Establishment and molecular cytogenetic characterization of non small cell lung cancer cell line, KU-T1 by multicolor FISH, comparative genomic hybridization and chromosome microdissection

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Abstract A human lung adenocarcinoma cell line, designated KU-T1, was established from a Japanese male in Kochi Medical School. Conventional banding and multicolor-fluorescence *in situ* hybridization (M-FISH) analyses of KU-T1 cells revealed that a chromosomal constitution was hyper-diploid and complex karyotypes. Comparative genomic hybridization (CGH) showed several chromosomal copy number changes, and five regions that were highly amplified. Two (1q and 3q) of five highly amplified regions were identified on the distributions of DNA sequences on a metaphase cell by FISH using chromosome microdissection (CMD) generated probes, which hybridized to the chromosomal regions of 1q32-34 and 3q26-28, respectively. The 3q probe depicted a homogenously staining region (hsr) in a derivative chromosome 3 of KU-T1. An hsr probe was regenerated by CMD and was hybridized back to KU-T1 and normal metaphases. This hybridization experiment confirmed the probe derived from an hsr and indicated original locations of DNA sequences of hsr on normal chromosome 3. Intense hybridized signals shown at three loci, 3p12, 3q26.3, and 3q28 suggested that some oncogenes may be involved in the hsr formation. The present study provides a comprehensive analysis of the chromosomal abnormalities, including hsr formation and related oncogenes, in the KU-T1 cell line.

1. Introduction

There is an increasing number of tumor-related deaths that can be attributed to carcinomas of the lung [1]. Non-small cell lung carcinomas (NSCLCs) represent 75-80% of all lung cancers. Despite the high incidence of these tumors, our understanding of the chromosome changes in NSCLC is very rudimentary compared to that for hematologic malignancies. Although lung cancer is the most common cause of cancer death for men and the second most common for women, followed by gastric cancer in Japan [2], the cytogenetic data available are still limited for this neoplasm, and the clinical significance of the cytogenetic findings has not been established. The identification of nonrandom chromosomal alterations has indicated an involvement of several oncogenes and tumor suppressor genes in lung carcinoma. However, lung tumors and cell lines exhibit karyotypes with a high degree of aneuploidy and unbalanced rearrangement that have made it difficult to identify specific recurrent chromosomal aberrations [3-5]. Conventional cytogenetic analyses have always revealed complex karyotypes with cryptic structural rearrangements and unidentifiable marker chromosomes in lung tumors and cell lines. To define complex karyotypes of tumors and cell lines, several molecular cytogenetic techniques have been developed. Initially, fluorescence in situ hybridization (FISH) was developed not only for performing gene mapping but also

allowing the delineation of whole chromosomes and chromosomal subregions [6, 7]. Secondly, comparative genomic hybridization (CGH) is a molecular cytogenetic method that detects and localizes gains or losses of genetic material across the entire tumor genome [8]. Furthermore, CGH assists in defining chromosomal regions that may harbor amplified oncogenes or deleted tumor suppressor genes. Thirdly, multicolor-FISH (M-FISH) has recently been used for detailed characterization of structural chromosomal rearrangements of malignant tumors, including lung carcinomas [9, 10]. One limitation of the M-FISH analysis is that it cannot assign chromosomal subregional identities, because of the high degree of structural complexity of lung carcinoma and equivocal chromosomal morphology. To solve this problem, chromosomal microdissection (CMD) is used, as a powerful technique to obtain specific probes from specified regions of chromosomes [11-13]. There is a complementary nature of M-FISH, CGH, and CMD analyses for detecting DNA amplification and chromosome alteration in tumors.

Lung carcinoma cell lines represent useful *in vitro* models for studies related to lung carcinogenesis, especially in the investigation of genetic changes and malignant transformation that are associated with development of lung carcinomas. In this report, the comprehensive karyotype of a newly established human NSCLC cell line, which originated from a Japanese male is herein presented and designated as KU-T1. A

conventional G-banding technique and a combination of molecular cytogenetic technologies, such as CGH, M-FISH, and CMD, permitted detailed characterization of the chromosomal aberrations and DNA rearrangements, including oncogene amplification as a homogenously staining region (hsr).

