al. 1996), cenM-FISH (Nietzel et al. 2001) and subcenM-FISH (Liehr et al. 2006b) can identify the chromosomal origin of sSMC, FISH-banding and use of locus specific probes enables a better breakpoint characterization than banding cytogenetics (Weise et al. 2002; Manvelyan et al. 2007), and aCGH can narrow down chromosomal breakpoints to some 10 kb or less (Weise et al. 2008). As aCGH has several limitations and is normally used for the analysis of multiple cells, it can be complemented easily by FISH, which is single cell directed. Also aCGH is an approach, not accessible by big parts of the worldwide cytogenetically working community. Thus, PCL-FISH was developed primarily to enable the size characterization of mosaic and non-mosaic sSMC based on a simple two- or three color molecular cytogenetic technique. Such a test system can be important especially in prenatal sSMC cases, where a comprehensive characterization is necessary to enable a sound genotype-phenotype correlation. As PCL-FISH was developed to be used in small derivative chromosomes, large chromosomes are not covered completely by the 10 Mb raster. Nonetheless, as demonstrated in cases A-E the new probe set can be helpful for breakpoint characterization there as well.

The PCL-FISH approach is specially suited for the characterization of derivative chromosomes present in mosaic, like those present in all sSMC cases from Table 2 besides cases 9, 10, 14–15, 17–19, 23 and 25 and cases B, D and E (Table 3). Especially in those cases with sSMC presence of below 50 % of the cells (cases 1, 2, 13, 16, 22, 24, 27 and 28) PCL-FISH is extremely helpful, as aCGH may be unable to detect these sSMC at all. Also PCL-FISH allows the characterization of sSMC present as cryptic mosaics (Liehr et al. 2010). In most such cases the different sSMC had different shapes but the breakpoints still were the same (e.g.,

cases 4–7, 20 and 29). However, in some instances the breakpoints can differ, like in case 13; here PCL-FISH could easily define all three involved breakpoints.

In general, PCL-FISH is a pericentromeric region directed bar-code FISH approach. However, to the best of our knowledge none of the previously published ones were created to constitute a 10 Mb raster along chromosomal regions. Also none of the locus-specific-probe-based bar-code FISH approaches were ever established for the whole human genome (Liehr et al. 2006a). PCL-FISH can be used immediately after identification of the chromosomal origin of a derivative; however, this was not done in the present study. Here all derivatives were previously characterized by well established molecular cytogenetic approaches and/or aCGH; those results are listed in Tables 2 and 3 and served to control the accuracy of the new established probe set.

Overall, it could be demonstrated that PCL-FISH characterizes chromosomal breakpoints reliably (Tables 2 and 3), as they were in concordance with previous FISH and aCGH results. In those breakpoints not characterized by aCGH, PCL-FISH not only confirmed the previous FISH-results but concretized them with an accuracy of 0.03 to ~10 Mb, as outlined in Results.

In conclusion we present a new FISH probe set easily and effectively applicable in clinical cytogenetic routine diagnostics. It could be enlarged by additional probes, e.g., BACs in 10 Mb distance covering all human genome and not only the pericentric regions. Also, applications of PCL-FISH in tumor cytogenetics, as well as in evolution research studies are principally possible.

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2.6. Article .5

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Somatic Mosaicism in Cases with Small Supernumerary Marker Chromosomes

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Abstract: Somatic mosaicism is something that is observed in everyday lives of cytogeneticists. Chromosome instability is one of the leading causes of large-scale genome variation analyzable since the correct human chromosome number was established in 1956. Somatic mosaicism is also a well-known fact to be present in cases with small supernumerary marker chromosomes (sSMC), i.e. karyotypes of 47,+mar/46. In this study, the data available in the literature were collected concerning the frequency mosaicism in different subgroups of patients with sSMC. Of 3124 cases with sSMC 1626 (52%) present with somatic mosaicism. Some groups like patients with Emanuel-, cat-eye- or i(18p)- syndrome only tend rarely to develop mosaicism, while in Pallister-Killian syndrome every patient is mosaic. In general, acrocentric and non-acrocentric derived sSMCs are differently susceptible to mosaicism; non-acrocentric derived ones are hereby the less stable ones. Even though, in the overwhelming majority of the cases, somatic mosaicism does not have any detectable clinical effects, there are rare cases with altered clinical outcomes due to mosaicism. This is extremely important for prenatal genetic counseling. Overall, as mosaicism is something to be considered in at least every second sSMC case, array-CGH studies cannot be offered as a screening test to reliably detect this kind of chromosomal aberration, as low level mosaic cases and cryptic mosaics are missed by that.

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SMALL SUPERNUMERARY MARKER CHROMOSOMES (sSMC)

In 1956, the exact chromosomal number in humans was established [1]. Since then it was possible to delineate numerical chromosomal aberrations in any body tissue where chromosomes could be prepared from, including clinical [2] and tumor cases [3]. After the introduction of molecular cytogenetics [4-7], it became even possible to analyze numerical chromosomal aberrations in non-dividing cells [8]. By that also low-level chromosomal aberrations could be detected in tumor [9-13], various clinical [14-18] and neuronal diseases [19-27], embryonic tissues [28-32] and different tissue types [9, 13, 15, 33-35]. Overall it can be stated that chromosome instability is one of the main causes of large-scale genome variation [36-39]. For review of cytogenetic and molecular cytogenetics see Refs. [4-5, 40].

Small supernumerary marker chromosomes (sSMC) are reported in 0.043% of newborn infants, 0.077% of prenatal cases, 0.433% of mentally retarded patients and 0.171% of subfertile people [41]. They 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; sSMC can either be present additionally in (1) an otherwise normal karyotype, (2) a numerically abnormal karyotype (like Turner- or Down-syndrome) or (3) a structurally abnormal but balanced karyotype with or without ring chromosome formation [42]. sSMCs are normally detected by banding cytogenetics in mentally retarded patients, in subfertile persons or during prenatal diagnosis and particularly prenatally ascertained ones, are not easy to correlate with a clinical outcome. It is known that ~30% of sSMCs are derived from chromosome 15; ~11% are i(12p) = Pallister-Killian, ~10% are der(22)-Emanuel-,~7% are inv dup (22)-cat-eye- and ~6% are i(18p)-syndrome associated sSMC [42].

sSMC are for several reasons still a problem in clinical cytogenetics: (i) they are too small to be characterized for their chromosomal origin by traditional banding techniques and require molecular (cytogenetic) techniques for their identification [41]; (ii) apart from the correlation of about one-third of the sSMC cases with a specific clinical picture, as mentioned above, most of the sSMCs have not been correlated with clinical syndromes, even though progress was achieved, recently [43, 44]; (iii) sSMC can be harmful due to different mechanisms like induction of genomic imbalance and/or uniparental disomy [42]; (iv) also sSMC can be found just by chance and cannot be correlated with the clinical problems of a patient [44]; finally (v) the percentage in

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which an sSMC is present can, but must not have an influence on the clinical outcome [42-44].

Here we focus on the latter mentioned problem – the regularly appearing somatic mosaicism in cases with an sSMC.

Mosaicism in association with sSMC is a well-known fact. Crolla (1998) [45] summarized 144 sSMC cases excluding those derived from chromosomes 15 and 22, 78 of which (54%) showed mosaic karyotypes. To get a more detailed view on mosaicism in sSMC the following subgroups are focused separately below: cases with sSMC duplication and multiple sSMC, cases with four known 'sSMC-syndromes' Pallister-Killian-, i(18p)-, Emanuel-, and cat-eye-syndrome, cases with sSMC and Prader-Willi- and Angelman-syndrome, cases with an sSMC present in a structurally abnormal but balanced karyotype, neocentric sSMC cases and patients with numerically abnormal basic karyotypes. The remaining sSMC with a normal basic karyotype of 46 chromosomes plus an sSMC are the group of patients this review starts with.

SOMATIC MOSAICISM IN sSMC PRESENT IN A NORMAL BASIC KARYOTYPE

According to Liehr (2010) [44] 731/1512 sSMC cases (52%) studied by cytogenetics are mosaic (see Table **1**).

Table 1. Cases with Mosaics 47,+mar, Excluding Cases with Known Syndromes, with Neocentric sSMC and such with Unclear Mosaicism Status

sSMC derived from chromosome	Number of cases with 47,+mar[100%]	Total number of sSMC cases	Cases with mos 47,+mar/46
1	6	67	91%
1/5/19	0	8	100%
2	6	36	83%
3	7	21	67%
4	7	21	67%
5	10	34	71%
6	2	14	86%
7	5	23	78%
8	11	92	88%
9	4	59	93%
10	5	18	72%
11	3	16	81%
12	6	29	79%
13	7	9	22%
13/21	54	84	36%
14	62	99	37%
14/22	31	49	37%
15	361	459	21%
16	4	46	91%
17	6	26	77%
18	14	43	67%
19	7	40	83%
20	7	33	79%
21	12	25	83%
22	78	115	32%
acro	3	6	50%
X	7	27	74%
Y	6	13	54%
overall	**731**	**1512**	**52%**
acrocentric	**608**	**846**	**28%**
non-acrocentric	**123**	**666**	**82%**

However, there is a strong difference between acrocentric and non-acrocentric derived sSMC: while 72% of acrocentric derived sSMC present no mosaic, 82% of non-acrocentric derived sSMCs are mosaic.

The real grade and complexity of mosaicism seems to be even slightly higher as recently repeatedly cryptic mosaicism was detected in sSMC cases by molecular cytogenetics. There were either cases showing an sSMC in all studied metaphase spreads, however, interphase-FISH in uncultured cells showed a mosaic situation like in case 16-CW-2 [44]. More often it is found that more than one variant of an sSMC is present in different studied cells of a patient. As summarized in Table **2**, at least 5% of sSMC cases have, after a detailed molecular cytogenetic analysis, a more complex mosaicism than suggested after simple cytogenetic diagnostics. In 20% of these cases, unexpected complex somatic mosaicism was detectable where cytogenetics did not suggest any mosaicism, i.e. in cases 04-U-7, 08-W-p11.2/1-2, 11-O-p11.1/2-1, 11-U-9, 13-U-13, 15-W-q11.1+q11.2/1-1, ?1-O-q11.21/1-1, 21-U-5, 22-O-q11.1/5-1, 22-O-q11.1/5-2, ƆX-W-p11.?3/1-1, 0X-W-p11.21/1-1 [44]. Interestingly, acrocentric derived sSMC are by far more stable than non-acrocentric derived ones (2% versus 9%, Table **2**).

Cryptic mosaicism appears as some sSMC tend to rearrange and/or be reduced in size during karyotypic evolution. This can lead to double ring formation or inverted duplica-

Table 2. Cases with Cryptic Mosaics 47,+mar, Excluding Cases with Known Syndromes, with Neocentric sSMC and such with Unclear Mosaicism Status

sSMC derived from chromosome	Number of cases with cryptic mosaicism	Number of cases with cryptic mosaicism
1	0/67	0%
1/5/19	0/8	0%
2	2/36	5%
3	5/21	24%
4	1/21	5%
5	5/34	15%
6	4/14	29%
7	4/23	17%
8	8/92	9%
9	6/59	10%
10	0/18	0%
11	4/16	25%
12	2/29	7%
13	2/9	15%
13/21	0/84	0%
14	2/99	2%
14/22	0/49	0%
15	4/459	1%
16	3/46	19%
17	0/26	0%
18	2/43	5%
19	6/40	15%
20	4/33	12%
21	2/25	8%
22	5/115	4%
acro	0/6	0%
X	2/27	7%
Y	0/13	0%
overall	**73/1512**	**5%**
acrocentric	**15/846**	**2%**
non-acrocentric	**58/666**	**9%**

tion starting from a centric minute-shaped chromosome and in the end to the formation of different variants and a highly complex mosaic as some of the new variants can also be degraded in a subset of the studied cells [46].

In summary, somatic mosaics are to be expected in at least 50% of sSMC cases with normal basic karyotype. More complex mosaics can be met in up to 10% of the cases; however, the overall rate of mosaic cases is not significantly altered by cryptic mosaicism, while the genetic complexity of individual cases may be severely influenced.

SOMATIC MOSAICISM IN CASES WITH sSMC DUPLICATION AND MULTIPLE sSMC

sSMC in a small subset of cases tend to duplicate, leading to a karyotype 48,+marx2 [42]. Up to now 64 such cases are reported [44] and 45% of those are derived from non-acrocentric chromosomes (Table **3**). While, cases with acrocentric derived sSMC tend to be by mosaic only in 54% of the cases, non-acrocentric derived ones are always mosaic with an exception of 1/29 reported patients (Table **3**). Thus, in sSMC duplication cases we find a similar situation as in

Table 3. Cases with Mosaics 48,+marx2 Excluding Cases with Known Syndromes, with Multiple and Neocentric sSMC and such with Unclear Mosaicism Status

sSMC derived from chromosome	Number of cases with 48,+marx2[100%]	Total number of sSMC cases	Cases with mosaic
1	n.a.	2 (diff. sizes)	100%
1/5/19	n.a.	n.a.	n.a.
2	n.a.	2	100%
3	n.a.	2 (diff. sizes)	100%
4	n.a.	1 / 1 (diff. sizes)	100%
5	n.a.	1 / 1 (diff. sizes)	100%
6	n.a.	1 / 1 (diff. sizes)	100%
7	n.a.	n.a.	n.a.
8	n.a.	2 / 1 (diff. sizes)	100%
9	n.a.	2 / 1 (diff. sizes)	100%
10	n.a.	n.a.	n.a.
11	n.a.	n.a.	n.a.
12	n.a.	n.a.	n.a.
13	1	1	0%
13/21	1	1 / 1 (diff. sizes)	50%
14	2	3 / 1 (diff. sizes)	50%
14/22	1	3	67%
15	11	22	50%
16	n.a.	1 / 1 (diff. sizes)	100%
17	n.a.	1 / 1 (diff. sizes)	100%
18	n.a.	n.a.	n.a.
19	1	1	0%
20	n.a.	3 / 1 (diff. sizes)	100%
21	n.a.	1	100%
22	n.a.	2	100%
acro	n.a.	n.a.	n.a.
X	n.a.	1	100%
Y	n.a.	1	100%
overall	**17**	**64**	**73%**
acrocentric	**16**	**35**	**54%**
non-acrocentric	**1**	**29**	**97%**

cases with one single sSMC and a karyotype 47, +mar concerning mosaicism.

Multiple sSMC cases [42] differ from sSMC duplication ones by the fact that the observed sSMC are not derived from the identical chromosome. Only 48 such cases are known by now [44], having between 2 and 7 sSMC of different origin, each; and all reported cases with multiple sSMC are mosaic. Formation of this rare cytogenetic condition is unclear, even though polysomic rescue or triploid rescue maybe suggested. As in most cases markedly chromosomal imbalances are induced by multiple sSMC presence, ~90-95% of them are correlated with clinical symptoms, irrespective of mosaicism status detectable in peripheral blood.

SOMATIC MOSAICISM PRESENT IN THE FOUR KNOWN 'sSMC-SYNDROMES': PALLISTER-KILLIAN-, I(18P)-, EMANUEL-, AND CAT-EYE-SYNDROME

Somatic mosaicism is reported to different extents in four sSMC-related syndromes.

Patients suffering from Pallister-Killian-syndrome (PKS) due to the presence of an additional isochromosome 12p are known to have somatic mosaicism in practically every case. In peripheral blood the +(12p) tends to be lost either already during pregnancy or shortly after birth in practically all cells. In the alternatively studied skin fibroblasts, the sSMC derived from chromosome 12 is normally easily to detect in >70% to 100% of the cells [47]. However, besides a mosaic of cells with 46 and 47 chromosomes exceptional cases also with two different shapes of sSMC (12-Wpks-4, 12-Wpks-159, [44]) or two isochromosomes 12p (12-Wpks-174 [44]) are also reported.

In isochromosome 18p syndrome mosaicism is rather rare. But also here exceptional cases are known having the full clinical phenotype but normal karyotype in some of the body cells (18-Wi-42, 18-Wi-153, 18-Wi-154, 18-Wi-157 [44]). In case 18-Wi-41 [44] the i(18p) was derived from the clinically normal mother; the latter had the i(18p) in only 4% of her peripheral blood cells. Also, an interesting case of somatic mosaicism is 18-Wi-158 [44] showing prenatally an i(18p) in 35% of the amnion cells but postnatal only normal cells in peripheral blood, being a normal child.

To the best of our knowledge no mosaic cases are known by now for Emanuel-syndrome (ES) [44]. Also in cat-eye-syndrome (CES) mosaicism is rather rare. sSMC derived from chromosome 22 having two different shapes were seen in CES (22-Wces-2 [44]) or minimal mosaicism with a normal cell line (22-Wces-3-03, 22-Wces-5, 22-Wces-5-119 [44]).

Overall, somatic mosaicism is, compared to other sSMC derived from the corresponding chromosomes, over-represented in PKS (100% vs. 79%) and under-represented in i(18p) syndrome (4% vs. 67%), ES (0% vs. 32%) and CES (3% vs. 32%).

SOMATIC MOSAICISM IN PRADER-WILLI- AND ANGELMAN-SYNDROME WITH sSMC

26 sSMC cases with Prader-Willi-syndrome (PWS) and 7 with Angelman-syndrome (AS) can be found in the literature [44]. 15 of these are PWS (58%) while only 1 of these AS cases (14%) is mosaic with respect to sSMC presence [44]. As the corresponding syndromes were caused either by uniparental disomy or microdeletion the sSMC presence has no direct influence on the clinical outcome; neither has mosaicism.

