Uporaba primerjalne genomske hibridizacije kot diagnostične metode v medicinskem genetskem laboratoriju: prva uporaba na različnih kliničnih vzorcih

The use of comparative genomic hybridization as a diagnostic tool in medical genetics laboratories: first application on different clinical samples

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Ključne besede:
primerjalna genomska hibridizacija, medicinska genetika, diagnostična metoda

Key words:
comparative genomic hybridization, medical genetics, diagnostic method

Članek prispel / Received
01.07.2010
Članek sprejet / Accepted
29.09.2010

Naslov za dopisovanje / Correspondence
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Izvleček

Namen: *Primerjalna genomska hibridizacija (PGH) je molekularno citogenetska tehnika za identifikacijo kromosomskih neravnovesij po celotnem genomu. Zaradi njene kompleksnosti jo kot rutinsko diagnostično metodo uporablja samo nekaj laboratorijev po svetu. Predstaviti želimo svoje izkušnje pri delu s tehniko PGH in njeno diagnostično uporabnost pri post–natalnih kliničnih vzorcih.*

Metode: *Validacijo PGH tehnike smo opravili na vzorcu 10 preiskovancev z diagnozo nepojasnjena mentalna retardacija in s predhodno določenimi subtelomernimi kromosomskimi spremembami v velikostnem razredu 3,9 do 37 Mbp. Kot potrditveno metodo za določitev kromosomske aneuploidije smo PGH uporabili pri petih vzorcih embrionalnega tkiva po spontanih splavih. Pri enajstih hematoloških onkoloških vzorcih smo PGH uporabili*

Abstract

Purpose: *Comparative genomic hybridization (CGH) is a molecular–cytogenetic technique used to identify chromosomal imbalances throughout a genome. Due to its complexity, the use of CGH as a regular diagnostic technique is limited to only a few diagnostic laboratories. In this study, we evaluated the potential applications of CGH as a diagnostic method in different post–natal clinical samples.*

Methods: *Ten patients were recruited with submicroscopic chromosomal abnormalities ranging in size from 3.9 to 37 Mb. For the purpose of confirmation, CGH was applied to five cases where molecular karyotyping with MLPA was previously utilized to detect chromosome aneuploidy. To date, CGH was largely used for the identification of the complex karyotype in haematological malignancies.*

Results: *In eight cases of haemato-*

pri razreševanju kompleksno preurejenih kariotipov.

Rezultati: *S PGH smo našli subtelomerne kromosomske spremembe, večje od 8 Mbp. Z metodo PGH smo potrdili vse z molekularno kariotipizacijo predhodno najdene kromosomske aneuploidije v embrionalnih tkivih po spontanih abortusih, kjer celice niso bile več mitotsko aktivne. Največja uporabnost PGH se je pokazala pri pojasnjevanju kompleksnih kromosomskih preureditev v primerih hematoloških malignih obolenj.*

Zaključek: *Čeprav je PGH tehnično zahtevna in zamudna tehnika in kot taka neprimerna za rutinsko diagnostično delo, je po naših izkušnjah nepogrešljiva v posameznih primerih, v katerih druge genetske analize niso uporabne, npr. pri mitotsko neaktivnem celičnem materialu ali pri kompleksno preurejenih kariotipih. Naše izkušnje in rezultati potrjujejo njeno uporabnost predvsem v tistih genetskih laboratorijih, kjer zaradi ekonomskih razlogov še niso uspeli vpeljati pregledovanja genoma na osnovi t.i. micro–array tehnologije.*

logical malignancies, we were able to resolve complex karyotypes with CGH. Utilizing CGH as a diagnostic tool, we detected chromosomal imbalances larger than 8 Mb. In addition, we confirmed all chromosomal aneuploidies that were previously detected with MLPA from embryonic tissues obtained from aborted fetuses. In this tissue, the cells were not mitotically active, and therefore, were inappropriate for the conventional cytogenetics.

