

# Chapter 23

## G-Banding: Fetal Chromosome Analysis by Using Chromosome Banding Techniques



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**Abstract** Clinical application of Next Generation Sequencing (NGS) technology is progressing. In the field of cytogenetics, NGS is used for noninvasive prenatal testing (NIPT) and preimplantation genetic testing for aneuploidy (PGT-A). Also, chromosomal microarray (CMA) testing is routinely performed in postnatal as chromosome testing for the patients with developmental delay, and in prenatal, it is widely used as testing for multiple fetal anomalies. Although prenatal screening for fetal aneuploidies by NIPT using cell-free DNA is gradually becoming more common, the standard for prenatal diagnostic testing is karyotype analysis by G-banding at this moment. This chapter outlines the necessary process of fetal chromosome analysis by G-banding, the features of other banding techniques, and points to consider in the interpretation issues regarding frequently encountered aneuploidy mosaic and structural variations such as chromosomal heteromorphisms.

**Keywords** Prenatal diagnosis · G-banding · Aneuploidy · Mosaicism  
Heteromorphism

The number of human chromosomes was determined in 1956, and the success of the culture of peripheral blood mononuclear cells with the addition of PHA was achieved in 1960, followed by the success of the culture of amniotic fluid cells in 1966 and placental villus cells in 1974. Chromosome analysis was established as clinical testing in the mid-1970s. Since then, various technological innovations occurred in the cytogenetics and cytogenomics field, and clinical applications have promoted in order. Among the various cytogenomic technologies, karyotyping by chromosome banding, fluorescence in situ hybridization (FISH), multiplex ligation-dependent probe amplification (MLPA), the chromosomal microarray (CMA), and the noninvasive prenatal testing (NIPT) are categorized as cytogenetic testing.

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309

In the prenatal diagnosis, the gold standard for the diagnosis of fetal chromosome abnormalities is karyotype analysis of G-banded cells, which were harvested after the cultivation of amniotic fluid, chorionic villus, and fetal umbilical cord blood cells.

CMA is used as the first choice only when multiple fetal morphological abnormalities are detected by ultrasound [1]. In the cytogenetic analysis, we need to choose a test method according to the size of the variant to be detected. Most prenatal diagnostic tests focus on chromosomal aneuploidies, and so cytogenetic testing by G-banding is an appropriate choice at this time.

### **23.1 The Procedure of the Chromosome Analysis by G-banding**

The chromosome analysis is usually started by appropriately culturing each of the aseptically collected materials. Please refer to other documents for the sampling of amniotic fluid, chorionic villi, umbilical cord blood, and precautions for that primary culture [2–4].

In the cell culture of umbilical cord puncture blood, phytohemagglutinin (PHA) is added in the same way as germline cytogenetic testing using peripheral blood lymphocytes to induce cell division, so that mitotic cell harvesting becomes possible in the short term of 48 or 72 h.

The cell culture for the chromosome analysis of amniotic fluid cells and chorionic villus cells is classified into an *in situ* method (directly harvesting the grown cell colonies) and a flask method (trypsinizing the grown cells and collecting them as free cells). In prenatal diagnosis, it is necessary to perform karyotyping by *in situ* cell culture and harvesting, which excels in discriminating between true mosaics and pseudo mosaics, except when performing molecular genetic diagnosis using DNA or biochemical genetic diagnosis via cell culture.

When amniotic fluid cells and chorionic villus cells are cultured by the *in situ* method, usually, it takes about 10 days until they are proliferated enough to harvest. The general procedure for testing amniotic fluid cells and chorionic villus cells is as follows.

Cell harvest:

1. To accumulate mitotic cells at the metaphase stage, a microtubule formation inhibitor, colcemid, is added and treated for several hours before harvest.
2. The culture medium in the container is put in the coverslips where the cells that have proliferated are removed by suction, and a hypotonic solution (75 mM KCl) is added to expose the cells, followed by treatment for 20 min.
3. Inject Carnoy's fixative (3:1 mixture of alcohol and acetic acid). While slowly injecting the fixative, the mixture of the hypotonic solution and Carnoy's solu-

**Fig. 23.1** A G-banded metaphase spread of the trisomy 21 cell. Band resolution is about 500 bands/haploid set. Arrowheads indicate chromosome 21



tion is removed by suction, and the concentration of Carnoy's solution is increased stepwise for fixation.

