

Universidade de Lisboa

Faculdade de Farmácia



***Molecular Cytogenetic Characterization of Murine Cell Line
SEWA***

Alice Da Cruz Madeira Borges

Mestrado Integrado em Ciências Farmacêuticas

2016/2017

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Monografia de Mestrado Integrado em Ciências Farmacêuticas apresentada à
Universidade de Lisboa através da Faculdade de Farmácia

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This work was developed at Friedrich-Schiller Universität Jena, Institut für Humangenetik, under the supervision of Dr. Thomas Liehr.

Resumo:

A hereditariedade e as alterações estruturais dos cromossomas são dois temas ligados à Citogenética Clínica que apresentam maior impacto aquando da tomada de decisão relativa a diagnósticos tumorais e pré/pós natais. Através de métodos de hibridação de fluorescência *in situ*, desenvolvidos ao longo dos anos, é possível obter informações relativas a alterações estruturais e variações numéricas no cariótipo celular, permitindo retirar conclusões relativamente à origem e prognóstico de cancros, e à viabilidade de fetos quando uma determinada doença congénita está presente numa família. Para que estes estudos sejam passíveis de ser realizados são necessárias linhas celulares modificadas. A linha celular SEWA é uma das mais usadas a nível de estudos carcinogénicos. Desenvolvida inicialmente a partir de um sarcoma osteogénico induzido por um políoma-vírus, o seu material carcinogénico foi posteriormente transformado em tumor ascítico e transplantado para ratinhos da linhagem A.SW. A sua importância para os estudos sobre tumores prende-se no facto de esta linha celular ser caracterizada pela presença *in vivo* de cromossomas *double minute*, de cromossomas *C-bandless* e *homogeneously staining regions*. Estas estruturas cromossómicas contêm o oncogene *c-myc*, cujo grau de amplificação está diretamente correlacionado com a gravidade do tumor. No entanto, até à data, nenhuma caracterização do seu cariótipo foi publicada. Este trabalho visa apresentar um possível cariótipo para esta linha celular, com uma descrição detalhada das suas alterações estruturais e numéricas. Para tal, técnicas como MCB, SKY e aCGH foram usadas. Qualquer uma destas técnicas é derivada de uma tecnologia desenvolvida no final dos anos 60, denominada de hibridação de fluorescência *in situ* (FISH). Neste tipo de métodos, diferentes sondas são marcadas com diferentes marcadores fluorescentes de modo a que seja possível visualizar regiões específicas do genoma. Os resultados, obtidos através de processamento de imagem, seriam posteriormente usados para encontrar homólogos no genoma humano e associar essas alterações a um tumor humano. A presença de cromotripsis, estruturas comuns em tumores agressivos, que ocorrem nas etapas iniciais de carcinogénese, seria também indício de mau prognóstico na sobrevivência ao cancro. Estudos como este permitem que modelos de origem tumoral sejam desenvolvidos, possibilitando um prognóstico mais prematuro relativamente ao desenvolvimento e cura de determinados tumores. Neste trabalho, 30 metafases foram analisadas, tanto para o método SKY, como para o MCB, sendo que no último a análise foi realizada cromossoma a cromossoma. No entanto, devido à má qualidade dos resultados obtidos e a alguma inexperiência do operador, não foi possível retirar conclusões concretas relativamente às alterações cromossómicas encontradas. Apenas se concluiu que as células SEWA apresentam um cariótipo instável, com diferentes alterações entre metafases. Além disso, resultados entre métodos não coincidiram o que levou à impossibilidade de criar um cariótipo representativo da população celular analisada, nem à descrição de *breakpoints*, que teriam significado para determinar uma futura homologia com o genoma humano. Será então necessária uma reavaliação dos resultados para que novas conclusões sejam obtidas.

Palavras chave: Citogenética, SEWA, Cariótipo, FISH, Tumor

Abstract:

Inheritance and structural changes of chromosomes are two themes related to Clinical Cytogenetics that present greater impact when making decisions regarding tumor and pre and post-natal diagnoses. Through *in situ* hybridization methods developed over the years, it is possible to obtain information on structural changes and numerical variations in the cellular karyotype, allowing investigators to draw conclusions regarding the origin and prognosis of cancers, and the viability of fetuses when certain congenital diseases are present in a family. The SEWA cell line, initially developed from osteogenic sarcoma induced by a polyoma virus, is one of the most widely used in carcinogenic studies. However, to date, no characterization of its karyotype has been published. This work aims to present a possible karyotype of this cell line, with a detailed description of its structural and numerical changes. For such, techniques like MCB, SKY and aCGH were used. The results would then be used to find homologs in the human genome and associate those changes with a human tumor. Studies like this allow models of tumor genesis to be developed, allowing an earlier prognosis regarding the development and cure of certain tumors. In this work, 30 metaphases were analyzed, both for SKY method and MCB. In the last one, the analysis was performed on each of the chromosomes. However, due to the poor quality of the results obtained and some inexperience of the operator, it was not possible to draw concrete conclusions regarding the chromosomal alterations that were found. It was only concluded that SEWA cells present an unstable karyotype, with several changes present in only one metaphase. Moreover, results between methods did not coincide, which led to the impossibility of creating a representative karyotype of the cell population and describing the breakpoints that would have meaning in homology with the human genome. It will then be necessary to re-evaluate the results so that new conclusions can be obtained.

Key-words: Cytogenetics, SEWA, Karyotype, FISH, tumor

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1. Introduction

1.1. Historical background

Clinical Cytogenetics is the field of Genetics which deals with chromosomes structure and inheritance, applying its knowledge to medical practice. It is known that changes in the chromosomal numbers and structure can be related to several clinical conditions which can be summarized as chromosomal disorders. Presently, chromosomal analysis is widely used in diagnostic procedures for clinical medicine (1) such as pre and postnatal diagnosis as well as tumour diagnosis.

Historically, the development of this field was marked by the constant innovations in technical procedures. Liehr et al. divided these technical developments in three major periods: the pre-banding era (1879-1970), characterized by the first visualization of human chromosomes (1879) (2) and the first published illustrations by Walther Flemming in 1882 (3), by the creation of the word "chromosome" in 1888 (2) by Waldeyer (3), the determination of the correct modal human chromosome number (1956) and the identification of the first inherited chromosomal abnormality – trisomy 21 – in 1959 (2). The banding era (1968-1986) started with the invention of Q-banding by Lore Zech (2). After this, many more improvements were made, including R-, T-, C- and G-banding technologies (3). Cytogenetics entered the last era, the molecular age, with the first molecular cytogenetic experiment on human chromosomes (1989) (2). This era was marked by the development of *in situ* hybridization, a procedure that allows tracking down specific DNA sequences in chromosomes (4) and that was first described 1969 (radioactive variant) (2). Since then, many variations of the method have been developed, and its sensitivity has increased enormously (4). The development of high resolution chromosome banding procedures, such as FISH, has greatly expanded both the range and precision of these routine analyses (1).

1.2. Fluorescence *in situ* hybridization (FISH)

FISH, standing for fluorescence *in situ* hybridization, is a series of techniques where probes are labelled with different coloured fluorescent tags to visualize one or more specific regions of the genome. It is named *in situ* because the DNA, either in interphase chromatin or in metaphase chromosomes, is fixed on a slide, denatured and finally visible in place – *in situ*. The hybridized labelled probes can be visualized with wavelength of light that excites the fluorescent dye (1). By using sophisticated image processing equipment and report-binding molecules carrying different fluorescent dyes, several DNA probes can be hybridized simultaneously so that the location of specific sequences can be identified in relation to each other.

There are three main types of probes used in FISH: whole chromosome painting (WCP, PCP) - used to discover chromosomal aberrations; repetitive sequence probes (centromeres=CEPs, telomeric probes) used either for chromosomal enumeration or chromosomal ends determination, and locus-specific identifier (LCI) probes for gene fusions, translocations, deletions or duplications (5). The probes can be labelled directly by incorporating a fluorescently labelled nucleotide precursor. Alternatively, a nucleotide containing a reporter molecule (e.g. biotin or digoxigenin) is incorporated into the DNA after which it can be specifically bound by a fluorescently

labelled affinity molecule that binds strongly and specifically to the reporter molecule.(6) One commonly used class of probes for FISH is a fragment of DNA derived from a unique location on a chromosome. Such probes hybridize and label the site on each homologous chromosome sequence. A FISH probe can also be a complex mix of DNA obtained from all or part of a chromosome arm or even from an entire chromosome. Depending on how the probe is designed, some part or the entire chromosome will be stained by the probe. Such probe mixtures are known as “whole chromosome painting” (1). (See Figure 1)

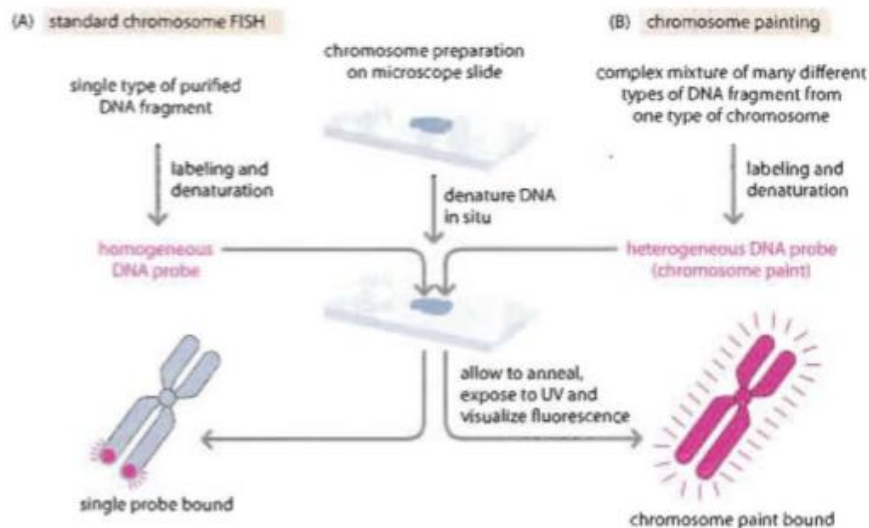


Figure 1 - Basis of the chromosome FISH and chromosome painting methods (in Read,A., Strachan, T. “ Human Molecular Genetics” 4th Ed)

An application designed for chromosome painting is widely used to investigate or define abnormal chromosome rearrangements in clinical and cancer cytogenetics. Also, it is particularly helpful with preparations from tumours, whose quality tend to often be poor (6). However, the use of this kind of probes in interphase chromosomes is very restricted, since they are dispersed inside the cell nucleus and do not form discrete units. Centromeric enumeration probes hybridize with alpha/beta or classical satellite repeat sequences within the specific centromeric regions of each chromosome, allowing enumeration. As to LSI FISH probes, there are two major kinds which are used in oncology: dual-colour translocation probes for chromosomal translocations involving known partner genes and dual-colour break-apart probes for chromosomal translocations involving genes with unknown or multiple translocation partners. These kinds of probes are also used to identify the origin of amplified DNA that belongs to homogeneously staining regions (HSRs) and double minute chromosomes (DMs) which occur frequently in tumour cells (5).

1.2.1. Spectral Karyotyping (SKY)

On the other hand, one can combine 24 different chromosome painting probes, one for each chromosome. Each probe is labelled with a different combination of fluorescent dyes that emit at different wavelengths. This technique is known as Spectral Karyotyping – SKY.

SKY is also based on the hybridization of fluorescently labelled DNA. These labels differentiate from the other ones used in conventional FISH because they are generally consisting of up to 55 individually generated chromosome-specific probes (7).

In SKY, because each chromosome specific probe emits its own signature combination of wavelengths, abnormal chromosomes consisting of pieces of different chromosomes are easily seen, and the chromosomes involved in the rearrangement can be readily identified (1).

SKY has proven to be a very useful technique in analysing and comprehending cytogenetic abnormalities associated with malignant diseases, and has been administered to a large selection of samples derived from haematological malignancies and solid tumours.

Another widely used method of chromosomal characterization is M-FISH (multiplex fluorescent *in situ* hybridization). Although the hybridization principle is similar between SKY and M-FISH, the last one uses a set of fluorochrome-specific optic filters, instead of the single custom-designed one used in SKY.

Constitutional chromosome abnormalities, originated by *de novo* balanced translocations (in which chromosomal regions are relocated in a reciprocal manner, and there is no loss of genetic material) and unbalanced translocations (associated with genomic imbalances), can be studied by using SKY or M-FISH procedures. This is extremely important in pre-natal diagnosis, since it may represent the difference of having a normal child, and one with mild to severe disease (7). Likewise, when it is not possible to identify the origin of a chromosome in a child, SKY/M-FISH has the ability to elucidate the unknown origin without the need to perform a karyotype test in the parents, which was the conventional way. This advantage is very useful when dealing with genetic counselling in families (8). However, even if this method enables an easier interpretation when analysing the results, it cannot detect balanced structural abnormalities such as inversions, or small changes like deletions, insertions and duplications in the same chromosome, since the colour scheme will be the same. This is only possible between different chromosomes (8). Also, the resolution limit of SKY is approximately 5-10Mb. This means that minor structural abnormalities with less than 5 Mb will not be detected.

As for applying SKY/M-FISH in cancer cytogenetics, this method allows the characterization of novel and hidden chromosomal translocations, identification of complex rearrangements and reconstitution of clonal evolution events during cancer progression. Also, SKY/M-FISH helps with the identification of homogenous staining regions and double minute chromosomes, regardless of size and numbers. It has, however, its limitations. Since most of the time these aberrations contain multiple gene and/or DNA regions that are tightly linked, additional FISH hybridization with gene-*loci* probes, specific chromosome or chromosome-arm paints, should be performed (7).

SKY/M-FISH can also be a useful tool for genome scanning in human carcinogenesis study. It is possible to determine and compare homologous regions of genomes between closely related species – cross-species hybridization. At present, commercially made SKY/M-FISH probes and analysis software are only available for human, mouse and rat. Murine models of human carcinogenesis are widely used to outline genetic mechanisms that control tumour initiation and progression (7).

1.2.2. Multicolour chromosome banding (MCB)

As described above, banding techniques allow a highly reproducible banding pattern, which leads to the identification of single chromosomes and structural aberrations. However, in numerous cases, the techniques mentioned above do not provide enough information about subtle rearrangements or chromosomal breakpoints.

Multicolour chromosome banding (MCB) is a method based on the use of differently labelled overlapping microdissection segments that are derived from partial chromosome painting libraries, where changes in the fluorescence intensity ratios are used to assign different pseudo-colours to specific chromosome regions.

With MCB, one can analyse a colour pattern for each chromosomal region and, by comparing it to normal chromosome pattern, find aberrations linked to specific chromosomal bands.

Also, the results obtained are independent from the chromosome condensation, since multicolour patterns are observed regardless the chromosomes' length.

Hence, MCB contributes to a more detailed information about chromosomal aberrations and their breakpoints.(9) It is available for human and mice (abbreviated mcb for distinguishing both probe sets).

1.3. Comparative genome hybridization (aCGH)

Even with all the new resources mentioned above, chromosomal karyotyping still had the disadvantage of being time consuming, laborious and demanding, with limited resolution and short-term cell culture needed (2).

In 1992, "comparative genomic hybridization" (CGH) was introduced for the first time to the scientific world by Kallioniemi *et al* (University of California, San Francisco) (10).

This method is based on the labelling of test and reference genomic DNAs with two different fluorochromes, followed by co-hybridization onto normal metaphase chromosomes. The chromosomes are then scanned and fluorescence intensity ratios measured in order to detect differences, leading to genomic imbalances findings (2).

aCGH has the advantage of not needing cell cultures, providing an overall view of gains and losses throughout the whole genome (11). These gains or losses are identified by changes from 1:1 ratio (2). (See Figure 2)

The sensitivity of aCGH depends on the level of copy number changes and is promptly altered by contamination. Also, aCGH can't detect balanced translocations, inversions and aberrations below its resolution. Facing these disadvantages, this technique should not be used as a solo technique, but as a supplementary one (11).

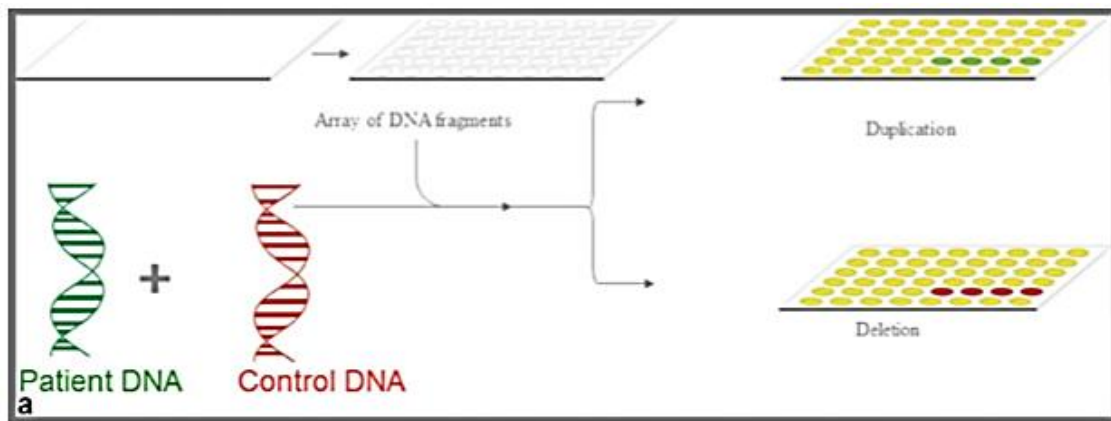


Figure 2 - aCGH scheme: test and reference DNA are labelled with different fluorochromes. Both samples are mixed and hybridized together on a slide containing arrays of spotted DNA fragments. The excess is washed away. The intensity ratios are calculated and normalized. If the DNA sample and test are equal in copy number, the intensities are the same. If there is a deletion or duplication, the intensities differ. (In “*Fluorescence in situ Hybridization*”, 2nd Ed, by Thomas Liehr, Chapter “Array CGH”, page 569)

By using this method as a guide, more specific techniques such as FISH can be used to identify oncogenes and/or tumour suppressor genes, allowing more accurate results about tumour genesis and development (11).

1.4. Murine cell line SEWA

One of the most broadly used murine cell line for cytogenetic work is probably SEWA murine cell line. The SEWA cell line is originally derived from a polyoma virus-induced osteogenic sarcoma (12). Material from the transplantable tumour was transformed into ascites tumour cell, that were carried in serial intraperitoneal transplantation in mice of the A.SW strain(13). This cell line is characterized by having a great variation of double-minute (DM) chromosomes per cell *in vivo*, but losing them over time when transplanted *in vitro*. They would, however, be restored after reinjection into animals. In 1977, some findings indicated that DM carried genes that were important for the *in vivo* growth of these types of cells, which suggested that DM had something to do with the malignancy of SEWA cells (Levan *et al.*, 1980). Indeed, years later it was shown that DM contained the *c-myc* oncogene (14) and that its level of amplification was correlated with the degree of tumorigenicity of SEWA (Martinsson *et al.*,1988) (13). On the other hand, cytogenetic analysis revealed another two typical chromosomal abnormalities related with the presence of amplified genes: C-bandless chromosomes (CM) and homogeneously staining regions (HSR)(14). It was observed that, under the same conditions, some subpopulations of SEWA lines would replace DM for CM. Like what happened to DM cells, CM cells were shown to have amplified *c-myc* (14). Likewise, there were other sublines that developed HSR instead of CM. It was also observed a certain “antagonism” between the simultaneous existence of these types of chromosomes, meaning that in most cell populations, there was either DM, or CM or HSR (13).

1.4.1. Chromothripsis and its impact on Cancer Development

Chromothripsis are defined as chromosomal structures derived from a massive *de novo* structural rearrangement, formed in one single catastrophic genomic event (see Annex 1 – Figure 1). Occasionally these fragments can lead to small circular DNA molecules, known as Double Minute Chromosomes, which, as described above, may

harbour oncogenes. Another characteristic feature is the oscillating copy-number pattern, instead of the typical 2-3 copy-number states usually found in cancer context (15).

FISH studies showed this type of structural rearrangements not only in tumour subclones but also in almost all tumour cells. This suggests that formation of chromothripsis occurs early on tumour events (16).

These formations have been reported through the years to appear in many tumour entities and have been linked to poor prognostic in cancer survival, indicating some relevance in using chromothripsis as a prognostic marker (16).

However, its detection is not a simple one. Many observational criteria are needed so that chromothripsis can be identified. Also, in tumours with extreme levels of genomic instability, these criteria may be difficult to recognise. Hence, tumour heterogeneity and ploidy may be factors that contribute to a mistakenly inference of chromothripsis (16).

2. Objectives

Since this cell line is so widely used for cancer studies, it is in our best interest to characterize its karyotype. The knowledge of the genomic content in detail enables the possibility of identifying a corresponding tumour model for human by translating the imbalances and chromosomal breakpoints found into human genome.

2.1. Line of work

To characterize the karyotype of this murine cell line, FISH procedures were performed.

The first step was to perform SKY (Spectral Karyotyping) to obtain a general view of the affected chromosomes.

After the identification of the altered chromosomes with SKY, MCB procedures were performed for each chromosome so that we could obtain more detailed genetic information.

The present work represents the firsts steps of the cytogenetic work needed to reach such conclusions. Until the present date, no other work for molecular cytogenetic characterization of SEWA was found published.

3. Experimental Methodology

3.1. Reagents:

For Mouse SKY procedure (post-hybridization washing) the following reagents were used:

- 0.4x SSC
- 4x SSC/Tw – 100 mL 20x SSC diluted in 400 mL of water (bi-dist) and 250 μ L Tween 20 (SIGMA);
- Blocking Reagent;
- Cy5-Antidigoxin Staining Reagent;
- SA-Cy5 Solution;
- Pacific Blue Staining;
- Anti-fade DAPI Solution;

For mcb Mouse procedure, the following reagents were used:

- 1x PBS – 47.75g PBS (Biochrom) diluted in 5L of water (bi-dist);
- 20x SSC (Invitrogen);
- 1x SSC – 25ml 20x SSC diluted in 475mL of water (bi-dist) pH7-7.5;
- 0.4x SSC ;
- 4x SSC/Tw – 100 mL 20x SSC diluted in 400 mL of water (bi-dist) and 250 μ L Tween 20 (SIGMA);
- 70% Formamide – 70mL Formamide + 10mL 20x SSC + 20mL of water (bi-dist);
- Ethanol 70%
- Ethanol 95%
- Ethanol 100%
- Post-Fix Solution – for 1mL solution: 500 μ L Paraformaldehyde (2% stock solution) + 450 μ L 1x PBS + 50 μ L 1M MgCl₂;
- Marvel Solution – 0.1g Milk + 2mL 4xSSC/Tw;
- Antibody solution – for each slide – SA-Cy5 5-8 μ L + 100 μ L Marvel;
- Anti-fade DAPI Solution;
- Probes

3.2. Samples:

- SEWA cell line suspension

3.3. Equipment:

For SKY/M-FISH and mcb mouse procedure:

- Incubator (humid chamber)
- Thermoshaker
- Hotplate
- Shaker

For image analysis:

- Fluorescence Microscope

4. Experimental Procedures

4.1. Mouse SKY/M-FISH

4.1.1. Execution of SKY/M-FISH procedure

The procedure, in general, is divided in six major parts: i) preparation of metaphase chromosome spreads; ii) slide pre-treatment and slide and probe denaturation; iii) hybridization; iv) detection of SKY probes; v) image acquisition; vi) image analysis (7).

For this experiment, SKY probes were used, but, instead of SKY protocol, a standard FISH protocol was performed. To perform a standard FISH procedure, one should fix the target DNA (cells, nuclei, fixed tissues sections, metaphase chromosomes or pure DNA) onto a slide surface. Next, the probes need to be prepared. After this denaturation of probes and target DNA needs to be promoted, followed by their combined renaturation. After renaturation, post-hybridization washes followed by detection wash are performed. For last, one needs to add the counterstain, antifade and coverslip to finish the procedure (2).

In this line of work, the SKY procedure and the metaphase image capturing were previously done by a team member, leaving only the analysis to be done.

A detailed protocol is available in annexes (see Annex 2 and 3)

4.1.2. Image acquisition and analysis

For SKY image analysis, normally a microscope equipped with SpectraCube and suitable for two filter sets is needed. (2)

Here, 40 metaphase images were captured in order to have 10 good metaphases for karyotype analysis by M-FISH system.

The correct way of scanning slides for microscope analysis is describes in the images below. (See Figures 3 and 4)

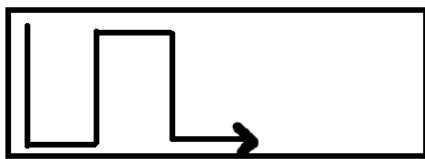


Figure 4 - example of slide scanning, down and then side

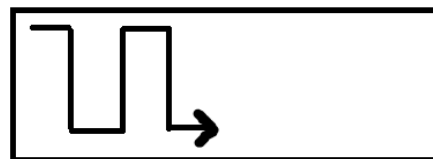


Figure 3 - example of slide scanning, side and then down

For imaging analysis, ISIS program was used.