2. Materials and Methods

2.1. Patient

Tumor lung tissue was obtained from a 70-year-old Japanese male patient undergoing a surgical restriction with curative intent. A portion of the lung tumor tissue was cultured for conventional cytogenetics and subsequent cell line development, while DNA extracted from the remainder was used for CGH studies. A pathological examination showed this tumor to be an adnocarcinoma of lung.

2.2. Cell culture and chromosome preparation

The lung tumor tissue was finely minced using sterile scalpels and digested in collagenase type II (400U/mL) at 37°C. The disaggregated cell suspension was seeded in complete medium containing D-MEM supplemented with 7% fetal bovine serum, 100 U/mL penicillin, 100U/mL streptomycin. Cultures were maintained in a humidified incubator at 37°C in an atmosphere of 5% CO_2, until successive passaging was possible.

For cell line development, contaminating fibroblasts were initially removed by differential subculture, using 0.5mM EDTA in plain DMEM medium. For the morphological study of KU-T1, cultured cells were observed by phase contrast microscopy. The population doubling time was determined by seeding cells at an initial density of 10^5 in 6-multiwell plates, and counting was performed up to 14 days, in triplicate, using a counting chamber (Erm, Tokyo) by dye exclusion.

2.3. Chromosome analysis

Chromosomes from this cell line were analyzed using G-banding, Q-banding, FISH, M-FISH, CGH, and CMD-FISH technologies. Metaphase spreads from the cell line were harvested, and slides were prepared according to standard methods [14]. Briefly, actively dividing cells were blocked in metaphase with 0.1 μg/ml of colcemid (Gibco) for 1 to 3 hr, incubated in 0.075 M KCl hypotonic solution for 20 minutes, and fixed in methanol-glacial acetic acid (3:1). G- and Q-bandings were performed using standard procedures. Representative images were captured using an Olympus (Japan) DP-70 system for G- and Q-bandings, and the Mac Probe analysis system (Applied Imaging Corporation, USA) for FISH and CGH. KU-T1 cells were karyotyped according to ISCN 2005 [15]

2.4. M-FISH study

For the M-FISH analysis, commercially available probes (SpectraVysion Assay; Abbot Laboratories, Des Plaines, IL, USA; Vysis, Downer's Grove, IL, USA) were used following the manufacturer's protocols.

2.5. CGH study and digital image analysis

CGH was performed according to the standard protocol [16], with minor modifications. Briefly, genomic DNA from the tumor specimens and peripheral blood lymphocytes from karyotypically normal male controls were isolated by standard techniques. Reference and tumor DNAs were labeled by nick translation with Rhodamine-dUTP (Amersham Pharmacia Biotech, USA) and fluorescein-12-dUTP (NEN Life Science Products, Boston, MA), respectively. The hybridization mixture consisted of 200 ng of tumor DNA, 200 ng of reference DNA, and 10μg of Cot-1 DNA (Roche Diagnostics Corporation, Indianapolis, USA) in 8μL of Hybridization solution, H-7782 (Sigma-Aldrich Co., St. Louis, USA). The probe mixture was hybridized to normal male metaphase spreads (46, XY) for 3 days at 37°C. These hybridized slides were post-washed in post-wash solution (50% formamide / 2xSSC) for 20 min at 43°C and washed twice in 2xSSC for 4 minutes at 37°C and once in 1xPBD (4xSSC/0.05% Tween20) at room temperature. After three 2-min washes in 1xPBD, the slides were mounted with a Vectashield (Vector Laboratories,

Burlingame, CA).