SOMATIC MOSAICISM IN sSMC PRESENT IN STRUCTURALLY ABNORMAL BUT BALANCED KARYOTYPE

Another rare cytogenetic variant of sSMC presence is that of a structurally abnormal but balanced karyotype (McClintock mechanism) [48]. Such cases can either be connected with a neocentromere formation (see section below) or both the derivatives share the available centromeric alpha-satellite sequences. If in such case mosaicism appears, i.e. loss of the sSMC, relevant genetic material is lost and this leads normally to clinical problems as described for the following cases: 03-W-p11/1-1, 04-W-p15.3/1-1, 04-W-p12/1-1 [44]. If no or only very low grade mosaicism is present the carrier of such a karyotype can be completely normal (e.g. 02-O-p12/1-1, 06-O-p22.3/1-1, 06-O-p22.3/1-1, 08-O-p11.1/2-1, 12-U-4, 17-O-p11.2/2-1, mother of 19-W-10/2-1, mother of 22-W-q11.2/2-1 [44]).

SOMATIC MOSAICISM IN NEOCENTRIC sSMC

For mosaicism in neocentric sSMC formed by McClintock mechanism, [48] the same holds true, like for the aforementioned centric sSMC present in structurally abnormal but balanced karyotype. If balanced and no or only minimal mosaicism is present, the carriers of such a chromosomal condition are clinically normal. If the neocentric sSMC is lost in a higher percentage of the body cells this has an adverse prognosis.

In general, in at least around 50% of the cases with a neocentric sSMC somatic mosaicism is observable (Table **4**). Strikingly, as in centric sSMC, mosaicism is more frequent in non-acrocentric derived compared to acrocentric derived ones (58% vs. 24%).

SOMATIC MOSAICISM IN sSMC PRESENT IN NUMERICALLY ABNORMAL BASIC KARYOTYPES

As above mentioned, sSMC can appear in a numerically normal basic karyotype of 46 chromosomes, but also in numerically abnormal basic karyotypes [42]. Up to now, sSMC are reported additionally to a basic karyotype 45, X (= Turner syndrome), 47, XXY (= Klinefelter syndrome), 47, XXX (triple-X syndrome) and 47, +21 (Down syndrome) [44, 49-51].

542 cases are available in the literature with a basic karyotype typical for Turner syndrome and an additional sSMC, i.e. 46,X,+mar [44, 49]. Only 73 of these are reported without mosaicism; thus, 76% of these Turner syndrome cases are mosaic [44].

Only three cases, each of them are known by now with Klinefelter- or triple-X syndrome and an additional sSMC. Concerning the Klinefelter-syndrome two cases of those are mosaic (07-U-6, 0X-U3) and one not (09-U5) [44]. For tri-

Table 4. Mosaicism in Cases with Neocentric sSMC

sSMC derived from chromosome	Number of cases with mosaicism	Percentage of cases with mosaicism
1	3/5	60%
2	3/4	75%
3	10/11	91%
4	1/1	100%
5	0/1	0%
6	1/2	50%
7	1/1	100%
8	7/9	77%
9	1/3	33%
10	1/2	50%
11	0/2	0%
12	2/3	75%
13	5/14	56%
14	1/1	100%
15	2/19	11%
16	1/1	100%
17	0/1	0%
18	1/1	100%
19	n.a.	n.a.
20	0/1	0%
21	n.a.	n.a.
22	n.a.	n.a.
X	0/1	0%
Y	0/1	0%
overall	**40/86**	**47%**
acrocentric	**8/34**	**24%**
non-acrocentric	**32/55**	**58%**

ple-X syndrome the same holds true: cases 09-U16 and 14-O-q11.1/1-5 are mosaic, case 14-U-5 is not [44].

For sSMC, at present additionally to a trisomy 21 (Down-syndrome), information on mosaic status is available in 16 cases; 7 of those (44%) have somatic mosaicism with a cell line 47, +21 without sSMC [44].

Overall, mosaicism is a frequent finding when an sSMC is present additionally to a numerically abnormal basic karyotype.

SOMATIC MOSAICISM IN sSMC AND THE RESULTING PITFALLS

Summarizing all above mentioned groups, 1626 of 3124 cases with sSMC (52%) present with somatic mosaicism. Even though, expressed to a different extent in various subgroups, mosaicism is something to be considered in at least every second such case. However, if a specific genetic imbalance caused by an sSMC is known to be harmful, in the overwhelming majority of the cases there is no influence of the grade of somatic mosaicism detectable in peripheral blood or amnion cells and the observed clinical effects. This seems to be due to the fact that the mosaicism rate in different human tissues is practically not predictable and very variable [52]. Only in exceptional cases the presence of specific sSMC with known adverse prognosis was reported which did not lead to clinical problems due to low somatic mosaicism; examples are 07-W-p10/1-1, 15-O-q13/1-1, 15-O-q13/1-2, 15-O-q13/2-1, 15-O-q13/3-1, 15-O-q13.1/1-1, 22-O-q11.21/4-2, 22-O-q11.21/4-3, 22-O-q11.21/5-1 [44]. Even though rare, this knowledge is extremely important for prenatal counseling.

Knowing that somatic mosaicism happens in ~50% of the cases with sSMC, array-CGH studies cannot be offered as a screening test to reliably detect this kind of chromosomal aberration. On the one hand, low level mosaic cases and on

the other hand, cryptic mosaics are missed. Thus, cytogenetics is still the gold-standard to detect any kind of chromosomal aberration, which then, in further steps can be characterized by molecular (cyto-) genetic approaches.

Interestingly, acrocentric and non-acrocentric derived sSMC are differently susceptible to mosaicism; acrocentric derived ones are hereby the more stable ones. This holds true for centric and neocentric sSMC, and an explanation is therefore at present not available.

CONCLUSION

Somatic mosaicism is a feature of the human body, which has to be considered much more than up to now in future. It is known as a fact, but not understood why man with age (in peripheral blood) develops something like a 'Turner-syndrome-mosaic' 46,XY/45,X. Similarly, in cases with sSMC it is known since years, that PKS patients lose the i(12p) in peripheral blood or that some inv dup(15) sSMC are stable and cytogenetically identical ones in another carrier are not. For all these facts to the best of our knowledge, no studies were undertaken to come closer to an understanding of these phenomena. Here we present, some kind of starting point for such studies, for the first time a detailed 'mosaicism map' for the different subtypes of sSMC.

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2.7. Article .6

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Pericentromeric BAC-probe set - thoughts about considering genedosage insensitive regions

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- Castronovo et al. (1) suggest an excellent probe set for the characterization of the centromere-near regions of all human chromosomes. This probe set is an important tool especially for the characterization of small supernumerary marker chromosomes (sSMC), as also outlined in (1). The probe set presented is a very good supplement for aCGH characterized sSMC with cryptic mosaicism (2). Also in case sSMC are present in such low mosaic rates that they may not be characterized by aCGH (3) this probe set might be helpful; also it can be used for larger derivative chromosomes with centromere-near break-events (including balanced rearrangements), as done already with comparable probe sets (4).

- Still, for sSMC we suggest, a clinically relevant pericentric probe set should be generated based on the thoughts outlined in the following. It is known that euchromatic imbalances induced by sSMC may cause clinical problems, but they not always do. Thus, it was hypothesized that centromere-near imbalances only then lead to clinical problems if the concerned region contain dosage sensitive genes (5). Regions being obviously free of such dosage sensitive genes and those containing such genes were narrowed down for most of the pericentric regions already (6-7). This knowledge should be included in an sSMC-specific pericentric probe set.

- We established such a probe set (unpublished data) and showed that it is suited for distinguishing between sSMC leading to clinical problems. For the pericentric region of chromosome 1p for example, it is known that the region free of dosage sensitive genes includes the stretches between 115.8 Mb down to the centromere starting at 121.1 Mb (NCBI 36.3/hg18); for the long arm of chromosome 1 such a region was not defined yet. Also it was shown that sSMC-induced trisomy of the euchromtic region 115.3-121.1 Mb of chromosome 1 lead to clinical problems. Thus, we selected 5 BAC probes spanning the region 114.4-118.5 Mb and another 5 BACs spanning 143.6 to

148.0 Mb starting from cytoband 1q12-q21.1. Similar probe sets are available now for the pericentric regions of all human chromosomes.

- A combination of BACs used in the present paper of Castronovo together with our probe set may be the best way to characterize sSMC in a clinically relevant way now and in future.

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Competing interests

No competing interests.

2.8. Article .7

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ORIGINAL ARTICLE

CLINICAL IMPACT OF PROXIMAL AUTOSOMAL IMBALANCES

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ABSTRACT

Centromere-near gain of copy number can be induced by intra- or inter-chromosomal rearrangements or by the presence of a small supernumerary marker chromosome (sSMC). Interestingly, partial trisomy to hexasomy of euchromatic material may be present in clinically healthy or affected individuals, depending on origin and size of chromosomal material involved. Here we report the known minimal sizes of all centromere-near, *i.e.,* proximal auto-somal regions in humans, which are tolerated; over 100 Mb of coding DNA are comprised in these regions. Additionally, we have summarized the typical symptoms for nine proximal autosomal regions including genes obviously sensitive to copy numbers. Overall, studying the carriers of specific chromosomal imbalances using genomics-based medicine, combined with single cell analysis can provide the genotype-phenotype correlations and can also give hints where copy-number-sensitive genes are located in the human genome.

INTRODUCTION

Autosomal Proximal Chromosome Imbalances. The finding of unbalanced chromosomal abnormalities (UBCA) was recently reviewed and summarized from a total of 200 families. The UBCA usually involve several megabases of DNA. Carriers of such UBCA are ascertained due to adverse reproductive effects or dysmorphic and/or mentally retarded offspring; the carriers themselves have an otherwise normal phenotype. Unbalanced chromosomal abnormalities have been reported for more than 50 euchromatic regions of almost all human autosomes [1,2].

Unbalanced chromosomal abnormalities leading to gain of genetic relevant material within the autosomal centromere-near region were not comprehensively followed in the above mentioned studies [1,2]. Such centro-mere-near, *i.e.,* proximal chromosomal imbalances (C-UBCA), can be induced by small supernumerary marker chromosomes (sSMCs) [3,4] and also by intrachromo-somal duplications [4]. While the latter are rare events and no reliable data on their frequency is available, sSMCs are present in 0.043% of human beings [5]. With a given population size of 7×10^9 individuals, 3×10^6 sSMC carriers are presently alive. As ~2/3 of these do not show any symptoms, $\sim 2 \times 10^6$ do not even know of their condition. Euchromatin is present in ~36.0% of those sSMC cases that do not lead to any clinical symptoms (Table 1) [6]. *N.B.*: sSMC, irrespective of origin and genetic constitution may cause fertility problems, especially in males [7]. Thus, infertility was not considered as an 'abnormal phenotype' in this study.

Even though partial trisomy is the most frequent imbalance induced by sSMC, tetra- or even hexasomy

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Table 1. Summarized here are 478 autosomal derived sSMC cases, which are characterized in detail for their size and genetic content; all of them can be found on the sSMC homepage [6]. All these cases are not associated with any clinical abnormalities. In 174, *i.e.,* 36.4%, proximal euchromatic material was present. As can be seen, the rates of cases with and without euchromatin vary from chromosome to chromosome. In general, in acrocentric derived sSMC, cases without euchromatin are in the majority, while it is the other way round in most non acrocentric derived sSMC.

Chromosomes	Cases With Euchromatin	Cases Without Euchromatin
1	4	11
2	9	2
3	10	2
4	1	0
5	8	5
6	1	1
7	1	0
8	9	2
9	18	1
10	6	1
11	2	1
12	6	2
13	1	0
14	6	53
15	35	136
16	11	8
17	2	0
18	9	2
19	5	1
20	6	6
21	8	1
22	16	69
Overall	**174**	**304**

of proximal euchromatin may be present in clinically healthy individuals [6]. Here we present the latest known proximal, centromere-near regions and their minimal molecular borders. The corresponding index cases were previously published and are summarized on the sSMC homepage [6]. This study intends to give a review on the clinical impact of proximal autosomal imbalances. A special focus is hereby laid on gain of copy numbers. For this, the following steps were necessary: ***i)*** define the pericentric regions that can be present as additional copy(ies) without causing any clinical phenotype. ***ii)*** After the definition of such copy number insensitive regions, in a second step, proximal autosomal regions including genes potentially sensitive to copy numbers can be defined. ***iii)*** Such copy number sensitive regions can be correlated with specific, typical symptoms; the latter already being possible for nine centromere-near regions in this study and there will be more in the future.

What Can be Learned From Cases With Chromosome Imbalances? It was nicely summarized back in 1993 [8] that structural autosomal imbalances may lead in 'typical cases' to syndromes with a complex of minor anomalies and/or congenital malformations. The latter 'suggests the importance of gene interaction in determining the phe-notypic picture of autosomal imbalance syndromes' [8]. Duplication-related syndromes are much more frequent than deletion-related ones, and thus, it is common sense that in general, duplications of several Mb in size are better tolerated by the human genome than deletions of the same size. This has also recently been confirmed on the level of micro-duplications and -deletions [9]. Overall, chromosomal imbalances can point towards dosage sensitive genes being responsible for specific syndromes or clinical features. A good example is the dosage sensitive peripheral myelin protein 22 (PMP22) gene in 17p11.2: a duplication of 1.4 Mb including PMP22 leads to the hereditary motor and sensory neuropathy type 1A and the reciprocal deletion to the hereditary neuropathy with liability to pressure palsies. However, also specific mutations in PMP22 itself can cause the identical syndromes [10].

Moreover, UBCA of several Mb in size have been reported, which surprisingly, do not have any clinical consequences [1,2]. At the same time, they are not pure copy number variants (CNV) such as those recently found for a cytogenetically visible amplified region in 8q21.2 [11]. In summary, there are genetically relevant regions which can be tolerated if 'amplified' as three or more copies; the reason for that is most likely that they do not comprise dosage sensitive genes. In summary, studying carriers of specific chromosomal imbalances can provide genotype-phenotype-correlations, and also give hints as to where copy-number-(in)sensitive genes are located in our genome.

Where to Find Proximal Chromosome Imbalances in Humans. Centromere-near imbalances may principally appear as deletions or duplications. However, practically no reports of proximal deletions are available in the literature. The only exceptions are offspring of carriers with an sSMC formed by the McClintock mechanism [12], *e.g.,* as reported for a child

having the karyotype 47,XY,del(2) (p12p11.1) due to a maternal cytogenetic condition (47,XX,del(2) (p12p11.1),+r(2)(::p12→p11.1::) [13]. Only eight corresponding cases are available in the literature [6] and all these patients were severely affected.

The best suited patients to study proximal duplications would be those with proximal intrachromosomal rearrangements, as direct or inverted duplications or unbalanced insertions, because these cases would be non mosaic [4]. However, such cases are scarce (summarized in Table 2). Most of these ~200 cases were only studied cytogenet-ically and no information on the molecular size of their duplicated region is available [6].

In contrast, the largest and best characterized group where to find proximal duplications are patients with sSMC [3-7,14-16]. Besides their cytogenetic characterization, more and more cases were characterized at the molecular level by array-comparative genomic hybridization (aCGH) studies [4,17,18]. However, when analyzing this group of patients one has to consider the following drawbacks: ***i)*** sSMC carriers may be mosaic with normal cell lines and/or may have different levels of mosaicism in different tissues; thus, harmful sSMC sizes may be rated as harmless [19], and ***ii)*** also harmless sSMC may be considered to be harmful if they appear together with a uniparental disomy (UPD) [20], or a mutation in a monogenic disorder gene [21]. Thus, results for regions including or excluding most likely dosage-sensitive genes, *i.e.,* C-UBCA, have to be handled carefully. Nevertheless, sSMC carriers are much more frequent and better characterized on the molecular level than intrachromosomal duplications, and are thus used here as a model system for proximal duplications.

MATERIALS AND METHODS

This study is based on the data summarized on the sSMC-homepage [6]. All raw data is freely available and can be followed down to each individual case. The data used for the present study is summarized in Tables 1 through 4.

Proximal Chromosomal Imbalances Without Clinical Consequences. The available in detail characterized sSMC cases [6] were studied by various approaches. In the majority of cases, the sSMC were characterized exclusively by molecular cytogenetics and the breakpoints are given as cytobands without molecular assessment of the exact breakpoint. In addition, there are already numerous sSMC cases characterized by well-defined locus-specific probes used in fluorescence *in situ* hybridization (FISH) and/or by aCGH [6]. In Table 3, the presently characterized C-UBCA are summarized. Overall, it could be shown that at least 96.8 Mb of the proximal chromosomal regions are tolerated as triplicates or more (Table 3). While for proximal 6q there is neither molecular nor cytogenetic hint for any dosage independent C-UBCA, in all other proximal autosomal parts at least cytogenetic evidence for C-UBCA in healthy individuals is there.

Except for proximal parts of 1q, 6p, 6q and 13q, there are molecular hints on C-UBCA for every chromosome arm, being at least between 0.07 and 10.23 Mb in size. According to cytogenetics, no less than 16 of the 39 autosomal proximal non dosage sensitive regions (= C-UBCA) are larger than already proven by aCGH, *i.e.,* 2p, 3p, 3q, 6p, 8p, 8q, 9p, 9q, 10p, 10q, 11p, 12p, 19p, 19q, 20p and 22q (Table 3).

Table 2. The ~200 case reports of proximal intra-autosomal duplications are summarized per autosome and distinguished in clinically normal and abnormal cases [6].