Conclusion: *Because CGH is technically demanding and time consuming, this technique is likely to be inappropriate for screening purposes. However, we found that CGH may be very useful in sporadic cases, where the sample material is not mitotically active or in cases with complex karyotypes. Therefore, our results confirmed that CGH may be useful in laboratories that are unable to use micro–array CGH for economic reasons.*

INTRODUCTION

Comparative genomic hybridization (CGH) is a molecular–cytogenetics technique that permits the detection of chromosomal imbalances throughout a genome without the need for the culturing of cells (1). In brief, the CGH method measures equal quantities of "tested" and normal "reference" genomic DNAs, which are labelled either directly or indirectly with different fluorescent dyes. Both samples of DNA are then mixed and hybridized in equal amounts to immobilized metaphase targets on microscope slides. The presence and location of chromosomal imbalances can then be detected by analyzing the ratio of fluorescence detected in "test" verses "reference" samples along the target chromosome using a digital image analysis (2). As ratio profiles are calculated along all chromosomes in the normal metaphase, an overview of the chromosomal imbalances throughout the entire genome is created (3). However, the highly polymorphic centromeric and heterochromatic regions are excluded from the CGH analysis due to technical reasons (4).

The CGH has been applied to different clinical samples during pre– and post–natal screening (5). However, only few diagnostic genetics laboratories perform CGH as a regular diagnostic technique due to its complexity (6). Thus far, the CGH has been successfully used to identify chromosomal regions involved in oncogenesis from a diverse spectrum of tumors (7, 8, 9, 10). Additionally, the CGH has been successfully utilized to identify genomic imbalances in mentally retarded patients with dysmorphic features and a normal karyotype (11, 12) as well as fetuses arising from recurrent spontaneous abortion (13). While the sensitivity of the CGH analysis can be hampered by the presence of variation in the amount of tumor cells in a respective test sample, the resolution of the CGH is usually in the range 8 – 10 Mb (14). Therefore, chromosomal imbalances, which are difficult or impossible to detect even with good quality R– and G–banding techniques, can be detected with the CGH. A high resolution CGH (HR–CGH) with increased sensitivity (3Mb) and specificity was developed by Kirchoff et

al. (15), and our laboratory of Medical Genetics has been using CGH as a diagnostic tool since 2003.

MATERIALS AND METHODS

Overall, this study was conducted to further assess CGH as a diagnostic tool. In the first part of this study, the CGH technique was tested on a set of patients with well–defined submicroscopic chromosome abnormalities. In the second part of this study, we used CGH to detect chromosomal abnormalities in embryonic tissues of aborted fetuses, and we utilized CGH for the clarification of the complex karyotype in hematological malignancies.

Ten patients with idiopathic mental retardation and dysmorphism were included in the "tested" CGH group. To perform CGH, T–FISH (simultaneous subtelomere fluorescent in situ hybridization) was utilized for the detection, and locus specific FISH (LS–FISH) was used for the confirmation and determination of subtelomeric chromosome rearrangements (16). The confirmed size of terminal chromosome rearrangements was between 3.9 and 37 Mb. The results from T–FISH and CGH are presented in Table 1.

In cases where cells were not mitotically active (i.e., embrionic tissue from aborted fetuses in our case), we first implemented multiplex ligation probe dependent amplification (MLPA) with subtelomeric probes

Table 1. *Group of patients with subtelomeric chromosomal rearrangements, where the resolution power of CGH (comparative genomic hybridization) experiments was determined. (FISH=fluorescent in situ hybridization; LS=locus specific).*

	Subtelomeric FISH	Size of anomaly (LS–FISH)	CGH
Patient 1	*Trisomy Xqter* *Monosomy Xpter*	*7 Mb* *8.8 Mb*	*+nd* *dim(X)(pter)*
Patient 2	*monosomy 2qter* *monosomy Xpter*	**nc (app. 0.24 Mb)* *9.2 Mb*	*+nd* *dim(X)(pter)*
Patient 3	*Trisomy 10qter* *Monosomy 13qter*	*37 Mb* *3 Mb*	*enh(10)(q23.3–>qter)* *+nd*
Patient 4	*Monosomy 2qter*	**nc (app. 0.24 Mb)*	*+nd*
Patient 5	*Trisomy 8qter* *Monosomy 21qter*	*8 Mb* *3.9 Mb*	*enh(8)(qter)* *+nd*
Patient 6	*Monosomy 2qter*	**nc (app. 0.24 Mb)*	*+nd*
Patient 7	*Monosomy 9pter*	*10 Mb*	*dim(9)(pter)*
Patient 8	*Monosomy 2qter*	**nc (app. 0.24 Mb)*	*+nd*
Patient 9	*Monosomy 2qter*	**nc (app. 0.24 Mb)*	*+nd*
Patient 10	*Trisomy 10qter* *Monosomy 11qter*	*8.3* *8*	*enh(8)(qtel)* *dim(11)(qtel)*

**nc – not confirmed, +nd – not detected*

(SALSA MLPA kit P0070 and P036B, MRC – HOLLAND) for molecular karyotyping. In five cases, we detected abnormal results; therefore, we performed the CGH as a confirmation method.