A CGH analysis was performed using an Olympus BX-50 fluorescence microscopy equipped with single band-pass filters for Fluorescein, Rhodamine, and DAPI and with a cooled CCD camera (KAF 1400, Photometrics, USA) and the Mac Probe version 3.4 analysis system (Applied Imaging Corporation, USA, Sekitechnotoron, Japan). More than five metaphases were combined for each case to obtain profiles of the mean ratio and standard deviation. The chromosomal regions where the green to red ratio exceeded 1.15 were considered over-represented (gained), whereas regions where the ratio was below 0.85 were considered under-represented (lost). If the mean green to red ratio exceeded 1.5 in a segment of the chromosome arm, these regions were considered to represent high-level amplification. The telomeric and heterochromatic regions were excluded from the analysis.

2.6. Molecular cytogenetic analysis by CMD-generated probe

Metaphase spreads for CMD and FISH were prepared on 24 x 60 mm cover-slips and glass slides, respectively. From normal 1q and 3q, and from the derivative chromosome 3, which is a probable hsr region of KU-T1, 2 to 20 chromosomes from metaphase preparations were scraped with a CMD technique [13]. In brief, glass needles with about 1 to 2 μm diameter tips were produced from glass capillaries (GD-1, Narishige, Tokyo,

Japan) using a pipette puller, PC-10 (Narishige). CMD was then performed under an inverted microscope (Olympus, Tokyo, Japan) equipped with a mechanical micromanipulator, Eppendorf 5171 (Hamberg, Germany). The scraped chromosome fragments were placed into a 0.5 ml tube under a binocular microscope. DOP-PCR was used to amplify DNA of the scraped chromosome segments in a thermal cycler, PTC-100 (MJ Research, Mass., USA). To verify DNA recovery from the PCR, electrophoresis for each sample was run on a 1.5% agarose gel at 100 V for 1 hr. When the appropriate sized products (300-800 base pairs) were obtained, a hapten of biotin- or digoxigenin-labeled nucleotide was incorporated into the DNA segments by the second-generation DOP-PCR using 2μl of the first-generation products as a template in the 50μl labeling reaction. To confirm the chromosome 3 of KU-T1 cells, a commercially available chromosome 3 α-satellite centromere probe (Qbiogene, MP Biomedical Japan) was used in the CMD-FISH experiment (**Fig. 6C**).

3. Results

3.1. Cell morphology, growth and KU-T1 karyotype by conventional G-banding

After the 10th passage, a more typical pavement arrangement of epithelial cells was observed. Cells of KU-T1 have a polygonal shape with small granules in the cytoplasm

(**Fig. 1**). They formed a monolayer and pile up after reaching confluence. Growth kinetics studies suggested exponential cell growth after 72 hours. The logarithmic growth was estimated to have a doubling time of approximately 70 hours. The average number of chromosomes was 53 and chromosome modal number of KU-T1 ranged from hyper-diploid to near-tetraploid (range 50-102). The karyotype of KU-T1 is based on the analysis of seven of G-banded metaphase spreads. More than ten aberrant chromosomes per metaphase were detected in most of the analyzed KU-T1 cells. A representative G-banded karyogram of KU-T1 is shown in **Figure 2**. The composite karyotype of KU-T1 was as follows; 51~53, X, -Y[7], del(1)(p13p22)[4], +der(1)t(1;2)(p34;q12)[7], -2[4], +3[2], +add(3)(q21)[2], +add(3)(q27)[7], +5[2], +6[2], -7[2], +7[2], add(7)(p13)[3], -8[2], del(9)(p13)x2[7], -10[3], +add(11)(q25)[7], -12[2], +12[3], -13[3], -14[2], -16[3], +16[3], -18[5], -19[3], +20[6], -21x2[5], +2~5mar[cp7]. Aberrant chromosomes, –Y, der(1)t(1;2)(p34;q12), add(3)(q27), del(9)(p13), and add(11)(q25), appeared clonally in most of KU-T1 cells.