Before analysing, image treatments were needed. This step is essential since it is when shadows and non-important signals are removed, and chromosome signals are enhanced, increasing the image quality.

When analysing it is important to know that the centromere gives no signal.

In this analysis, each chromosome has a colour pattern. This way, it is possible to compare the sample with the pattern corresponding to normal chromosome, and take conclusions about the existence (or not) of aberrations.

The colour scheme used for comparison is showed below. (See Figure 5)

Mouse SKY paint Colour Code

	FITC	Orange	Texas Red	Cy-5	Pacific Blue
1	Green	Orange		Yellow	
2				Yellow	Blue
3		Orange			Blue
4	Green	Orange	Pink		
5		Orange		Yellow	
6			Pink		Blue
7	Green		Pink		
8		Orange	Pink		
9			Pink	Yellow	
10				Yellow	
11					Blue
12	Green			Yellow	Blue
13	Green				
14		Orange		Yellow	Blue
15	Green	Orange			
16		Orange			
17	Green		Pink	Yellow	
18			Pink		
19	Green				Blue
X		Orange	Pink	Yellow	
Y	Green	Orange			Blue

Figure 5 - Mouse SKY colour scheme

4.2. Mouse mcb Procedure

4.2.1. Execution of mcb procedure

Murine multicolour chromosome banding (mcb) is an assay that uses the principle of Multicolour FISH (mFISH) method and applies it in detection of intra-chromosomal rearrangements (17).

In this work, before performing a mcb procedure for each of the 21 chromosomes of the cell line, a “dropping” technique (preparation of the slides) was executed. In each slide two chromosomes were added, one to each edge. The slides were firstly cooled for 2h in a coplin jar with distilled water in the fridge. Before performing the dropping, if needed, a FIX solution was prepared. The whole technique was executed on a 50°C hot plate. In the bench, the sample was dropped firstly on the slide, followed by the FIX solution. After this, the slide was left on top of a wet paper (placed on the hot plate) for about two minutes, until steam is formed, and then dried directly on the hot plate. The spread of the metaphases was checked on a microscope.

The mcb procedure available was divided in four major phases: pre-treatment of slides with pepsin; probe denaturation and pre-hybridization; denaturation of slides and post washing.

A detailed protocol is available in annexes (See Annex 4).

4.2.2. Imaging acquisition and analysis

As it was done for SKY/M-FISH, around 40 to 50 metaphases for each chromosome were captured, this time in order to have 30 good metaphases to analyse.

For imaging analysis, ISIS program was used once more.

The method is very similar to the one applied for SKY. A comparing pattern for each chromosome was used, and differences between control and sample were registered as inversions, deletions or translocations.

The colour schemes for each chromosome are available in annexes (see Annex 5).

5. Results

5.1. SKY/M-FISH Results

Performing SKY had the objective of searching for a global karyotype that could describe better this cell line. For that purpose, 30 metaphases were selected to be analysed. In each metaphase, the karyotype was organized and for each chromosome, general structural aberrations were searched and noted. From there, similar single karyotypes were compared and placed together as subclones and clones.

After 30 metaphases were analysed, most of the chromosomes in this cell line showcased diploidy (30/30 metaphases). Therefore, it can be named as near diploid cells (chromosome numbers 35-57).

Clone 1:

Clone 1 and its subclones were found in 12 out of 30 metaphases. They appear to have the following karyotype:

39~43, XX, der(X)t(8;x), +del(2), +idic(3;5), der(4)t(4;15), -5, +del(6) x2, der(7)t(7;17), +der(8)t(8;14), del(17), der(17)t(4;15;17), -18x2

Subclone 1a:

Subclone 1a was found in 6 out of 30 metaphases. It can be described with the following karyotype:

42~43, XX, der(X)t(8;x), +del(2), +idic(3,5), der(4)t(4;15), -5, +del(6)x2, der(6)t(6;14), der(7)t(7;17), +der(8)t(8;14), del(17), der(17)t(4,15,17), -18x2

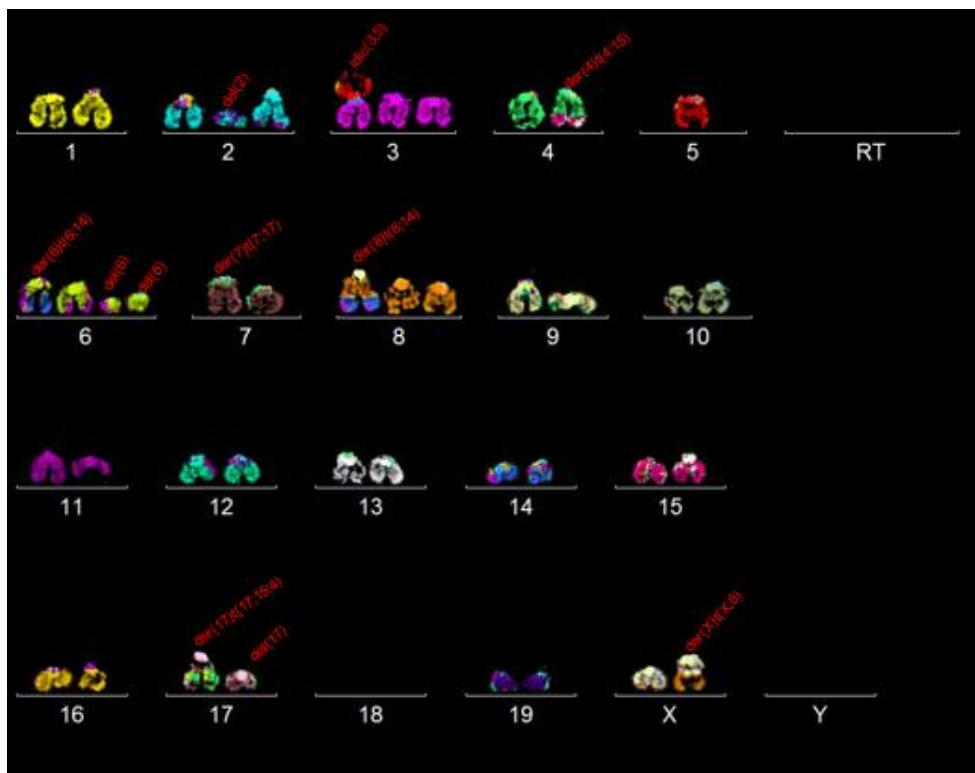


Figure 6 - SKY result for SEWA cell line, subclone 1a

For single cell aberrations see Annex 6.1.1.

Subclone 1b:

Subclone 1b was found in 2 out of 30 metaphases. It can be described with the following karyotype:

39~42, XX, der(X)t(8;X), del(2), +idic(3;5), der(4)t(4;15), -5, +del(6), +der(6)t(6;10;14), der(7)t(7;17), der(8)t(2;8), + der(8)t(8;14), del (17), der(17)t(4;15;17), -18x2

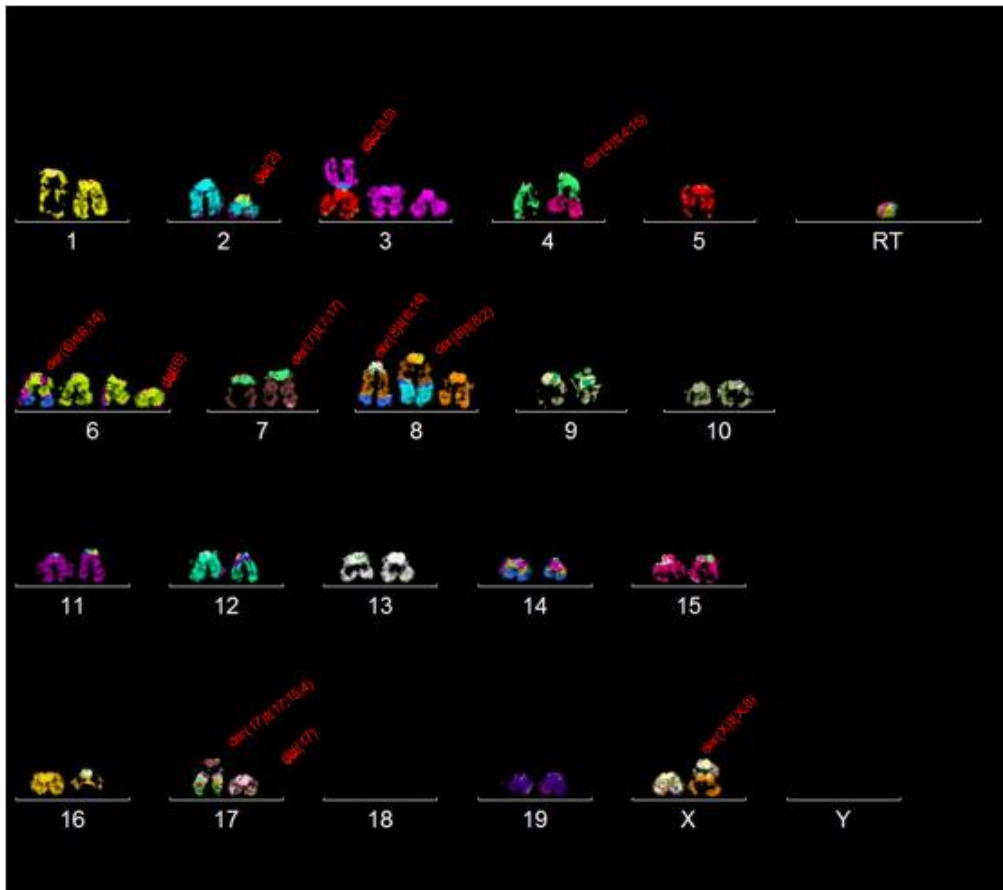


Figure 7 - SKY result for SEWA cell line, subclone 1b

For single cell copy number variations and aberrations see Annexes 6.1.2. and 6.1.3.

Subclone 1c:

Subclone 1c was found in 2 out of 30 metaphases. It can be described with the following karyotype:

40~42, XX, der(X)t(8;X), del(2), +idic(3;5), der(4)t(4;15), -5, del(6), +der(6)t(6;10;14), der(7)t(7;17), +der(8)t(8;14), del(17), der(17)t(4;15;17), -18x2

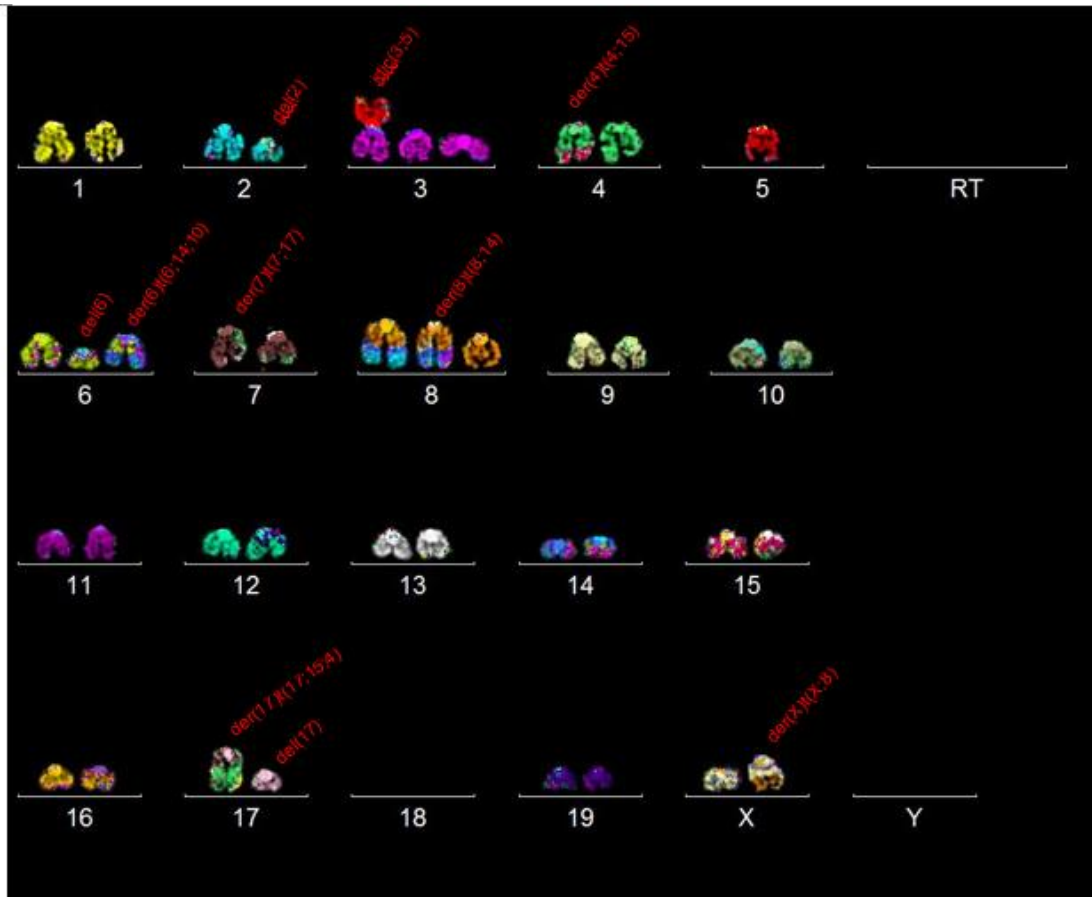


Figure 8 - SKY result for SEWA cell line, subclone 1c

For single copy number variations and aberrations see Annexes 6.1.4 and 6.1.5.

Subclone 1d:

Subclone 1d was found in 2 out of 30 metaphases. It can be described with the following karyotype:

41-43, XX, der(X)t(8;X), +del(2), +idic(3;5), der(4)t(4;15), -5, +del(6)x2, der(6)t(6;10;14), der(7)t(7;17), +der(8)t(8;14), der(9)t(9;15), del(17), -18x2

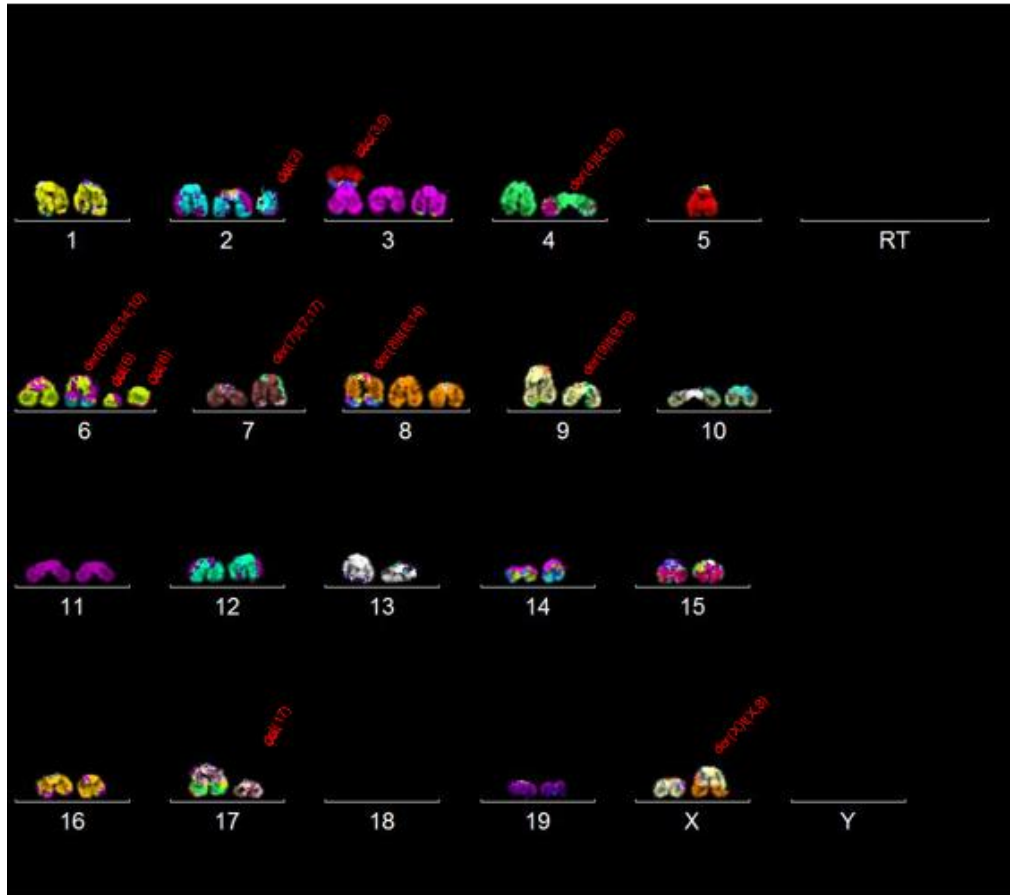


Figure 9 - SKY result for SEWA cell line, subclone 1d

For single cell aberrations see Annex 6.1.6.

Clone 2:

Clone 2 and its subclones were found in 9 out of 30 metaphases. They appear to have the following karyotype:

40~43, XX, der(X)t(8;X), del(2), +idic(3;5), der(4)t(4;15), -5, +del(6), +der(6)t(6;14), +der(8)t(8;14), del(17), -18x2

Subclone 2a:

Subclone 2a was found in 3 out of 30 metaphases. It can be described with the following karyotype:

41~43, XX, der(X)t(8;X), del(2), +idic(3;5), der(4)t(4;15), -5, del(6), +del(6), +der(6)t(6;14), +der(8)t(8;14), del(17), der(17)t(8;15;17), -18x2

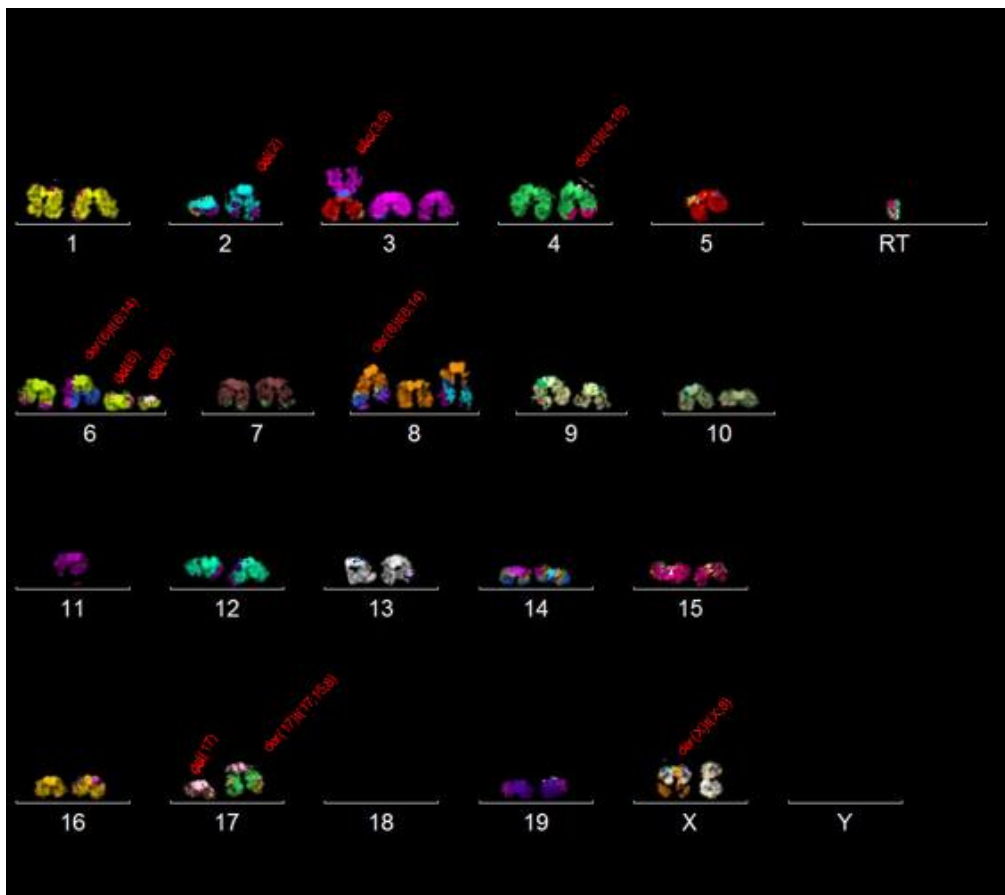


Figure 10 - SKY result for SEWA cell line, subclone 2a

For single cell aberrations see Annex 6.2.1.

Subclone 2b:

Subclone 2b was found in 2 out of 30 metaphases. It can be described with the following karyotype:

40~43, XX, der(X)t(8;X), del(2), +idic(3;5), der(4)t(4;15), -5, del(6), +der(6)t(6;14), der(7)t(7;17), +der(8)t(8;14), del(17), -18x2

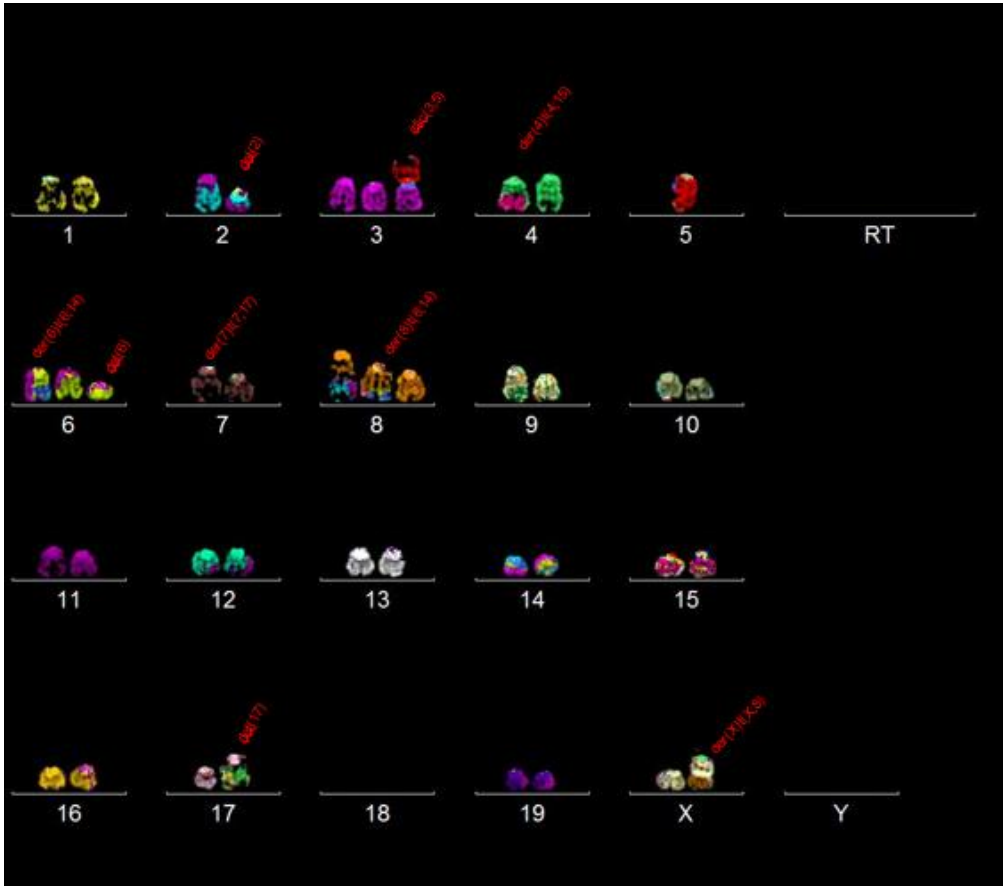


Figure 11 - SKY result for SEWA cell line, subclone 2b

For single cell aberrations see Annex 6.2.2.