4. After completely replacing the hypotonic solution with Carnoy's solution, remove the coverslip with forceps, carefully absorb excess Carnoy's droplets with filter paper, and slowly air dry to make chromosome preparation (press onto glass surface with surface tension).
5. Perform solid Giemsa staining and G-banding (Fig. 23.1).

Chromosome analysis:

Regarding images of a metaphase spread taken under an image analysis system or a microscope are analyzed by following procedures.

1. Count the chromosome number of a minimum of 15 cells from at least 15 colonies, distributed as equally as possible between at least two or more independently established cultures.
2. Analyze five cells, each from a different colony, preferably from two independently established cultures.
3. Karyotype 2 cells, these cells can be from the analyzed five cells. If more than one abnormal cell line is found, karyotype is at least one cell representative of each cell line.

When using fresh chorionic villus cells, mitotic cells can be harvested by short-term culture (direct method), but it is difficult to obtain a morphologically well-metaphase. Therefore, in principle, the results of karyotyping must be obtained by long-term cultured cells [5].

## 23.2 Chromosome Banding

The chromosome banding is a general term for a method of performing various processes to making a chromosome preparation, displaying striped patterns (bands and sub-bands) on the chromosome. The primary method of chromosome banding is the G-banding, which is simple to operate and provides a clear staining image. The chromosomes are grouped by relative size and shape (depending on the position of constriction of kinetochore region), and band patterns identify homologous chromosomes and then are compared with each of the homologous chromosomes.

The number of appearing bands determines the resolution, and the required resolution depends on the reason for the referral of the testing. The principle of the G-banding depends on the resistance to the digestion of nonhistone proteins to proteases such as trypsin so that the difference in chromatin condensation is detected as the difference of Giemsa staining (Table 23.1).

According to the latest International system for Human Cytogenomic Nomenclature (ISCN2016) [6], idiograms (schematic diagrams of normal karyotypes by G-banding) are expressed in 300, 400, 550, 700, and 850 bands per haploid set. In the report of the result of chromosome analysis, it must be specified, which band level of the test is performed. Chromosome analysis for the prenatal diagnosis requires a minimum of 400 bands for advanced maternal age and positive screening cases and a minimum of 500 bands for fetal morphological abnormalities.

When XX cells were found to be mixed in XY cells, the analysis should be performed on XY cells, considering that XX cells were caused by maternal tissue contamination. However, karyotype analysis of a small number of cells is required for XX cells for confirmation purposes. The attending physician consults with the laboratory staff and considers whether or not maternal tissue contamination was detected at the time of cell culture. If XY/XX cells were detected in CVS, additional tests using amniotic fluid cells should be considered. If XY/XX cells were detected in amniotic fluid cells, it is necessary to confirm the fetal genitalia by ultrasonography.

**Table 23.1** Features of G-banding

Official name	Principal reagent	Staining pattern	Structural features	Features of the nonhistone protein
G-bands by trypsin using Giemsa (GTG)	Trypsin, Giemsa	G-dark band	Heterochromatic region, A-T rich, late S-phase replication	Tightly condensed region, rich in protein disulfide cross-links hydrophobic regions that have a high affinity with eosin and thiazine.
		G-light band	Euchromatic region, G-C rich, early S-phase replication	Relatively loose structure, rich in protein sulfur as sulfhydryls less hydrophobic region that does not have a affinity with eosin and thiazine.

In addition to routine G-banding, various banding techniques such as Q-, R-, C-, and N-banding and Alu I-digested C-like banding are used to identify specific chromosome variants and/or abnormalities (Table 23.2), [6]. Based on the results of the G-banding, other banding techniques and FISH are added as necessary. Interpretation and cytogenetic diagnoses are made based on the results of those obtained.