The complex karyotypes were resolved in eight cases of hematological malignancies using CGH. For instance, Case 3 represents a patient with acute leukemia and a complex karyotype. In this case, CGH was utilized because the tissue sample from his bone marrow did not yield chromosomes of sufficient quality for conventional cytogenetic analysis (i.e., only a few metaphases were found).

CGH Methodology

Preparation of CGH probe

The CGH was performed as described previously (1) with some modifications according to Jeuken (17). Briefly, 1 µg of DNA from the tested patients was isolated either from fixed cells in Carnoy fixative (ethanol:acetic acid, 3:1) or from peripheral blood lymphocytes. 1 µg of DNA was also isolated from normal (reference) patients, and included DNA from males or females isolated from peripheral blood lymphocytes of donors showing a normal karyogram. The test DNA was directly labeled with Spectrum Green, and the reference DNA was labeled with Spectrum Red (Vysis) by nick translation. For both groups, the CGH probe was prepared by an ethanol precipitation, and the DNA was differentially labeled either green (test) and red (reference) in the presence of an excess of human Cot1 DNA (Roche Diagnostic). Probes were prepared one day prior to the CGH experiment.

CGH hybridization

The CGH probes were hybridized to a slide with normal metaphase chromosomes, which were prepared from phytohaematoglutinin–stimulated peripheral blood lymphocyte cultures of a karyotypic normal female or male donor (18). Previous studies have established that the quality of the chromosomes during metaphase highly affects the quality of the CGH experiment (17). Therefore; strong requirements of the spreads were considered as described (19). To optimize the hybridization, the slides for CGH were pre–treated with pepsin, and were incubated twice in 2SSC (3 min), 10 minutes in formalin/MgCl2/2SSC, and 3x2 SSC for 3 min. The slides were then dehydrated in an ethanol series, and were air dried. Denaturation of slides was carried out in 70% formamide (2xSSC, 3 min, 72°C) followed by rehydration in ethanol at –20°C and air drying.

For the CGH, the DNA probe was resuspended in a hybridization buffer, and denaturated at 72°C for 10 min. The DNA probe was then applied to the prepared slide, and incubated for three days at 37°C in a moist chamber. Post–hybridization washes were carried out in 0.4 SSC at 72°C for 2 min, and then in 2 SSC/0.05% Tween at room temperature for 1 min. Following post–hybridization, the samples were counterstained with 4.6–diamidino–2–phenylindol (DAPI). The CGH image capture was performed with a CytoVision program (Applied Imaging, Sunderland, UK) interfaced to axiophot fluorescence microscope (Zeiss, Jena, Germany). To test the quality of the CGH hybridization, two CGH hybridizations were prepared for each sample, and each hybridization was performed with a different gender of the reference DNA.

CGH analysis

The primary aim of analysis was to produce an accurate profile of all of the chromosomes in the genome in order to indicate areas of deletions and amplifications in the test DNA. The average green to red fluorescence ratio measured along the chromosomal axis represents loss where the ratio is <1 (colored red), and the gain (ratio >1) of genetic material at specific chromosome or locus in the tested genome is colored green. For each case, we analyzed at least 10 inverted DAPI metaphases. For the analyses, the first step was to karyotype the chromosomes with inverted DAPI metaphases chromosomes, and a computer program then separately calculated the CGH profile for the p and q arms of the chromosome. Detection thresholds for losses and gains for chromosomal regions were performed by fixed reference intervals, as previously described (0.8 to 1.2)(3).