Chromosomes	Clinically Normal	Clinically Abnormal
1	1	8
2	0	5
3	0	1
4	0	2
5	0	4
6	0	3
7	0	2
8	0	2
9	4	2
10	1	4
11	3	3
12	0	11
13	0	2
14	0	1
15	32	>50
16	2	16
17	0	5
18	22	3
19	0	0
20	0	4
21	0	3
22	3	3
Overall	**68**	**>134**

Table 3. All 39 proximal autosomal regions containing no copy number-sensitive genes are summarized. According to the sSMC-homepage [6], the positions and sizes of duplications are given in columns 2 and 3. Column 4 summarizes if the C-UBCA may be larger according to non molecular cytogenetic results. Additionally, in the last two columns it is indicated if the C-UBCA is based on mosaic or non mosaic sSMC cases, and if more than three copies were present in the corresponding index cases. (UCSC: University of California Santa Cruz genome browser; http://genome.ucsc.edu).

Chromosomes	Molecular Bands (UCSC hg18, 2006)	Size (Mb)	Region Expected to be Larger According to Molecular Cytogenetic Results	Mosaic	>Three Copies
1p	118.33-121.10	2.80	[+/–]	(–)	–
1q	142.40-??.??	n.a	[++]	+	–
2p	89.60-91.00	1.40	[++]	+	{+;4}
2q	95.70-101.58	5.88	[+/–]	+	{+;4}
3p	87.60-89.40	1.80	[++]	–	{+;4}
3q	93.20-96.01	2.81	[++]	+	{+;4}
4p	44.03-48.70	4.67	[+/–]	+	–
4q	52.40-62.63	10.23	[+/–]	+	–
5p	37.21-45.80	1.41	[+/–]	–	{+;4}
5q	50.50-55.27	4.77	[+/–]	+	{+;4}
6p	??.??-58.40	n.a.	[++]	+	–
6q	63.40-??.??	n.a	[+/–]	n.a.	n.a.
7p	56.45-57.40	0.95	[+/–]	+	–
7q	61.10-67.00	5.90	[+/–]	+	–
8p	42.50-43.20	0.70	[++]	+	{+;4}
8q	48.10-48.30	0.20	[++]	+	–
9p	42.96-46.70	3.74	[++]	+	{+;4}
9q	70.00-70.50	0.50	[++]	+	–
10p	34.75-38.80	4.05	[++]	(–)	–
10q	42.10-43.82	1.72	[++]	–	–
11p	50.95-51.40	0.45	[++]	–	–
11q	56.40-60.23	3.83	[+/–]	+	{+;4}
12p	28.47-33.20	4.73	[++]	+	–
12q	36.50-39.90	3.40	[+/–]	+	–
13q	18.40-??.??	n.a.	[++]	–	+;4
14q	19.10-19.88	0.78	[+/–]	–	+;4
15q	18.40-21.05	2.65	[+/–]	–	+;4 +;6
16p	28.86-34.40	5.54	[+/–]	–	–
16q	45.50-46.02	0.52	[+/–]	–	{+;4}
17p	18.68-22.10	3.42	[+/–]	+	–
17q	23.20-23.32	0.12	[+/–]	+	–
18p	12.80-15.40	2.60	[+/–]	(–)	{+;4}
18q	17.30-18.12	0.82	[+/–]	+	–
19p	22.98-26.70	3.72	[++]	+	{+;4}
19q	30.20-36.90	6.70	[++]	+	–
20p	24.96-25.70	0.74	[++]	+	{+;4}
20q	28.40-29.93	1.53	[+/–]	+	{+;4} {+;6}
21q	13.20-14.85	1.65	[+/–]	–	{+;4}
22q	16.30-16.37	0.07	[++]	–	–

+/–: no larger C-UBCA expected; (–): in part mosaic index cases; n.a.: not available; ++: larger according to molecular cytogenetic results; {}: mosaic; –: no mosaic; +: mosaic; +;4: four copies; +;6: six copies.

Table 4. Clinical consequences of larger proximal autosomal imbalances for nine corresponding regions are summarized [6]. Common specific clinical symptoms were observed in crucial parts of the 5-12 cases, each; for 4q only two cases were available, both showing overgrowth. Unspecific symptoms such as mental retardation developmental delay or dysmorphic face were neglected but normally also present in these cases. For 22q cases with cat eye syndrome were excluded.

Symptom – Chromosome Region	1p	1q	4q	5q	7q	10p	17p	18q	22q
Autism	+	–	–	–	–	–	–	–	–
Finger/toe/foot malformations	+	–	–	–	+	–	–	–	–
Growth retardation	–	+	–	–	–	+	–	–	–
Heart defects	–	+	–	–	–	–	–	–	–
Hernia	–	–	–	+	–	–	+	–	–
Hypotonia	+	–	–	+	–	–	–	–	+
Macrocephaly	–	–	–	+	–	–	–	–	–
Overgrowth	–	–	+	–	+	–	–	–	–
Seizures	–	–	–	–	–	–	–	–	–
Urethral problems	–	–	–	–	–	–	–	+	+

Twenty-four of the 38 informative proximal autosomal regions are based on mosaic sSMC cases. Thus, the data summarized in Table 3 is still to be considered as preliminary in those cases, even though in >99.0% of sSMC cases, mosaicism detected in peripheral blood plays a minor role for the clinical outcome [22]. Mosaicism may play a role for the phenotype if its rates are variant in different tissues of the body [23]. The C-UBCA regions 1p, 3p, 5p, 10p, 10q, 11p, 13q, 14q, 15q, 16p, 16q, 18p, 21q and 22q were reported in non mosaic cases. The remaining regions await such proof.

Another issue to be reflected is the copy number of a C-UBCA tolerated by the human genome. At least, for 15 C-UBCA low mosaics (maximum 20.0%) of cells having four (or in one case of 20q up to six) copies of the corresponding regions are tolerated. The C-UBCA of chromosomes 13q, 14q and 15q can be present in four copies in normal carriers in 100.0% of the studied cells. For 15q, even six copies are possible (Table 3).

Autosomal Proximal Imbalances Leading to Clinical Consequences. In case an sSMC or an intrachromo-somal duplication is larger than the critical region for harmless sSMC, as summarized Table 3, a variety of clinical problems can be the consequence for the sSMC carrier. Besides well-known syndromes such as isochromosome-12p (Pallister-Killian syndrome) [24], -15q [25], -18p [26] or -22q (cat-eye-syndrome) [27], a variety of symptoms can be associated with an sSMC-induced imbalance [3,6]. In most cases the correlated symptoms are rather non specific. However, first potentially specific symptom combinations for nine corresponding imbalances are summarized in Table 4. In future, it should be possible for at least some of these proximal autosomal imbalances to define new, possibly even clinically recognizable, syndromes [3].

CONCLUSIONS

The sSMC are a long time underestimated source for the understanding of proximal chromosomal imbalances in humans. New information on regions of the human genome, possibly inert to copy number changes, can be acquired from this group of patients. Moreover, effects such as heterochromatization [3] or feedback-loops in gene regulation [28] might also be considered for the understanding of the effects of such imbalances. Comprehensive studies of more aberrant cases will also lead to new genotype-phenotype correlations and to the possibility of a clinical sub-differentiation of more sSMC cases. All these goals can only be achieved by a sophisticated balance of single cell analysis (such as in mosaic cases) and genomics-based medicine (such as for array-based approaches).

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2.9. Article .8

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A New Multicolor Fluorescence In Situ Hybridization Probe Set Directed Against Human Heterochromatin: HCM-FISH

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Summary

A new multicolor fluorescence in situ hybridization (mFISH) probe set is presented, and its possible applications are highlighted in 25 clinical cases. The so-called heterochromatin-M-FISH (HCM-FISH) probe set enables a one-step characterization of the large heterochromatic regions within the human genome. HCM-FISH closes a gap in the now available mFISH probe sets, as those do not normally cover the acrocentric short arms; the large pericentric regions of chromosomes 1, 9, and 16; as well as the band Yq12. Still, these regions can be involved in different kinds of chromosomal rearrangements such as translocations, insertions, inversions, amplifications, and marker chromosome formations. Here, examples are given for all these kinds of chromosomal aberrations, detected as constitutional rearrangements in clinical cases. Application perspectives of the probe set in tumors as well as in evolutionary cytogenetic studies are given. (J Histochem Cytochem 60:530–536, 2012)

Keywords

multicolor fluorescence in situ hybridization (mFISH), heterochromatin-M-FISH (HCM-FISH) probe set, heteromorphism, small supernumerary marker chromosome (sSMC), insertion, translocation

A detailed characterization of chromosomal rearrangements detected in routine banding cytogenetics can nowadays be done easily by fluorescence in situ hybridization (FISH) and/or array-comparative genomic hybridization (aCGH) (Manolakos et al. 2010; Weimer et al. 2011). While in aCGH, a higher resolution may be achieved, FISH still has several advantages over the array-based approaches (Manolakos et al. 2010). FISH allows, for example, the analysis of balanced rearrangements, of chromosomal aberrations present only in low mosaic levels, and of the large heterochromatic regions of the human genome. The acrocentric short arms; the centric and the large pericentric regions of chromosomes 1, 9, and 16; as well as the band Yq12 cannot be analyzed by aCGH.

A multitude of multicolor FISH (mFISH) probe sets have been developed in the last decades (Liehr 2012a). They were implemented for use in one experiment: 1) all 24 human whole chromosome painting probes (multiplex FISH = M-FISH [Speicher et al. 1996]; spectral karyotyping = SKY [Schröck et al. 1996]) or 2) all centromeric probes (centromere-specific M-FISH = cenM-FISH [Nietzel et al. 2001]). Also, 3) various FISH banding

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approaches (Liehr et al. 2002a) were introduced as well as 4) combinations of centromeric with locus-specific and/or partial chromosome painting probes (e.g., subcentromere-specific M-FISH = subcenM-FISH [Liehr et al. 2006]). These probe sets are highly suited to characterize simple and complex chromosomal aberrations (approaches 1 and 3) or small supernumerary marker chromosomes (sSMC) (Liehr et al. 2004, 2006) (approaches 2 and 4). Recently, even a probe set was introduced to substantiate indirectly epigenetic changes (parental origin determination FISH = POD-FISH [Weise et al. 2008]).

Here, we present a new mFISH probe set specifically directed against the large heterochromatic regions within the human genome. This so-called heterochromatin-M-FISH (HCM-FISH) set was successfully established and applied already in 30 cases, where its application saved sample material and time. We present 25 representative cases studied by HCM-FISH and discuss the possible applications of this new probe set.

Materials and Methods

HCM-FISH Probe Set

The HCM-FISH probe set (Fig. 1) is based on eight glass-needle microdissection (midi)–derived and one P1 artificial chromosome (PAC) probe (RP5–1174A5 = dj1174A5); the latter was kindly provided by Dr. M. Rocchi (Bari, Italy). The latter probe is specific for the nucleolus organizer region (NOR), which contains several tandem copies of ribosomal RNA genes and in humans is clustered on the short arms of chromosomes 13, 14, 15, 21, and 22; that is, the acrocentric chromosomes (Trifonov et al. 2003). Midi was done as previously reported (Liehr et al. 2002b). Midi probes for the regions 1q12, 9q12, 15p12~11.2 (i.e., a β-satellite–specific probe), 16q11.2, 19p12~19q12, and Yq12 were established for this probe set, while those probes for 9p12/9q13 (midi 36) (Starke et al. 2002) and for all acrocentric short arms (midi 54) were as previously reported (Mrasek et al. 2003).

The DNA of the nine probes was amplified in vitro and labeled by degenerated oligonucleotide primer polymerase chain reaction (DOP-PCR) according to standard procedures (Telenius et al. 1992). The amplification procedure followed a published scheme (Fig. 2A in Liehr et al. 2002b). The used fluorochromes Spectrum Green (SG), Spectrum Orange (SO), Texas Red (TR), cyanine 5 (CY5), and diethylaminocoumarin (DEAC) were applied for the nine DNA probes as depicted in Figure 1A. Thus, each DNA probe obtained its unique fluorochrome combination, which could be transformed into pseudocolors (Fig. 1) using the software mentioned below.

Twenty metaphase spreads were analyzed, each using a fluorescence microscope (Axioplan 2 MOT; Carl Zeiss, Oberkochen, Germany) equipped with appropriate filter sets to discriminate between all five fluorochromes and the counterstain 4',6-diamidino-2-phenylindole (DAPI). Image capturing and processing were carried out using an Isis mFISH imaging system (MetaSystems; Altlussheim, Germany).

Clinical Cases

Overall, 30 clinical cases were studied already by HCM-FISH (Table 1). The clinical indications were infertility, repeated abortions, dysmorphic features and/or mental retardation, or a prenatal cytogenetic study due to advanced maternal age (Table 1). In all studied cases, apart from cases 1 and 1a to 1d, which were normal controls, banding cytogenetics revealed an aberrant karyotype. M-FISH was not informative in cases 2, 4, 6, 7, 8, 10, 11, and 14 (results not shown). In cases 3, 5, 12, 13, and 15, heteromorphisms were suggested after Giemsa stained chromsomes banding. In the additional redundant 11 cases (Table 1), similar observations were made. In case 9, HCM-FISH was applied directly, as an sSMC derived from an acrocentric chromosome was suggested.

Results

In the present study, it could be demonstrated that HCM-FISH can be used to characterize within one single step chromosomal rearrangements with gross involvement of heterochromatic material. The HCM-FISH probe set was established first in five control cases (result shown for case 1 in Fig. 1A and for case 1b in Fig. 1B). The probe mix appeared to work reliably and stably and stained the foreseen chromosomal regions as expected. Afterwards, it was applied in the five groups of patients listed below (Table 1). All chromosomal aberrations in cases 2 to 14 were initially detected by GTG banding.

1. Heterochromatic material attached to the tip of a nonacrocentric chromosomal arm: In cases 2, 4, 4a, and 4b, the short arm of a acrocentric chromosome unable to be further characterized was attached to the short arm of a chromosome 1 (Fig. 1C) or the long arm of a Y chromosome (Fig. 1E).
2. Heterochromatic material attached to the end of an acrocentric chromosomal arm: In cases 5-8, 12, 13, and 15, the short arms of different acrocentric chromosomes were enlarged. Chromosome 15p–specific β-satellite DNA was amplified in one chromosome 15 of case 5 (Fig. 1F); additionally, double satellites (dss) were present on the second chromosome 15 and one chromosome 22 in case 5. Furthermore, chromosome 22 of case 5 with dss had a so-called increase in the length of the stalk of the short arm (pstk+) (Fig. 1F). Similar heteromorphisms were

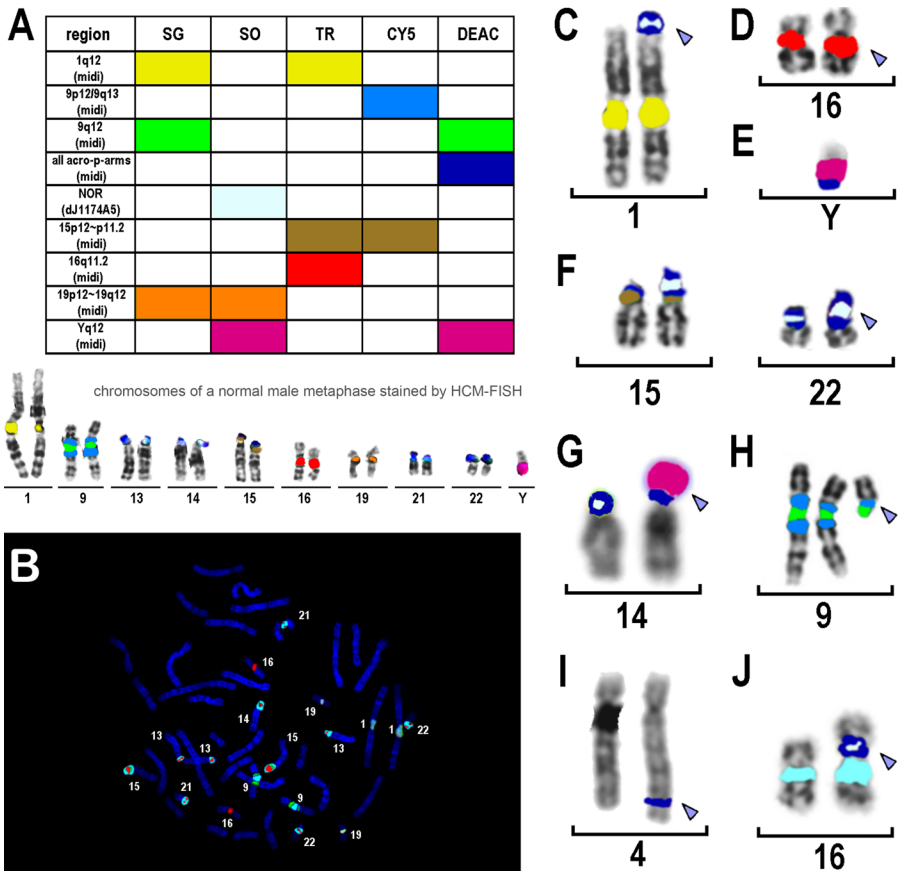

Figure 1. (A) Label scheme used for the heterochromatin multicolor fluorescence in situ hybridization (HCM-FISH) probe set. The pseudocolors used for the corresponding region-specific DNA probes in C through J are used to indicate the fluorochromes applied to generate the HCM-FISH probe set. Also, the nine labeled chromosome pairs and the Y chromosome are shown in pseudocolor depiction below the scheme. Eight microdissection (midi)–derived and one cosmid probe (dj1174A5) were labeled by Spectrum Green (SG), Spectrum Orange (SO), Texas Red (TR), cyanine 5 (CY5), and diethylaminocoumarin (DEAC) as depicted. acro-p-arms, short arms of all acrocentric human chromosomes; NOR, nucleolus organizer region. (B) Real color depiction of a female metaphase after HCM-FISH. All labeled chromosomes are highlighted by the chromosome numbers. Chromosomes are counterstained in dark blue by 4',6-diamidino-2-phenylindole (DAPI), CY5 and SG are depicted in greenish colors, TR and SO are in reddish ones, and DEAC is in light blue. (C-J) Typical FISH results after application of the HCM-FISH probe set on a metaphase of a normal control (see Table 1). (C) HCM-FISH revealed in one hybridization step the nature of the derivative chromosome 1 (arrowhead) in case 2 (Table 1), that is, der(1)t(1;acro)(p36.33;p11.2). (D) In case 3 (Table 1), a suggested 16qh+ (arrowhead) could be confirmed. (E) der(Y)t(Y;acro)(q11.2;p12) was characterized in case 4 (Table 1). (F) In case 5 (Table 1), the short arms of both chromosomes 15 and one chromosome 22 looked abnormal. By HCM-FISH, the following could be defined: one chromosome 15 has an enlarged β-satellite–positive region (left chromosome 15), the second chromosome had double satellites (right chromosome 15), and the chromosome 22 in question had an enlarged midi-positive region plus double satellites (arrowhead). (G) In case 7 (Table 1), the extremely enlarged short arm of one chromosome 14 (arrowhead) derived from Yq12 and a final karyotype of 46,XX,der(14)t(Y;14)(q12;p13) was characterized. (H) A small supernumerary marker chromosome (sSMC) was present in case 9 (Table 1). It was initially suggested to be derived from an acrocentric chromosome; however, HCM-FISH characterized the sSMC as a derivative of the short arm of chromosome 9 (arrowhead): del(9)(q11.1~12). (I) The unknown material inserted in 4q34.2 of case 10 (Table 1) was characterized by HCM-FISH as derived from the short arm of an acrocentric chromosome (arrowhead). (J) In case 11 (Table 1), short arm material derived from an acrocentric chromosome was inserted in a derivative chromosome 16 in p11.2 (arrowhead).