Subclone 2c:

Subclone 2c was found in 2 out of 30 metaphases. It can be described with the following karyotype:

40~41, X, der(X)t(8;X), +del(2), +idic(3;5), der(4)t(4;15), -5, del(6), +der(6)t(6;14), -7, der(8)t(8;2), del(17), der(17)t(4;15;17), -18x2

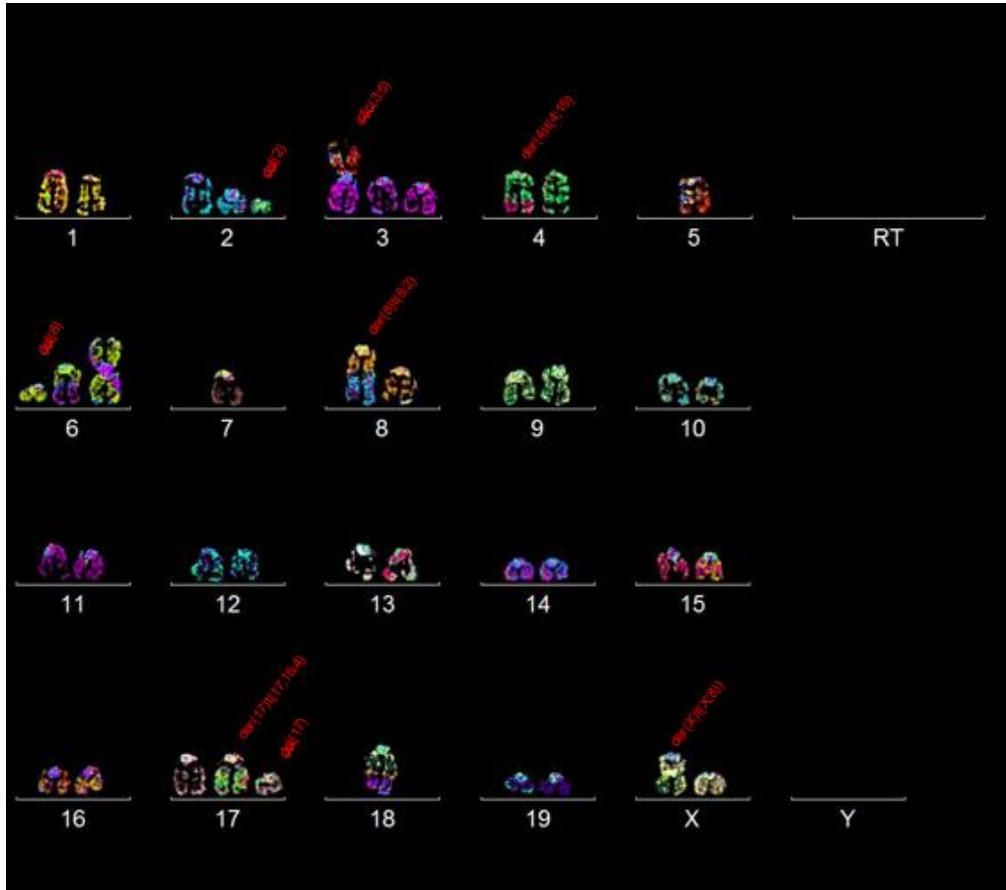


Figure 12 - SKY result for SEWA cell line, subclone 2c

For single cell copy number variations and aberrations see Annexes 6.2.3. and 6.2.4.

Subclone 2d:

Subclone 2d was found in 2 out of 30 metaphases. It can be described with the following karyotype:

40~43, X, der(X)t(8;x), del(2), +idic(3;5), der(4)t(4;15), -5, del(6), +der(6)t(6;14), der(7)t(7;17), +der(8)t(8;2), del(17), der(17)t(5;14;17), -18x2

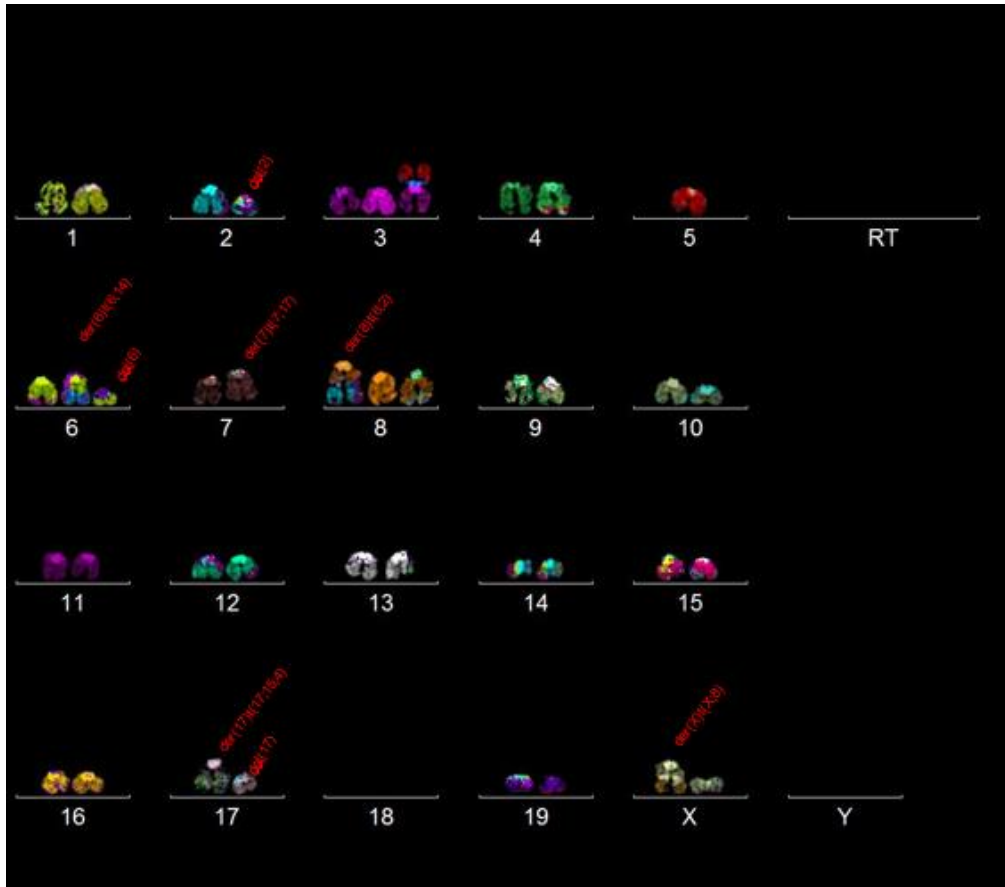


Figure 13 - SKY result for SEWA cell line, subclone 2d

No random aberrations were found.

Clone 3:

Clone 3 was found in 3 out of 30 metaphases. It appears to have the following karyotype:

40~43, X, der(X)t(8;X), del(2), +idic(3;5), der(4)t(4;15), -5, +del(6), +der(6)t(6;14), der(7)t(7;17), der(8)t(8;2), +der(8)t(8;14), del(17), der(17)t(8;15;17), -18x2

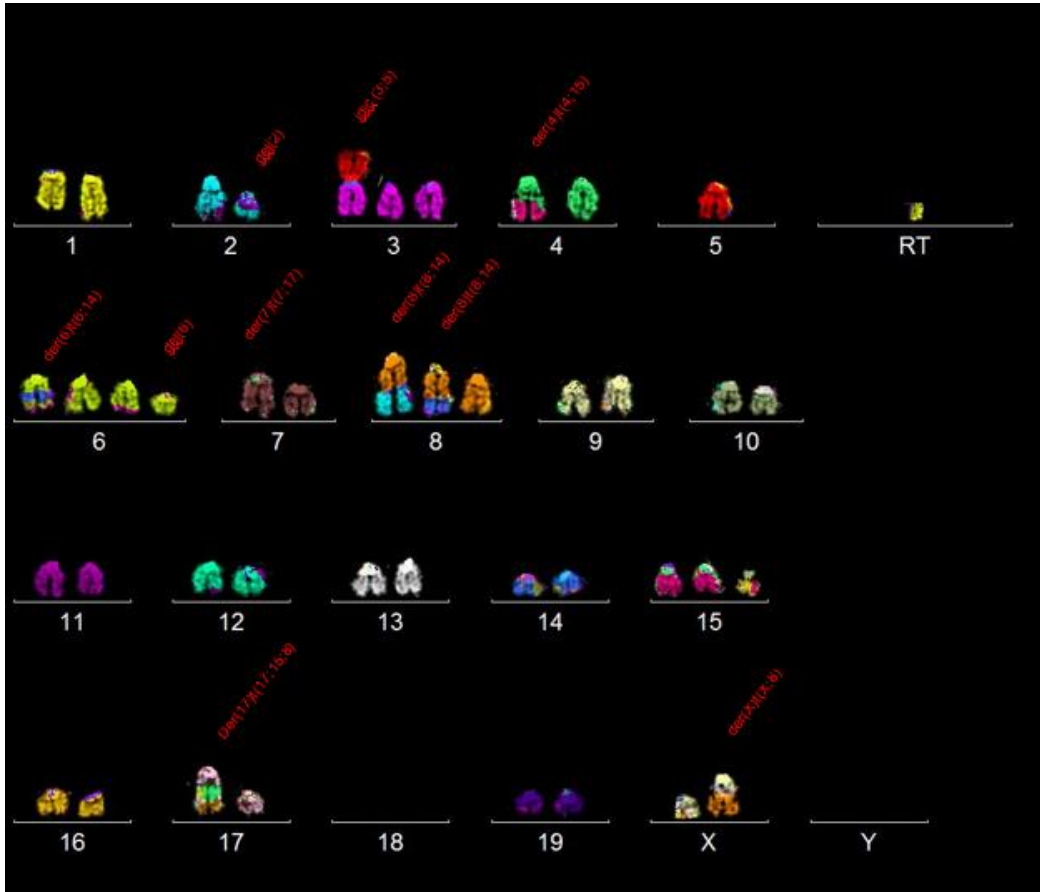


Figure 14 - SKY result for SEWA cell line, clone 3

For single copy number variations and aberrations see Annex 6.3.

Clone 4:

Clone 4 was found in 4 out of 30 metaphases. It appears to have the following karyotype:

41~44; X,X, der(X)t(8;X), del(2), +idic(3;5), der(4)t(4;15), -5, del(6), +der(6)t(6;14), der(7)t(7;17), +der(8)t(8;2), der(8)t(8;14), del(17), der(17)t(4;15;17), -18x2



Figure 15 - SKY result for SEWA cell line, clone 4

For single copy number variations and aberrations see Annex 6.4.

Clone 5:

Clone 5 was found in 1 out of 30 metaphases. It appears to have the following karyotype:

41, X, -X, del(2), der(3)t(3;9), +idic(3;5), der(4)t(4;15), -5, +del(6), +der(6)t(6;14), der(8)t(8;2), +9, del(17), +der(17)t(8;15;17), -18



Figure 16 - SKY result for SEWA cell line, clone 5

For single cell aberrations and copy number variations see Annex 6.

5.1.1. Chromosome markers

There were some cases in which the signals had no clear order and/or pattern and did not correspond to any chromosome described. However, it was clear that they were formed by DNA material clustered together. Therefore, these were considered DNA markers and analysed as such.

1-2 chromosome markers were found in 11 metaphases.

5.2. Mcb Results

Mcb was performed with the purpose of describing all chromosomal alterations meticulously. For that, also 30 metaphases were selected, this time for each chromosome, and only signals belonging to the chromosome selected were analyzed and written down.

For all chromosomes represented, always 30 metaphases were analyzed.

Mcb 1:

Two normal chromosomes 1 were found in 24 metaphases.

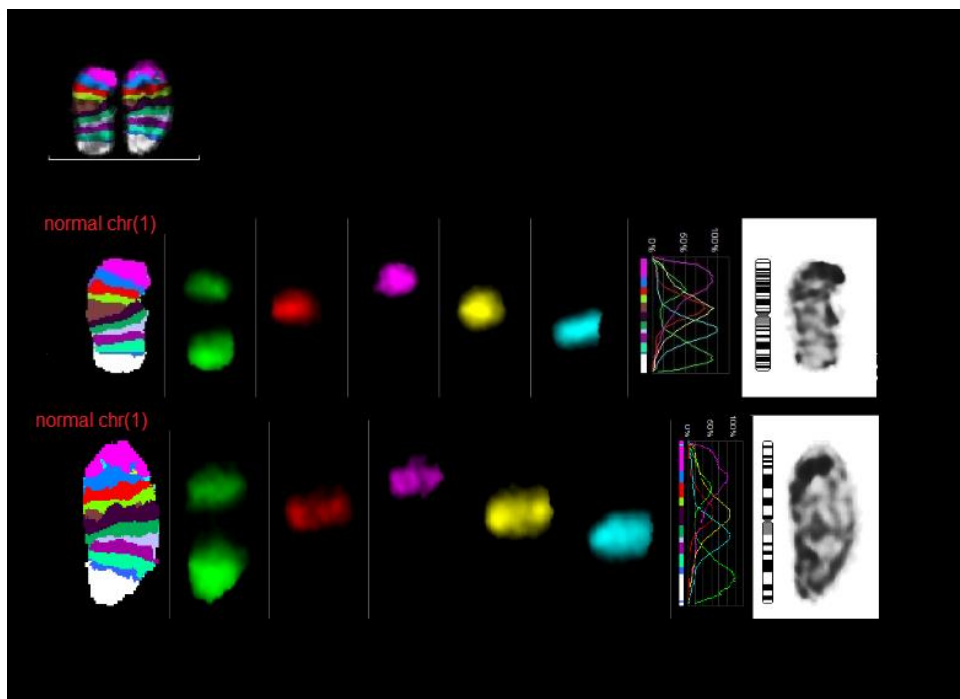


Figure 17 - mcb result for chromosome 1, two normal chromosomes

One normal chromosome 1 and one del(1)(H) were found in 5 metaphases.

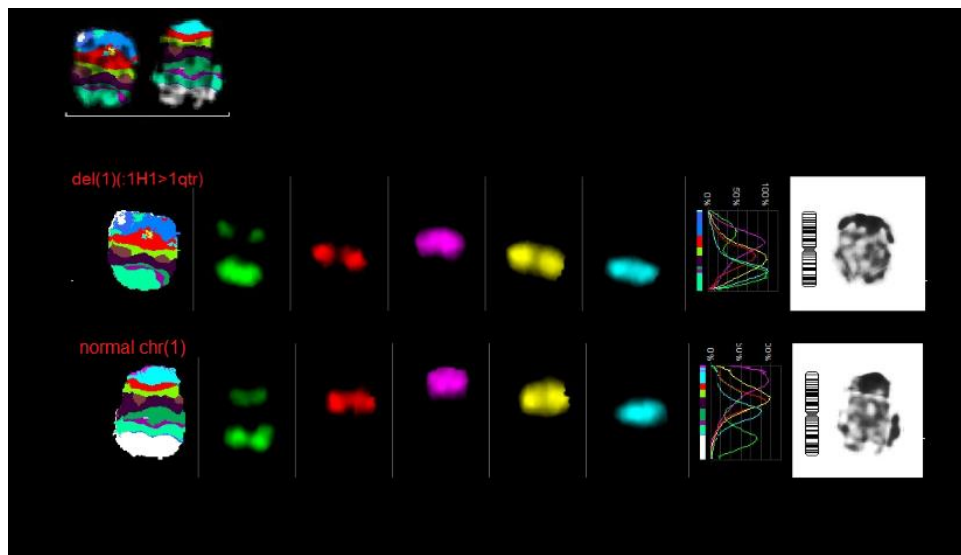


Figure 18 - mcb result for chromosome 1, one normal chromosome and 1 del(1)(:1H1>1qter)

Two del(1)(1H1) were found in 1 metaphase.

Mcb 2:

Two normal chromosomes 2 and one del(2)(2A1→2F3:) were found in 6 metaphases.

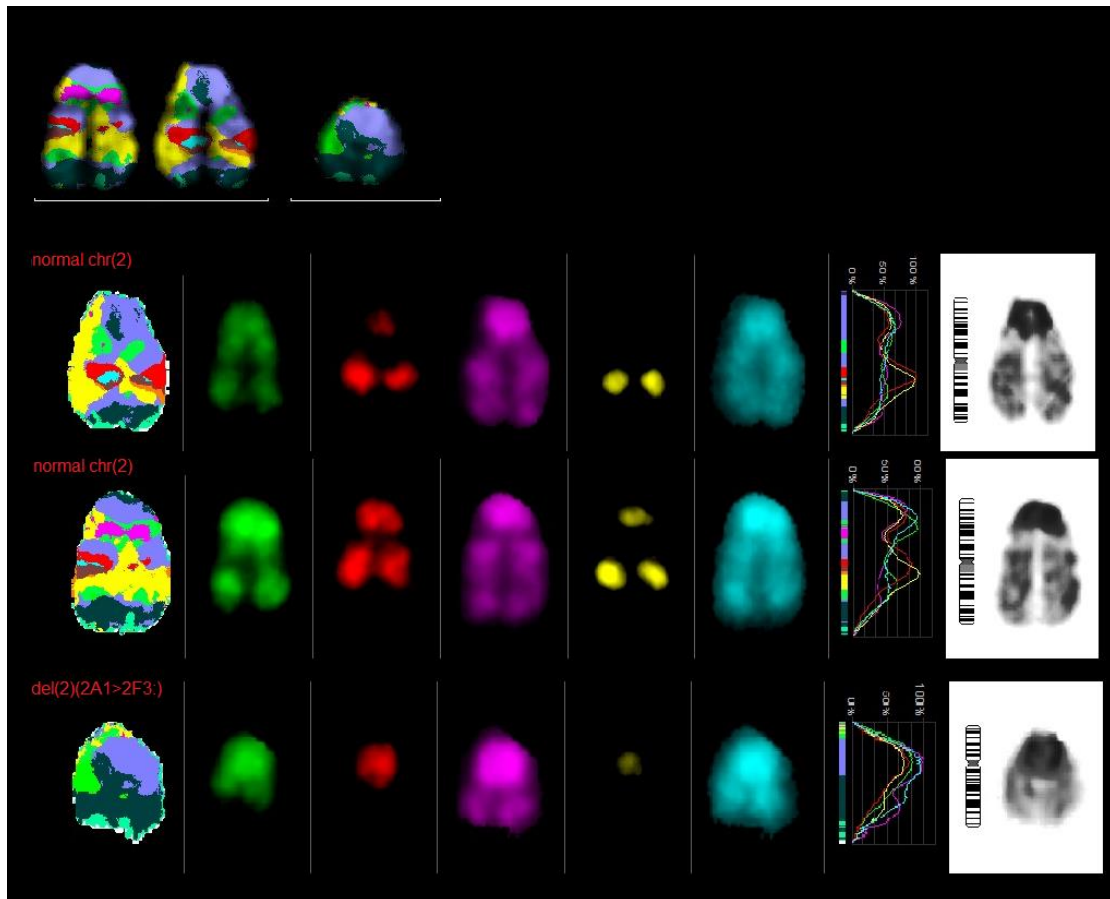


Figure 19 - mcb result for chromosome 2, two normal chromosomes and on del(2)(2A1>F3:)

One normal chromosome 2, one del(2)(2A1::2H3→2qter) and one del(2)(2A1→2F3:) were found in 2 metaphases.

Two normal chromosomes were found in 2 metaphases.

One normal chromosome 2, one del(2)(2A1→2F3:) and one der(?)t(?;2)(?:2E5→2F1::2A2→2qter) were found in 2 metaphases.

For single aberrations see Annex 7.1

Mcb 3:

One $\text{idic}(3;?)(3A1 \rightarrow 3qter:cen:?)$, one normal chromosome 3 and one $\text{del}(3)(3A1 \rightarrow 3A2:)$ were found in 26 metaphases

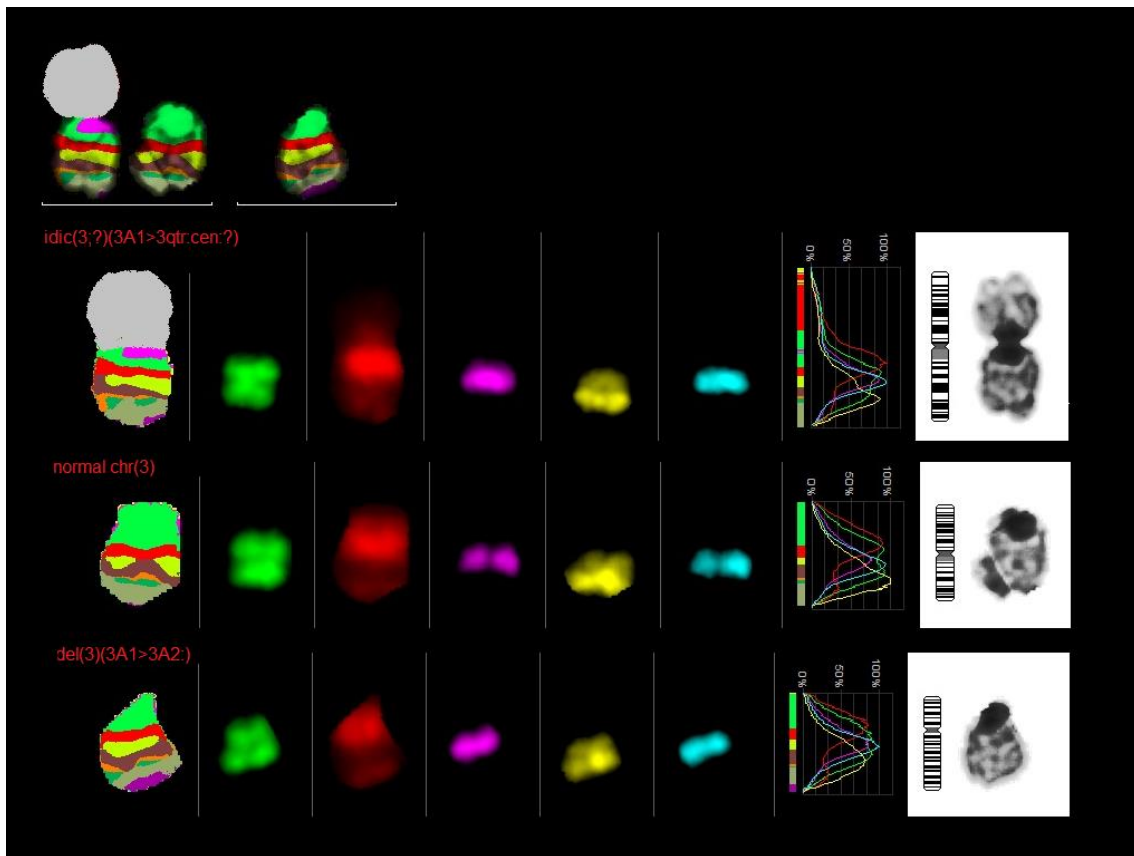


Figure 20 - mcb result for chromosome 3, one $\text{idic}(3;?)(3A1 \rightarrow 3qter:cen:?)$, one normal chromosome and one $\text{del}(3)(3A1 \rightarrow 3A2:)$

One $\text{idic}(3;?)(3A1 \rightarrow 3qter:cen:?)$ and one normal chromosome 3 were found in 2 metaphases.

Three $\text{idic}(3;?)(3A1 \rightarrow 3qter:cen:?)$, one normal chromosome 3 and one $\text{del}(3)(3A1 \rightarrow 3A2:)$ were found in 1 metaphase.

Two $\text{idic}(3;?)(3A1 \rightarrow 3qter:cen:?)$, one normal chromosome 3 and one $\text{del}(3)(3A1 \rightarrow 3A2:)$ were found in 1 metaphase.

Mcb 4:

One der(4)t(4;?)(:4C2~C3→4qter::?) and one der(4)t(4;4)(4A1→4B3::4B1→4qter) were found in 10 metaphases.

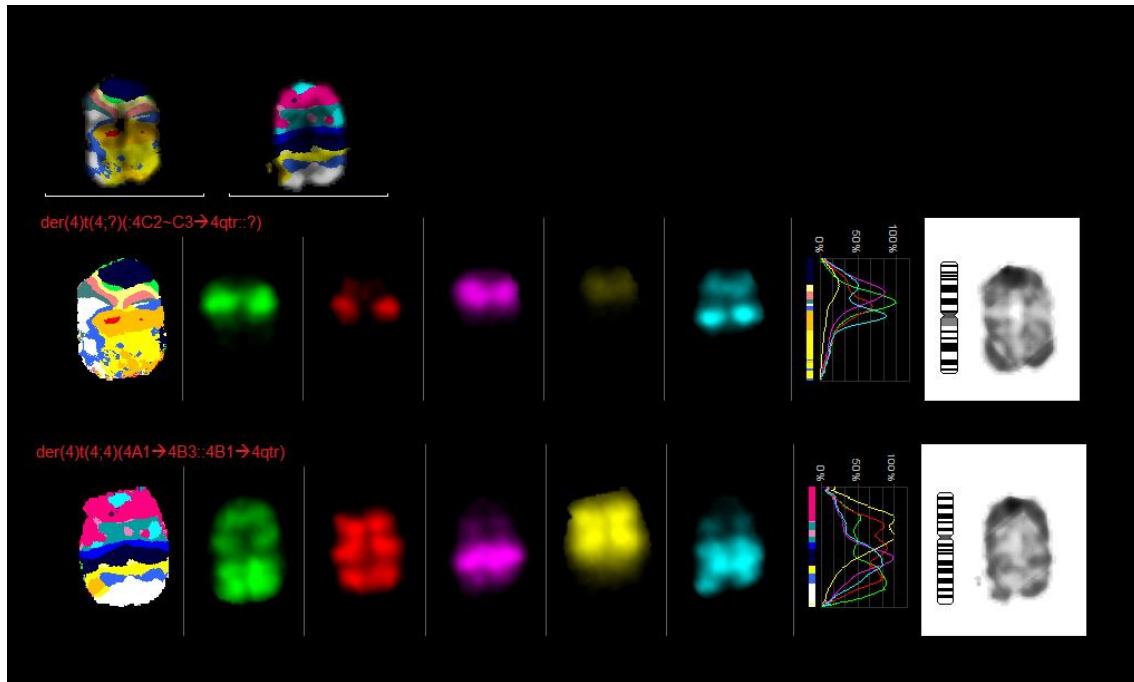


Figure 21 - mcb result for chromosome 4, one der(4)t(4;?)(:4C2~C3>4qter::?) and one der(4)t(4;4)(4A1>4B3::4B1>4qter)

One der(4)t(4;?)(:4C2~C3→4qter::?) and one del(4)(4A1→4A5::4qter) were found in 9 metaphases.