RESULTS

For this study, the patients phenotype and genotype have been previously described (20). As an example, we present case 1 with the following karyotype:46,XY,inv(9)(p11q13)ishrec(X)(qter+, pter–). In the present study, we re–examined the case utilizing the CGH method. For instance, Case 1 exhibited a deletion of terminal chromosome region Xpter, and the duplication of the region Xqter, previously detected by T–FISH and LS–FISH. Only a gain of the chromosomal material of Xp22.3 region was detected by the CGH (8.8 Mb), while an amplification of the region Xqter was not detected (7 Mb) (Figure 1). Based on the results of the whole group (see Table 1), we determined the approximate resolution of the CGH, and revealed that only imbalanced chromosome rearrangements larger than 8 Mb were detected. Additionally, aberrations on chromosome X were detectable only on male metaphases due to the comparative nature of the CGH technique.

For case 2, a duplication of both subtelomeric chromosomal regions of chromosome 16 was detected by MLPA on embryonic tissue of aborted fetuses. Similar to the MLPA findings, an amplification of the whole chromosome 16 was confirmed by CGH (Figure 2). For this experiment, we used female DNA as a reference DNA, and we hybridized this DNA on female metaphase spreads. Thereby, the loss of one chromosome X was detected in the test sample, as compared to the reference sample. The sex of embryo was male (XY), and the karyotype (21) of case 2 after the CGH was **ish cgh XY, +16.**

Case 3 was a male patient with acute leukemia, and a complex karyogram (Figure 3a). After combining the results by GTG–banding and the CGH (Figure 3b), the karyotype (21) was written as follows: **40~46, XY, der(1), der (4), –5, –7, –9, –10, der (11), der (13), der (15), der (16), i(21), + 5mar, inc. (9) / ish cgh XY, del(5q), del(7q), +8, del(13q), del(15q), del(16q), amp(21q).**

DISCUSSION

CGH can be used to screen different types of genomes, and produces consistent chromosomal regions associ-

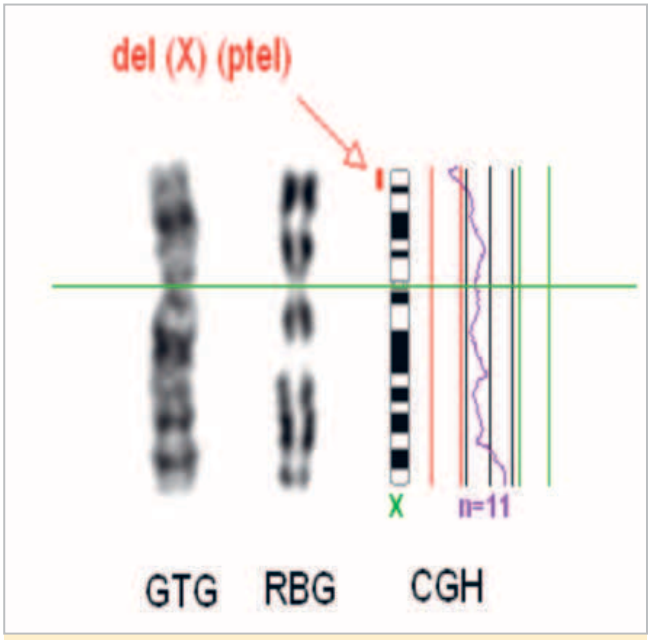
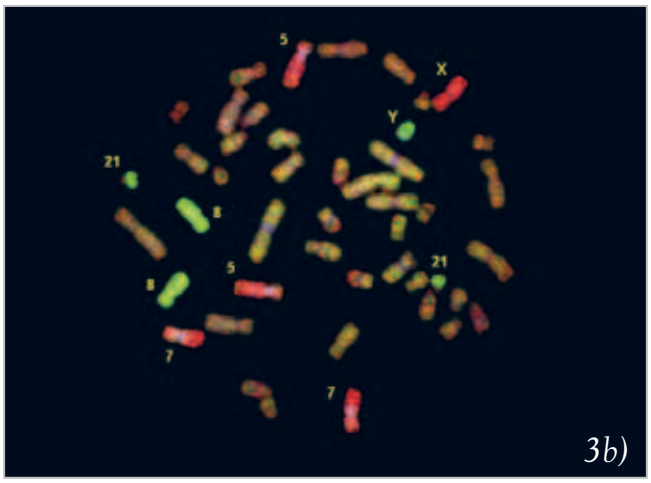