3.2. M-FISH analysis

An M-FISH analysis identified fourteen structural rearrangements in the KU-T1 cell (**Fig. 3**). M-FISH revealed that chromosome 10 was most frequently involved in rearrangements. Chromosome 10 had unbalanced translocations with 5 different

chromosomes. It fused with chromosomes 3, 7, 16 and 21 at one breakpoint, and with chromosome 5 at 2 breakpoints (due to insertion), respectively (**Fig. 3C**). Chromosomes 3, 11, and 16 had unbalanced translocations with two different chromosomes. They fused with chromosomes 10 and 11, 3 and 5, and 9 and 10, respectively.

3.3. CGH Findings

In KU-T1 cells, CGH revealed copy number gains on the following chromosomal regions, Xq13, 1p13.3-31, 1q22-44, 3p12-14.3, 3p23-25, 3q13.3-28, 5p, 5q34-35, 6p12-22, 7p21-22, 10q22, 11q12-25, 12p12-13, 12q14-23, 16q22-24 and 17q21.2-23 (the green and red fluorescent signal ratio of more than 1.15 and less than 1.3). Five highly amplified regions with a peak (more than 1.5) were detected on 1q, 3q, 5p, 5q, and 11q, with peaks of 1q43, 3q26.2, 5p12-14, 5q35 and 11q14-25, respectively (**Figs. 4 and 5**). Losses were found on chromosome arms, 21q21 and whole Y (less than 0.85) (**Table 1**). **Figure 5** displays the CGH profile of KU-T1 cells.

3.4. CMD-FISH analysis

The region-specific chromosome probes, 1q41-qter and 3q24-26, from the chromosomes of normal lymphocytes and the hsr-like region of derivative chromosome 3 of KU-T1 cells, were successfully regenerated by CMD (**Fig. 6**). Then, these probes were used for the FISH analysis of KU-T1. **Figure 6A** illustrates that six chromosomes

contained part of 1q41-qter, and three had 3q24-26 in a KU-T1 metaphase by FISH. The hybridization of regional probe of 3q24-26 to KU-T1 cells highlighted the large painted portion, suggesting an hsr-structure (**Fig. 6A**). Hence, a CMD-probe was also regenerated from the hsr-like region of derivative chromosome 3 of KU-T1. This probe visualized an hsr-like region and two other chromosomes containing the 3q24-26 region in a KU-T1 metaphase cell (**Fig. 6C**). To determine the origin of DNA sequences of this probe, it was hybridized back to normal metaphases. Consequently, specific intense fluorescent signals were seen at 3p12, 3q26 and 3q28 on normal chromosome 3 from normal lymphocytes, which revealed the original locations of this probe (**Fig. 6E**).

4. Discussion

KU-T1, like many other NSCLC-derived cell lines, showed aneuploidy, and was comprised of many numerical and structural abnormalities indicated by conventional G-banding. M-FISH depicted precise chromosomes which had joined rearrangements in KU-T1 cells. Especially, chromosome 10 was the most frequently involved in rearrangements seen in this cell line. Rearrangements of chromosome 10 with other chromosomes by unbalanced translocations may establish fusion genes, resulting in the production of chimera proteins which play an important role in pathogenesis of this lung cancer. Recently, Sakai *et al.* reported that *C10orf3* sequences located on chromosome

10q23.3, might play an essential role in the growth of cancer cells including a lung cancer [17]. Chromosomes 3 and 5 were also frequently rearranged. In either gains or losses, chromosome 3 was probably involved in the tumorigenesis of NSCLC [3-5]. In chromosome 5, conventional cytogenetic studies have shown that chromosomal alterations occur on the short arm in all major tumor types [4, 18].