One der(4)t(4;?)(:4C2~C3→4qter::?) and one del(4)(4A1→A5::4D1→4qter) were found in 2 metaphases.

One der(4)t(4;?)(:4C2~C3→4qter::?) and one del(4)(4A1→4A5::4D2→4qter) were found in 2 metaphases.

One der(4)t(4;?)(:4C2~C3→4qter::?) and one del(4)(:4D1→4qter) were found in 2 metaphases.

For single aberrations see Annex 7.2

Mcb 5:

One $\text{idic}(?;5)(?:\text{cen}:5\text{A1}\rightarrow 5\text{qter})$ and one normal chromosome 5 were found in 13 metaphases.

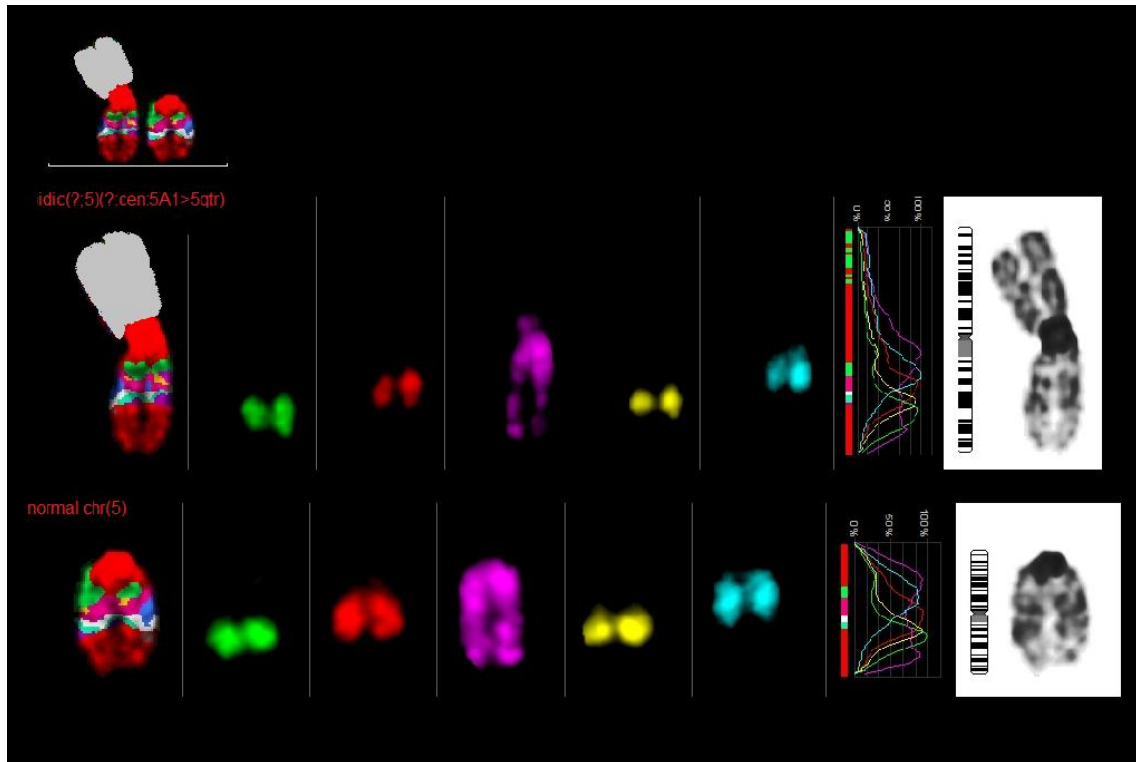


Figure 22 - mcb result for chromosome 5, one $\text{idic}(?;5)(?:\text{cen}:5\text{A1}\rightarrow 5\text{qter})$ and one normal chromosome 5 were found in 5 metaphases.

One $\text{idic}(?;5)(?:\text{cen}:5\text{A1}\rightarrow 5\text{F:})$ and one normal chromosome 5 were found in 5 metaphases.

One $\text{idic}(?;5)(?:\text{cen}:5\text{A1}\rightarrow 5\text{F:})$ and one $\text{del}(5)(5\text{G1}\rightarrow 5\text{qter:})$ were found in 4 metaphases.

One $\text{idic}(?;5)(?:\text{cen}:5\text{A1}\rightarrow 5\text{qter})$ and $\text{del}(5)(5\text{G1}\rightarrow 5\text{qter:})$ were found in 2 metaphases.

One $\text{idic}(?;5)(?:\text{cen}:5\text{A1}\rightarrow 5\text{F:})$ and one $\text{del}(5)(5\text{A1}\rightarrow 5\text{A2::}5\text{G}\rightarrow 5\text{qter})$ were found in 2 metaphases.

For single aberrations see Annex 7.3

Mcb 6:

One $\text{der}(6)\text{t}(6;?)(6A1\rightarrow 6C2::?)$, one $\text{del}(6)(6A1\rightarrow 6D:)$, one $\text{del}(6)(6A1\rightarrow 6D::6F2\rightarrow 6\text{qter})$, one $\text{del}(6)(6A1\rightarrow 6E\sim F1:)$ and one normal chromosome 6 were found in 6 metaphases.

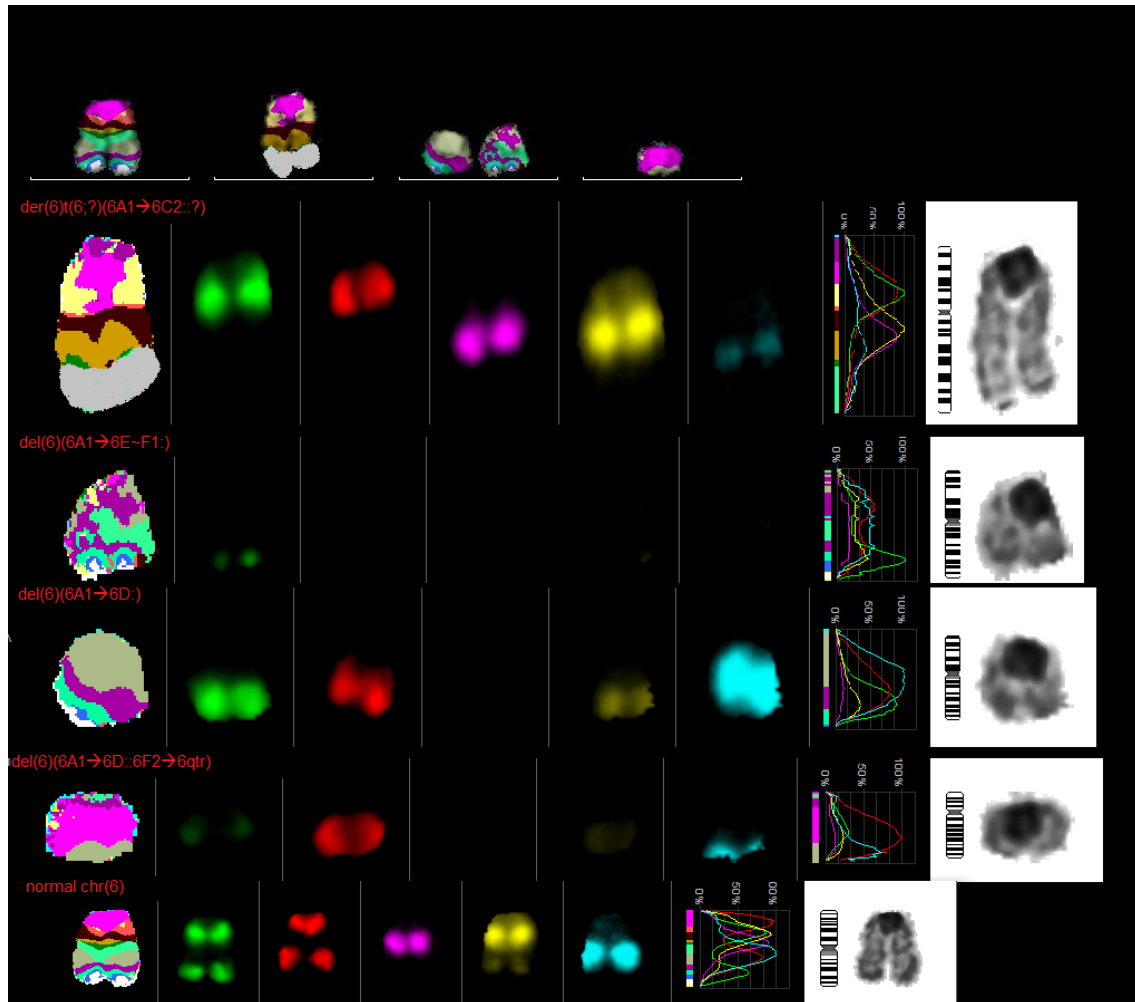


Figure 23 - mcb result for chromosome 6, one $\text{der}(6)\text{t}(6;?)(6A1\rightarrow 6C2::?)$, one $\text{del}(6)(6A1\rightarrow 6D:)$, one $\text{del}(6)(6A1\rightarrow 6D::6F2\rightarrow 6\text{qter})$, one $\text{del}(6)(6A1\rightarrow 6E\sim F1:)$ and one normal chromosome

One $\text{der}(6)\text{t}(6;?)(6A1\rightarrow 6C2::?)$, one $\text{del}(6)(6A1\rightarrow 6D:)$, one $\text{del}(6)(6A1\rightarrow 6D::6F2\rightarrow 6\text{qter})$ and one normal chromosome 6 were found in 3 metaphases.

One $\text{der}(6)\text{t}(6;?)(6A1\rightarrow 6C2::?)$, one $\text{del}(6)(6A1\rightarrow 6D:)$, one $\text{del}(6)(6A1\rightarrow 6D::6F2\rightarrow 6\text{qter})$ and one $\text{del}(6)(:6F2\rightarrow 6\text{qter})$ were found in 2 metaphases.

For single aberrations see Annex 7.4

Mcb 7:

One der(7)t(7;?)(7A1→7qter::?) and one normal chromosome 7 were found in 20 metaphases

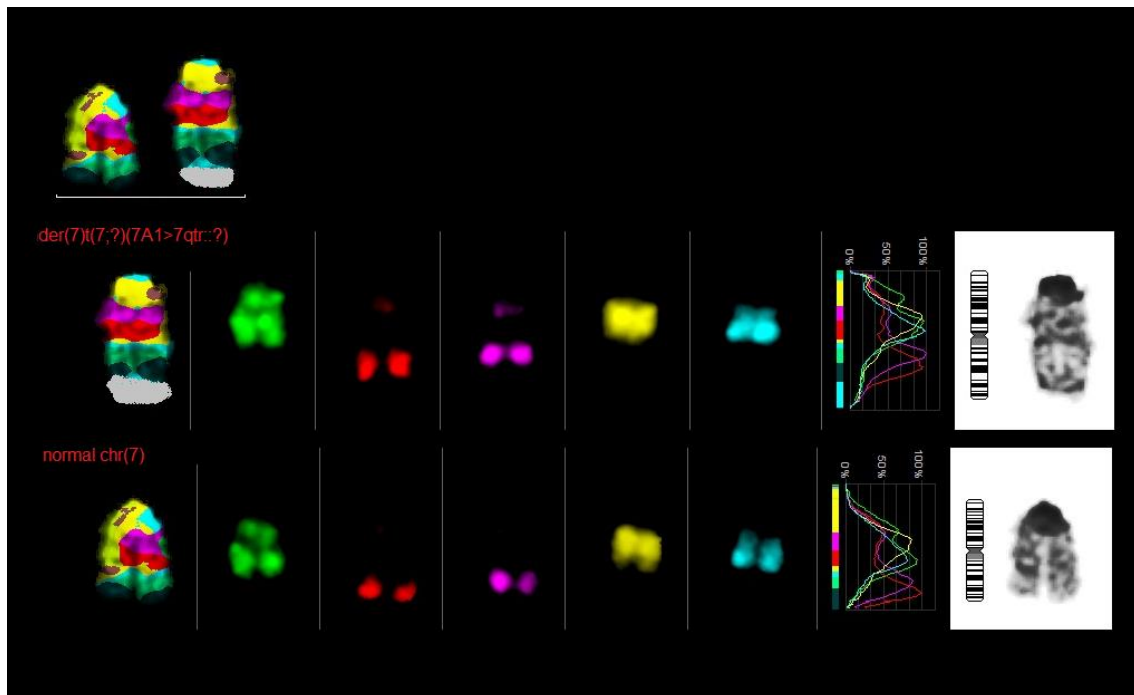


Figure 24 - mcb result for chromosome 7, one der(7)t(7;?)(7A1→7qter::?) and one normal chromosome 7

Two normal chromosomes were found in 6 metaphases.

For single aberrations see Annex 7.5

Mcb 8:

One $\text{der}(8)t(8;?)(8A1\rightarrow 8D1::?)$, one $\text{der}(?)t(?;8)(?::8C3\rightarrow 8D1:)$ and one $\text{inv}(8qter\rightarrow 8A1)$ were found in 11 metaphases.

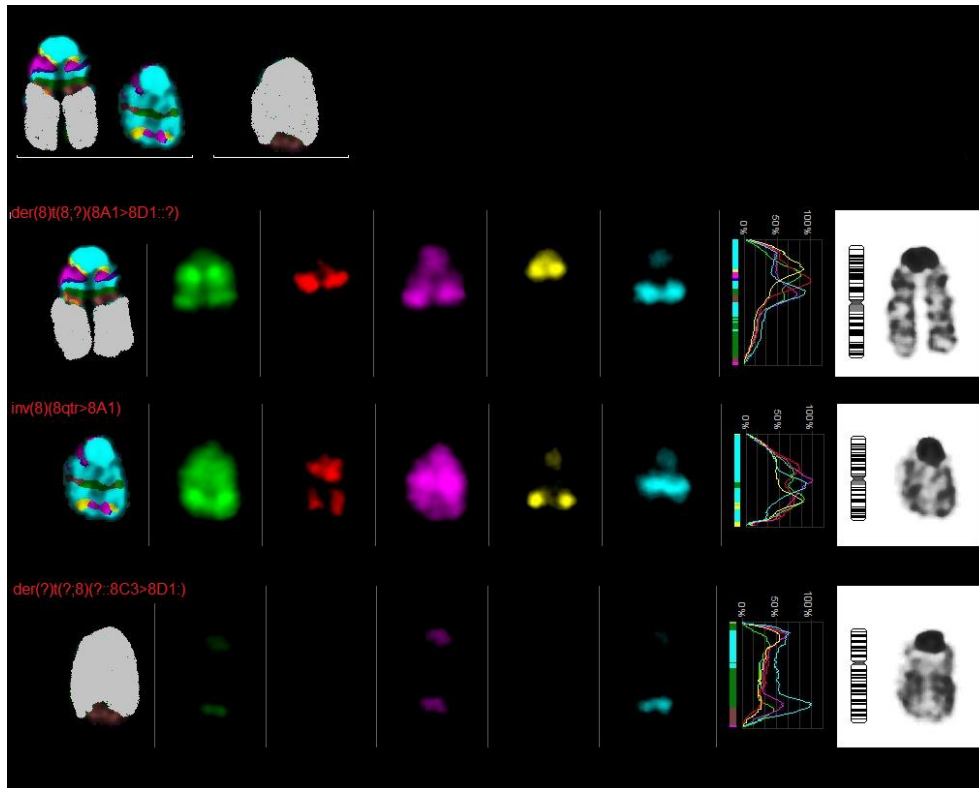


Figure 25 mcb result for chromosome 8, one $\text{der}(8)t(8;?)(8A1>8D1::?)$, one $\text{der}(?)t(?;8)(?::8C3>8D1:)$ and one $\text{inv}(8qter>8A1)$

One $\text{der}(8)t(8;?)(8A1\rightarrow 8D1::?)$, one $\text{der}(?)t(?;8)(?::8C3\rightarrow 8D1:)$ and one $\text{inv}(:8D1\rightarrow 8A1)$ were found in 6 metaphases.

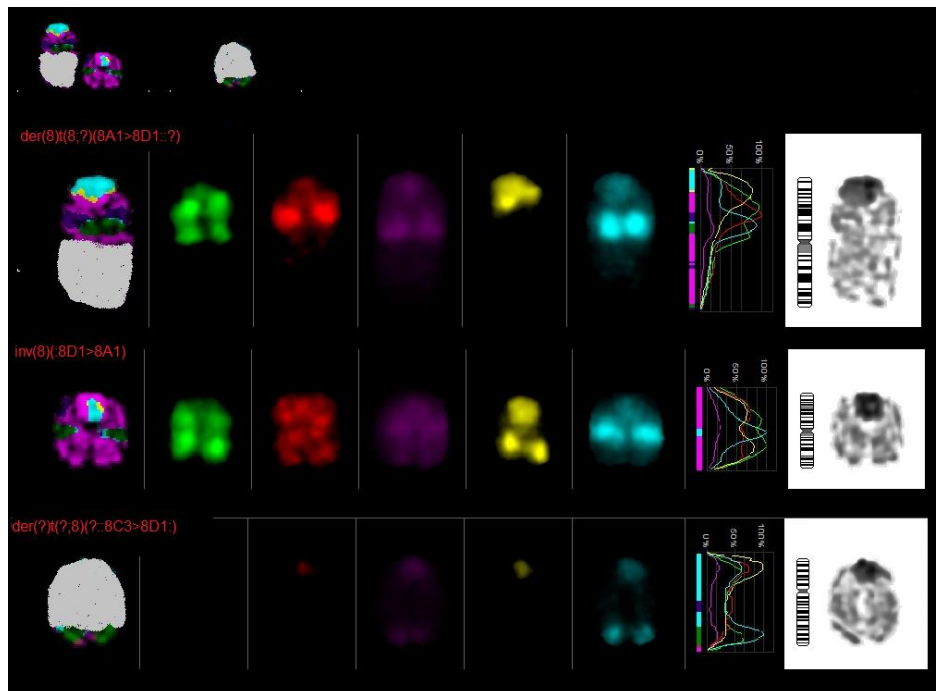


Figure 26 - mcb result for chromosome 8, one $\text{der}(8)t(8;?)(8A1\rightarrow 8D1::?)$, one $\text{der}(?)t(?;8)(?::8C3\rightarrow 8D1:)$ and one $\text{inv}(:8D1\rightarrow 8A1)$

For single aberrations see Annex 7.6

Mcb 9:

One normal chromosome 9 and one del(9)(:9F2~F3→9qter) were found in 8 metaphases.

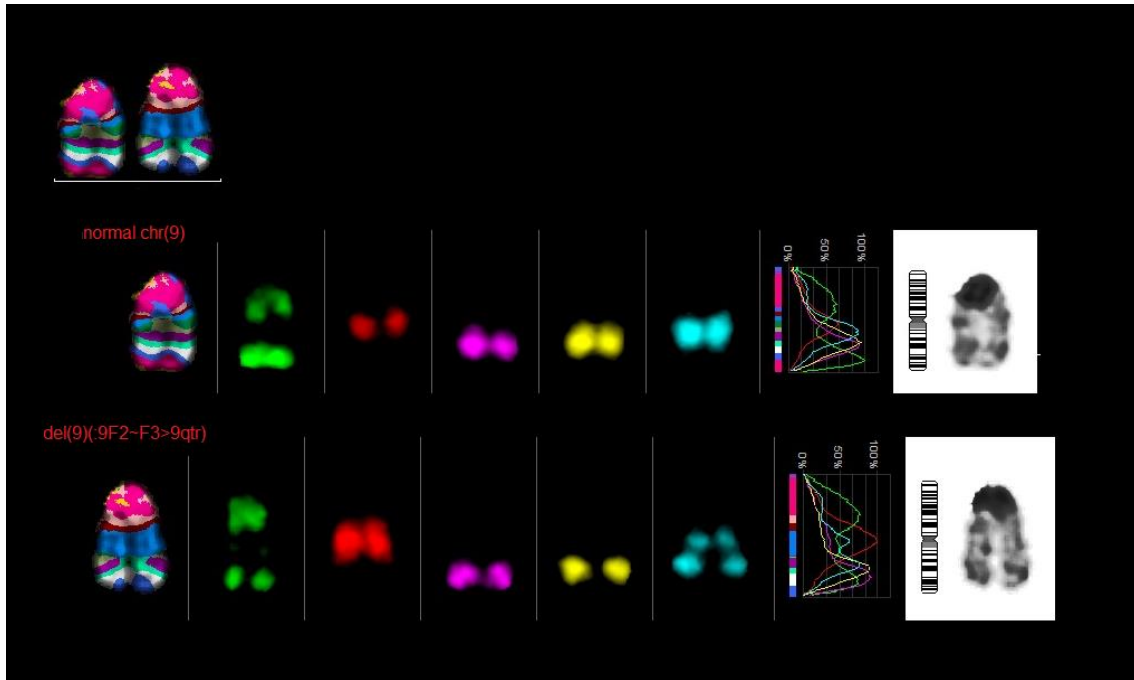


Figure 27 - mcb result for chromosome 9, one normal chromosome 9 and one del(9)(:9F2~F3→9qtr)

One del(9)(9A1→9A2::9F2~F3→9qter) and one del(9)(:9F2~F3→9qter) were found in 5 metaphases.

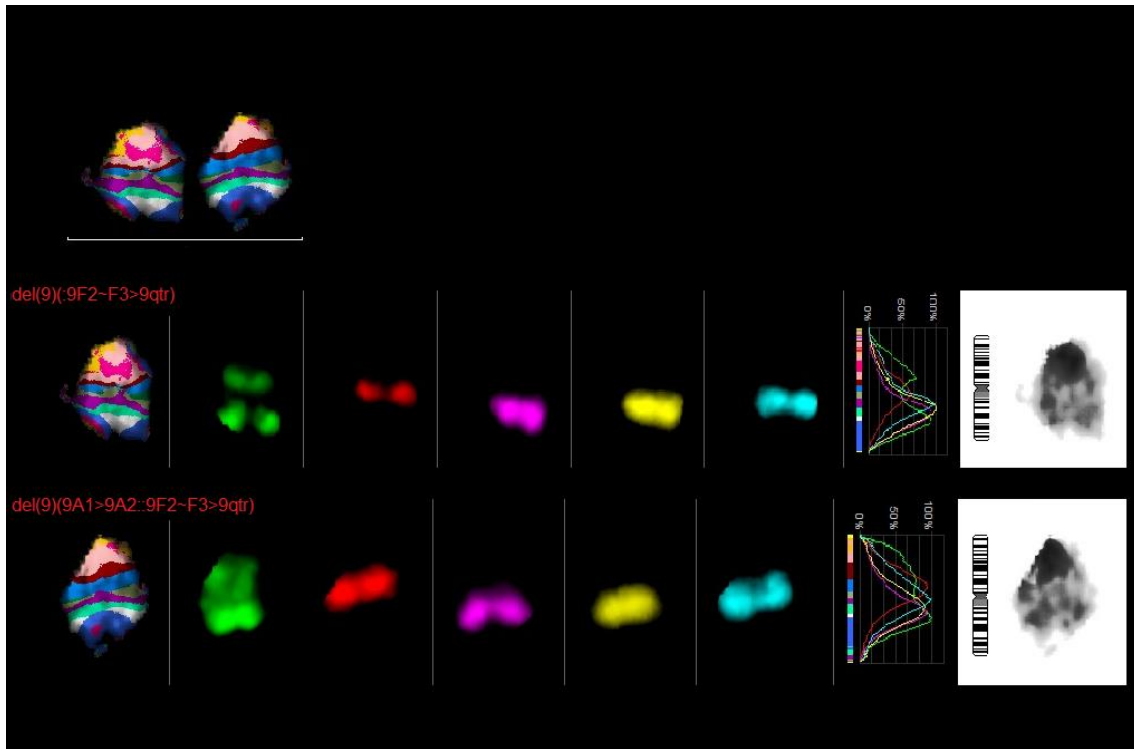


Figure 28 - mcb result for chromosome 9, one del(9)(9A1>9A2::9F2~F3>9qter) and one del(9)(:9F2~F3>9qter)

One del(9)(:9F2~F3→9qter) and one del(9)(9A1→9A2:) were found in 4 metaphases.
One normal chromosome 9 and one del(9)(9A1→9A2:) were found in 4 metaphases.
Two del (9)(:9F2~F3→9qter) were found in 4 metaphases.

For single aberrations see Annex 7.7

Mcb 10:

One normal chromosome 10, one $\text{der}(?)\text{t}(?;10)(?::10\text{D}2\rightarrow 10\text{D}3:)$ and one $\text{del}(10)(10\text{A}1\rightarrow 10\text{A}2:)$ were found in 28 metaphases.