the reason for the enlargements of acrocentric p-arms in cases 12 (including cases 12a and 12b), 13 (including cases 13a and 13b), and 15: dss, pstk+, or double stalks (pstkpstk) were characterized (Table 1). In cases 6, 7, and 8 (including cases 6a and 8a–8d), heterochromatic material derived from Yq12 was added to the short arms of a chromosome 13, 14 (Fig. 1G), or 15.

3. Heterochromatic material inserted in an autosome: In cases 10 and 11, undefined additional material

Table 1. Cases Solved by HCM-FISH

Case No.	Clinical Indication	Final Cytogenetic Result
1	None: normal control	46,XY
2	Infertility	46,XX,der(1)t(1;acro)(p36.33;p11.2)
3	Prenatally detected; advanced maternal age	46,XY,16qh+
4	Infertility	46,X,der(Y)t(Y;acro)(q11.2;p12)
5	Dysmorphic features	46,XY,15βsat+,15pss,22pstk+pss
6	Infertility	46,XY,der(13)t(Y;13)(q11.2;p12)
7	Prenatally detected; advanced maternal age	46,XX,der(14)t(Y;14)(q12;p13)
8	Infertility	46,XY,der(15)t(Y;15)(q12;p13)
9	Dysmorphic features, mentally retarded	47,XX,+del(9)(q11.1~12)
10	Dysmorphic features, mentally retarded	46,XX,der(4)ins(4;acro)(q34.2;p11.2p12)
11	Dysmorphic features, mentally retarded	46,XX,inv(2)(q31q37.3),ins(16;acro)(p11.2;p11.2p12)
12	Repeated abortions	46,XX,14pstkpstk,21pstk+
13	Repeated abortions	46,XX,22pstk+pss
14	Repeated abortions	46,XX,inv(9)(var5)
15	Infertility	46,XY,21pstkpstk
15 Additional Redundant Cases		
1a	None: normal control	46,XY
1b–1d	None: normal control	46,XX
4a–4b	Infertility	46,X,der(Y)t(Y;acro)(q11.2;p12)
6a	Infertility	46,XX,der(13)t(Y;13)(q11.2;p12)
8a–8b	Infertility	46,XY,der(15)t(Y;15)(q12;p13)
8c–8d	Infertility	46,XX,der(15)t(Y;15)(q12;p13)
12a	Repeated abortions	46,XY,14pstkpstk
12b	Repeated abortions	46,XY,21pstk+
13a–13b	Repeated abortions	46,XX,22pstk+pss

was inserted into a chromosome 4 and 16, respectively. By HCM-FISH, this material was defined to be derived from an acrocentric short arm (Fig. 1I and 1J).

4. Enlargement of heterochromatic blocks in autosomes: In cases 3 and 14, the heterochromatic blocks of one chromosome 16 and 9, respectively, were enlarged. In case 3, it was an enlargement of 16q11.2, describable as 16qh+ (Fig. 1D). In case 14, the enlargement resulted from an additional band derived from DNA homologous to midi 36 (specific for 9p12/9q13).
5. Potentially heterochromatic sSMC: Case 9 was studied by the HCM-FISH probe set, as a heterochromatic; it was most likely that acrocentric chromosome–derived sSMC was expected according to GTG banding. Surprisingly, this sSMC turned out to be del(9)(q11.1~12), also describable as der(9)(pter->q11.1~12:) (Fig. 1H).

Discussion

During the last decades, numerous mFISH approaches have been developed (Liehr 2012a): M-FISH/SKY is able to characterize the origin and/or composition of larger euchromatic-derivative chromosomes (Speicher et al. 1996; Schröck et al. 1996); cenM-FISH can identify the chromosomal origin of sSMC (Nietzel et al. 2001); FISH banding and the use of locus-specific probes enable a better breakpoint characterization than banding cytogenetics (Weise et al. 2002; Manvelyan et al. 2007); and POD-FISH is able to determine the parental origin of derivative chromosomes on a single cell level (Polityko et al. 2009). Even though there were already probe sets specific for some of the large heterochromatic human chromosomal regions, like pericentromere of chromosome 9 (Starke et al. 2002), or short arms of all acrocentric chromosomes (Trifonov et al. 2003), no probe set was available up to now that was directed against all of them. The HCM-FISH probe set closes this gap in mFISH approaches; within one single step, chromosomal rearrangements with gross involvement of heterochromatic material can be characterized, as shown for cases 2 to 15.

Here, HCM-FISH was applied for the characterization of five different kinds of chromosomal rearrangements and proved to be a helpful tool in clinical cytogenetic diagnostics. However, the HCM-FISH probe set could also be used to answer questions in other fields, such as tumor cytogenetics

or evolutionary studies. Examples would be interstitial heterochromatin in tumor-associated derivative chromosomes (Doneda et al. 1989) or studies on evolutionarily conserved heterochromatin (Mrasek et al. 2003).

If heterochromatic material is attached to the tip of a nonacrocentric chromosomal arm, the carrier can be clinically normal and only detected due to infertility or clinically affected due to essential loss of subtelomeric material in the "receiving" chromosome. There are cases reported with attached heterochromatin derived from an acrocentric short arm, similar to the present cases 2 and 4 (Weise et al. 2002), or derived from Yq12 (de Ravel et al. 2004; Hiraki et al. 2006). Yet, there are no other terminal additions of heterochromatic material reported as inborn rearrangements. However, in tumor cytogenetics, terminal translocations with breakpoints in 16q11.2 (Tsuda et al. 1999) or the pericentric region of chromosome 19 (Nagel et al. 2009) are reported.

Heterochromatic material attached to the end of an acrocentric chromosomal arm can have various sources. In general, such derivative acrocentric chromosomes are considered to be heteromorphic variations without any clinical meaning. They can be found in infertility patients and in those with clinical problems. In rare cases, the clinical phenotype of a patient is due to euchromatin translocated to an acrocentric short arm (Trifonov et al. 2003). The cases included in this study had only heterochromatic variants, considered to have no clinical meaning. However, their influence on fertility is still a matter of discussion (Codina-Pascual et al. 2006). In cases 5, 12, 13, and 15, the enlargement of one or more acrocentric short arms was due to double satellite formation (dss), increase in the length of the stalk of the short arm (pstk+), or double stalks (pstkpstk). These are well-known length variations in heterochromatic segments described in the corresponding standard literature (Shaffer et al. 2009). In most of them, the NOR is involved; however, systematic studies aligning results from NOR silver staining (Goodpasture et al. 1976) and FISH studies using a NOR-specific or an rDNA probe are still lacking. In case 5 also, 15p-specific β-satellite DNA was amplified on one chromosome 15, a variant less frequently observed (Acar et al. 1999) and not yet included in Shaffer et al. (2009). Finally, the short arm of an acrocentric chromosome can be enlarged due to an unbalanced translocation of Yq12 material (cases 6–8). Most frequently observed are der(15)t(Y;15)(q12;p13) (Chen et al. 2007), while corresponding derivatives of chromosomes 13 (Morris et al. 1987), 14 (Buys et al. 1979), 21 (Ng et al. 2006), or 22 are rarely or have not been seen up to now.

Insertion of heterochromatic material into a chromosome arm of an autosome was present in cases 10 and 11 of this study. HCM-FISH showed in one step that this material was derived from an acrocentric short arm, once with and once without the NOR region. Similar reports are scarcely available in the literature (Watt et al. 1984; Reddy and Sulcova 1998; Guttenbach et al. 1998; Chen et al. 2004). However, even such an insertion in an X chromosome was seen once (Tamagaki et al. 2000). Also, heterochromatic material from the pericentric region of chromosome 9 may be inserted into euchromatic (own unpublished observation) of heterochromatic material of other chromosomes (Doneda et al. 1998). Furthermore, Yq12 (Ashton-Prolla et al. 1997) and 16q11.2 material (McKeever et al. 1996) were observed to be inserted in another chromosome. Moreover, heterochromatic insertions such as Yq12 have been observed in tumor cytogenetics (Sala et al. 2007).

Enlargement of heterochromatic blocks in autosomes, specifically in chromosomes 1, 9, and 16, is well known and described in Shaffer et al. (2009) and elsewhere (Starke et al. 2002). Variants such as qh+, ph+, and qh– can be easily characterized by HCM-FISH. Also, the variants of chromosome 9 reported in Starke et al. (2002) can be visualized, similar to here in case 14.

Finally, HCM-FISH is suited to be used for the one-step characterization of potentially heterochromatic sSMC cases. In case 9, an acrocentric chromosome–derived sSMC was expected but turned out to be del(9)(q11.1~12). Thus, sSMC, being largely C banding-positive, are good candidates to be tested by HCM-FISH. sSMC derived from chromosomes 1, 9, 16, 19, or any acrocentric chromosome, can be determined or at least narrowed for their origin using this probe set, including such sSMC being Yq12 positive. Thus, over 55% of sSMC can be characterized with this simple probe set (Liehr 2012b).

In conclusion, we present a new mFISH probe set easily and effectively applicable in clinical cytogenetic routine diagnostics. It could be enlarged by additional probes, for example, an rDNA probe (Muravenko et al. 2001), midi probes of obviously heterochromatic sSMC of unclear origin within the human genome (Mackie Ogilvie et al. 2001), or regions of cytogenetically visible copy number variants (Manvelyan et al. 2011). The application of HCM-FISH will be helpful in tumor cytogenetics as well as in evolution research studies; for the latter, the addition of species-specific heterochromatic DNA probes would also be recommended.

Declaration of Conflicting Interests

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2.10. Article .9

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Clinical Impact of Somatic Mosaicism in Cases with Small Supernumerary Marker Chromosomes

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Key Words
Molecular cytogenetics · Mosaicism · Small supernumerary marker chromosome

Abstract
Somatic mosaicism is present in slightly more than 50% of small supernumerary marker chromosome (sSMC) carriers. Interestingly, non-acrocentric derived sSMC show mosaicism much more frequently than acrocentric ones. sSMC can be present in different mosaic rates, which may go below 5% of the studied cells. Also cryptic mosaicism can be present and mosaics may be differently expressed in different tissues of the body. Even though in the overwhelming majority of the cases somatic sSMC mosaicism has no direct clinical effect, there are also cases with altered clinical outcomes due to mosaicism. Also clinically important is the fact that a de novo sSMC, even present in mosaic, may be a hint of uniparental disomy (UPD). As it is under discussion to possibly replace standard karyotyping by methods like array-CGH, the impracticality of the latter to detect low-level sSMC mosaics and/or UPD has to be considered as well. Overall, sSMC mosaicism has to be studied carefully in each individual case, as it can be extremely informative and of importance, especially for prenatal genetic counseling.

Small Supernumerary Marker Chromosomes

Small supernumerary marker chromosomes (sSMC) are defined as structurally abnormal chromosomes that cannot be identified or characterized unambiguously by conventional banding cytogenetics alone; they are generally equal in size or smaller than a chromosome 20 of the same metaphase spread. sSMC can either be present additionally in (1) an otherwise normal karyotype, (2) a numerically abnormal karyotype (like Turner- or Down-syndrome) or (3) a structurally abnormal but balanced karyotype with or without ring chromosome formation. Overall, sSMC are too small to be considered for their chromosomal origin by traditional routine banding techniques, and molecular cytogenetic approaches are needed for their exact characterization [Liehr et al., 2004]. The general risk for an abnormal phenotype in prenatally ascertained de novo cases with sSMC is given as 26–30% [Liehr and Weise, 2007].

Different factors have to be considered to establish a potential clinical impact of a prenatally ascertained de novo sSMC case. The sheer size of the extra chromosome is less important; rather, the question if the sSMC consists of hetero- or euchromatin has to be answered first, together with the characterization of its chromosomal origin. As shown first in 2006 [Liehr et al., 2006], a genotype-phenotype correlation can be based on the re-

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gions and sizes of chromosomal imbalances induced by the sSMC [Liehr, 2012]. However, even if an sSMC is, according to that, considered to be harmless, it still is important to test for a uniparental disomy (UPD) of the corresponding sister chromosomes [Liehr et al., 2011], as around 5% of de novo sSMC are correlated with a UPD [Liehr et al., 2004]. Besides, in ~14% of the cases, an sSMC is present additionally to a numerical chromosomal aberration like trisomy 13, 18 or 21, monosomy X, or any other numerical aberration of the gonosomes. For these latter cases, it is common sense that the sSMC is more or less negligible for clinical outcome, as the effects of a whole chromosome gain or loss are much stronger than that of an sSMC.

One of the most puzzling problems in sSMC cases is mosaicism, as in general, the percentage in which an sSMC is present can, but must not, have an influence on the clinical outcome [Liehr et al., 2004, 2006]. Thus, this review is focusing on this special question and tries to give an answer on the clinical impact of somatic mosaicism in cases with sSMC.

Somatic Mosaicism in sSMC Cases

Somatic mosaicism is not only observed in the everyday-life of cytogeneticists [Gebhart and Liehr, 1999; Yurov et al., 2007; Iourov et al., 2008], but also in recent research projects [Yurov et al., 2009; Mkrtchyan et al., 2010]. However, of the almost 400 different human body tissues, normally only one tissue, i.e. blood, amnion or chorion cells, or fibroblasts are studied cytogenetically. Thus, nobody can know the real rate of somatic mosaicism in any studied individual. Though, few postmortem studies in carriers of sSMC indicate up to now that frequency of sSMC-carrying cells is highly variable from tissue to tissue [Fickelscher et al., 2007]. This has to be considered for the following thoughts, especially, as mosaicism ranges from very low, i.e. less than 0.5% of studied cells with an sSMC, to very high, i.e. (practically) all cells of the studied tissue with sSMC [Liehr et al., 2011].

Frequency and Clinical Impact of Somatic Mosaicism in sSMC Cases

Basic Karyotype 47,XN,+mar

In sSMC, carriers having karyotypes of 47,+mar a mosaic of 47,+mar/46 is present in 52% of the cases, excluding the well-defined sSMC-related syndromes mentioned in the following paragraph. However, there is a difference between acrocentric and non-acrocentric derived sSMC: 28% of acrocentric derived sSMC, compared to 82% of non-acrocentric derived sSMC are mosaic [Liehr et al., 2010].

Somatic mosaicism is reported also in the known sSMC-related syndromes isochromosome-5p-syndrome (92%), isochromosome-8p-syndrome (95%), isochromosome-9p-syndrome (~90%), isochromosome-12p-syndrome = Pallister-Killian-syndrome (100%), isochromosome-15q-syndrome (15%), isochromosome-18p-syndrome (4%), Emanuel-syndrome (0%), and cat-eye-syndrome (3%) [Liehr et al., 2010; Liehr 2012].

If a specific genetic imbalance caused by an sSMC is known to be harmful [Liehr et al., 2006; Liehr 2012], in the overwhelming majority of the cases there is no influence of the grade of somatic mosaicism detectable in peripheral blood or amnion cells and the observed clinical effects. This is suggested to be due to the fact that the mosaicism rate in different human tissues is, as aforementioned, not predictable and very variable [Fickelscher et al., 2007].