The CGH study revealed that DNA gains, rather than DNA losses, were more frequently observed in the KU-T1 cell line. In five of the regions of gains, high level amplifications were detected on 1q, 3q, 5p, 5q, 11q, with peaks of 1q41, 3q26.2, 5p12-14, 5q34, and 11q14.1-25, detected in KU-T1. A high level of amplification suggested an oncogene amplification and locus which may be the candidate regions for the isolation of genes related to NSCLC. The loss of 21q and Y is a common finding in NSCLC [3-5]. Missing the Y chromosome may be indicative of some tumorigenesis [4, 19, 20]. Though deletion 9p has been repeatedly reported in lung cancer [3, 5, 21-23] and was also indicated on KU-T1 by the G-banding analysis, CGH did not reflect it. This may be because the short arm of chromosome 9 was translocated on chromosomes 8 and 16 as shown by M-FISH. However, the genes that reside in the breakpoint, 9p21, may contribute to this lung carcinogenesis. High-level gains of 1q and 3q are frequently observed in lung cancer [3-5, 24]. The amplification of some sequences of chromosome

11q13 is a common event in lung and esophageal tumors, where *CTTN*, *FGF3* and *CCND1* (*CoAA)* genes resided in [25-27]. Previous chromosomal CGH studies, including an array CGH analysis, have identified 5p amplification as a common event in NSCLC [4, 25, 28-31]. However, copy number reduction of 5p and alterations of 5q are rarely described.

Chromosome painting by CMD-regenerated sub-regional painting probes appears to be an indispensable technique for cytogenetic description of a complex karyotype as well as detecting amplified oncogenes. The CMD probes were regenerated from normal chromosomes 1 and 3, and from an hsr region of derivative chromosome 3 of KU-T1 cell, because 1q and 3q are frequently altered regions associated with lung cancinogenesis and CGH results indicated highly amplified regions in this cell line. The original CMD-regenerated probes, which painted, 1q41-qter, and 3q24-26, respectively, revealed the distribution of chromosome regions of 1q41-qter and 3q24-26 on KU-T1 chromosomes. Consequently, a CMD-regenerated probe, which hybridized to 3q24-26, painted a large part of a marker chromosome with the chromosome 3 centromere, demonstrated by a dual hybridization with the α-satellite probe of chromosome 3, in KU-T1. This finding revealed an amplification of DNA sequences of 3q24-27, thus suggesting the presence of an hsr. The CMD-regenerated probe, painted 1q41-qter, and

showed its distribution on six chromosomes, however, M-FISH detected only three chromosome 1. This may be due to the tiny portion of chromosome 1q which may not be detected by M-FISH. The DNA probe from the dissected hsr portion hybridized intensely to the derivative chromosome 3 (der(3)t(3;10)(?;?) hsr(3)(?;?)), with numerous signals distributed over the entire hsr region, whereas some hybridization signals were intensely seen at three locations, 3p12, 3q26.3, and 3q28, on a normal chromosome 3. These three loci suggested that this hsr region might contain some putative oncogenes and their co-amplifications. One of the locations, 3p12, which is reported as the locus, contains a tumor suppressor gene in several tumors [5] and the disruption of the 3p12 gene, *DUTT1*, by homozygous deletion, may be related to lung tumorigenesis [32]. An amplification n of 3q26-28 was reported in lung and other tumors, and the region harbors many interesting genes, such as *EVI1* (3q26.2), *LPP* (3q27-28), and *FHF-1* (3q28) [33-35]. These findings further augment the results of experimental studies aimed at gene discovery.

The study of this cell line could provide insight into the mechanisms leading to rearrangements involving oncogenes and an hsr formation. Further studies will be required to elucidate the oncogenes involved in the formation of other high-level of gains as well as the hsr of this cell line.

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Table 1. Regions of gain and loss of sequences in a KU-T1 cell line

Gains	High level of gains (a peak)	Losses
Xq13, 1p13.3-31, 1q22-44, 3p12-14.3, 3p23-25, 3q13.3-28, 5p, 5q34-35, 6p12-22, 7p21-22, 10q22, 11q12-25, 12p12-13, 12q14-23, 16q22-24 17q21.2-23	1q(1q43), 3q(3q26.2), 5p(5p12-14), 5q(5q35), 11q(11q14-25)	21q, Y

Figure legends

Figure 1: Morphology of the KU-T1 cell line, photographed by phase-contrast invert microscopy. Scale bar = 200μm.

Figure 2: Karyotype of a representative G-banded metaphase cell from KU-T1 cell line.