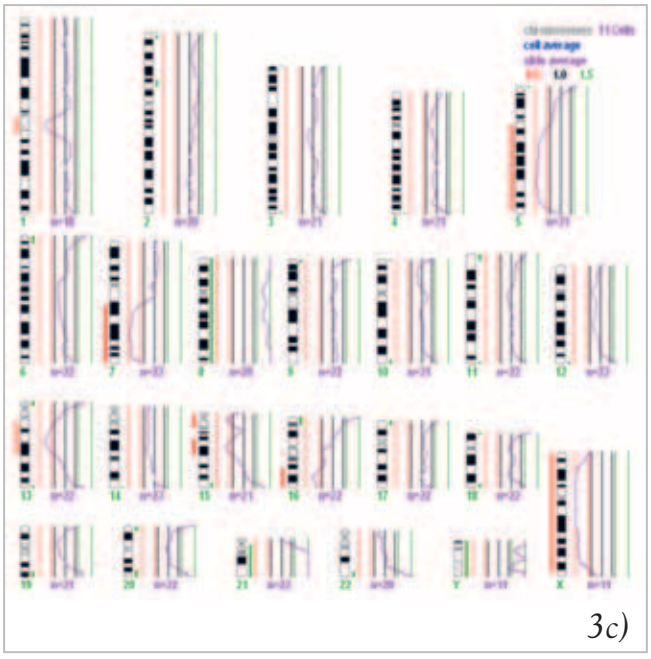
Figure 1. *Results by case 1 derived by chromosome banding techniques GTG, RBG and CGH karyogram on chromosome X: with CGH only deletion of subterminal region Xp was detected.*

Figure 2. *CGH results of genome analysis by case 2: the average ratio profile of at least ten metaphases. The whole chromosome material from chromosome 16 is amplified.*

3a)



3b)



3c)

Figure 3. *Results by case 3: a) Karyogram of the patient after chromosome GTG banding analysis; b) CGH Metaphase fluorescence image after Triple filter (DAPI/TRITC/FITC); and c) The CGH average ratio profile.*

ated with the amplification or the deletion of genes. To date, CGH represents a first line analysis in tumor genetics, and can globally identify chromosomal regions of interest that can be later examined more precisely using other techniques. Despite the broad applicability of the CGH (5, 6), only a few diagnostic genetics laboratories perform CGH as a regular diagnostic tool. The limited use of CGH is likely due to its complexity.

In this study, we presented three examples of cases where whole genomes were investigated with the CGH. CGH was applied for the re-examination of the whole genome of mentally retarded patients, in cases where cells were not mitotically active, and as a supplemented method for resolving complex karyotypes. We found that the CGH method has several advantages compared to other techniques for analyzing genetic changes. For example, CGH does not require cells to be mitotically active, and CGH is not dependent on the DNA source. Additionally, CGH only requires a small amount of the tested DNA. Furthermore, the CGH method produces detailed information on genome-wide gains and losses, and can be utilized to screen the whole genome in a single experiment. However, compared to other techniques (cytometry, cytogenetics, fluorescent in situ hybridization, PCR-based techniques), the CGH method has some disadvantages, including a lower sensitivity than PCR-based methods. In addition, CGH is more time consuming than other techiniques like cytometry and PCR, and the equipment for the CGH is still rather costly (22). Finally, balanced chromosomal changes, which are frequently found, can not be detected with the CGH.

Based on our experience, we conclude that the CGH method is a useful tool in medical genetic laboratories as a diagnostic tool, especially as a supplemental method to the current methods. While a well-defined array of DNA sequences has replaced the metaphase chromosomes as the hybridization targets on glass slides, a new microarray CGH is currently being developed in various laboratories (23). Furthermore, array-based genome-wide screening techniques are also still rather costly. Until the genome-wide screening techniques, like array-CGH, are more accessible for routine cytogenetic diagnostics (both financially and technologically), the combination of different complementary molecular and molecular-cytogenetic techniques can currently provide a precise interpretation of genetic results. Ultimately, the CGH still represents a method of significant importance among other genomic strategies.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. Mariano Rocchi for the locus-specific FISH probes. This work was supported by the Ministry of High Education, Science and Technology; contract number RP L3-4549.

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