However, in exceptional cases the presence of an sSMC with known adverse prognosis was reported, which did, surprisingly, not lead to clinical problems. Most likely explanation for this finding is somatic mosaicism; examples are listed in table 1. Especially noteworthy is that by now 2/23 isochromosome-5p-, 5/51 isochromosome-9p-, 1/271 isochromosome 12p-, 4/400 isochromosome-15q-, 1/229 isochromosome-18p, and 4/192 cat-eye-chromosome-carriers showed no clinical symptoms due to low-grade-mosaicism. Besides, there are other cases reported for sSMC derived from chromosomes 5, 7, 8, and 20 which should lead to clinical symptoms, but did not, as a large cell line with normal karyotype was predominant. In case of sSMC (7), the father had the extra chromosome in only 35% of his blood lymphocytes, while the phenotypically impaired son was carrier in 100% of his blood cells. A similar example with good outcome is case 15-O-q13.1/1-1, in which a r(15)(p11.2q13.1) was present in mother and daughter in mosaic and did not harm any of them. Also possible, as among the cases listed in table 1, the sSMC may not be the reason for clinical signs and symptoms [Nelle et al., 2010]. Overall, even though rare, considering mosaicism as a possibility with clinical impact in comparable sSMC cases is extremely important for prenatal counseling.

Basic Karyotype 46,X,+mar

Basically, a karyotype 45,X may be connected with a Turner syndrome [Liehr et al., 2007]. In patients with a karyotype 45,X,+mar, mosaics like 45,X/45,X,+mar/46,XN are found in 87% of the cases. If the corresponding

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Table 1. sSMC cases with clinical consequences of mosaicism: pathogenic sSMC but normal clinical phenotype due to mosaicism

Chromosomal origin	Karyotype (mosaic in [%])	Material studied	Case number acc. to Liehr [2012]
5	47,XY,+del(5)(q11.1)[3]	blood	05-O-pter/1-1
5	47,XY,+i(5)(p10)[16]	blood	05-O-pter/2-1
5	47,XX,+i(5)(p10)[7] interphase: 47,XX,+i(5)(p10)[70]	amnion buccal mucosa	05-O-pter/2-2
7	47,XY,+r(7)(p10→q11.2)[35]	blood	father of 07-W-p10/1-1
8	47,XX,+r(8)(p23.1q1?1)[27]	blood	08-O-p23.1/1-1
9	47,XY,+i(9)(p10)[20]	blood	09-O-pter/1-1
9	47,XX,+i(9)(p10)[100] 47,XY,+i(9)(p10)[65]	blood buccal mucosa	09-O-pter/1-2
9	48,XXX,+i(9)(p10)[40]/47,XXX[40]/ 47,XX,+i(9)(p10)[7]/46,XX[13]	blood	09-O-pter/1-3
9	47,XX,+i(9)(p10)[?]	blood	09-O-pter/1-4
9	47,XX,+i(9)(p10)[72]	blood	09-O-pter/1-5
12	47,XX,+i(12)(p10)[37]	skin	12-Wpks-1
15	47,XX,+inv dup(15)(q13)[56]	blood	15-O-q13/1-1
15	47,XY,+inv dup(15)(q13)[23] 47,XY,+inv dup(15)(q13)[27]	amnion blood	15-O-q13/1-2
15	47,XX,+inv dup(15)(:p11.1→q12~13: :q12~13→p11.1:)[7]/47,XX,+min(15) (:p11.1→q11.2:)[22]/47,XX,+r(15) (p11.1q11.2)[30]/47,XX,+r(15) (p11.1q12)[15]/46,XX[26]	blood	15-O-q13/2-1
15	47,XX,+inv dup(15)(pter→q13::q12→pter)[6] 47,XX,+inv dup(15)(pter→q13::q12→pter)[15] 47,XX,+inv dup(15)(pter→q13::q12→pter)[25] 47,XX,+inv dup(15)(pter→q13::q12→pter)[8]	amnion blood skin buccal mucosa	15-O-q13/3-1
15	47,XX,+r(15)(p11.2q13.1)[79] maternal – there 10% of blood cells	amnion	15-O-q13.1/1-1
18	47,XY,+i(18)(p10)[35]	amnion	18-Wi-158
20	47,XY,+r(20)(p12.2~12.3q11.1)[15]/ 47,XY,+r(20)(p12.1q11.1q11.1p12.1)[3]/ 47,XY,+min(20)(:p12.1→q11.1: :q11.1→p12.1:)[2]/46,XY[80]	amnion	20-O-p12.2/1-1
22	47,XX,+inv dup(22)(q11.21)[9]	PBL	22-Wces-3-22
22	47,XX,+inv dup(22)(q11.21)[?]	PBL	22-Wces-3-22a
22	47,XN,+inv dup(22)(q11.21)[?]	PBL	22-Wces-5-140
22	47,XN,+inv dup(22)(q11.21)[?]	PBL	22-Wces-5-149

sSMC is derived from the Y-chromosome, mosaic is present in 83%; if derived from the X-chromosome, it is present in 94% of the cases [Liehr et al., 2010]. For patients with dysgenetic gonads, the presence of Y-chromosome material detected during cytogenetic analysis indicates an increasing risk of gonadal tumors, especially gonadoblastoma, estimated to be ~30%. Gonadoblastoma is a benign tumor, but it can undergo transformation into invasive dysgerminoma in 60% of the cases and also into other malignant forms of germ cell tumors [Liehr et al., 2007]. Prenatally, in case of a 45,X/46,X,+der(X) karyotype, it is important to test for the ability of the derivative X-chromosome to be inactivated, i.e. for the presence of the X-inactivation center-gene [Agrelo and Wutz, 2010]. An sSMC derived from the X-chromosome can only be inactivated if the X-inactivation center-region is preserved on it. Presence or absence of the X-inactivation center in the sSMC may lead to different clinical outcomes, especially with respect to mental development [Liehr et al., 2007].

Table 2. sSMC cases with mosaicism and uniparental disomy

Origin of UPD	Karyotype (mosaic in [%])	Material studied	Case number acc. to Liehr [2012]
1 mat	47,XX,r(1)(::p21.1→q12)[3]	amnion	01-W-p21.1/1-1
4 mat	48,XY,+21,+min(4)(:p12→q11:)[80]	blood	04-U-1
6 mat	48,XXY,+mar(6)[60]	blood	06-CW-3
6 pat	47,XX,+r(6)(p21.2q10)[74]	blood	06-W-p21.2/1-1
7 mat	47,XY,+min(7)(p12→p11.1:)[8]	blood	07-W-p12/1-1
7 mat	47,XY,+min(7)(:p11.2→q11.21:)[36]	blood	07-W-p11.2/1-3
7 mat	47,XY,+min(7)(:p11.2→q11.21:)[56]	blood	07-W-p11.2/1-4
7 mat	47,XN,+r(7)(p11.2q21)[4]	blood	07-W-p11.2/2-1
7 mat	47,XX,+r(7)(p11.1q11.2?2)[27]	blood	07-W-p11.1/2-2
9 mat	47,XX,+r(9)(p12q10)[36]	blood	09-W-p12/1-1
10 mat	47,XX,+min(10)(:p12.31→q11.1:)[88]	blood	10-U-2
12 mat	47,XX,+min(12)(:p11→q11:)[53]	amnion	12-O-p11/1-1
12 mat	47,XX,+12/47,XX,+i(12)(p10)/46,XX	amnion	12-Wpks-159
14 mat	47,XY,+del(14)(q11.1)[87]	blood	14-W-q11.1/3-1
14 pat	47,XX,+inv dup(14)(q11)[88]	blood	14-W-q11.1/2-1
15 mat	47,XY,+r(15)(p11.1q11.1∼q13)[16]	blood	15-W-q11.1∼13/1-1
15 mat	47,XX,+mar(X)[50]	blood	15-P-2
15 mat	47,XX,+mar(15)[25]	blood	15-P-3
15 mat	47,XY,+inv dup(15)(q11)[70]	blood	15-P-q11/1-2
15 mat	47,XY,+inv dup(15)(q11)[45]	blood	15-P-q11/1-5
15 mat	47,XY,+del(15)(q11.1)[70]	blood	15-P-q11.1/1-1
15 mat	47,XX,+inv dup(15)(q11.1)[55]	blood	15-P-q11.1/2-2
15 mat	47,XN,+inv dup(15)(q11.1)[?]	amnion	15-P-q11.1/2-4
15 mat	47,XN,+inv dup(15)(q11.1)[?]	amnion	15-P-q11.1/2-5
15 mat	47,XY,+inv dup(15)(q11.1)[39]	amnion	15-P-q11.1/2-6
15 mat	47,XX,+inv dup(15)(q11.1)[50]	amnion	15-P-q11.1/2-7
15 mat	47,XY,+inv dup(15)(pter→q11::q13→pter)[85]	blood	15-P-q11∼13/1-1
15 mat	47,XY,+r(15)(p11.1q11.1∼q13)[16]	blood	15-P-q11∼13/1-2
15 mat	47,XX,+inv dup(15)(q12∼13)[20]	blood	15-P-q12/2-3
15 mat	47,XN,+mar[8]	blood	15-P-4
15 mat	47,XY,+inv dup(22)(q11.1)[46]	blood	22-U-40
15 pat	47,XY,+inv dup(15)(q11)[60]	blood	15-A-q11/1-1
15 pat	47,XY,+inv dup(15)(q11.2)[32]	amnion	15-A-q11.2/1-1
16 mat	47,XY,+r(16)(p11.1q11.2)[84]	amnion	16-W-p11.1/3-1
16 mat	47,inv(X)(p11.4p22.3)Y,+min(16)(:p11.21→q11.1:)[72]	amnion	16-O-p11.21/1-1
20 mat	47,XY,+min(20)(:p11.1→q11.1:)[42]	blood	20-W-p11.1/2-1
20 pat	47,XY,+min(20)(:p11.21∼11.22→q11.1:)[17]	amnion	20-O-p11.21∼11.22/1-1
22 mat	47,XX,+min(22)(:p11.1→q11:)[22]	blood	22-O-q11/2-1
Other sSMC cases with UPD, without mosaic	03-U-8; 07-W-p11.2/1-1; 14-W-q11.1∼11.2/1-1; 14-CW-2; 15-P-q11/1-4; 15-P-q11/1-13; 15-P-q11.1/1-2, 15-A-q11/1-2; 20-W-p13/3-1; 22-Wces-5-81		

UPD = Uniparental disomy; mat = maternal; pat = paternal.

Thus, in such cases, even if an sSMC is present only in a subset of the cells, its characterization has high clinical impact for the individual pregnancy and/or patient.

Basic Karyotype 48,XN,+21,+mar

In Down-syndrome cases, a mosaic status is known for only 16 of 40 reported cases; 7 of those (44%) have somatic mosaicism with a cell line 47,+21 without sSMC [Liehr et al., 2010]. Neither a correlation of clinical outcome of cases with nor without mosaicism was done yet for this rare subgroup of sSMC-carriers.

sSMC in Klinefelter- and Triple-X-Syndrome

There are 3 reported sSMC-cases, each for Klinefelter- or triple-X syndrome. Two of 3 cases, each, is mosaic and one not [Liehr et al., 2010]. Here the same holds true as for the aforementioned Down-syndrome cases.

sSMC and UPD

Forty-eight cases with sSMC and UPD are reported and summarized in table 2. 80% of them are mosaic cases, i.e. it is a statistically significant difference (t-test: $p = 0.001$) for appearance of mosaicism in sSMC without

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Table 3. Cases with sSMC, formed by the McClintock mechanism, with low level mosaicism and normal outcome

Chromosomal origin	Karyotype (mosaic in [%])	Material studied	Case number acc. to Liehr [2012]
1 neo	47,XY,del(1)(p32p36.1),+r(1)(p32p36.1)[87]/ 47,XY,del(1)(p32p36.1),+r(1)(p32→p36.1::p23→p36.1)[10]/ 46,XY,del(1)(p32p36.1)[3]	blood	McCl-01-N-p32/1-1
6	47,XX,del(6)(p11.2∼p11.1q12),+r(6)(p11.2∼p11.1q12)[80]	blood	McCl-06-O-p11.2∼p11.1/1-1
8	47,XY,del(8)(p11.1q12.1),+r(8)(p11.1q12.1)[90]	blood	McCl-08-O-p11.1/2-1
13 neo	47,XX,del(13)(q12.3q22),+r(13)(q12.3q22)[97]	blood	McCl-13-N-p12.3/1-1
17	47,XX,del(17)(p11.2q10)+min(17)(:p11.2→q10:)[89]	blood	McCl-17-O-p11.2/2-1
22	47,XX,del(22)(p11.1q11.2)+mar[80]/	blood	McCl-22-O-q11.1/1-1

neo = Neocentromere.

Table 4. Neocentric sSMC cases with clinical consequences of mosaicism: pathogenic sSMC but normal clinical phenotype due to mosaicism

Chromosomal origin	Karyotype (mosaic in [%])	Material studied	Case number acc. to Liehr [2012]
1	47,XY,+r(1)(q43q44)[50]	amnion	01-N-q43/1-1
2	47,XN,+mar(2)[12]	amnion	02-N-1
3	47,XY,+inv dup(3)(qter→q27.1: :q27.1→qter)[30]	blood	03-N-qt27.1/1-1
	47,XY,+inv dup(3)(qter→q27.1: :q27.1→qter)[6]	skin (pigmented)	
8	47,XY,+inv dup(8)(pter→p23.2∼23.1: :p23.2∼23.1→pter)[47]	amnion	08-N-pt23.2∼23.1/1-1
	47,XY,+inv dup(8)(pter→p23.2∼23.1: :p23.2∼23.1→pter)[21]	blood	
15 neo	47,XX,+mar(15)(:q11.2→q13.1: :q11.2→q13.1::)[76]	blood	15-N-q11.2/1-1

(52%) and such cases with UPD (80%). Looking closer, one can find that the mosaic-rate of acrocentric sSMC without UPD is 28% compared to 75% in sSMC with UPD (table 2). The mosaic-rate of non-acrocentric sSMC does not differ significantly in both groups 82% versus 85%.

Overall, it may be concluded that acrocentric derived mosaic sSMC present a UPD much more likely than non-mosaic ones. For non-acrocentric derived sSMC, there is no such correlation with mosaicism. [Liehr et al., 2011].

Multiple sSMC

There are ∼65 cases reported with a karyotype 48,+marx2 and ∼50 cases with multiple sSMC derived from different chromosomes [Liehr 2012]. In these cases, 73% or 100% are mosaic, respectively [Liehr et al., 2011]. Again we have to state that there is no clinical impact known for mosaicism, due to low case numbers.

sSMC Formed According to McClintock Mechanism

Slightly over 30 cases with sSMC presence but balanced karyotype (McClintock mechanism) are reported [Baldwin et al., 2008]. Here either a neocentromere is formed or the both derivatives share the available centromeric alpha-satellite sequences. If mosaicism appears, i.e. loss of the sSMC, relevant genetic material is lost and this normally leads to clinical problems. If no or only very low grade mosaicism is present, the carrier of such a karyotype can be completely normal; 5 such cases are summarized in table 3.

Neocentric sSMC

In at least 50% of the known ~100 neocentric sSMC cases, somatic mosaicism is present. Strikingly, as in centric sSMC, mosaicism is more frequent in non-acrocentric derived compared to acrocentric derived ones (58% vs. 24%) [Liehr et al., 2010]. In table 4, the 5 known neocentric sSMC cases present in mosaic and normal clinical outcome are collected (i.e. in ~8% of the mosaic neocentric sSMC carriers, a normal clinical outcome is reported).

sSMC Carriers with Cryptic Mosaicism

The real grade and complexity of mosaicism may be even higher in ~5% of the sSMC cases, considering that recently cryptic mosaicism was repeatedly detected, which means sSMC cases can have more complex rearranged sSMC in mosaic than expected after cytogenetic analysis. Acrocentric derived sSMC are by far more stable than non-acrocentric derived ones (2 vs. 9%) [Liehr et al., 2010]. With this knowledge, clinical consequences are to be expected because cryptic mosaics may lead e.g. to partial tetra- instead of trisomies. As it is known that trisomy 18p is tolerated hardly without any clinical signs, tetrasomy 18p, i.e. isochromosome-18p-syndrome, is associated with severe mental and physical problems. However, the finding of cryptic mosaics maybe to new, and thus, no correlation with this fact is possible in sSMC by now.

Summary and Conclusion

Somatic mosaicism is present in ~50% of the cases with sSMC. Acrocentric and non-acrocentric derived sSMC are differently susceptible to mosaicism. Acrocentric derived are the more stable ones, and surprisingly, this holds true for centric and neocentric sSMC. Also, there is an enhanced susceptibility for UPD formation in mosaic acrocentric- than in non-acrocentric-derived sSMC.

It has to be stressed that the only reliable approach to detect sSMC present in (low-level) mosaic is banding cytogenetics. Array-CGH studies cannot be offered as a screening test to reliably detect this kind of chromosomal aberration. Thus, especially when considering somatic mosaicism, cytogenetics is still the gold-standard to detect any kind of chromosomal aberration, which afterwards may be characterized in detail by molecular (cyto) genetic approaches.

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Fernández-Toral et al. Journal of Medical Case Reports 2010, 4:239
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JOURNAL OF MEDICAL CASE REPORTS

CASE REPORT Open Access

Four small supernumerary marker chromosomes derived from chromosomes 6, 8, 11 and 12 in a patient with minimal clinical abnormalities: a case report

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Abstract

Introduction: Small supernumerary marker chromosomes are still a problem in cytogenetic diagnostic and genetic counseling. This holds especially true for the rare cases with multiple small supernumerary marker chromosomes. Most such cases are reported to be clinically severely affected due to the chromosomal imbalances induced by the presence of small supernumerary marker chromosomes. Here we report the first case of a patient having four different small supernumerary marker chromosomes which, apart from slight developmental retardation in youth and non-malignant hyperpigmentation, presented no other clinical signs.