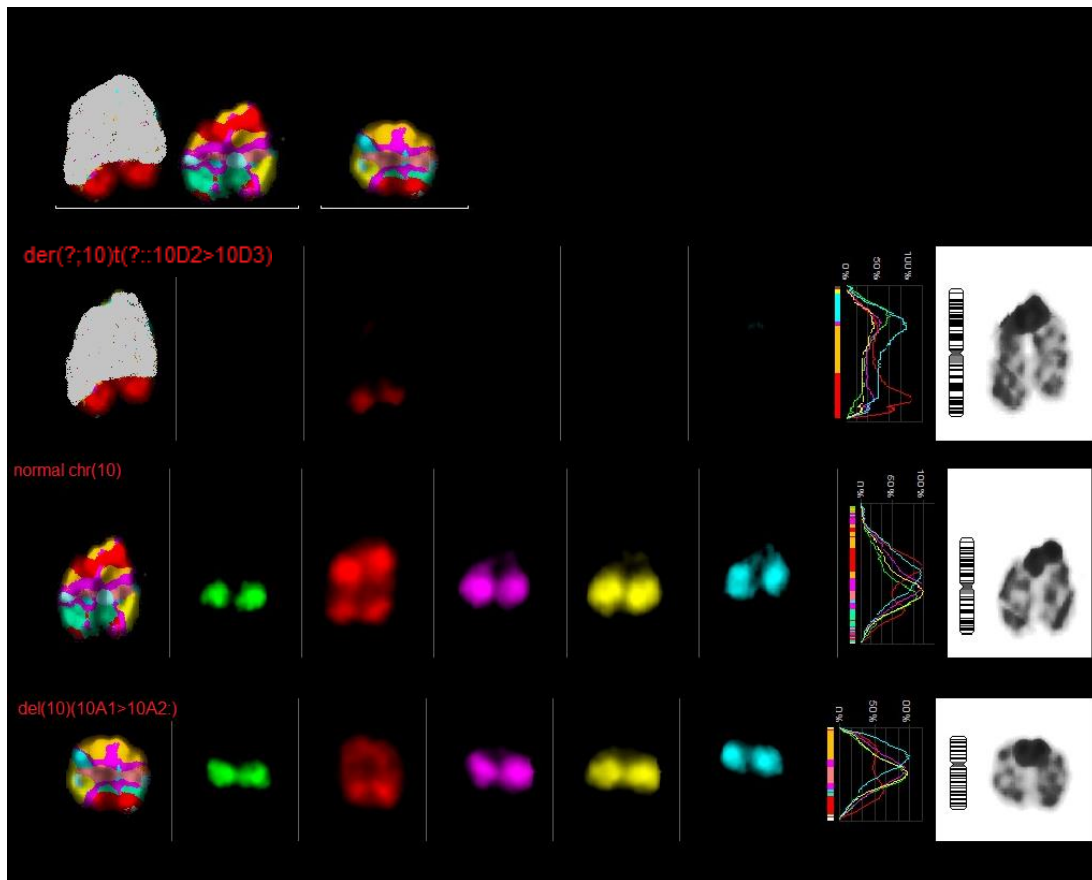


Figure 29 - mcb result for chromosome 10, one normal chromosome 10, one $\text{der}(?)\text{t}(?;10)(?::10\text{D}2\rightarrow 10\text{D}3:)$ and one $\text{del}(10)(10\text{A}1\rightarrow 10\text{A}2:)$

For single aberrations see Annex 7.8

Mcb 11:

Two normal chromosomes 11 were found in 7 metaphases.

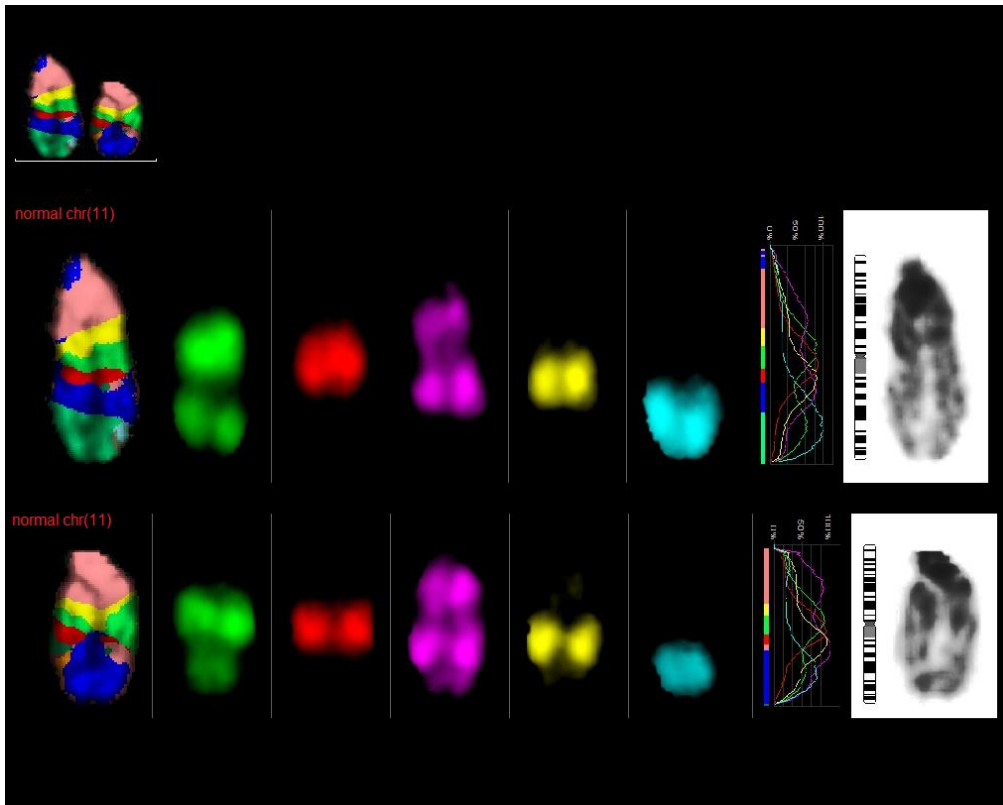


Figure 30 - mcb result for chromosome 11, two normal chromosomes 11

One del(11)(11A1→11A4:) and one del(11)(11A1→11A3::11E1→11qter) were found in 4 metaphases.

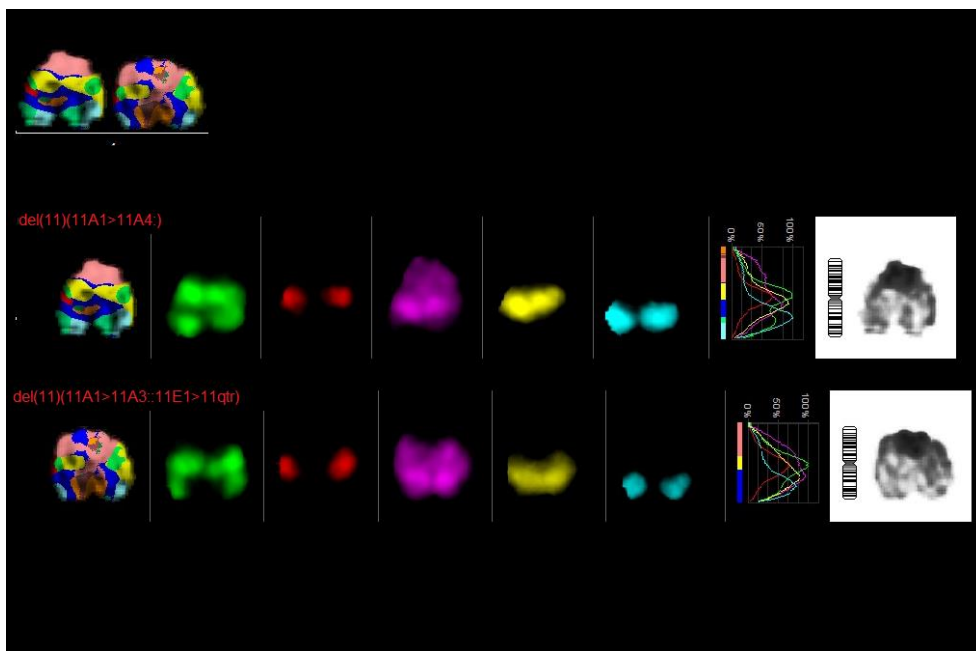


Figure 31 - mcb result for chromosome 11, one del(11)(11A1>11A4:) and one del(11)(11A1>11A3::11E1>11qter)

For single aberrations see Annex 7.9

Mcb 12:

Two $\text{der}(12)t(12;12)(12A1 \rightarrow 12C2::12F1 \rightarrow 12qter)$ were found in 27 metaphases.

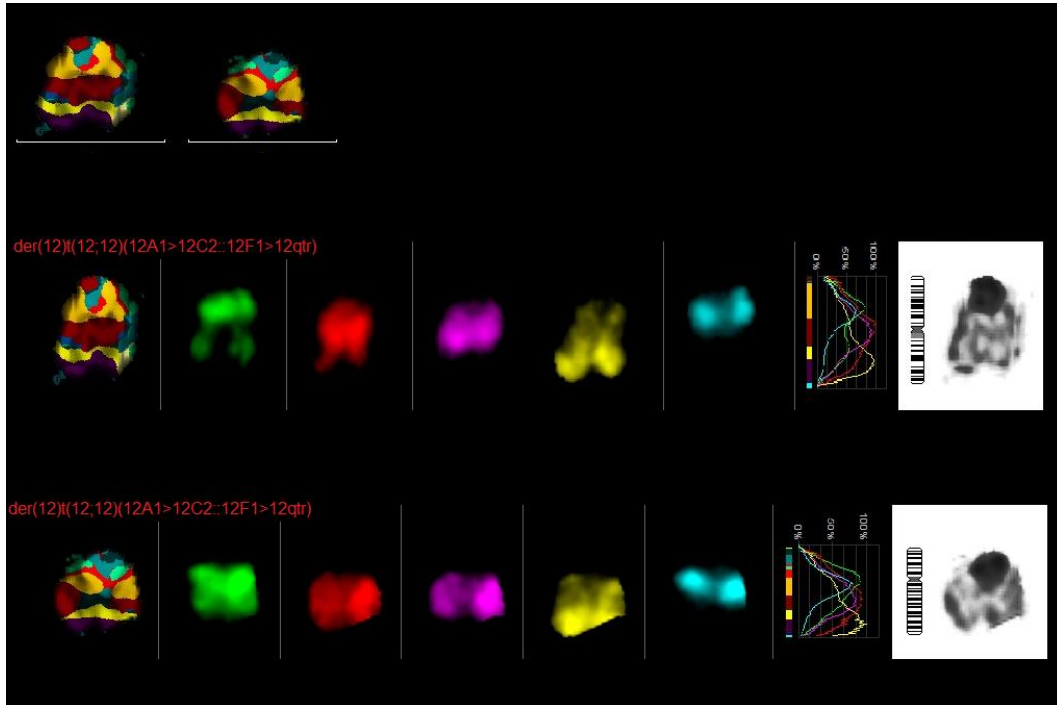


Figure 32 - mcb result for chromosome 12, two $\text{der}(12)t(12;12)(12A1 \rightarrow 12C2::12F1 \rightarrow 12qtr)$

One $\text{der}(12)t(12;12)(12A1 \rightarrow 12C2::12F1 \rightarrow 12qter)$ was found in 2 metaphases.

Three $\text{der}(12)t(12;12)(12A1 \rightarrow 12C2::12F1 \rightarrow 12qter)$ were found in 1 metaphase.

Mcb 13:

One normal chromosome 13 and one del(13)(:13D2) were found in 9 metaphases.

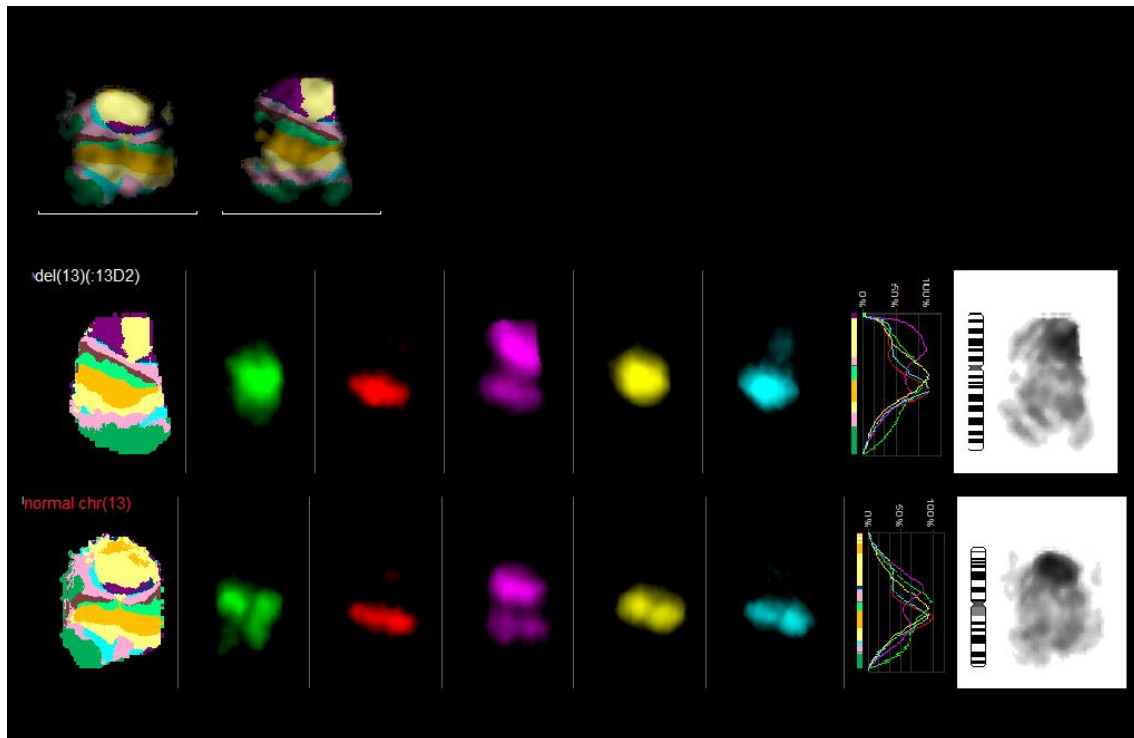


Figure 33 - mcb result for chromosome 13, one normal chromosome 13 and one del(13)(:13D2)

Two normal chromosomes 13 were found in 7 metaphases.

Two del(13)(:13D2) were found in 6 metaphases.

For single chromosome aberrations see Annex 7.10

Mcb 14:

One $\text{der}(?)\text{t}(?;14)(?::14\text{A1}\rightarrow 14\text{D1}:)$, one $\text{der}(?)\text{t}(?;14)(?::14\text{D3}\rightarrow 14\text{C2}\sim\text{C3}:)$ and two $\text{del}(14)(:14\text{D2}\rightarrow 14\text{qter})$ were found in 16 metaphases.

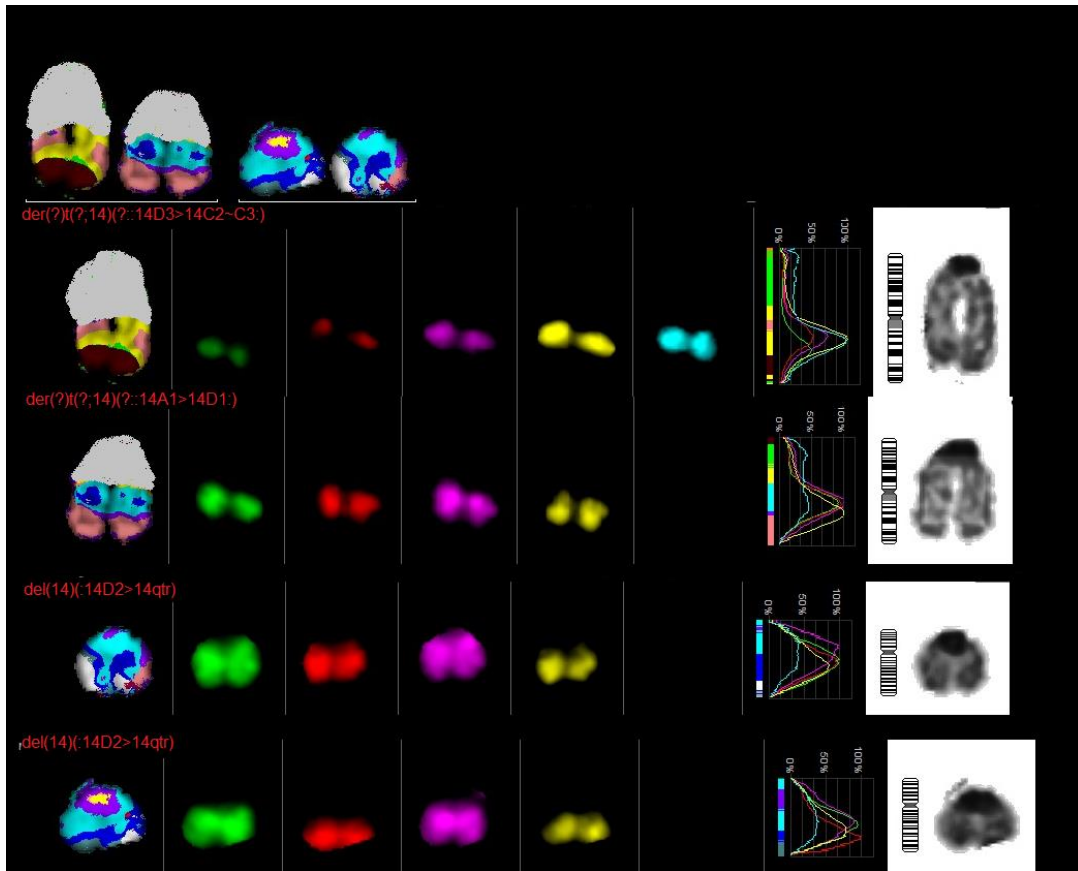


Figure 34 - mcb result for chromosome 14, one $\text{der}(?)\text{t}(?;14)(?::14\text{A1}\rightarrow 14\text{D1}:)$, one $\text{der}(?)\text{t}(?;14)(?::14\text{D3}\rightarrow 14\text{C2}\sim\text{C3}:)$ and two $\text{del}(14)(:14\text{D2}\rightarrow 14\text{qter})$

One $\text{der}(?)\text{t}(?;14)(?::14\text{D2}\rightarrow 14\text{qter})$, one $\text{der}(?)\text{t}(?;14)(?::14\text{D3}\rightarrow 14\text{C2}\sim\text{C3}:)$ and two $\text{del}(14)(:14\text{D2}\rightarrow 14\text{qter})$ were found in 6 metaphases.

For single chromosome aberrations see Annex 7.11

Mcb 15:

Two normal chromosomes 15, one $\text{der}(?)\text{t}(?:15)(?:15\text{D1}\rightarrow 15\text{D3})$ and one $\text{der}(?)\text{t}(?:15;?)(?:15\text{D1}\rightarrow 15\text{D3}::?)$ were found in 13 metaphases

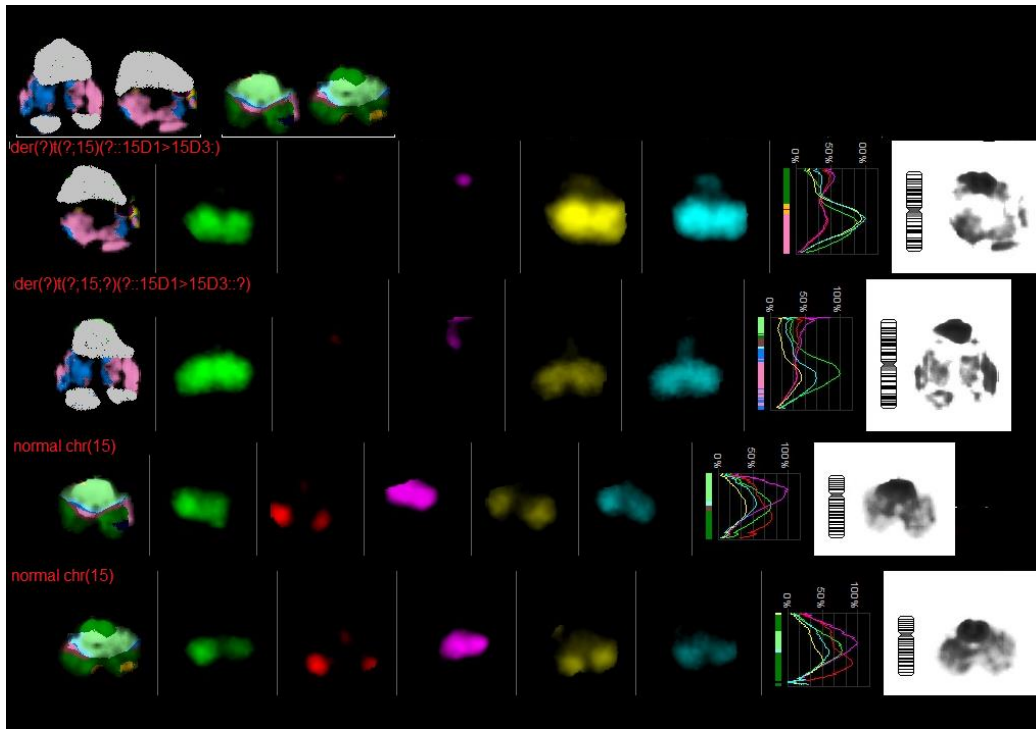


Figure 35 - mcb result for chromosome 15, two normal chromosomes 15, one $\text{der}(?)\text{t}(?:15)(?:15\text{D1}\rightarrow 15\text{D3})$ and one $\text{der}(?)\text{t}(?:15;?)(?:15\text{D1}\rightarrow 15\text{D3}::?)$

Two normal chromosomes 15 and two $\text{der}(?)\text{t}(?:15;?)(?:15\text{D1}\rightarrow 15\text{D3}::?)$ were found in 6 metaphases.

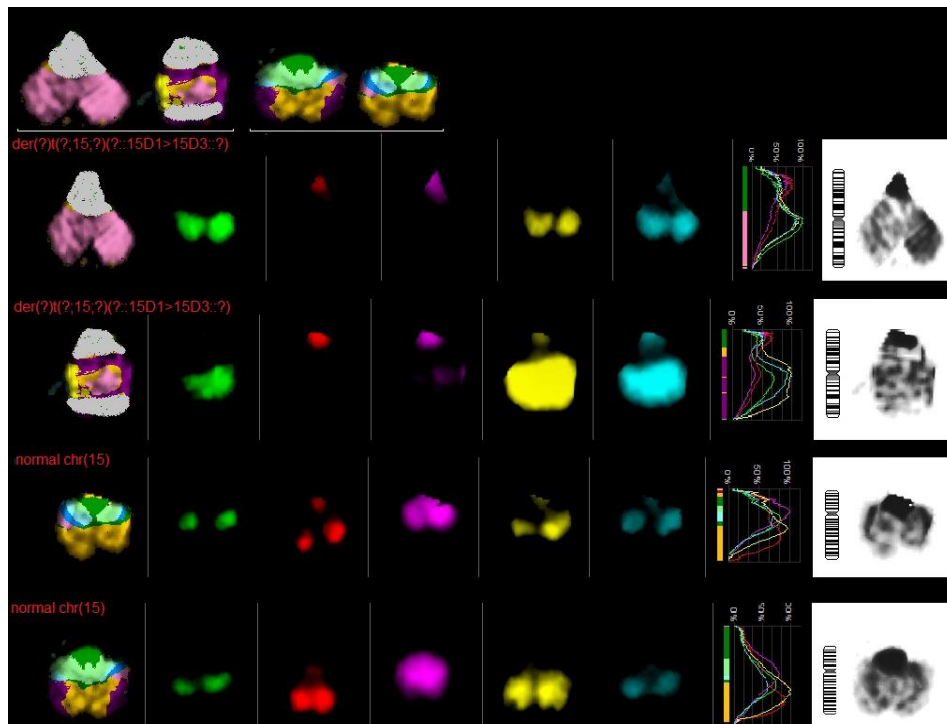


Figure 36 - mcb results for chromosome 15, two normal chromosomes 15 and two $\text{der}(?)\text{t}(?:15;?)(?:15\text{D1}\rightarrow 15\text{D3}::?)$

Two normal chromosomes 15, two der(?)t(?;15)(?::15D1→15D3:) and one der(?)t(?;15;?)(?::15D1→15D3::?) were found in 5 metaphases.

For single aberrations see Annex 7.12.

Mcb 16:

Two normal chromosomes 16 were found in 27 metaphases.