**Table 23.2** Features and applications of various banding techniques

Banding (idiom)	Official name	Principal reagent(s)	Features/application/target
Q-	Q-bands by fluorescence using quinacrine (QFQ)	Quinacrine mustard	Specific staining of A-T bases pairs, similar pattern as G-banding Yq12, satellites, centromeric heterochromatin
R-	R-bands by BrdU using Giemsa (RBG) R-bands by BrdU using acridine orange (RBA)	5-Bromodeoxyuridine (BrdU), Hoechst 33258, Giemsa, Acridine orange	The addition of BrdU before harvest to incorporate BrdU into late-replication region. R-bands (inversion of the G-bands) appears by the sequential staining with Giemsa or acridine orange after staining with Hoechst 33258, which has a strong affinity to BrdU.
N-	—	Silver nitrate, Giemsa	Silver staining to the region of satellite stalk of acrocentric chromosomes correspond to Nucleolus Organizing Region (NOR)
C-	C-bands by barium-Hydroxide using Giemsa (CBG)	Barium hydroxide, Giemsa	Specific staining of heterochromatin brocks (1q12, 9q12, 16q11.2, Yq12)
Alu I digested C-like-	—	Restriction enzyme Alu I, Giemsa	Frequent digestion of euchromatin with the enzyme brings staining of heterochromatin brocks (1q12, 9q12, 16q11.2, Yq12)
Sister chromatid exchange (SCE)	—	BrdU, Hoechst 33258, Giemsa	The addition of BrdU to incorporate for two cell cycles to bring identification of each chromatid. It allows detecting sister chromatid exchange. Diagnostic testing for Bloom syndrome.
High resolution	—	Ethidium Bromide	The addition of ethidium bromide to inhibit chromosome condensation brings harvesting the cells at prophase to prometaphase stage. Thymidine synchronization of cell division frequently used together. It used for the detection or identification of the breakpoints of rearrangement.

### 23.3 Precise Investigation of Mosaicism and Points of That Interpretation

Anaphase lags frequently occur at the very early stage of embryogenesis so that fetal chromosomal mosaicism becomes not rare [7]. On the other hand, growth factors are fundamentally added to the culture medium used for cell culture of fetal tissue, which enhances cell proliferation while increasing the possibility of generating aneuploidies that do not exist in the original cells (artifacts). Therefore, additional workup needs to be carefully and rationally performed on the mosaic detected in the prenatal chromosome testing, to determine whether the mosaic is a true or a pseudo mosaic.

Mosaics detected by the in situ culture method are classified as follows, and the level 3 mosaic is determined to be a true mosaic.

- Level 1 mosaic: detected in only one cell in one colony or only in a part of one colony
- Level 2 mosaic: detected in all cells in one colony
- Level 3 mosaic: commonly detected in multiple colonies in multiple cultureware

Workups proposed by Hsu and Benn are widely used in the additional mosaic analysis for cultured amniotic fluid cells and chorionic villus cells [8].

Report of the test result should mention what level of the mosaic is determined as a result of the additional workups. The attending physician would be expected to interpret the results appropriately and explain it precisely to the couple.

In the culture of chorionic villus cells, mosaic confined to placental tissue is observed to be about 2%. Depending on the developmental stage of the mosaic that was arisen, trophoblast cells and villous stromal fibroblasts may have different detection patterns and classified into the following types of confined placental mosaicism (CPM).

Type I CPM:

Limited to trophoblast cells. Detected by the direct method (short-term culture), but not detected in fibroblasts derived from the villous stroma (with long-term culture).

Type 2 CPM:

Limited to fibroblasts derived from the villous stroma, not detected in trophoblast cells.

Type 3 CPM:

Detected in both trophoblast cells and fibroblasts derived from the villous stroma.

Since CPM cannot be definite at the step of villus cell examination alone, when mosaicism is detected in villous cells, it is necessary to confirm it with the examination of amniotic fluid cells.