Case presentation: Our patient was a 30-year-old Caucasian man, delivered by caesarean section because of macrosomy. At birth he presented with bilateral cryptorchidism but no other birth defects. At age of around two years he showed psychomotor delay and a bilateral convergent strabismus. Later he had slight learning difficulties, with normal social behavior and now lives an independent life as an adult. Apart from hypogenitalism, he has multiple hyperpigmented nevi all over his body, short feet with pes cavus and claw toes. At age of 30 years, cytogenetic and molecular cytogenetic analysis revealed a karyotype of 50,XY,+min(6)(:p11.1-> q11.1:),+min(8)(:p11.1->q11.1:),+min(11)(:p11.11->q11:),+min(12)(:p11.2~12->q10:), leading overall to a small partial trisomy in 12p11.1~12.1.

Conclusions: Including this case, four single case reports are available in the literature with a karyotype 50,XN, +4mar. For prenatally detected multiple small supernumerary marker chromosomes in particular we learn from this case that such a cytogenetic condition may be correlated with a positive clinical outcome.

Introduction

Multiple small supernumerary marker chromosomes (sSMC) with diverse sSMC derived from different chromosomal origin are rarely reported. According to Liehr [1], up to now 46 such cases were reported: 33 cases with two different sSMC, four cases each with three or four different sSMC, two each with six and seven sSMC, and one case with five sSMC. Overall, only seven of the 46 cases (= 15%) were reported as without clinical signs (according to Liehr [1] cases 2-14, 2-17, 2-23, 2-26, 2-29, 3-3 and 7-1).

Patients with multiple sSMC constitute a sub-group of patients with sSMC [2,3]. Little is known about the formation of sSMC in general [1-3] or about multiple sSMC specifically [4]. As reported previously, chromosomes 6, 3, 5, X, 1, 7, and 12 are over-represented in multiple sSMC compared to their contribution to single sSMC [4].

Here we report the first case with four sSMC derived from chromosomes 6, 8, 11 and 12, with almost no clinical signs.

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Fernández-Toral *et al. Journal of Medical Case Reports* 2010, **4**:239
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Case presentation

Our patient was a 30-year-old Spanish Caucasian man; the third child from healthy and non-consanguineous parents. The first child was a healthy boy and the second child was also a boy who died after two days due to hyaline membrane disease and prematurity. Our patient was delivered by caesarean section after 39 gestational weeks because of macrosomy, with a weight of 4250 g and an Apgar score of three, thus, intensive reanimation was required. Within five hours of life he suffered apnea. He was also hypoglycemic and hypocalcemic, but responded well to treatment without suffering a recurrence. Clinical examination showed bilateral cryptorchidism. During her pregnancy our patient's mother was treated with diazepam towards the end of the pregnancy.

When our patient was 19 months old, his weight and length were two standard deviations below normal. During further development, he showed psychomotor delay and a bilateral convergent strabismus; also he started walking when he was 22 months old. At the age of 10 years, his testes were surgically descended. And at the age of 13 years the strabismus was corrected. At school he had slight learning difficulties, with normal social behavior. He later left studying to become a painter.

When he was 22 years old, he had no facial dysmorphism, he weighed 89 kg, his height was 165 cm and he had a corporal index mass of 32.7. He had hypogenitalism, with a short thick penis (6 cm), and testes of 8 and 10 cc. He has multiple hyperpigmented nevi all over his body, showing no sign of malignancy after biopsy (Figure 1A,C). He also had a left vesicoureteral reflux grade III, with normal renal function. His cardiac, audition and fundus of the eye examinations were normal, as was his blood biochemistry. His feet are short with a pes cavus and claw toes (Figure 1B,C). At this time, he was referred to a Genetic Laboratory and one sSMC was found in his karyotype, which was considered to be *de novo* because his parents had normal karyotypes. Now, at the age of 30 years a new blood sample for cytogenetic analysis was requested. Surprisingly, the high resolution G-band karyotype attained from this sample showed the presence of a relatively big SMC, together with the presence of three additional tiny SMCs in most cells. This cytogenetic analysis revealed a karyotype of 50,XY,+mar1,+mar2,+mar3,+mar4.

To further characterize the sSMC centromere-specific multicolor fluorescence *in situ* hybridization (cenM-FISH [5]) was carried out. From this the chromosomal origin of the sSMC was determined as 6, 8, 11 and 12 (Figure 2A). By sub-centromere specific M-FISH (subcenM-FISH [6,7]) (Figure 2B-E) it was shown that the sSMC derived from chromosomes 6, 8 and 11 do not

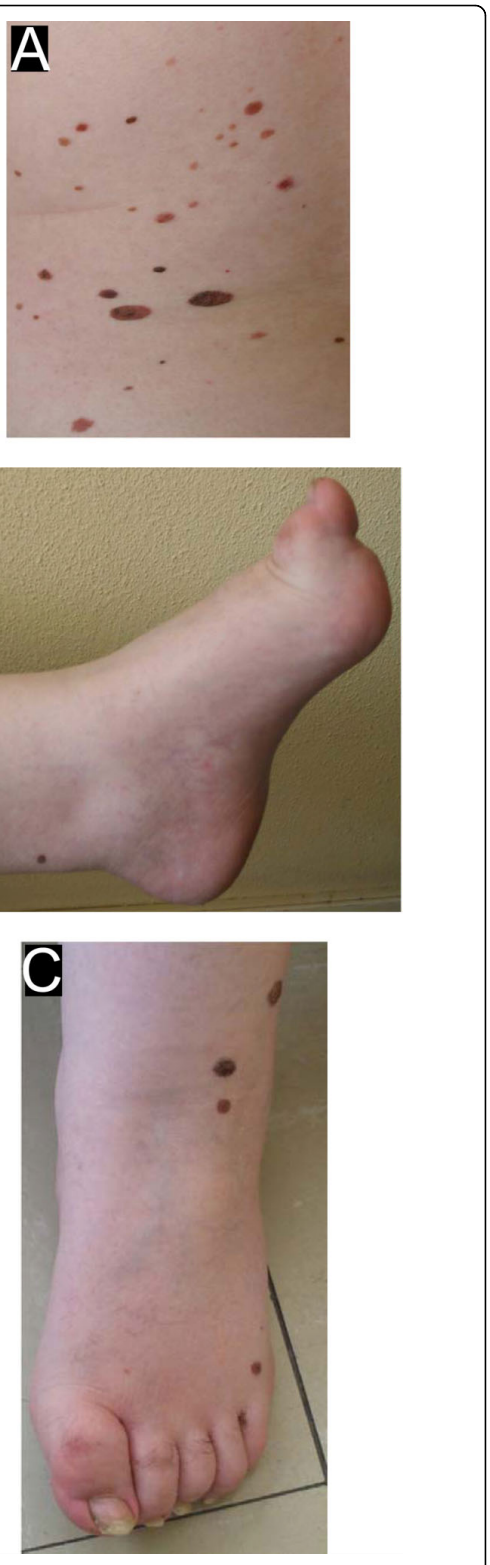

Figure 1 View of the patient at age of 30 years. (A) Multiple hyperpigmented nevi at the trunk. (B,C) Multiple hyperpigmented nevi at the foot which was too short, showed a pes cavus and claw toes.

Fernández-Toral *et al. Journal of Medical Case Reports* 2010, **4**:239
http://www.jmedicalcasereports.com/content/4/1/239

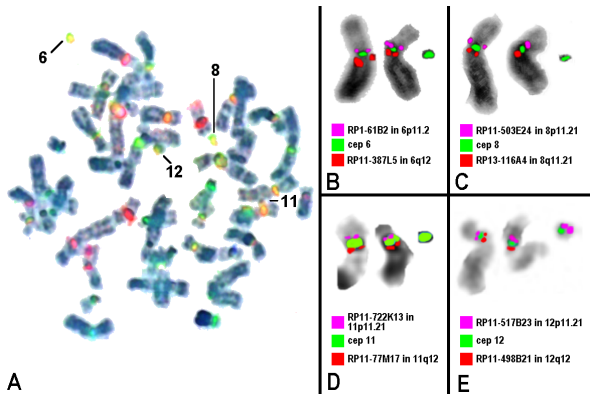

Figure 2 FISH results obtained on the chromosomes of the reported patient. (A) cenM-FISH revealed that the four sSMC were derivatives of chromosomes 6, 8, 11, and 12. (B-E) subcenM-FISH revealed absence of euchromatic material in sSMC derived from chromosomes 6, 8 and 11 and presence of centromere near material on the sSMC(12).

contain any detectable euchromatic material. Only for the derivative of chromosome 12 centromere-near material in 12p12.1 could be detected. The final karyotype was 50,XY,+min(6)(:p11.1->q11.1:),+min(8)(:p11.1->q11.1:),+min(11)(:p11.11->q11:),+min(12)(:p11.2~12->q10:).

Discussion

Here we report the fourth unusual case with four different sSMC and the 34th case with multiple sSMC. It is the eighth case with no or only minor clinical signs due to the sSMC presence. The only detectable sSMC-related chromosomal imbalance is a small partial trisomy 12p11.2~12.1. According to Liehr [8] there are several cases with a partial trisomy 12p12 due to an sSMC which were all clinically normal. Thus, this region seems to be a potentially transmittable unbalanced chromosomal abnormality (UBCA) without causing clinical problems (see case 12-O-p11.1/1-1 [8]). Similar UBCA were recently reported for a multitude of chromosomal regions [9] and especially for the centromere near regions [3]. Thus, it is not clear if the sSMC have a positive correlation with the observed clinical symptoms.

Moreover, it is interesting that the multiple sSMC derive in the present case from chromosomes 6, 8, 11 and 12. Chromosomes 6 and 12 are over-represented in multiple sSMC cases reported to date compared to their contribution to single sSMC [4]. This might point towards a specific way of formation of multiple sSMC during meiosis [10].

Conclusions

The present case confirms that multiple sSMC may be correlated with an almost normal clinical outcome. This is especially important for the correct genetic counseling of similar pre-natal cases. Furthermore, a small partial trisomy

12p11.2~12.1 seems to correlate largely to no clinical effects. Finally, involvement of chromosome 6 in sSMC formation seems to be correlated with the tendency of multiple sSMC formation.

Consent

Written informed consent was obtained from the patient for publication of this case report and any accompanying images. A copy of the written consent is available for review by the Editor-in-Chief of this journal.

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Authors' contributions
LR performed the cytogenetic studies in the present case. JFT and AP collected the data relative to this case report and provided genetic counseling to the parents. MLMF supervised the cytogenetic analysis as Director of the ECEMC. EE, ABH, MZ and TL did the molecular cytogenetic analysis and interpretation. TL drafted the paper and all authors contributed to the finalizing of the manuscript.

Competing interests
The authors declare that they have no competing interests.

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Tetrasomy 9p Mosaicism Associated with a Normal Phenotype in Two Cases

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Key Words
Mosaicism · Normal phenotype · Tetrasomy 9p

Abstract
Tetrasomy 9p is a rare chromosomal syndrome and about 30% of known cases exhibit mosaicism. Approximately 50 of the reported cases with tetrasomy 9p mosaicism show a characteristic facial appearance, growth failure, and developmental delay. However, 3 patients with mosaicism for isochromosome 9p and a normal phenotype have also been reported. We report 2 additional cases of clinically normal young females with tetrasomy 9p mosaicism, one of whom also exhibited X chromosome aneuploidy mosaicism leading to an overall of 6 different cell lines. STR analysis performed on this complex mosaic case indicated that the extra isochromosome was of maternal origin while the X chromosome aneuploidy was of paternal origin, indicating a postzygotic event.

Small supernumerary marker chromosomes (sSMCs) are reported in 0.044% of newborn infants and in 0.125% of subfertile individuals [Liehr and Weise, 2007]. sSMCs are structurally abnormal chromosomes that cannot be identified or characterized unambiguously by conventional cytogenetics alone, and they are generally equal in size or smaller than chromosome 20 of the same metaphase spread [Liehr et al., 2004]. To date, only one report is available for a triple-X syndrome patient with an additional sSMC [Lee-Jones et al., 2004], and only 3 cases have been reported so far with mosaic tetrasomy 9p that present no clinical symptoms [Sait and Wetzler, 2003; McAuliffe et al., 2005; Baronchelli et al., 2011].

In the present study, we report 2 patients with tetrasomy 9p mosaicism and an apparently normal phenotype. The first individual was cytogenetically studied because of a de novo inversion in a chromosome 7, observed in a previous pregnancy. The second proband was referred for cytogenetic studies as part of in vitro fertilization (IVF) pre-testing due to her husband's azoospermia. The results of the molecular, clinical, and cytogenetic findings are presented and compared to reports previously published.

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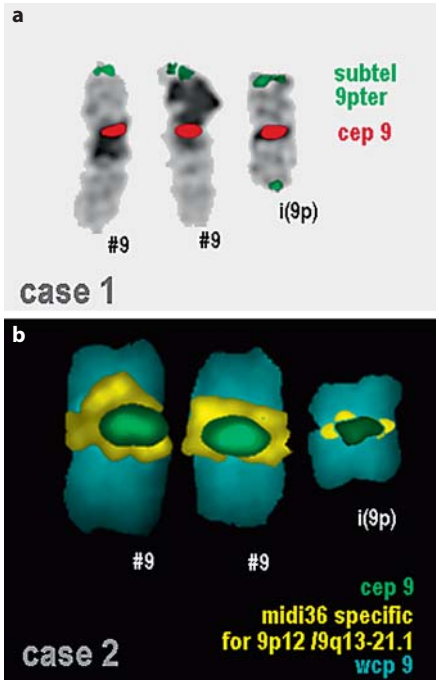

Fig. 1. a Partial karyogram of case 1 showing both normal chromosomes 9 and the isochromosome 9p in inverted DAPI-banding and after FISH. An alpha-satellite-specific probe for chromosome 9 (cep 9) and a subtelomeric probe for 9pter (subtel 9pter) were applied. **b** Both normal chromosomes 9 and the isochromosome 9p found in case 2 after FISH using an alpha-satellite-specific probe for chromosome 9 (cep 9) together with a microdissection-derived probe (midi36) for the pericentric region of chromosome 9 (9p12/9q13-21.1) and a whole chromosome painting (wcp) probe.

Case Reports

Case 1

A 20-year-old female was studied cytogenetically due to a previous pregnancy with a de novo pericentric inversion in a chromosome 7. The patient presented no dysmorphic features and/or mental abnormalities, and there was no family history of miscarriages and/or genetic abnormalities. Peripheral blood and buccal mucosa were available for cytogenetic studies.

Case 2

A 28-year-old female, the second child of healthy non-consanguineous parents, was studied cytogenetically before starting IVF treatment due to her husband's azoospermia. The family history was unremarkable. The patient had a height of 169 cm, head circumference of 55 cm, weight 63 kg, had normally developed genitalia, normal menstrual cycle, and an average mental condition. Endocrinological studies revealed no abnormal values (PRL 23.3 ng/ml, FSH 9.7 mIU/ml, LH 3.3 mIU/ml, E_2 74.7 pg/ml, PRG 0.41 ng/ml, 17-OH PRG 0.53 ng/ml, and DHEA 946.4 ng/ml).

Methods and Results

Metaphase chromosome preparations were obtained from PHA-stimulated lymphocyte cultures according to standard procedures [Verma and Babu, 1998].

In case 1, the cytogenetic analysis of stimulated blood cells revealed a non-mosaic karyotype of 47,XX,+mar. Multiplex-fluorescence in situ hybridization (M-FISH) [Speicher et al., 1996] showed that the sSMC was a derivative of chromosome 9. Application of a centromeric probe for chromosome 9 (cep 9) in combination with a subtelomeric probe for the short arm of chromosome 9 (9pter) identified the sSMC as an i(9)(p10) (fig. 1a). However, in buccal mucosa, interphase-FISH, using a centromeric probe for chromosome 9, confirmed the presence of the sSMC only in 65% of the examined cells. This finding in association with the normal clinical phenotype of the patient indicates that it is possible that most of the tissues of the patient present a mosaicism for isochromosome 9p rather than a full tetrasomy 9p. According to ISCN [2009], the karyotype was mos 47,XX,+i(9)(p10)/46,XX. Follow-up cytogenetic studies of the patient's parents were not possible.

In case 2, a routine cytogenetic analysis on peripheral blood revealed a mosaic karyotype mos 48,XXX,+mar[14]/47,XX,+mar[14]/49,XXXX,+mar[4]/47,XXX[2]/46,X,+mar[2]/46,XX[4]. Parental chromosome analysis revealed normal karyotypes. Application of an alpha-satellite-specific probe for chromosome 9 (cep 9) together with a microdissection-derived probe (midi36) for the pericentric region of chromosome 9 (9p12/9q13–21.1), and a whole chromosome painting probe identified the sSMC as an i(9)(p10) (fig. 1b). Accordingly, the karyotype was designated as mos 48,XXX,+i(9)(p10)[14]/47,XX,+i(9)(p10)[14]/49,XXXX,+i(9)(p10)[4]/47,XXX[2]/46,X,+i(9)(p10)[2]/46,XX[4]dn.