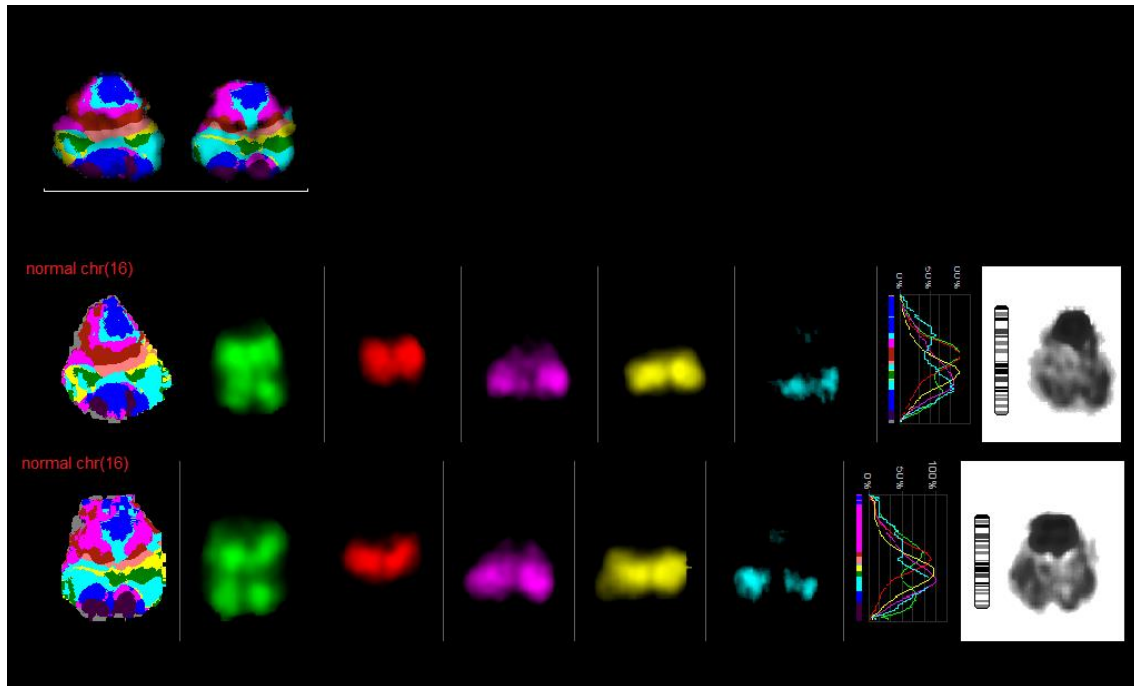


Figure 37 - mcb result for chromosome 16, two normal chromosomes 16

For single aberrations see Annex 7.13

Mcb 17:

One normal chromosome 17, one der(?)t(?;17)(?:17E1→17E2:) and one del(17):(17C~D→17qter) were found in 28 metaphases.

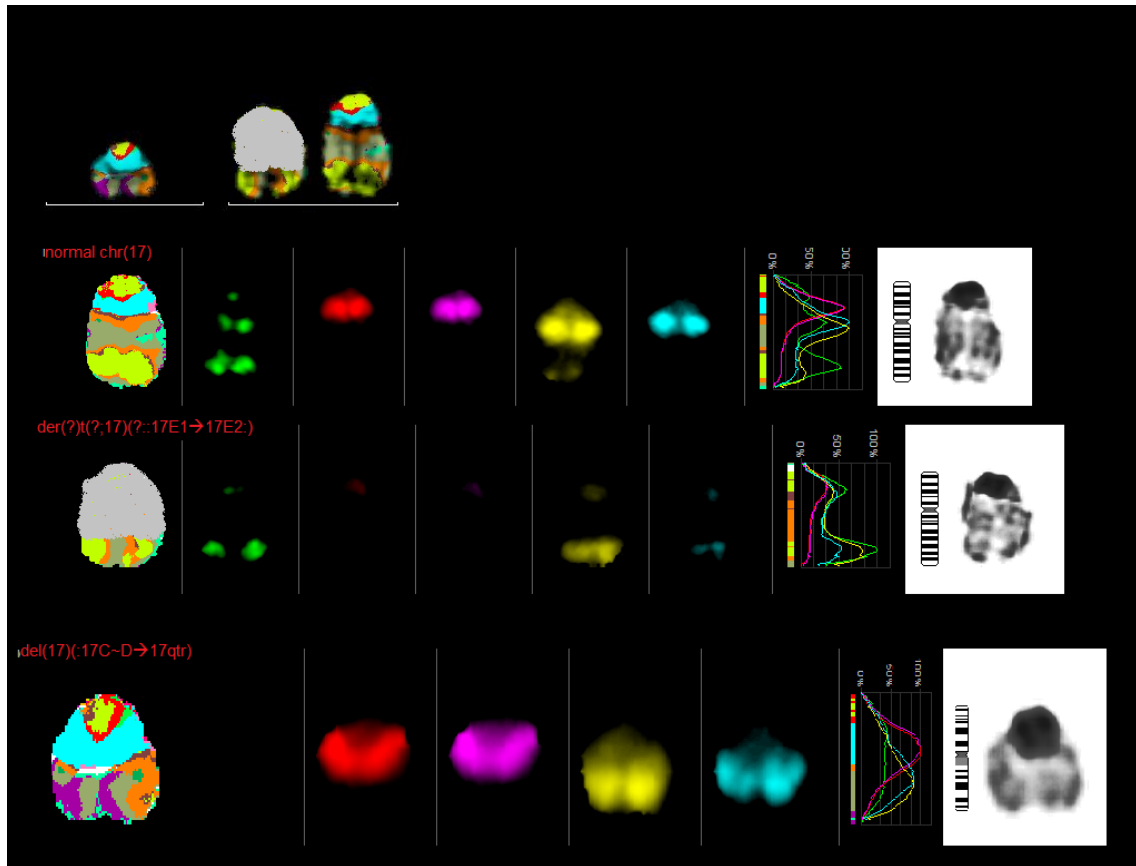


Figure 38 - mcb result for chromosome 17, one normal chromosome 17, one der(?)t(?;17)(?:17E1>17E2:) and one del(17):(17C~D>17qter)

For single aberrations see Annex 7.14

Mcb 18:

One $\text{der}(?)\text{t}(?:18)(?::18\text{A1}\rightarrow 18\text{qter})$ and one $\text{der}(18)\text{t}(18;?)(18\text{A1}\rightarrow 18\text{qter}::?)$ were found in 29 metaphases.

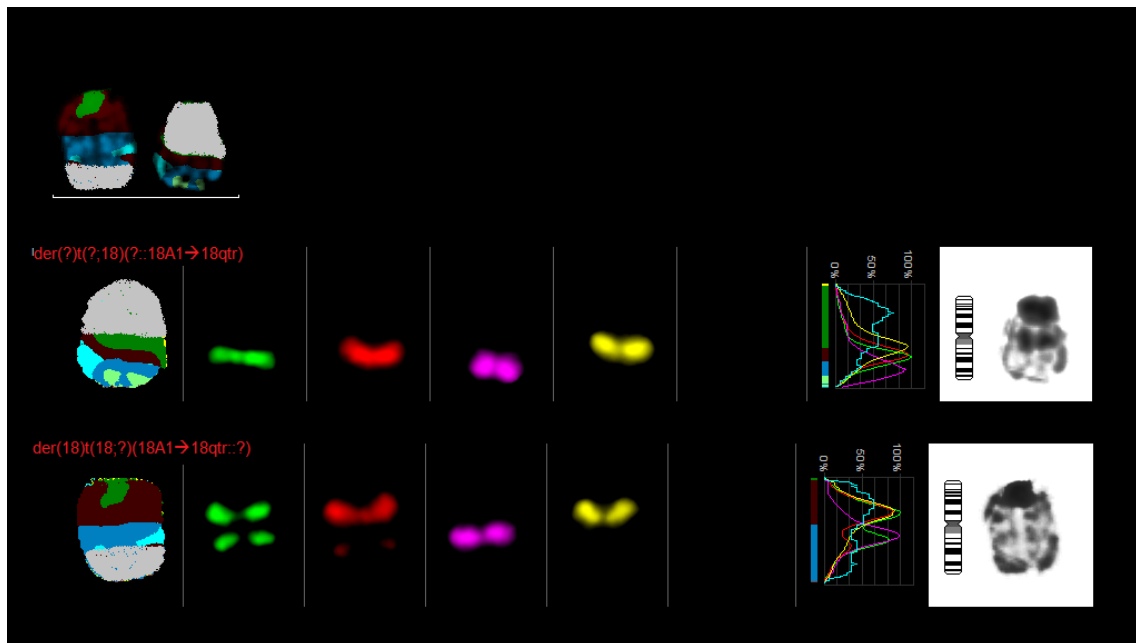


Figure 39 - mcb result for chromosome 18, one $\text{der}(?)\text{t}(?:18)(?::18\text{A1}\rightarrow 18\text{qtr})$ and one $\text{der}(18)\text{t}(18;?)(18\text{A1}\rightarrow 18\text{qtr}::?)$

For single aberrations see Annex 7.15

Mcb 19:

Two normal chromosomes 19, one der(?)t(?;19)(?::19D1→19qter) and one der(?)t(?;19;?)(?::19D1→19qter::?) were found in 28 metaphases.

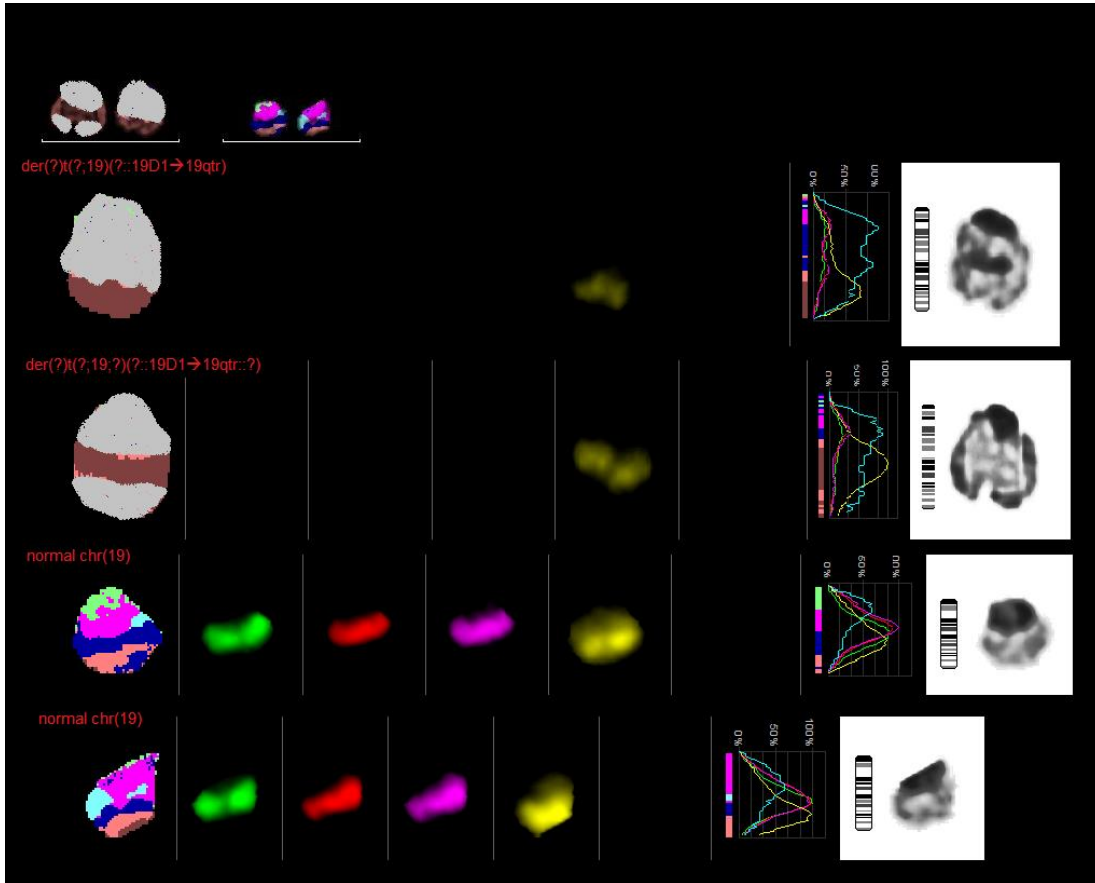


Figure 40 - mcb result for chromosome 19, two normal chromosomes 19, one der(?)t(?;19)(?::19D1>19qter) and one der(?)t(?;19;?)(?::19D1>19qter::?)

For single aberrations see Annex 7.16

Mcb X:

One $\text{der}(X)t(X;?)(XA1 \rightarrow XA6::?)$, one $\text{del}(X)(XA1 \rightarrow XA6::XE \rightarrow Xqter)$ and two $\text{del}(X)(XA1 \rightarrow XA6::XC \rightarrow Xqter)$ were found in 17 metaphases.

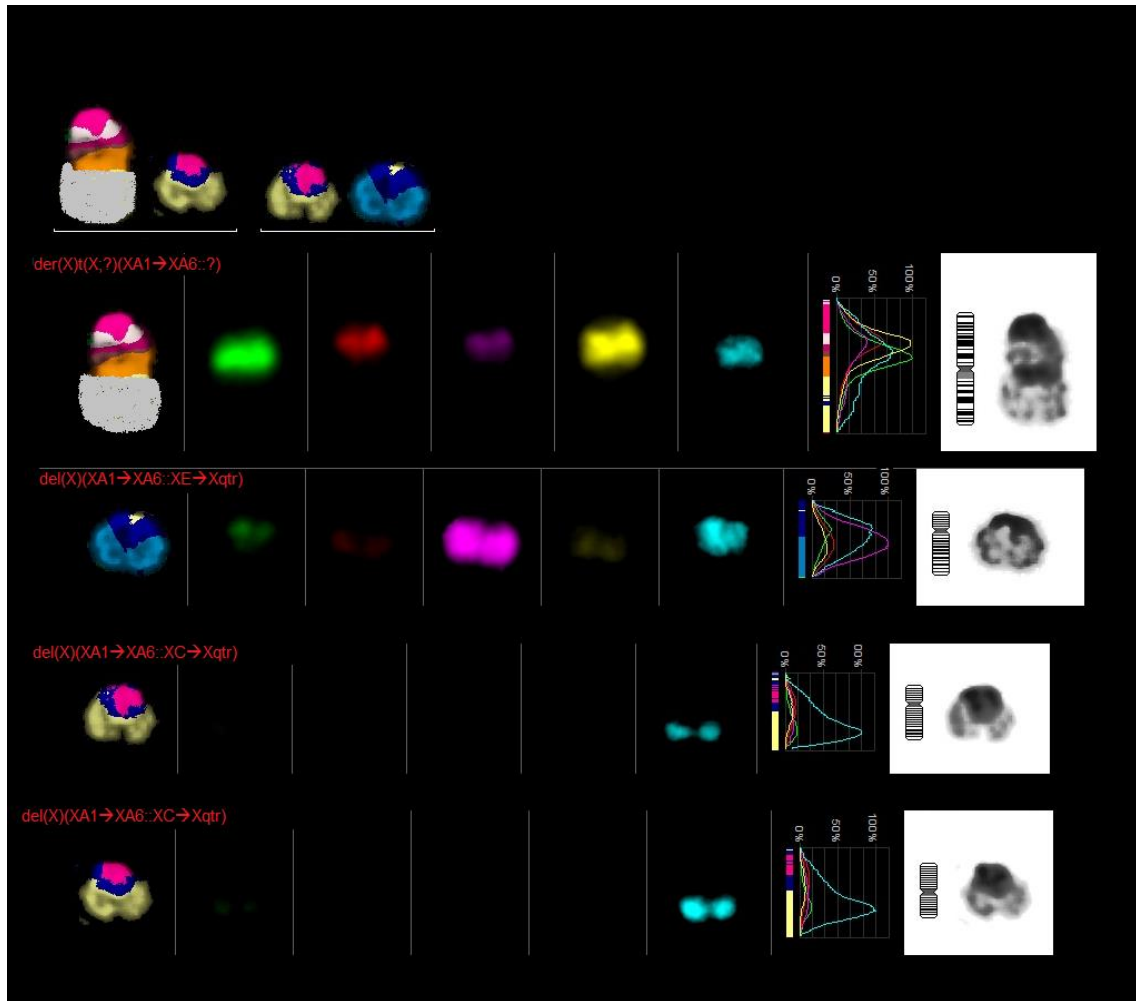


Figure 41 - mcb result for chromosome X, one $\text{der}(X)t(X;?)(XA1 \rightarrow XA6::?)$, one $\text{del}(X)(XA1 \rightarrow XA6::XE \rightarrow Xqter)$ and two $\text{del}(X)(XA1 \rightarrow XA6::XC \rightarrow Xqter)$

One $\text{der}(X)t(X;?)(XA1 \rightarrow XA6::?)$, one $\text{del}(X)(XA1 \rightarrow XA2::XE \rightarrow Xqter)$ and two $\text{del}(X)(XA1 \rightarrow XA6::XC \rightarrow Xqter)$ were found in 5 metaphases.

One $\text{der}(X)t(X;?)(XA1 \rightarrow XA6::?)$, one $\text{del}(X)(XA1 \rightarrow XA6::XE \rightarrow Xqter)$ and one $\text{del}(X)(XA1 \rightarrow XA6::XC \rightarrow Xqter)$ were found in 5 metaphases.

5.3. aCGH Results

aCGH results were asked to a collaborating laboratory in order to verify all of the results obtained from both sky and mcb procedures. In this case, multiple aCGH reports were sent, and the one which more closely represented the population studied was selected to compare results. All the aberrations were checked for each chromosome.

Table 1 - aCGH resumed results: green stands for observed aberrations; red stands for non-observed aberrations

Chromosome	Cytoband	Gain(+)/Loss(-)
Chr 1	qD	-
Chr 2	qA1	-
Chr 2	qE5-qF1	+
Chr 2	qG1-qH4	+
Chr 3	qA3-qH4	+
Chr 5	qC1-qC3.2	+
Chr 6	qA1-qA3.1	+
Chr 6	qD2-qE1	+
Chr 6	qG1-qG3	-
Chr 8	qA4-qB1.3	+
Chr 8	qC3-qD1	+
Chr 8	qE1	-
Chr 9	qA3-qB	+
Chr 9	qA5.3-qB	+
Chr 10	qA1-qA4	-
Chr 10	qD2-qD3	+
Chr 11	qD-qE2	+
Chr 14	qA1-qB	+
Chr 14	qA1-qA2	+
Chr 14	qB-qC1	+
Chr 14	qD2-qE5	-
Chr 15	qD1	+
Chr 15	qE3	+
Chr 17	qB3-qE2	+
Chr 18	qB1-qE3	+
Chr 18	qB1-qB3	+
Chr 18	qE3-qE4	-
Chr X	qE1-qE3	-

5. Discussion

After analysing SKY results, SEWA cell line was characterized by having near diploid cells, which means that most of the metaphases analysed had between 35 and 37 chromosomes.

The cell line divided itself in 5 major clones, presenting similar karyotypes for each group.

Clone 1 appears to have the main karyotype, representing 40% of the metaphases studied. It is divided in four subclones:

- I. The difference between **subclone 1a** and clone 1 is that 1a has another recurring aberration that doesn't exist in clone 1: $\text{der}(6)\text{t}(6;14)$. Plus, one of the metaphases did not have the aberration $\text{der}(7)\text{t}(7;17)$ that is characteristic of clone 1 karyotype.
- II. Additionally to the main karyotype, **subclone 1b** also has as main aberration $\text{der}(8)\text{t}(8;2)$. In this karyotype the original aberration $\text{der}(6)\text{t}(6;14)$ is replaced by $\text{der}(6)\text{t}(6;14;10)$.
- III. Instead of original aberration $\text{der}(6)\text{t}(6;14)$, **subclone 1c** has as main aberration $\text{der}(6)\text{t}(6;14;10)$. The difference between this one and subclone 1b is that 1c lacks the aberration $\text{der}(8)\text{t}(8;2)$.
- IV. The main difference between **subclone 1d** and the original one, is that it has another two main aberrations in its karyotype: $\text{der}(9)\text{t}(9;15)$ and $\text{der}(6)\text{t}(6;14;10)$. The last one is replacing the original aberration $\text{der}(6)\text{t}(6;14)$. One of the metaphases lacked one of the main aberrations from clone 1: $\text{der}(17)\text{t}(17;15;4)$. Instead, it was replaced by the aberration $\text{der}(17)\text{t}(17;15;8)$.

Clone 2 was present in 30% of the metaphases, being also divided in four subclones:

- I. The main difference between **subclone 2a** and its original is the presence of main aberration $\text{der}(17;15;8)$ that replaces the original one $\text{der}(17;15;4)$ and the presence of aberration $\text{der}(8)\text{t}(8;14)$. One of the metaphases didn't showcase the aberration $\text{der}(17;15;8)$, having it replaced by the aberration $\text{der}(17)\text{t}(17;15)$.
- II. **Subclone 2b** differs from the original one since, apart from the main karyotype, it also has aberrations $\text{der}(7)\text{t}(7;17)$ and $\text{der}(8)\text{t}(8;14)$.
- III. **Subclone 2c** differs from the previous subclones since it has as main aberrations $\text{der}(8)\text{t}(8;2)$ and $\text{der}(17)\text{t}(17;15;4)$. It is, however, lacking the aberration $\text{der}(8)\text{t}(8;14)$.
Also, one of the metaphases had normal X chromosomes, hence lacking the main aberration $\text{der}(X)\text{t}(X;8)$ that occurs in the original clone 2.
- IV. **Subclone 2d** also has as main aberrations: $\text{der}(8)\text{t}(8;2)$, $\text{der}(6)\text{t}(6;14)$ and $\text{der}(7)\text{t}(7;17)$. These aberrations are not found in the original clone 2.

Clone 3 was found in 3 out of 30 metaphases, having no subclones associated. Clone 4 and 5 were found in four and one metaphases, respectively, having also no subclones associated. Clone 5 was the only one to show loss of sexual chromosome X.

While analysing, some metaphases had non-identifiable DNA structures, cluster – like, with no recognizable colour pattern. These structures were named chromosome markers. However, there could be a possibility for them to be chromothripsis.

As for mcb results, 30 metaphases were studied for each chromosome. Chromosomes 1, 3, 10, 12, 16, 17, 18 and 19 were those whose aberration development was the most stable, having major aberrations in most of the metaphases analysed. Chromosomes 2, 4, 8, 9 and 11 were the most unstable ones, showing mostly single aberrations.

When asked for the aCGH, the results obtained with the *in situ* hybridization procedures did not always correspond to the ones observed in the array (mismatched breakpoints and aberrations).

As can be seen in Table 1 (see subchapter 5.3), chromosomes 1, 4, 9, 11 and 18 were completely mismatched comparatively to the aberrations found with mcb. There were cases where no significant aberration was detected with aCGH, whereas in mcb they were observed – example of chromosome 4, where there should be marked deletion of cytoband A1 to B1; chromosome 12, where it was expected to see significant deletion of cytoband E and chromosome 19, with amplification of D1 to qtr.

For chromosome 5, M-FISH/SKY reveals that one chromosome 5 is missing, however that is not detected in mcb and aCGH. This is due to the fact that the missing chromosome is forming and idic with chromosome 3, and thus, while making a karyotype, it was annexed to chromosome 3 instead of 5.

In chromosome 6, aCGH reveals deletion of the final cytoband, which makes sense since most of metaphases had at least one chromosome without the final cytoband.

In chromosome 7, no major breakpoints or aberrations were found. However, in SKY as well as mcb, derivative chromosomes were found. This can be explained by the fact that aCGH detected all the cytobands and they were indeed present in the right “amount”. The aberrations were not detected because no copy number was altered, and no DNA material was added or deleted, just moved.

As to chromosomes 14 and 17, in Table 1 they were considered mismatched. However, due to the proximity of the breakpoints detected, and the bad quality of the signals, it is possible to consider that while capturing, the signals detected moved, giving false results.

Surprisingly, chromosome 2, which was one of the most unstable chromosomes, was the one with the more matches between mcb and aCGH results.

One factor that did not match between SKY results and MCB results, was that in SKY no chromosome 18 was observed, whereas in MCB 30 metaphases with chromosome 18 were analysed. No reason for these observations was found.

A resumed evaluation of SKY, mcb and aCGH is shown in annex 8.