### 23.4 Points to Care in the Interpretation of Chromosomal Aberrations

When mosaic aneuploidy is detected, it is necessary to examine the fetal structural abnormalities by ultrasonographic examination precisely. Even if a Level 3 mosaic is detected in the culture of chorionic villi or amniotic fluid cells, it might not be detected in the somatic cells of the fetus. Depending on what chromosome is detected as mosaic, the empirical risk of true mosaicism on the fetal somatic cells and the presence of disease complications might have varying degrees, so that the reexamination by invasive sampling should be carefully considered.

Benn carefully reviewed the pregnancy outcomes, and the results of confirmatory testing of the aneuploid mosaic are detected in the amniotic fluid cells [4]. About autosomal aneuploidy mosaic: In CVS, the mosaic containing chromosomes 2, 3, and 7 is common, but is very unlikely to be confirmed by amniocentesis. Mosaics containing chromosomes 8, 9, 18, and 21 are infrequent but are often confirmed by amniocentesis. In Amniotic fluid cells, it is recommended to take into account empirical risks. Mosaic trisomies 2, 4, 9, and 16 have very high risk (>60%) of abnormal consequences; mosaic trisomies 5, 13, 14, 15, 18, and 21 have high risk (40–59%); mosaic trisomies 6, 7, 12, and 17 have moderately high risk (20–39%). Except for mosaic trisomies 8, 9, 13, 18, and 21, additional confirmation by PUBS is not recommended. If a mosaic of chromosomes with imprinting effects (chromosome 6, 7, 11, 14, 15, and 20) is detected, genetic testing of uniparental disomy (UPD) by DNA polymorphism analysis with parental samples is recommended. About sex chromosome aneuploidy mosaic: Mosaic of sex chromosomal aneuploidy is detected at a higher rate than autosomal abnormalities, and almost no abnormalities are observed at birth. For 45, X/46, XY mosaics, it is necessary to confirm the sex of the fetus by ultrasonography and to examine the presence or absence of internal genitals at birth. Long-term prognosis is unknown in many prenatally diagnosed cases.

Caution should be exercised when Robertson translocation is detected during prenatal testing. One-third of the Robertson translocation detected through prenatal testing is known as de novo origin, but when Robertson translocation of the nonhomologous chromosome is detected, the risk with UPD is estimated at 0.6% even if it is de novo or not. The risk with UPD of Robertson translocation of the homologous chromosome (isochromosome) is estimated at 66% [9]. It is necessary to consider the examination of diagnostic testing of UPD when the Robertson translocation involves chromosome 14 or 15 [10].

When a structural chromosome abnormality is detected, whether it is a balanced type or not becomes a problem, and it is necessary to identify breakpoints as precise as possible. However, it is not easy to identify the breakpoints precisely by the proband's G-banding alone. So it is crucial to obtain results in a short period by adding a chromosome analysis of the parents and/or FISH analysis by using appropriate DNA probe such as subtelomeric clones. Sometimes, a confusion might occur in the case identified with de novo morphologically/or apparently balanced structural

rearrangement. Additional CMA testing will consider clarifying whether the rearrangement accompanied a genomic deletion or not. Prenatal detection of small supernumerary marker chromosomes (sSMCs) is occasionally detected [11]. sSMC is often derived from acrocentric chromosomes, especially chromosome 15, and a stepwise workup is needed to perform in consideration of the parental origin, which also has the possibility that intact homologous chromosomes 14 and 15 might be UPD [12].

The additional workups consist of chromosome analysis of the parents, FISH, CMA, and UPD tests that need to use DNA extracted from not only fetal tissue but also the parents [13]. It is necessary to proceed while confirming the turnaround time of this testing strictly.

### 23.5 Normal Chromosome Variants

Normal chromosome variants are morphological abnormalities that are manifested by the chromosome banding and do not affect phenotype or reproduction. It is divided into heteromorphism (Table 23.3) and euchromatic variant [14]. These variants are inherited from one of the parents in principle.