DNA was extracted from blood samples using the NucleoSpin blood extraction kit (Macherey-Nagel, Düren, Germany). Uniparental disomy (UPD) of the normal chromosomes 9 was excluded by means of parent-to-patient segregation analysis using a panel of 8 short tandem repeat (STR) markers located along the length of chromosome 9 (D9S103, D9S117, D9S199, D9S194, D9S195, D9S109, D9S193, D9S200). A set of 4 STR markers was also used for the determination of the origin of the X chromosome aneuploidy (DXS990, DXS987, DXS8091, DXS1047). Quantitative fluorescence (QF) PCR was performed to amplify the repeat sequences at the above polymorphic loci, and the primer sequences were probed with fluorescent labels as described elsewhere [Mann et al.,

Table 1. Cytogenetic findings and clinical data in 4 mosaic cases with a supernumerary i(9p)

	McAuliffe et al., 2005	Sait and Wetzler, 2003	Baronchelli et al., 2011	Case 1	Case 2
Age, years	37	41	adult	20	28
Sex	male	male	female	female	female
Phenotype	normal	normal/skin lesions/hypereosinophilia	normal	normal	normal
Reason for investigation	oligospermia	hypereosinophilia in bone marrow and peripheral blood film/skin lesions	premature ovarian failure	previous pregnancy inv(7)	IVF
Karyotype GTG-banding	47,XY,+i(9)(p10)[4]/46,XY[16]	47,XY,+i(9)(p10)[?100%]	47,XX,+i(9)(p10)[72]/46,XX[28]	47,XX,+i(9)(p10)[100%]; in buccal mucosa marker only in 65% of cells	mos 48,XXX,+i(9)(p10)[14]/47,XX,+i(9)(p10)[14]/49,XXXX,+i(9)(p10)[4]/47,XXX[2]/46,X,+i(9)(p10)[2]/46,XX[4]dn
Origin	n.a.	n.a.	n.a.	n.a.	de novo
FISH method	cep 9; subtel 9p	n.a.	n.a.	M-FISH; cep 9; subtel 9p	cep 9; subtel9p
Identified sSMC	i(9)(p10)	i(9)(p10)	i(9)(p10)	i(9)(p10)	i(9p) maternal
Studied material	PBL, skin	PBL	PBL	PBL, buccal mucosa	PBL

PBL = Peripheral blood lymphocytes.

2001]. The fluorescent QF-PCR products were analyzed by capillary electrophoresis on an automated DNA sequencer (ABI 3100, Applied Biosystems, Carlsbad, Calif., USA). STR analysis indicated that the extra isochromosome was of maternal origin, while the X chromosome aneuploidy observed in case 2 was of paternal origin (all extra copies of chromosome X).

STR analysis was not performed in case 1 as no parental DNA material was available.

Discussion

Tetrasomy 9p is a rare syndrome, and about 30% of known cases exhibit chromosome mosaicism [Stumm et al., 1999]. Reports in the literature of cases with tetrasomy 9p, about 50 including mosaic and non-mosaic cases [Liehr, 2011], showed characteristic facial appearance with hypertelorism (72%), broad nasal root or bulbus/beaked nose (69%), cleft lip or palate (78%), ear anomalies (88%), micrognathia (59%), developmental delay (94%), central nervous system anomaly (89%), limb defects (88%), postnatal growth failure (71%), congenital heart disease (62%), small gestational age (57%), renal anomalies (57%), wide sutures/large fontanelle (56%), and short neck/excess nuchal skin (53%) [Dhandha et al., 2002].

There are 3 patients depicted in the literature with mosaicism for isochromosome 9p and a normal phenotype [Sait and Wetzler, 2003; McAuliffe et al., 2005; Baronchelli et al., 2011] (table 1). Sait and Wetzler [2003] described a healthy 41-year-old male with mosaicism of isochromosome 9p who was referred for cytogenetic analysis because of skin lesions; the only abnormality found was hypereosinophilia in the bone marrow and peripheral blood film. McAuliffe et al. [2005] reported a 37-year-old male patient with isochromosome 9p mosaicism with oligospermia who had fathered 2 normal children, and Baronchelli et al. [2011] found an i(9p) in 72% of peripheral blood cells studied cytogenetically in an adult female with premature ovarian failure.

In the 2 additional cases reported here, the chromosomal imbalance of chromosome 9 was not associated with any prominent phenotypic abnormality in the apparently healthy 20- and 28-year-old females. It has been proposed that the degree of phenotypic involvement can be associated with the degree of mosaicism, the size of the isochromosome involved, and the extent of tissue involvement [Grass et al., 1993].

Although a correlation between the level of mosaicism and phenotypic abnormalities has been described, there was no such evidence in our 2 cases. Interestingly, similar findings have been reported for other sSMC cases usually known to have an adverse prognosis but instead presented a mild phenotype, such as additional isochromosome 18p [Kim et al., 2009], inv dup(15)(q13) [Bonati et al., 2005; Loitzsch and Bartsch, 2006], inv dup(22)(q11.21)

leading generally to cat eye syndrome [Lin et al., 2006], or even isochromosome 12p leading to Pallister-Killian syndrome [Genevieve et al., 2003]. In our case 1, no mosaicism was evident after studying blood lymphocytes; it became obvious only after interphase-FISH of the buccal mucosa. Still, only a few tissues were studied in both of our cases.

The supernumerary isochromosome 9p in case 2 was a de novo finding as in all of the so far described cases [Dutly et al., 1998; Eggerman et al., 1998; Wyandt et al., 2000] and of maternal origin. It seems that errors in maternal meiosis may be responsible for the origin of the isochromosome and that non-disjunction during meiosis II could be followed by rearrangements leading to duplication of the short arm and loss of the long arm in the majority of cases [Dutly et al., 1998].

For sSMCs in general, the predominant mechanism of origin has been shown to be ring chromosome formation by centromere misdivision, the so-called McClintock mechanism [Baldwin et al., 2008].

Trisomy X occurs from a non-disjunction event in which the X chromosomes fail to properly separate during cell division, either during gametogenesis or after conception [May et al., 1990]. Studies made to determine the parental origin of the additional X chromosome demonstrated that in 58–63% of cases the extra X chromosome derived from maternal meiosis I errors, in 16–17% from maternal meiosis II errors, and in 18–20% from post-zygotic non-disjunction [Hall et al., 2006; Hassold et al., 2007]. One study [Wallerstein et al., 2004] with mosaic trisomy X (such as 45,X/47,XXX) suggested that cases of mosaicism may result from a post-zygotic non-disjunction event as could be the cause in our case 2. This case presented with a normal stature, while women with a mosaic karyotype of 45,X/47,XXX generally develop a short stature [Syber and McCauley, 2004]. The severity of the short stature has been correlated with the distribution of cell lines in 47,XXX/45,X/46,XX mosaicism [Partsch et al., 1994].

Mosaicism for tetrasomy 9p is a challenging issue in terms of prenatal diagnosis and genetic counseling as the abnormality may not be detectable in the amniotic fluid and fetal ultrasound assessment can be normal throughout pregnancy. In one reported case, amniocentesis due to advanced maternal age showed a normal fetal karyotype. However, further cytogenetic analysis due to postnatal developmental delay revealed mosaic tetrasomy 9p in blood and skin cells [Eggermann et al., 1998].

Our 2 cases of healthy females can be regarded as representing the one end of the spectrum of karyotype-phenotype correlation in chromosomal aneuploidies [Avramopoulos et al., 1997]. In most such cases, however, tissue-specific mosaicism has not been fully investigated.

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Notes Added in Proof

A more intense literature search revealed four (4) additional i(9p) cases with normal phenotype. For more details visit: http://www.fish.uniklinikum-jena.de/sSMC/sSMC+by+chromosome/sSMC+9.html#i9p).

3. Discussion

As mentioned in Introduction part: Detection of an sSMC is nearly always unexpected by the clinician and more or less an accidental result in cytogenetics; the origin of an sSMC is almost impossible to establish by routine cytogenetics alone, whereas FISH methods are highly suited for this (Starke et al. 2003a). The majority of sSMC comprise exclusively of material derived from one chromosome. Of those, only a very small subset does not consist of consecutive chromosomal material, but has complex intrachromosomal rearrangements (Liehr et al. 2004a). Approximately 30% of the marker chromosome cases are familial and they impose a low risk of abnormality (Crolla et al. 1998, Hastings et al. 1999), whereas ~30% *de novo* cases present statistically an increased risk of inborn defects due to sSMC (Warburton 1984, Liehr 2014a). In prenatal clinical cytogenetics, sSMC are still a problem: the question is, has it been correlated with clinical syndromes and is it harmful? Thus, in this study I worked on

1) better sSMC characterization approaches,
2) characterization of chromosomal breakpoints involved in sSMC formation,
3) on mosaicism in sSMC, and overall,
4) on a refinement of the genotype-phenotype correlation in sSMC.

3.1. Development of probe sets for detection of euchromatic presence in sSMC

To obtain additional information regarding genotype-phenotype correlations, sSMC need to characterized as precisely as possible. Several FISH-based techniques have been developed during the last decades to achieve this end (Liehr 2014b). Specific probe sets were suggested to detect the presence of euchromatic on an sSMC after identification of its chromosomal origin (e.g. by cenM-FISH (Nietzel et al. 2001)). Strikingly euchromatin can be present on an sSMC and must not cause any harm in the carrier; it depends which exact genetic imbalance was induced. As above mentioned, a detailed sSMC characterization is especially necessary in prenatal cases (Liehr 2014a).

In (**article 1**) we reviewed the effectiveness of multicolor FISH (mFISH) methods in current clinical diagnostics. mFISH is defined as the simultaneous use of at least three different ligands or fluorochromes for the specific labeling of DNA – excluding the counterstain (Liehr et al. 2004c). Due to this definition, the first successful mFISH experiments were performed in 1989 (Nederlof et al. 1989). aCGH is an efficient and sensitive technique for detecting genome-wide copy number alterations at high resolution (Shaffer et al. 2007), and can narrow down chromosomal breakpoints to some 10 kb or less (Weise et al. 2008). Also aCGH now

provides accurate characterization of sSMCs in terms of chromosomal origin, gene content, and other concomitant imbalances elsewhere in the genome (Reddy et al. 2013). Moreover sSMCs have to be differentiated from insertions or unbalanced translocations, and individual combinations of locus-specific (BAC) probes are used to prove or contradict a gain or loss suggested after aCGH (Liehr et al. 2006a, Kumar et al. 2010). All available probe sets like whole chromosome painting mFISH probe sets (Liehr and Claussen 2002a,b, Liehr et al. 2004c), cenM-FISH (Nietzel et al. 2001), subcenM-FISH (Liehr et al. 2006c) or FISH-banding (Weise et al. 2002, Starke et al. 2003a) and use of locus specific probes enabled a better sSMC characterization than banding cytogenetics (Weise et al. 2002, Manvelyan et al. 2007). Also sSMCs have been successfully characterized by glass needle-based chromosome microdissection and reverse chromosome painting (Starke et al. 2001) or use of the sSMC-derived DNA in aCGH (**article 6**).

For the present work combinations of these approaches were very successfully applied for the characterization of a complex sSMC derived from chromosomes 14 and 8, as der(14)t(8;14)(p23.2;q22.1)dn in (**article 2**). And also they were applied for our comprehensive study (**article 3**) in 412 reported complex sSMC. Also major parts of the 5,200 sSMC cases collected in our sSMC database (Liehr 2014a) were characterized by the standard approaches mentioned in the previous paragraph.

Still aCGH and mentioned mFISH-approaches and probes-sets were not suited for the comprehensive characterization of sSMC in each case as each technique has its limitations. M-FISH include the inability to detect most intra-chromosomal abnormalities such as inversions and inter-chromosomal anomalies especially if they are 3 Mb or less (Fan 2002). And although aCGH is a more sensitive technique, which can significantly, narrow down sSMC breakpoints, available ‘chips’ often do not completely cover the pericentromeric regions and furthermore cannot detect low-level and/or cryptic mosaic sSMCs (Ballif et al. 2006, Ballif et al. 2007, Baldwin et al. 2008, Li and Andersson 2009, Sheth et al. 2011, Vetro et al. 2012, Reddy et al. 2013). Thus, part of the present work was to establish the pericentric-ladder-FISH (PCL-FISH) probe set (**article 4**), which is especially suited to narrow down chromosomal breakpoints in derivative chromosomes of known origin, including sSMC. This probe set has been used in dual-color/multicolor-FISH approaches, and it is a specific tool for the pericentromeric regions, which enables sSMC breakpoint characterization with a resolution between 1 and ~10 Mb. Pericentromeric regions of human chromosomes are transitional territories between centromeric heterochromatin and euchromatic regions. They represent complex mosaic structures, including coding sequences interspersed with non-

coding sequences (She et al. 2004). Therefore, sequencing of these regions is technically difficult, and a complementary approach is necessary to clarify their role in human disease. In particular, the PCL-FISH probe set is a bar-code FISH assay that constitutes a 10 Mb raster along pericentromeric chromosomal regions, allowing the determination of mosaic and non-mosaic sSMC breakpoints within genomic regions of 1–10 Mb in size. In addition, this approach has been particularly useful in characterizing cryptic mosaic sSMCs (Liehr et al. 2010/**article 5**), and for easily defining all involved breakpoints.

To further characterize sSMC with respect to their clinical impact we established another pericentromeric BAC-probe set (unpublished data / **article 6**). This yet unnamed probe set consists of 10 BACs euchromatic chromosome-arm with a distance of about 1 Mb between each probe. It is directed towards distinguishing between sSMC leading to clinical problems and such which are non-deleterious. This probe set is based on the assumption that centromere-near imbalances only then lead to clinical problems if the concerned region contain dosage sensitive genes (**article 7)**. An obvious example; the pericentric region of chromosome 1p, is known that the region free of dosage sensitive genes includes the stretches between 115.8 Mb down to the centromere starting at 121.1 Mb (NCBI 36.3/hg18); for the long arm of chromosome 1 such a region was not defined yet. Also it was shown that sSMC-induced trisomy including the euchromatic region starting at 115.3 Mb (NCBI36/hg18) of chromosome 1 lead to clinical problems. Similar probe sets are available now for the pericentric regions of all human chromosomes (Castronovo et al. 2013), and a combination of BACs used in the present paper of Castronovo together with our probe set may be the best way to characterize sSMC in a clinically relevant way now and in future (**article 6**).

Finally, one more probe set was established to close another gap in the available mFISH probe sets. The latter do normally not cover the heterochromatic regions of the human genome, i.e. the acrocentric short arms; the large pericentric regions of chromosomes 1, 9, and 16; as well as the band Yq12. Here in (**article 8**), we developed the so-called heterochromatin-M-FISH (HCM-FISH) probe set, which enables a one-step characterization of the large heterochromatic regions within the human genome. It was applied for the characterization of five different kinds of chromosomal rearrangements including sSMC (Table 1, **article 8**), and proved to be a helpful tool in clinical cytogenetic diagnostics. However, the HCM-FISH probe set could also be used to answer questions in other fields, such as tumor cytogenetics or evolutionary studies. Examples would be interstitial heterochromatin in tumor-associated derivative chromosomes (Doneda et al. 1989) or studies on evolutionarily conserved heterochromatin (Mrasek et al. 2003).

Summary: The present work provided 3 FISH-probe sets for more comprehensive sSMC-characterization.

3.2. sSMC and localization of chromosomal breakpoints

A yet only poorly understood point in sSMC is how and why they form. One possibility to approach this is to study their breakpoints as detailed as possible (Liehr 2014b). Here aCGH (**articles 2, 6, 7** and 50 unpublished cases) and FISH (**articles 1-11**) were used to do this.

PCL-FISH (**article 4**) was applied successfully to characterize sSMC breakpoints on single cell level in mosaic and non-mosaic sSMCs. In some cases the same breakpoints were determined in sSMC of different shapes (e.g., **article 4**, cases 4–7, 20 and 29, Tables 2); however, in some instances the breakpoints differed in sSMC of the same case (e.g. case 13). Such constitutions are called cryptic mosaicism (Liehr et al. 2010/**article 5**).

Comparable studies as ours based on PCL-FISH were done yet only for selected sSMC like e.g. such derived from chromosome 15 and showed that the majority of the sSMC(15) have asymmetrical breakpoints, with the two inverted arms of the SMC being unequal in length (Roberts et al. 2003). For PCL-FISH establishment sSMC cases previously studied by aCGH were used, like also done in comparable cases published by, Pietrzak and colleagues (2007) or Lu and colleagues (2009) using a BACs and DNA probe pooling strategy like ours.

Besides the PCL-FISH study (**article 4**) in this work chromosomal breakpoints were also narrowed down for complex sSMC (**articles 2 and 3**) and to delineate the genedosage insensitive regions surrounding the human centromeric regions (**articles 6 and 7**). For the latter it is necessary to know that unbalanced chromosomal abnormalities (UBCA) have been reported for more than 50 euchromatic regions of almost all human autosomes and leading among other regions also to gain of genetic relevant material within the autosomal centromere-near region (Barber 2005), Such centromere-near, i.e., proximal chromosomal imbalances (C-UBCA), can be induced by sSMCs (Liehr et al. 2006c, 2009b) and also by intrachromosomal duplications (Liehr et al. 2009b). In (**article 7**) we reported the known minimal sizes of all C-UBCA in humans. In general, C-UBCA could be shown that at least 96.8 Mb of the proximal chromosomal regions are tolerated as triplicates or more (Table 3).There are molecular hints on C-UBCA for every chromosome arm, being at least between 0.07 and 10.23 Mb in size. Also, copy number variant regions are thought to be causative at least for a certain number of chromosomal rearrangements (Mefford and Eichler 2009, Zhang et al. 2009). It was confirmed that more than 99% of the sSMC breakpoints are located within copy number variant regions and/or segmental duplications. Moreover, approximately 75% of

the breakpoints were concordant with so-called fragile sites of the human genome (Mrasek et al. 2010) and still there was an approximately 10% overlap of the observed breakpoints and interspersed telomeric sequences (Liehr 2012). In (**article 6**) a corresponding probe set was suggested to distinguish better harmless from deleterious sSMC.