A general view of the aberrations found can be seen in the figure below. See *Figure 42*)

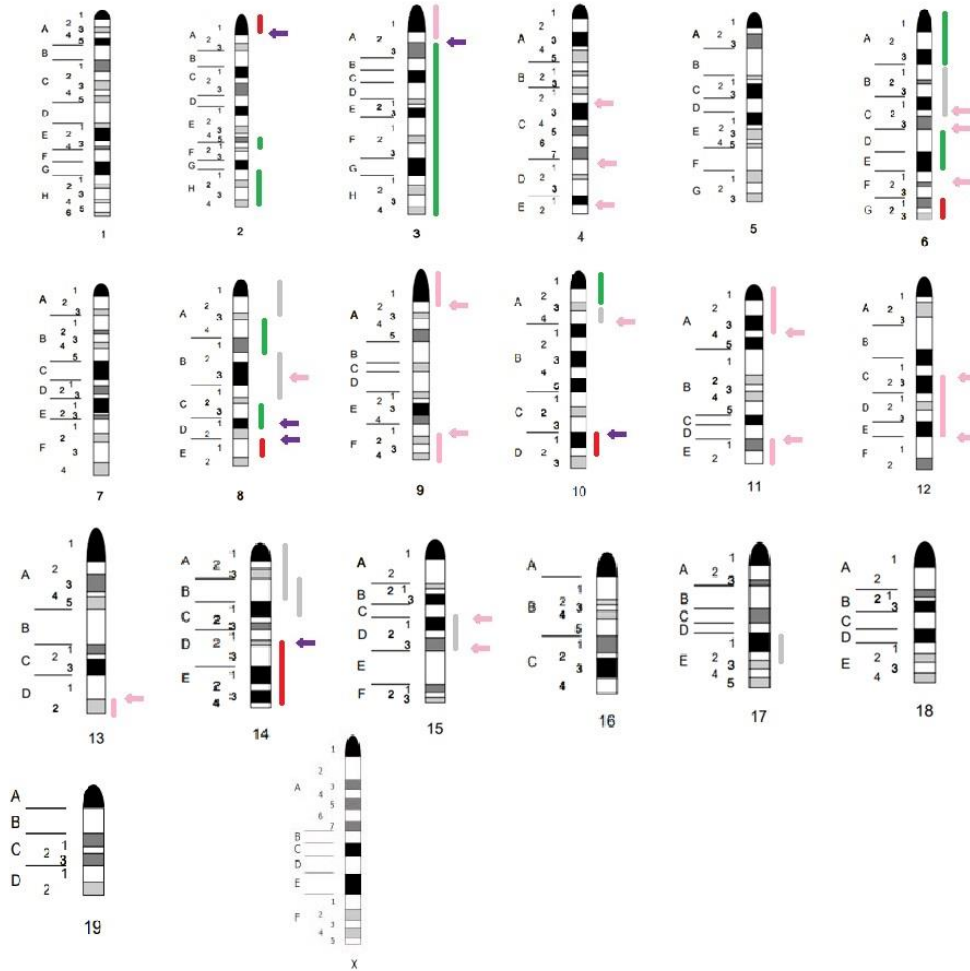


Figure 42 - overall view of all karyotype structural aberrations: deletions found in mcb and aCGH marked as red bars; amplifications found in mcb and aCGH marked as green bars; matched breakpoints marked as purple arrows; amplifications found only in mcb marked as light grey bars; deletions found only in mcb marked as pink bars; possible breakpoints marked as pink arrows (18).

6. Conclusions

SEWA cell line is one murine cell line which is mainly used for cytogenetic researches. Along the line of this work it was clear that this cell line was rather unstable.

After SKY/M-FISH analyses, five clones were determined, with a wide number of associated subclones.

Some chromosomal clusters were also found. It would not be strange to regard these structures as chromothripsis since they are common in many tumour forms, and can be found not only in subclones but also in most of the primary cells. In this work, only 11 metaphases out of 30 studied had this kind of aberrations, but there could be more and not have been recognised because of inexperience in identifying and acknowledging their existence. Also, as described in Chapter 1.4.1, it is mostly difficult to infer the existence of chromothripsis when there is marked genetic instability, which is one of the main characteristics of this cell line. It was also pointed that, sometimes, chromothripsis may form DMs, which are a common feature of SEWA. However, these formations get lost when transferring from *in vivo* cell cultures to *in vitro* cell cultures. This could be another explanation for the unusual low number of chromothripsis identified.

Concerning mcb results, most chromosomes had single anomalies (found in only one metaphase), not being possible to define major aberrations.

The major obstacle this cell line presented was the bad quality of the chromosomes, since they were too small to get completely clear probe signals. This problem was observed both with the results from SKY and MCB.

aCGH showed a very different reality of this cell line aberrations, when compared to mcb and SKY. It was, however, possible to discern major breakpoints on few chromosomes.

If the results were conclusive enough to get a clear genomic description of SEWA cell line, the next step would be to translate chromosomal breakpoint to its homologous in human genome by using databases and bioinformatics. Then, a comparison between human cancers of different origins would be performed and find the one with the higher level of concordance. This would allow to identify SEWA cell line as a model for studies involving a specific human tumour.

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Annexes

Annex 1 – Tumorigenesis models and chromothripsis formation

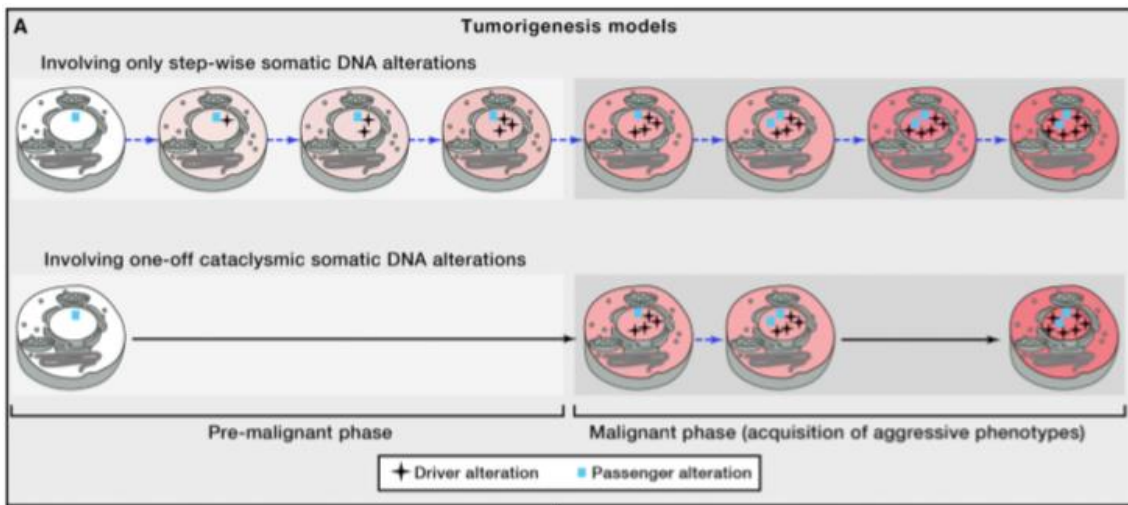


Figure 43 – Classical theory for tumorigenesis: stepwise acquisition of somatic DNA driver alterations (dashed blue arrows). Cellular crisis, as chromothripsis may accelerate this process by resulting in several DNA alterations at once (solid black arrows). The colour symbolize the malignancy of the cells: white = non-malignant, red = aggressive/highly malignant. (in O.Korbel, et al, 2013)

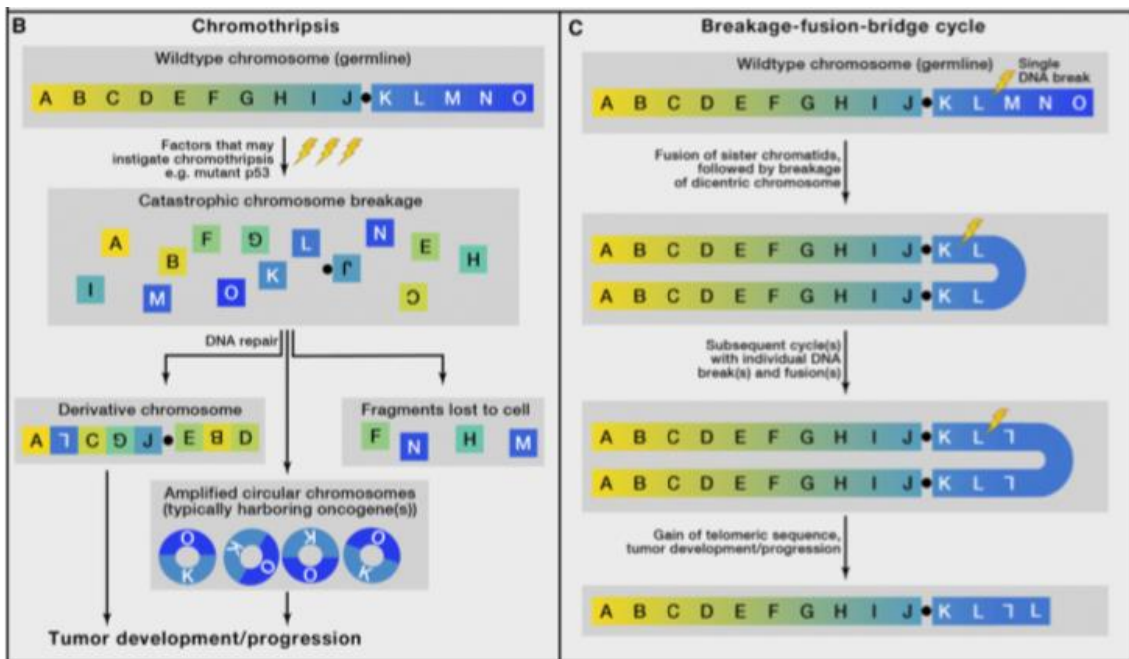


Figure 44 – B) chromothripsis and tumour development. C) classical approach to breakage-fusion-bridge cycle that involves several subsequent cell cycles. It is however hypothesised that chromosome end-to-end fusions may also lead to chromothripsis events. (in O.Korbel, et al.,2013)

Annex 2 - MOUSE SKY - Post-hybridization wash (Modified protocol 20.07.2016)

1. Wash in 0.4x SSC at 66-68°C for 4 minutes
2. Wash in 4x SSC/Tween, in a different coplin jar, for 5 minutes with agitation (use shaker)
3. Block with 80 µL of Marvel solution with a coverslip and incubate at 37 °C for 10 minutes.
4. Remove coverslips and dip the slides in 4x SSC/Tween for about 1 minute.
5. Apply 80-100 µL of Cy5/Antidigoxin staining reagent per slide and incubate at 37°C for 30-40 minutes.

Note 1: for 1 slide → 100 µL of Marvel solution + 2,5 µL Anti-digoxin (stored at -20°C) + 5 µL SA-Cy5

Note 2: Use filter tips for Anti-digoxin!

Post-detection wash I

1. 4xSSC/Tween, RT with agitation for 5 minutes – 3 times
2. Apply 80-100 µL of Pacific Blue staining reagent per slide and
3. Incubate at 37°C for 30-40 minutes

Note 1: for 1 slide → 100 µL of Marvel solution + 4 µL of Pacific Blue (Pacific Blue™ F(ab')₂ Frag of Goat Anti-mouse IgG (H+L), 2mg/mL, stored at +4°C)

Note 2: Use filter tips for Pacific Blue!

Post-detection wash II

1. 4xSSC/Tween, RT with agitation for 5 minutes – 3 times
2. Rinse slides briefly in milliQ water.
3. Dehydrate slides in ethanol series, 2-3 minutes each and air dry the slides.
4. Apply 20 µL of “home-made” Anti-fade DAPI or Anti-fade DAPI (vial 5) provided by the kit.

Annex 3 - "Dropping" – slides preparation

1. Firstly, put the slides in the fridge for about 2h before starting.
2. Preparation of FIX solution: add 100 μ L of acetic acid to 300 μ L of methanol (1:3).
3. Pre-heat hot plate to 50°C and put on top of the plate a wet paper. Shape it so it looks like an intestine surface, in order to have more steam surface.
4. Drop 20 μ L of sample into the slide. Place the pipet some centimetres above the slide and let one drop fall in one edge, one drop in the other and the rest directly into the slide centre.

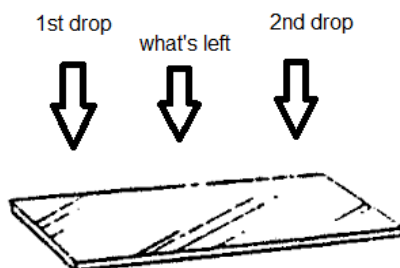


Figure 45 - Dropping scheme

5. Add 20 μ L of FIX solution the same way as the previous step.
 6. Leave the slide on top of the wet paper in the hot plate for about 2 minutes, and when all the slide is filled with water drops (from steam), let it dry on the dry surface of the hot plate.
 7. Observe the distribution of the metaphases in a microscope.
 8. If the metaphases are not evenly spread on both edges of the slide, dilute the sample. Since the sample is diluted, there is no need to drop firstly the FIX solution. Drop immediately 20 μ L of the MIX.
- Note:** in this work, the sample was diluted 1:1 with FIX solution (100 μ L sample + 100 μ L FIX).

Annex 4 – MCB procedure

Pepsin pre-treatment of slides

1. Pass slides through Ethanol series (70%, 95%, 100%) 3 minutes each.
2. Air dry.
3. Wash 1 min in 1xPBS at RT.
4. For Post-Fixation: incubate for 10 minutes at RT under coverslip with 100 μ L Post-Fix solution (1% Formaldehyde solution).

Note 1: post-fix solution is toxic!

Note 2: 1mL post-fix solution = 500 μ L Paraformaldehyde (2% stock solution) + 450 μ L 1xPBS + 50 μ L 1M MgCl₂ solution.

5. Remove coverslip.
6. Wash for 5 min in 1xPBS at RT and rinse with distilled water multiple times.
7. Pass slides through Ethanol series (70%, 95%, 100%) 3 minutes each.
8. Air dry.

Probe denaturation and pre-hybridization

1. Mix the probes solution for about 5-10min at 37°C on the thermoshaker.
2. For denaturation/pre-hybridization use following program: 85Pre Hyp (5 min at 85°C + 2 min at 4°C + 30 min at 37°C). Choose thick container, 20 μ L and heated lid for the options of type, volume and lid.

Denaturation of slides

For steps 1-6 work at the hotte.

1. Heat hotplate to 72-73°C.

Note: since the lid from the hotte was open, the working temperature of the hotplate was around 80°C to compensate the warm loss from the open lid.

2. Put 100 μ L 70% Formamide on slide and cover with coverslip.
3. Denature for 3 min on the hotplate.
4. Remove coverslip and place the slide in 70% Ethanol at -20°C (previously cold) for 3 min.
5. Pass the slides through Ethanol series (95% and 100%) for 3 min each.

Note: since 70% was already done in the previous step, there is no need to repeat it.

6. Air dry.
7. Add 10 μ L of probe-solution onto the denaturated slide, cover with 24x24 coverslips on the edges and seal with rubber cement (Fixogum, Marabu).
8. Incubate slides overnight at 37°C in a humid chamber.

Post washing – direct washing

1. Take slides out of the humid chamber, remover rubber cement and the coverslips with forceps.
2. Postwash slides for 3-4 min in 0.4xSSC at 64-65°C in a 100mL coplin jar.
3. Put slides in 4xSSC/0.2%Tween (100mL, RT), for 4 min, in the shaker.
4. Block with 100 μ L marvel solution (0.1g Milk + 2mL 4xSSC/0.2%Tween) under coverslip at 37°C for 15 min.
5. Remove coverslip and wash the slide for 1-2 min in 4xSSC/0.2%Tween at RT.
6. Detection with 100 μ L antibody solution for 35-45 min at 37°C.
7. Remove coverslip and put slides in 4xSSC/0.2%Tween (100mL, RT), for 5 min on

shaker. Do this step 3 times, each time with new solution.

8. Put slides in 1xPBS (100mL, RT) for 3-5min. rinse with distilled water.

9. Pass slides through Ethanol series (70%, 95%, 100%), 3 min each.

10. Air dry in dark chamber and cover with 20 μ L of antifade-DAPI-solution (24x60 coverslips).

Annex 5 – MCBs colour schemes

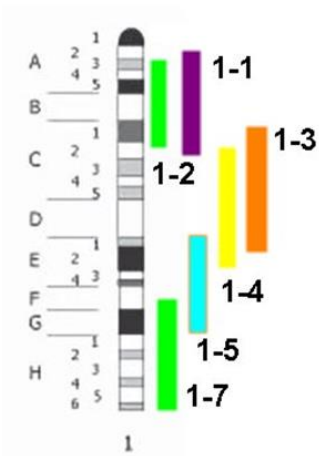


Figure 46 - MMU mcb #1

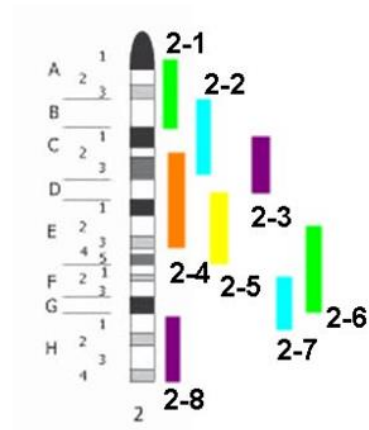


Figure 47 - MMU mcb #2

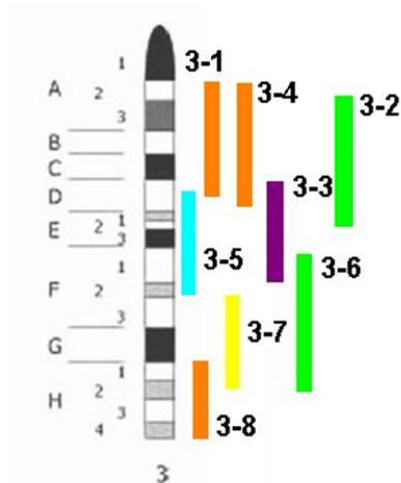


Figure 48 - MMU mcb #3

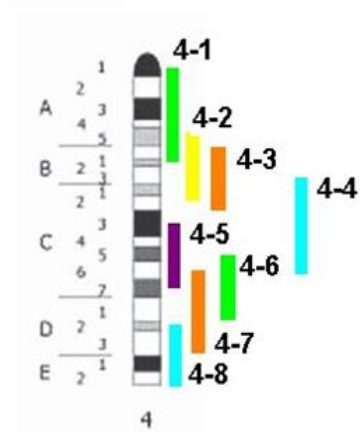


Figure 49 - MMU mcb #4

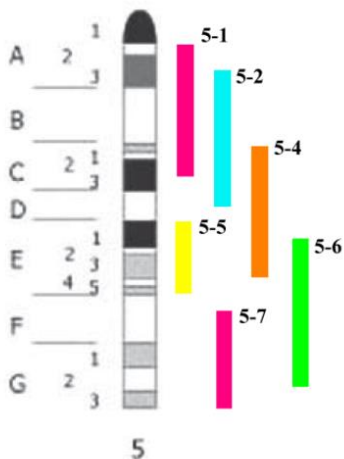


Figure 50 - MMU mcb #5

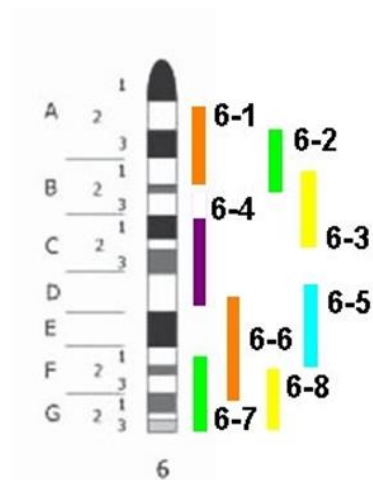


Figure 51 - MMU mcb #6

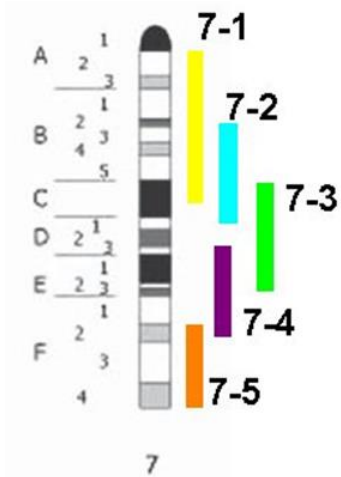


Figure 52 - MMU mcb #7

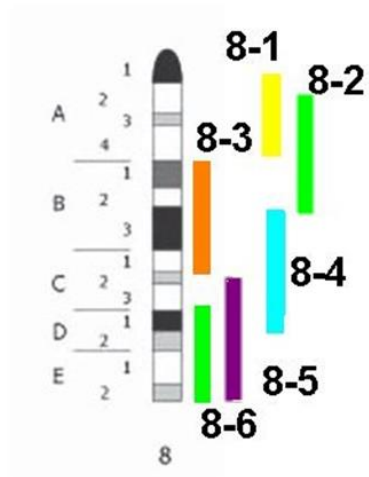


Figure 53 - MMU mcb #8

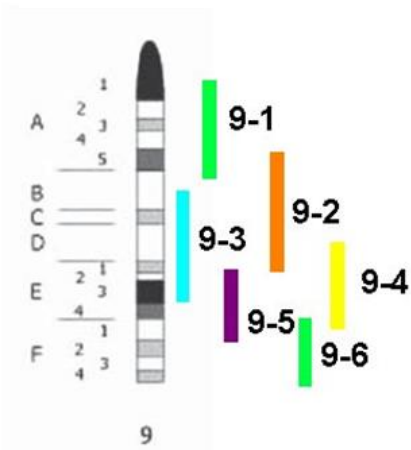


Figure 54 - MMU mcb #9

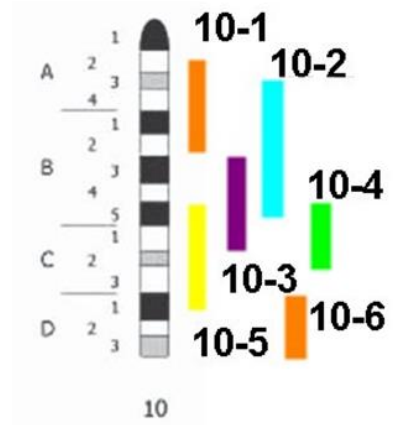


Figure 55 - MMU mcb #10

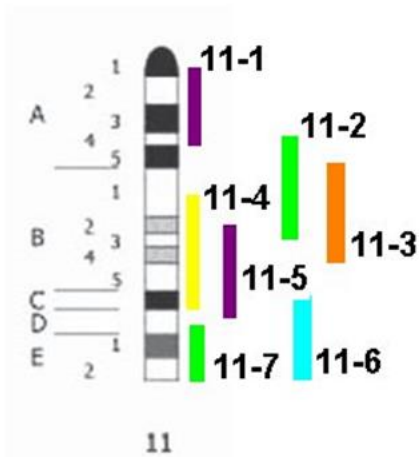


Figure 56 - MMU mcb #11

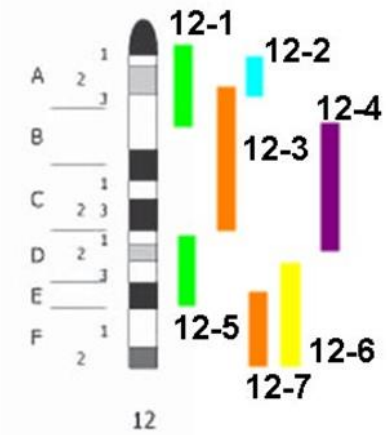


Figure 57 - MMU mcb #12

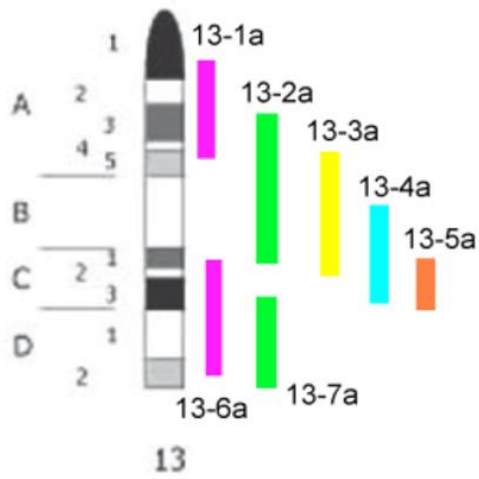


Figure 58 - MMU mcb #13

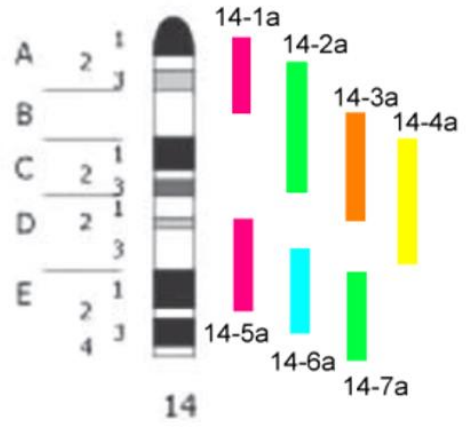


Figure 59 – MMU mcb #14

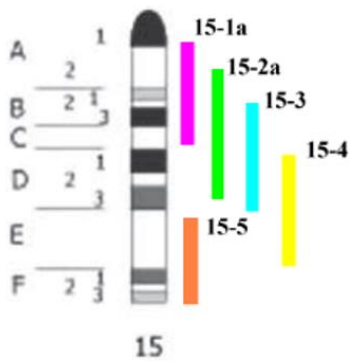


Figure 60 - MMU mcb #15

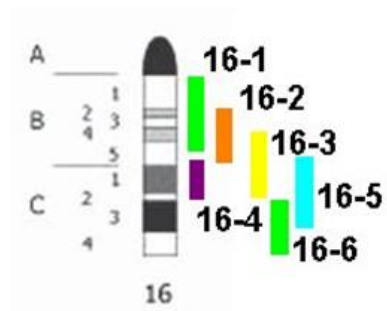


Figure 61 – MMU mcb #16

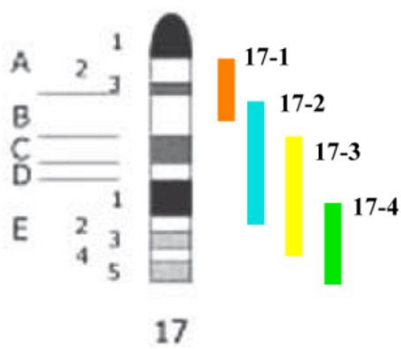


Figure 62 – MMU mcb #17

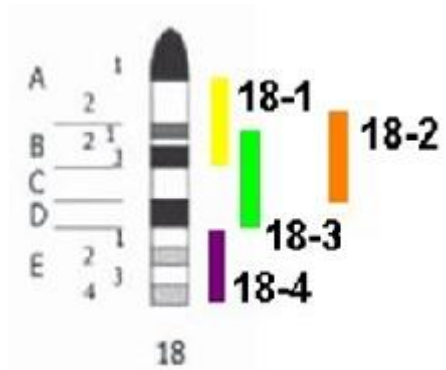


Figure 63 – MMU mcb #18

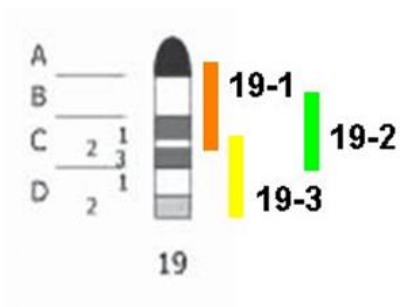


Figure 64 - MMU mcb #19

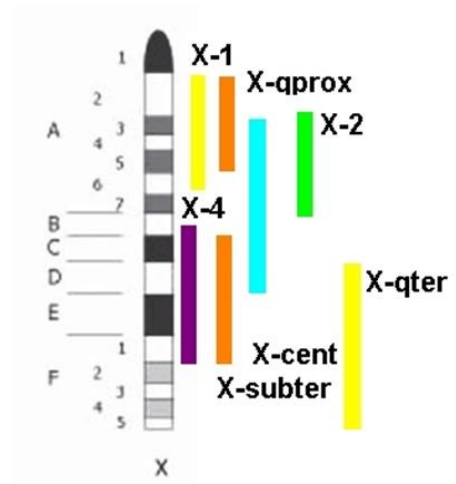


Figure 65 - MMU mcb #X

Annex 6 – SKY single cell aberrations

Annex 6.1: Clone 1 single copy number variations

- +2
- - 2
- +3
- +4
- +6
- +6x2
- +8
- - 9
- +10
- +12
- - 15
- + del (2)