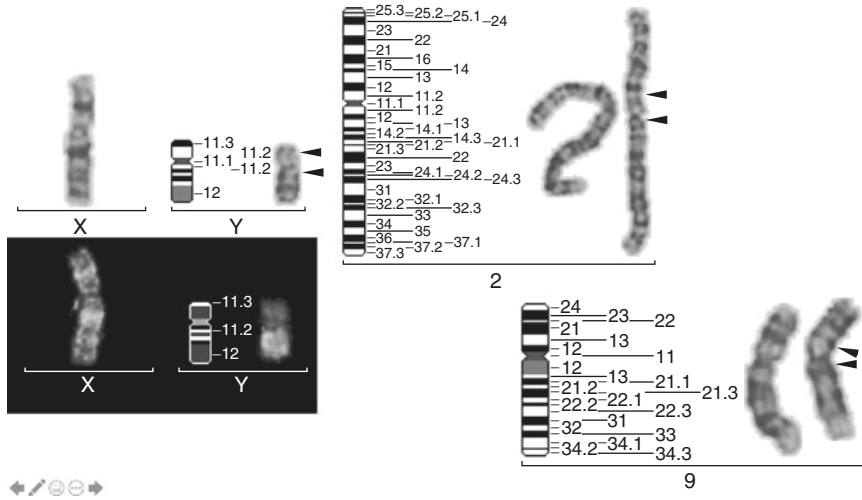
Heteromorphism is the size diversity and pericentric inversion of highly condensed constitutive heterochromatin. The most frequently observed one is *inv(9)(p12q13)* that is detected in about 2% of the general population (Fig. 23.2). Euchromatin variant is an inversion, deletion, or duplication of euchromatin.

Except for *inv(9)(p12q13)*, in many cases, it is difficult to identify heteromorphism or euchromatic variant by G-banding alone so that we need to identify them by adding various chromosome banding techniques properly. Parental chromosome analysis should also be used to identify the carrier status for these normal variants.

**Table 23.3** The representative normal chromosomal variation

Variant	Chromosome	Locus/karyotype
<i>Heteromorphism</i>		
Length or size	1, 9, and 16	Constitutive heterochromatin (1q12, 9q12, and 16q11.2)
	Acrocentric-	Short arm, satellite stalk, satellite
	Y	Yq12
Pericentric-inversion	Y	<i>inv(Y)(p11.2q11.23)</i>
	1	<i>inv(1)(p11q12), inv(1)(p13q21)</i>
	3	<i>inv(3)(p11.2q12)</i>
	9	<i>inv(9)(p12q13)</i>
	16	<i>inv(16)(p11.2q12.1)</i>
<i>Euchromatin</i>		
Pericentric-inversion	2	<i>inv(2)(p11.2q13)</i>
	10	<i>inv(10)(p11.2q21.2)</i>





**Fig. 23.2** Partial karyotypes of the carriers of pericentric inversion polymorphisms. (From left to right) Chromosome X and  $inv(Y)(p11.2q11.2)$ ,  $inv(2)(p11.2q13)$ , and  $inv(9)(p12q13)$ . The inverted chromosome is on the right side of the homologous chromosome, and arrowheads indicate breakpoints of the inversion. The lower part on the left side (with the dark background) is a Q-banded partial karyotype, and the others are G-banded partial karyotypes

### 23.6 Conclusion

Although the chromosome analysis by using the banding technique is classic genetic testing with a half-century of history, it is still essential clinical testing for a definitive diagnosis of chromosomal abnormalities. Various genetic and genomic analysis technologies are being applied to the medical field. Karyotyping of G-banded cells is having critical advantages of the ability to detect intercellular heterogeneity by a cell-by-cell basis analysis and easily detect balanced chromosome rearrangements. Most chromosome analyses are performed in reference laboratories. All of the testing laboratories are expected to report the results accurately and in an easy-to-understand way when they identified the rare chromosome abnormalities. On the other hand, health-care professionals such as physicians need the skills to choose additional testing appropriately and to interpret them accurately based on clinical information. Rapid and accurate communication between the two professional parties is required, and collaboration with specialists in clinical cytogenetics is also needed.

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