Interestingly, there are hot spots for the chromosomal breakpoints involved in sSMC formation. Ballif and coworkers (2007) proposed pericentromeric region BAC clone set for characterization and detection of sSMC besides aCGH findings and to distinguished between the involvement of the short arm and/or the long arm of each chromosome, defined the sizes of many of the markers, and revealed complex rearrangements or multiple markers in single individuals (Ballif et al. 2007). Own unpublished data supported these findings and showed correlations of copy number variant, gene-poor regions and breakpoints involved in sSMC formation.

According to the result of (**articles 2 and 3**) in the 73 different complex sSMC only 67 breakpoints were involved. 44/67 breakpoints were unique; the remainder observed two to 14 times (**article 3**, Table 2). At present it seems, complex sSMC fall into two major groups: such with unique and such with (more) common breakpoints. The latter group comprises at present 23 different breakpoints involved 2 to 14 times in one of the 73 complex sSMC. As reason for this preference several mechanisms are discussed, including palindrome mediated recurrent translocations (Sheridan et al. 2010), homologous recombination between olfactory receptor gene clusters (Maas et al. 2007) or an involvement of fragile sites in the formation of constitutional breakpoints (Liehr et al. 2011).

Summary: Chromosomal breakpoints involved in sSMC formation appear at preferential sites. Further detailed studies are necessary to reveal their features in more detail.

3.3. Mosaicism in association with sSMC

Mosaicism is an important factor for clinical manifestation of symptoms (Sarri et al. 2006), and it is a problem often associated with sSMC, not only the new discovered “cryptic mosaicism” of sSMC, but also cell mosaicism with a normal cell line (Liehr et al. 2006c). Mosaicism is present in 52.3% - 61.9% of phenotypically normal sSMC carriers, and in 56.3% - 56.6% in phenotypically abnormal sSMC carriers (Liehr 2014a). According to (**article 5**) previous studies on lower case numbers are included in this data (Crolla et al. 1998, Starke et al. 2003a). Thus, now we know that 52 % of sSMC cases are mosaic and there is a strong difference between acrocentric and non-acrocentric derived sSMC. In general,

acrocentric- and non-acrocentric-derived sSMC are differently susceptible to mosaicism; non-acrocentric-derived ones are the less stable ones: 28% of acrocentric derived sSMC and 82% of non-acrocentric-derived sSMC are mosaic. Cryptic mosaicism appears as some sSMC tend to rearrange and/or be reduced in size during karyotypic evolution. This can lead to double-ring formation or inverted duplications starting from a centric minute-shaped chromosome and in the end to formation of different variants and a highly complex mosaic, as some of the new variants can also be degraded in a subset of the cells studied (Liehr 2009). More confusing examples of familial sSMC can be found in the literature. Similar grades of mosaicism in two generations but variations in the clinical outcome have been reported (Tan-Sindhunata et al. 2000), as well as great variations in mosaicism with no phenotypic consequences (Anderlid et al. 2001). Manolakos and colleagues (2010) characterized sSMC in their study with regard to mosaicism, in incidence rate was 39% and the majority of the mosaic cases (7/9) had a normal outcome (Manolakos et al. 2010).The question of whether an sSMC is familial or derived de novo is easy to answer for most clinical cases. The problem of mosaicism and its consequences for the phenotype are still not solved. Applying sophisticated molecular cytogenetic methods often leads to detection of more complex mosaics than initially detected by banding cytogenetics alone (Starke et al. 2003a, Bartels et al. 2003). In the overwhelming majority of the cases there is no influence of the grade of somatic mosaicism detectable in peripheral blood or amnion cells and the observed clinical effects (**articles 9 and 11**). And depending on the SMC is present in 10% to 86% of the peripheral blood cells, and to arrive at a final assessment concerning the real mosaic status of all those cases, examination of different tissues could be helpful (Liehr et al. 1996). This was underlined by a previous study by Fickelscher and coworkers (2007) who found 11 different levels of mosaicism of a de novo sSMC derived from chromosome 1 in the 11 postmortem studied tissues (Fickelscher et al. 2007).

Summary: Mosaicism is one of the many factors to be determined in sSMC, as mosaicism in rare cases has been shown to have an impact on the clinical outcome – especially in such cases where an adverse prognosis was to be expected due to the sere size of an sSMC, but a normal outcome was observed nonetheless (**article 11**).

3.4. sSMC and genotype-phenotype correlation

As mentioned in 3.3. mosaicism is one factor influencing genotype-phenotype correlations for sSMC. Thus, it has to be mentioned here, that twenty-four of the 38 informative proximal autosomal regions used for genotype phenotype correlations in (**article 7**) are based on mosaic sSMC cases; thus, the data summarized in (**article 7**, Table 3) is still to be considered as preliminary in those cases. Also to be mentioned is that there are sSMC cases inducing mosaics of partial trisomy combined with partial tetra- or even hexasomy of proximal euchromatin (Liehr 2014a); genotype-phenotype correlations are not available for such cases, yet. The best suited patients to study proximal duplications would be those with intrachromosomal rearrangements, as direct or inverted duplications or unbalanced insertions, because these cases would be non mosaic (Liehr et al. 2009b). However, patients with sSMC are the largest and best characterized group where to find proximal duplications (Liehr et al. 2006c, Liehr and Weise 2007, Manvelyan et al. 2008, Rodríguez et al. 2008, Sheth et al. 2009, Liehr et al. 2009b, Liehr 2014a). Another issue to be reflected is the copy number of a C-UBCA tolerated by the human genome. At least, for 15 C-UBCA low mosaics (maximum 20.0%) of cells having four (or in one case of 20q up to six) copies of the corresponding regions are tolerated. The C-UBCA of chromosomes 13q, 14q and 15q can be present in four copies in normal carriers in 100.0% of the studied cells. In fact chromosome 15 is one of the seven human chromosomes with a high rate of segmental duplication (regions >1 kb that are not high copy repeats and have >90% identity to another genomic region). These duplications are clustered in two regions located on proximal and distal 15q (Bailey et al. 2002, Zody et al. 2006).

It was reported that only in 1/3 of the cases the sSMC is associated with clinical abnormalities (Liehr 2012). Besides some specific syndromes, i.e. Pallister-Killian {= i(12p), OMIM #601803}, isochromosome 18p {i(18p), OMIM #614290}, cat-eye {i(22p ~ q), OMIM #115470}, idic(15) {no OMIM number} and Emanuel or derivative chromosome 22 {der(22)t(11;22), OMIM #609029} syndromes (Liehr 2012), for the remaining sSMC cases only first steps towards genotype-phenotype correlations were achieved (Liehr 2012, Liehr 2014a). Using established and new approaches (see 3.1, **articles 1-8**) progress on the genotype-phenotype correlation for sSMC could be achieved (**articles 2-4, 6-7 and 10-11**). Especially, data which was directly implemented into the sSMC-database (Liehr 2014a) has been provided to better characterize the genedosage insensitive regions (see also Lurie 1993; Roa and Lupski 1994, Barber 2005, 2008) around the human centromeres. This data was used for the establishing of the yet only exemplarily published 1MB-probe set (**article 6**).

Besides the genedosage other parameters are influencing the clinical outcome in sSMC cases, thus hampering a simple genotype-correlation. There are the discussed above mosaicism, but also other factors like uniparental disomy of the sSMC's sister chromosomes (Kotzot 2002b, Liehr et al. 2004b), which was not a specific topic of the present work. Still, our results go together well with the result of Starke and colleagues (2003a) who stated that sSMC without euchromatic content and without UPD can be considered as harmless, independent of mosaicism. Also there is an influence of parental origin and clinical outcome: > 99% of inherited sSMC are harmless and basically only such are a problem which formed in connection with the so-called McClintock mechanism (Liehr 2012).

Besides sSMC derived due to McClintock mechanism, there are a few other rare sSMC groups for which genotype-phenotype correlations are scarce. These are so-called neocentric sSMC (not part of this work; for details see Liehr et al. 2007), sSMC going together with trisomy 21 (not part of this work; for details see Starke et al. 2003b), multiple sSMC and complex sSMC.

One case with multiple sSMC was studied in this work (**article 10**). As the sSMC could be characterized in detail it could be shown that the clinical symptoms present in this case were not due to the heterochromatic sSMC derived from chromosome 6, 8 or 11, but due to the partial trisomy 12p11.1~12.1 induced by the fourth sSMC. Clinical features of that patient were similar to those with similar imbalances of proximal chromosome 12p, showing that the general dosage-dependant genotype-phenotype correlation can also be applied to multiple sSMC. Also it is striking that the multiple sSMC derive in the present case from chromosomes 6, 8, 11 and 12 as chromosomes 6 and 12 are over-represented in multiple sSMC cases reported to date compared to their contribution to single sSMC (Liehr et al. 2006a). This might point towards a specific way of formation of multiple sSMC during meiosis, perhaps involving complex rearrangements, resulting in a germ cell containing all markers, with subsequent loss of markers during cell division (Mackie-Ogilvie et al. 1997).

Complex sSMC were studied and summarized in (**articles 2 and 3**). Besides Emanuel syndrome (Trifonov et al. 2008) recently a der(22)t(8;22)(q24.1;q11.1) syndrome was reported (Sheridan et al. 2010). Besides in the present study it became obvious that there is at least one more syndrome present among the patients with complex sSMC – nine patients with a der(13 or 21)t(13 or 21;18) were reported yet. It is not known yet if it is always de novo or can also be due to a balanced t(13;18)(q11;p11.21) or t(18;21)(p11.21;q11.1) in one of the parents. However, in contrast to most other complex-sSMC associated syndromes symptoms are very variable, even though a trisomy 18p is induced (Liehr 2014a). As complex sSMC

comprise in most cases besides centromeric material also chromosomal parts from gene-rich subtelomeric regions, it is not surprising that in the majority of the cases the clinical consequences are adverse. Interestingly there are also seven cases with complex sSMC and no clinical signs. In concordance with the dosage dependant genotype-phenotype-correlation those only comprised genomic regions without dosage-dependant genes or even only heterochromatin. Overall, most complex sSMC are inherited form a balanced translocation in one parent and are also non-mosaic.

Summary: The genedosage dependent genotype-phenotype correlation was verified as a general mechanism in simple, complex and multiple sSMC. Also the size of the euchromatin is the most important one on the clinical outcome, however, UPD, parental origin and mosaicism have also to be considered.

4. Conclusions and Outlook

The present work provided 3 FISH-probe sets for more comprehensive sSMC-characterization, which, together with microdissection based aCGH and literature review provided insights in size and borders of potentially dosage-insensitive regions around the human centromeres. It could be shown that chromosomal breakpoints involved in sSMC formation appear at preferential sites, which are gene-poor and contain copy number rather than single copy DNA-stretches.

The genedosage dependent genotype-phenotype correlation was verified as a general mechanism in simple, complex and multiple sSMC. Also the size of the euchromatin is the most important one on the clinical outcome, however, UPD, parental origin and mosaicism have also to be considered as well as mosaicism, which was also studied in detail here.
Overall the questions studied in this thesis could be answered:

1. sSMC can best be characterized quickly and comprehensively using FISH-approaches;
2. FISH-probe-sets could be developed and can now be used to distinguish straight forward between benign and harmful sSMC, as
3. for most pericentric regions borders of dosage-sensitive could be defined.
4. Also it could be shown that sSMC break preferentially in gene-poor regions.

Even though during the last years and also in the present study already major progress was achieved, still lots of work is necessary for better possibilities of prenatal predictions of clinical outcomes due to sSMC presence. Future studies should also focus on sSMC formation and possibly in vitro models of sSMC.

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6. Appendix

6.1. List of own publications

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Jena, 28. 05. 2014

6.2. Curriculum Vitae

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- Education

- **1979 – 1985** Al-Rifaie Primary school, Thi-Qar, Iraq.
- **1985 – 1991** Al-Rifaie Secondary school, Thi-Qar, Iraq.
- **1991 – 1995 B.Sc.** in Biology from College of Education, Baghdad University, Iraq
- **2000 – 2003 M.Sc.** degree in Zoology / Cytogenetics from College of Science, Al-Mustansiriya University, Iraq

- Award

DAAD fellowship for Ph.D. study in Friedrich Schiller University of Jena (2009-2013)

-Work experience

- Cell Culture and Chromosomal analysis with GTG Banding.
- Molecular Cytogenetics; Fluorescent in-situ hybridization (FISH) techniques.
- Array- based Comparative Genomic Hybridization (a-CGH) technique.
- Glass Needle Base Microdissection technique.
- Degenerate Oligonucleotide-Primed (DOP-PCR) technique.
- DNA Reamplification and Labelling technique.
- Plasmid DNA Purification.

- Record of Employment

- **1998 – 1999** Asst. researcher, Department of Cancer Research, Iraqi Centre for Cancer & Medical Genetics Research (**ICCMGR**).
- **1999 – 2000** Chief of laboratories / ICCMGR.

- **2000 – 2003** M.Sc. Student in College of Science/Al-Mustansiriya University / Iraq, in Cytogenetics field and the thesis title "***A Study of The Frequency of Y Chromosome Loss in Some Cancers and Elderlies"***
- **2003 – 2004** Researcher, Department of Medical Genetics, ICCMGR.
- **2004** Vice-Chairman and Researcher, Department of Medical Genetics, ICCMGR.
- **2004 – 2007** Chairman and Researcher, Department of Medical Genetics, ICCMGR
- **2007 – 2008** Lecturer in Cytology, Genetic and Ecology laboratories / Biology Department / College of science / Thi-Qar University.
- **2008 – 2009** Researcher, Department of Medical Genetics, ICCMGR.
- **October 2009 till now** Ph.D. student in group of PD Dr. rer. nat./ med.habil. Thomas Liehr / Molecular Cytogenetics Department / Institute for Human Genetics / Jena University Hospital / Friedrich Schiller University of Jena.
- **October 2013 till now** scientist co-worker in group of PD Dr. rer. nat./ med.habil. Thomas Liehr / Molecular Cytogenetics Department / Institute for Human Genetics / Jena University Hospital / Friedrich Schiller University of Jena.

- <u>Contributions in the international conferences and workshops</u>

- **21st Annual Meeting of the German Society of Human Genetics** – Hamburg 2-4 March 2010.
- **Annual Conference of the German Genetic Society (GfG) Evolution of Primates**, 16-18 September 2010, Jena, Germany.
- **12th Day of Microscopy** Jena 9th November 2011 (Zeizz Workshop).
- **3rd B-Chromosome Conference**, Leibniz Institute of Plant Genetics and Crop Plant Research (IPK)- Gatersleben, Germany, 7 – 9 April 2014.
- National Research Center of Mental Health, RAS, Moscow, Russia, 27 – 31 August 2012 (Workshop).

- <u>Personal skills and competences</u>

- <u>Training Courses</u>

1. Academic Teaching Course from 13 to 29 October 2003 / Teaching Methods Unit / College of Education / Al-Mustansiriya University.
2. First International Course on Small Supernumerary Marker Chromosomes (sSMC) - Institute for Human Genetics / Jena University Hospital, 2009.

3. International Course on Fluorescence in Situ Hybridization (FISH, a-CGH, Microdissection) of marker chromosomes - Institute for Human Genetics / Jena University Hospital, 2009.

- Languages

- **Mother tongue:** Arabic
- **Other languages:** English
  German **(**Basic user / A2)

Language certificates:

1. English Language Test Certificate at University of Baghdad in 2000.
2. TOEFL. ITP- English Language Test Certificate 2009.
3. DUO Deutsch – Uni Online Certificate (13 March 2009 – 30 June 2009)
4. Intensive German language course – interDaf e.V. am Herder – Institute of Leipzig University (02 June2009 – 30 September 2009).

Computer certificates:

1. Proficiency Certificate in using the computer from 1 to 30 August 2000, Consulting Bureau / College of Science / Al-Mustansiriya University.
2. Proficiency Certificate in using the computer from 3 to 20 July 2005 / Consultation Bureau of Information Systems and Computers / Baghdad University.

Jena, 28. 05. 2014

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6.4. Ehrenwörtliche Erklärung

Hiermit erkläre ich, dass mir die Promotionsordnung der Medizinischen Fakultät der Friedrich-Schiller-Universität bekannt ist,

ich die Dissertation selbst angefertigt habe und alle von mir benutzten Hilfsmittel, persönlichen Mitteilungen und Quellen in meiner Arbeit angegeben sind,

mich folgende Personen bei der Auswahl und Auswertung des Materials sowie bei der Herstellung des Manuskripts unterstützt haben: PD Dr. rer. nat./ med. habil. Thomas Liehr.

die Hilfe eines Promotionsberaters nicht in Anspruch genommen wurde und dass Dritte weder unmittelbar noch mittelbar geldwerte Leistungen von mir für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen,

dass ich die Dissertation noch nicht als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung eingereicht habe und

dass ich die gleiche, eine in wesentlichen Teilen ähnliche oder eine andere Abhandlung nicht bei einer anderen Hochschule als Dissertation eingereicht habe.

Jena, 28. 05. 2014

Ahmed Basheer Hamid