Annex 6.1.1: Subclone 1a single cell aberrations

- del(12)
- der(9)t(9;15)
- der(3;6)

Annex 6.1.2: Subclone 1b single cell copy number variations

- +12
- +6
- - 2

Annex 6.1.3: Subclone 1b single cell aberrations

- idic (6,6)
- del (10)
- del (16)

Annex 6.1.4: Subclone 1c single cell copy number variations

- +2
- +4
- +6
- -1

Annex 6.1.5: Subclone 1c single cell aberrations

- der(6)t(6;1)
- der(1)t(1;6)
- der(4)t(4;8)

- der(17)t(17;15)
- der(8)t(8;2)

Annex 6.1.6: Subclone 1d single cell aberrations

- der(17)t(17;15;8)

Annex 6.2: Clone 2 single cell copy number variations

- + 2
- + 3x2
- + 6x2
- + 6x3
- - 7
- - 11
- +12
- + idic(3;5)
- + del (6)
- + del(6) x2
- + del(2)
- +18

Annex 6.2.1: Subclone 2a single cell aberrations

- der(8)t(8;2)
- der(9)t(9;15)
- der(17)t(17;15)
- del (10)
- der(10)t(10;19)

Annex 6.2.2: Subclone 2b single cell aberrations

- der(8)t(8;2)
- der(17)t(17;15;8)

Annex 6.2.3: Subclone 2c single cell copy number variations

- -18
- +idic(3;5)
- +12
- +3x2
- +17

Annex 6.2.4: Subclone 2c single cell aberrations

- idic (6;6)

Annex 6.3: Clone 3 single cell copy number variations and aberrations

The following copy numbers were found:

- +2
- +6x2
- +7
- +15

The following random aberrations was found:

- del (16)

Annex 6.4: Clone 4 single cell copy number variations and aberrations

The following copy number variations were found:

- -7
- +13
- +15

The following random aberrations were found:

- idic (3;6)
- der(10)t(10;19)

Annex 7 – MCB single cell aberrations

Annex 7.1: Chromosome 2 single aberrations

- One der(?)t(?;2)(?::2A1→2qter) and one der(?)t(?;2)(?::2E5→2F1::2A2→2qter) were found in 1 metaphase.
- Two del(2)(2A1:) were found in 1 metaphase.
- One der(?)t(?;2)(?::2A1→2qter) was found in 1 metaphase.
- One der(?)t(?;2)(?::2C1→2H1~H2:) and one del(2)(:2H3→2qter) were found in 1 metaphase.
- One del(2)(2A1:) and one der(?)t(?;2)(?::2A2→2H1~H2:) were found in 1 metaphase.
- One del(2)(2A1→2B:) and one der(?)t(?;2)(?::2E5→2F1::2A2→2qter) were found in 1 metaphase.
- One del(2)(2A1→2B::2H3→2qter) and one der(?)t(?;2)(?::2C1→2G~H1:) were found in 1 metaphase.
- Two der(?)t(?;2)(?::2C1→2H1~H2:) and one del(2)(2A1→2F3:) were found in 1 metaphase.
- One normal chromosome 2, one del(2)(2A1→2B::2H3→2qter) and one del(2)(2A1→2F3:) were found in 1 metaphase.
- One normal chromosome 2 and one der(?)t(?;2)(?::A1→G~H1:) were found in 1 metaphase.
- One normal chromosome 2, one der(?)t(?;2)(?::2E5→2F1::2A2→2qter), one der(?)t(?;2)(?::2G→2qter) and one del(2)(2A1→2F3:) were found in 1 metaphase.
- One normal chromosome 2 and one der(?)t(?;2)(?::2A1→2qter) were found in 1 metaphase.
- One normal chromosome 2, one del(2)(2A1::2qter) and one del(2)(2A1→2F3:) were found in 1 metaphase.
- Two del(2)(2A1→2B:) were found in 1 metaphase.
- One del(2)(2A1:), one del(2)(2A1→2F3:) and one der(?)t(?;2)(?::2E5→2F1::2A2→2qter) were found in 1 metaphase.
- One der(?)t(?;2)(?::2A2→2qter), one der(?)t(?;2)(?::2A1→2qter) and one del(2)(2A1→2F3:) were found in 1 metaphase.
- One der(?)t(?;2)(?::2E5→2F1::2A2→2qter) and one der(?)t(?;2)(?::A2→2qter) were found in 1 metaphase.

- One der(?) (?:2A1→2qter), one del(2)(2A1:) and one del(2)(2A1→2F3:) were found in 1 metaphase.

Annex 7.2: Chromosome 4 single aberrations

- One der(4)t(4;?)(:4C2~C3→4qter::?) and one idic(?;4)(?:cen:4A5→4C6:) were found in 1 metaphase.
- One del(4)(4A1→4C1~C2:) and one del(4)(4A1→4A5::4qter) were found in 1 metaphase.
- One del(4)(4A1→4A5::4qter) and one idic(?;4)(?:cen:4C2~C3→qter::?) were found in 1 metaphase.
- One der(4)t(4;?)(:4C2~C3→4qter::?), one der(?)t(?;4)(?:4D1→4C3:) and one del(4)(4A1→4A5::4qter) were found in 1 metaphase.
- One del(4)(4A1→A5::4qter) was found in 1 metaphase.
- One del(4)(4A1→4A5::4D1→4qter) was found in 1 metaphase

Annex 7.3: Chromosome 5 single aberrations

- One idic(?;5)(?:cen:5A1→5qter) and one del(5)(5A1→5A2:) were found in 1 metaphase.
- Two idic(?;5)(?:cen:5A1→5F:) and one normal chromosome 5 were found in 1 metaphase.
- One idic(?;5)(?:cen:5A1→5qter) and one idic(5;5)(:5F→5A1:cen:5A3→5F:) were found in 1 metaphase.
- One normal chromosome 5 was found in 1 metaphase.

Annex 7.4: Chromosome 6 single aberrations

- One der(6)t(6;?)(6A1→6C2::?), two der(?)t(?;6)(?:6E→6qter), one del(6)(6A1→6D::6F2→6qter) and one normal chromosome 6 were found in 1 metaphase.
- One der(6)t(6;?)(6A1→6C2::?), one der(?)t(?;6)(?:6E→6qter), one del(6)(2A1→2E:), one inv(6)(:6F3→6F1:) and one normal chromosome 6 were found in 1 metaphase.
- One del(6)(6C3→6qter), one del(6)(6A1→6D:), one del(6)(6A1→6D::6F2→6qter), one del(6)(6F2→6qter:) were found in 1 metaphase.

- One der(6)t(6;?)(6A1→6C2::?), two del(6)(6A1→6E::6F3→6qter) and one normal chromosome 6 were found in 1 metaphase.
- One der(6)t(6;?)(6A1→6C2::?), one del(6)(6A1→6D:) and one normal chromosome 6 were found in 1 metaphase.
- One idic(6;6)(qtr→6A1:cen:6A3→6F3:), one der(6)t(6;?)(6A1→6C2::?), one del(6)(6A1→6D:) and one inv(6)(:6F3→6F1:) were found in 1 metaphase.
- One der(6)t(6;?)(6A1→6C2::?), one der(?)t(?;6)(?::6E→6qter), one del(6)(6A1→6D:), one del(6)(6A1→6E::6F3→6qter) and one normal chromosome 6 were found in 1 metaphase.
- One der(6)t(6;?)(6A1→6C2::?), one der(?)t(?;6)(?::6E→6qter), one del(6)(6A1→6D:) and one normal chromosome 6 were found in 1 metaphase.
- One der(6)t(6;?)(6A1→6C2::?), one del(6)(6A1→6D:), one inv(6)(:6F3→6F1:) and one normal chromosome 6 were found in 1 metaphase.
- One idic(6;6)(qtr→6A1:cen:6A1→6qter:), one der(6)t(6;?)(6A1→6C2::?), one del(6)(6A1→6D:), one del(6)(6A1→6C3::6F1→6qter) were found in 1 metaphase.
- One der(6)t(6;?)(6A1→6C2::?), one der(?)t(?;6)(?::6E→6qter), one del(6)(6A1→6D:), one del(6)(6A1→6D::6F2→6qter) and one normal chromosome 6 were found in 1 metaphase.
- One der(6)t(6;?)(6A1→6C2::?), one del(6)(6A1→6D:), one del(6)(6A1→6E~F1:) and one normal chromosome 6 were found in 1 metaphase.
- One der(6)t(6;?)(6A1→6C2::?), one der(?)t(?;6)(?::6E→6qter), one del(6)(6A1→6D:), one del(6)(6A1→6D::6F2→6qter) and one normal chromosome 6 were found in 1 metaphase.
- One del(6)(:6C3→6qter), one del(6)(6A1→6D:), one del(6)(6A1→6D::6F2→6qter), one del(6)(6A1→6E::6G1→6qter) and one normal chromosome 6 were found in 1 metaphase.
- One der(6)t(6;?)(6A1→6C2::?), one der(?)t(?;6)(?::6D→6qter), one del(6)(6A1→6C3:), one del(6)(6A1→6C3::6F1→6qter) and one normal chromosome 6 were found in 1 metaphase.
- Two der(6)t(6;?)(6A1→6C2::?), one del(6)(6A1→6D:) and one normal chromosome 6 were found in 1 metaphase.
- One der(6)t(6;?)(6A1→6C2::?), one del(6)(6A1→6D:), one del(6)(6A1→6D::6F2→6qter), one del(6)(6A1→6E~F1:) and two normal chromosome 6 were found in 1 metaphase.

- One der(6)t(6;?)(6A1→6C2::?), one der(?)t(?;6)(?::6E→6qter), one del(6)(6A1→6D:), one del(6)(6A1→6D::6F2→6qter) and one normal chromosome 6 were found in 1 metaphase.
- One der(6)t(6;?)(6A1→6C2::?), one del(6)(6A1→6E~F1:), one del(6)(6A1→6D:), one del(6)(6A1→6E::6G1→6qter), one inv(6)(:6F3→6F1:) and one normal chromosome 6 were found in 1 metaphase.

Annex 7.5: Chromosome 7 single aberrations

- One normal chromosome, one der(7)t(7;?)(7A1→7qter::?), and one der(7)t(7;?)(:7E1→7F2::?) were found in 1 metaphase.
- One normal chromosome, one der(7)t(7;?)(7A1→7D1::?), and one del(7)(7A1→7D3:) were found in 1 metaphase.
- One normal chromosome and one del(7)(7A1→7B4::7F3→7qter) were found in 1 metaphase.
- One normal chromosome and one del(7)(7A1→7B4:) were found in 1 metaphase.

Annex 7.6: Chromosome 8 single aberrations

- One normal chromosome, one der(?)t(?;8)(?::8C3→8D1:) and one inv(8qter→8A1) were found in 3 metaphases.
- One der(8)t(8;?)(8A1→8D1::?), and one inv(:8D1→8A1) were found in 3 metaphases. One del(8)(:8D2→8qter), one der(?)t(?;8)(?::8C3→8D1:) and one inv(8qtr→8A1) were found in 2 metaphases.
- One del(8)(:8D2→8qter), and one inv(8qter→8A1) were found in 2 metaphases.
- One der(8)t(8;?)(8A1→8D1::?), and one inv(8qter→8A1) were found in 1 metaphase.
- One der(8)t(8;?)(8A1→8qter::?), one der(?)t(?;8)(?::8C3→8D1:) and one inv(:8D1→8A1) were found in 1 metaphase.
- One der(8)t(8;?)(8A1→8D1::?), one der(?)t(?;8)(?::8C3→8D1:) and one inv(8qter→8A1) were found in 1 metaphase.

Annex 7.7: Chromosome 9 single aberrations

- One del(9)(9A1→9A2::9F2~F3→9qter) and one der(9)t(9;?)(:9A3→9qter::?) were found in 2 metaphases.
- One del(9)(9A1→9A2::9F2~F3→9qter) and one del(9)(9A1→9A2:) were found in 1 metaphase.

- One der(9)t(9;?)(:9A3→9qter::?) and one del(9)(:9F2~F3→9qter) were found in 1 metaphase.
- One normal chromosome 9 and one del(9)(9A1→9A2::9F2~F3→9qter) were found in 1 metaphase.

Annex 7.8: Chromosome 10 single aberrations

- One normal chromosome 10 and one der(?)t(?;10)(?:10D2→10D3:) were found in 1 metaphase.
- Two normal chromosomes 10, one der(?)t(?;10)(?:10D2→10D3:) and one del(10)(10A1→10A2:) were found in 1 metaphase.

Annex 7.9: Chromosome 11 single aberrations

- One normal chromosome 11 and one del(11)(:11E1→11qter) were found in 2 metaphases.
- One del(11)(11A1→11A4:) was found in 2 metaphases.
- One normal chromosome 11 and one del(11)(11A1→11A3:) were found in 2 metaphases.
- One del(11)(11A1→11A4:) and one del(11)(11A1→11A5::11E1→11qter) were found in 2 metaphases.
- One normal chromosome 11 and one del (11)(11A1→11A2::11E1→11qter) were found in 2 metaphases.
- One del(11)(11A1→11A3::11E1→11qter) and one del(11)(11A1→11A3:) were found in 2 metaphases.
- One normal chromosome 11 was found in 2 metaphases.
- One normal chromosome 11 and one del(11)(11A1→11A5:) were found in one metaphase.
- Two del(11)(11A1→11A3:) were found in one metaphase.
- One del(11)(11A1→11A3:) and one del(11)(:11E1→11qter) were found in one metaphase.
- One del(11)(11A1→11A3:) and one del(11)(11A1→11B1::11E1→11qter) were found in one metaphase.
- One del (11)(11A1→11A4::11E1→11qter) and one del(11E1→11qter) were found in one metaphase.

Annex 7.10: Chromosome 13 single aberrations

- One del(13)(:13D2) was found in 2 metaphases.
- Two inv(13)(13qtr→13D1:) were found in 2 metaphases.
- One inv(13)(13qtr→13D1:) and one del(13)(:13D2) were found in 1 metaphase.
- One inv(13)(13qtr→13D1:) was found in 1 metaphase.
- One normal chromosome 13 was found in 1 metaphase.
- Two normal chromosomes 13 and one del(13)(13A1→13B:) were found in 1 metaphase.

Annex 7.11: Chromosome 14 single aberrations

- One der(?)t(?;14)(?:14D3→14qter), one der(?)t(?;14)(?:14D3→14C2~C3:) and two del(14)(:14D2→14qter) were found in 2 metaphases.
- One der(?)t(?;14)(?:14B1→14D1:) and one del(14)(:14D2→14qter) were found in 1 metaphase.
- One der(?)t(?;14)(?:14A1→14D1:), one der(?)t(?;14)(?:14D1→14qter) and two del(14)(:14D2→14qter) were found in 1 metaphase.
- One der(?)t(?;14)(?:14D1→14qter), one der(?)t(?;14)(?:14D3→14C2~C3:), one der(14)t(14;?)(:14D3→14C2~C3::?) and one del(14)(:14D2→14qter) were found in 1 metaphase.
- One der(?)t(?;14)(?:14D1→14qter), one der(?)t(?;14)(?:14D3→14C2~C3:), one del(14)(14A1→14C3:) and two del(14)(:14D2→14qter) were found in 1 metaphase.
- One der(?)t(?;14)(?:14D1→14qter), one der(?)t(?;14)(?:14D3→14C2~C3:) and two del(14)(:14D2→14qter) were found in 1 metaphase.

Annex 7.12: Chromosome 15 single aberrations

- Two normal chromosomes 15 and two der(?)t(?;15)(?:15D1→15D3:) were found in 3 metaphases.
- Two normal chromosomes 15, one der(?)t(?;15)(?:15D1→15D3:) and one der(?)t(?;15;?)(?:15D1→15D3::?) were found in 1 metaphase.
- One normal chromosome 15, one der(?)t(?;15;?)(?:15D1→15D3::?), one der(15)(:15qtr→15D1::15F1→15qter) and one der(15)t(15;?)(:15E→15D1::?) were found in 1 metaphase.
- One normal chromosome 15 and two der(?)t(?;15;?)(?:15D1→15D3::?) were found in 1 metaphase.

Annex 7.13: Chromosome 16 single aberrations

- One normal chromosome 16 were found in 2 metaphases.
- Three normal chromosomes 16 were found in 1 metaphase.

Annex 7.14: Chromosome 17 single aberrations

- One normal chromosome 17 and one der(?)t(?;17)(?::17E1→17E2:) were found in 1 metaphase.
- One normal chromosome 17, one der(?)t(?;17)(?::17E1→17E2:), one del(17)(:17C~D→17qter) and one del(17)(17A1→17A3::17E1→17qter) were found in 1 metaphase.

Annex 7.15: Chromosome 18 single aberrations

- One der(18)t(18;?)(18A→18qtr::?) was found in 1 metaphase

Annex 7.16: Chromosome 19 single aberrations

- One normal chromosome 19, one der(?)t(?;19)(?::19D1→19qter) and one der(?)t(?;19;?)(?::19D1→19qter::?) were found in 1 metaphases.
- Two normal chromosomes 19 and one der(?)t(?;19;?)(?::19D1→19qter) were found in 1 metaphases.

Annex 7.17: Chromosome X single aberrations

- One der(X)t(X;?)(XA1→XA6::?), one del(X)(XA1→XA2::XE→Xqter) and one del(X)(XA1→XA6::XC→Xqter) and one der(?)t(?;X)(?::XA7→XB:) were found in 2 metaphases.
- One der(X)t(XA1→XA6::?), two del(X)(XA1→XA6::XE→Xqter) and two del(X)(XA1→XA6::XC→Xqter) were found in 1 metaphase.

Annex 8 – Comparison of results obtained by different methods

Table 2 – Review of all results from all methods

Chromosome	M-FISH/SKY	Mcb	aCGH
2	All clones had at least one del(2) Clone 1 had one extra #2 Subclone 2c and 2d and Clones 3,4 and 5 had one derivative chromosome with 8 + 2	73% metaphases with A1 deleted 40% metaphases had at least one extra E5→F1	del(A1) amplf (E5→F1)
3	All clones had idic(3;5) No deletion was detected	All metaphases with idic (? :cen:A1→qtr) 93% metaphases with del(A1→A2)	amplf A3→H4
4	All clones had two #4 All clones had a derivative chromosome 4+15 Subclones 1b, 1c and 2c had one derivative chromosome 17+15+4	90% metaphases with der(C2-3→qtr::?) 20% metaphases had a breakpoint at cytoband D1 No terminal deletion detected	No significant amplifications or deletions detected. Possible breakpoints in C3, D1 and E2
5	All clones had one missing #5	No results can justify the amplification found in aCGH	amplf C1→C3.2
6	All clones had at least one extra #6 All clones had one derivative chromosome 6	93% metaphases with der(A1→C2::?) 80% metaphases with del(A1→D) 60% metaphases with del(A1→D::F2→qtr)	amplf A1→A3.1 del G1→G3 amplf D2→E1

Table 2 – Review of all results from all methods (cont.)

Chromosome	M-FISH/SKY	Mcb	aCGH
7	All clones except 2c had two #7 All clones except 2c and 5 had one derivative chromosome 7+17	70% metaphases with a der (7A1→qtr::?)	No major amplifications or deletions found
8	All clones had one extra #8 All clones had one derivative chromosome with 8 All clones except 5 had one derivative chromosome with X + 8	66,7% metafases with Inv(D1→A1) 33,3% metafases with Inv(qtr→A1) 70% metaphases with der(A1→D1::?) 80% metaphases with der(?::C3→D1)	del E1 amplf A4→B1 amplf C3→D1
9	All clones had always 2 chromosomes Subclone 1d had one derivative chromosome 9+15	73,3% metaphases with del(F2-3→qtr) 30% metaphases with del(A1→A2::F2-3→qtr)	amplf A3→B
10	All clones had 2 chromosomes Clones 1 had a derivative chromosome with terminal 10	93% metaphases with: der(?::D2→D3) del(A1→A2)	amplf D1→D2 del A1→A4
11	All clones, except for one metaphase of 2a, had 2 chromosomes	27% metaphases with del(A1→A4) 20% metaphases with del(A1→A3::E1→qtr) No metaphases had one extra or one less chromosome	amplf D→E2
12	All clones had 2 chromosomes	All metaphases with der(A1→C2::F1→qtr)	No significant amplifications or deletions were found
13	All clones had 2 chromosomes	60% metaphases with del(D2)	No significant amplifications or deletions found
14	All clones had 2 chromosomes All clones had at least one derivative of another chromosome with 14	All metaphases had del(D2→qtr) 57% metapgases with der(?::A1→D1) 90% metaphases with der(?::D3→C3)	del(D2→E5) amplf(A1→B) amplf(B→C1)

Table 2 – Review of all results from all methods (cont.)

Chromosome	M-FISH/SKY	mcb	aCGH
15	All clones had 2 chromosomes All clones had at least one derivative chromosome with 15	57% metaphases with der(?::D1→D3) 73% metaphases with der(?::D1→D3::?)	No major amplifications or deletions found
16	All clones had 2 chromosomes	90% metaphases had two normal chromosomes	No major amplifications or deletions found
17	All clones had always 2 chromosomes All clones had one derivative chromosome 17+.. All clones except 5 had one derivative 7+17	der(E1→E2) del(C~D→qtr)	amplf B3→E2
18	No chromosomes 18 were observed	97% metaphases had: der(?::A1→qtr) der(A1→qtr:?)	amplf(B1→E3) del(E3→E4)
19	All clones had 2 chromosomes No derivatives with 19 where found	93% metaphases had: der(?::D1→qtr) der(?::D1→qtr::?)	No significant amplifications or deletions were found
X	All clones except 5 had 2 chromosomes All clones except 5 had one derivative X+8	der(A1→A6::?) del(A1→A6::E→qtr) del(A1→A6::C→qtr)	del(E1→E3)