Karyotype of KU-T1 cells; 53, -Y, +der(1)t(1;2)(p34;q12), add(3)(q27), add(5)(p14), +add(6)(q24)x2, +del(6)(q21), add(7)(p13)x2, +7, +9, del(9)(p13)x2, +add(11)(q25), +12x2, -14, add(16)(q12), +20, -21, -22, +2mar.

Figure 3: M-FISH of KU-T1. (A) Aberrant chromosomes are displayed in classified color image. (B) DAPI staining image of the same metaphase cell. (C) A representative M-FISH karyogram of cell line, KU-T1.

Figure 4: CGH image of KU-T1. CGH using DNA from KU-T1 cells (labeled in green) and normal DNA (red) onto a normal metaphase cell counterstained with DAPI (blue). A yellow/greenish color indicates chromosomal sequences that are overrepresented and reddish color sequences that are underrepresented in the tumor cells. The arrowheads with the chromosome number are indicated in several overrepresented chromosomes.

Figure 5: The average ratio profiles of chromosomes from KU-T1 cells, obtained from more than 5 metaphase cells. The numbers under the profiles are the chromosome numbers analyzed. Dark thick and open bars to the left of the chromosome ideograms represent gains and losses. The arrowheads indicate high-level amplification. Middle lines (blue) indicate average ratios.

Figure 6: FISH image of dual hybridization of DNA probes to KU-T1 metaphase cells.

CMD probes (1q32-34 and 3q26-28) from normal chromosomes. (A) The arrowheads indicate pale blue fluorescent signals hybridized on 1q32-34. The arrows show parts of chromosome 3q26-28 (red color). The inset shows the original location of CMD probes of 1q32-34 (pale blue signals) and 3q26-28 (red signals), hybridized chromosome images were put next to DAPI staining chromosomes side by side. (B) DAPI staining image of the same metaphase. (C) Dual FISH image of KU-T1, regenerated probe made from an hsr-like region of derivative chromosome 3 of KU-T1. Three chromosomes are partially painted (red color) (arrows). One of them was painted on a large region, supposedly, an hsr-like portion. The arrowheads indicate centromeres of chromosome 3, repetitive α-satellite DNA sequences, were visualized by green fluorescent signals. Two of them had chromosome 3 centromere sequences. (D) DAPI staining image of the same metaphase cell. (E) A hybridization image of normal chromosomes. The probe regenerated from an hsr-like region was hybridized on normal metaphase cell. Each arrow shows the original locations of the probe derived from a derivative chromosome 3 of KU-T1 cells. (F) DAPI staining image of the same metaphase cell.

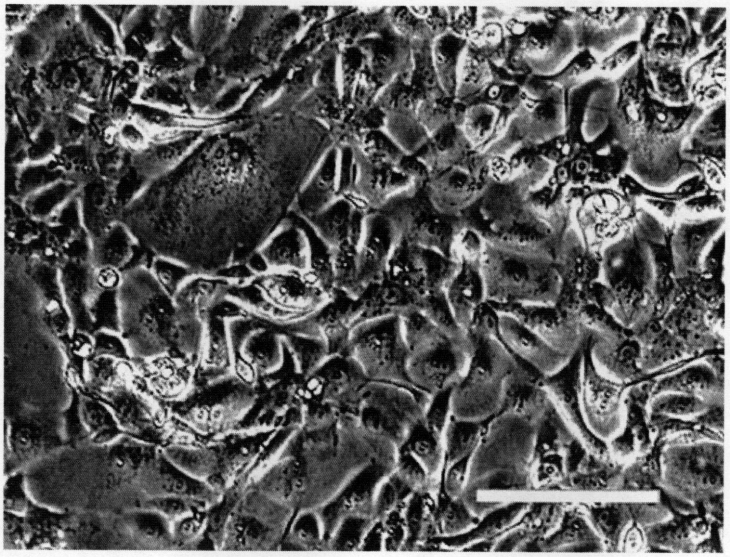


Fig. 1

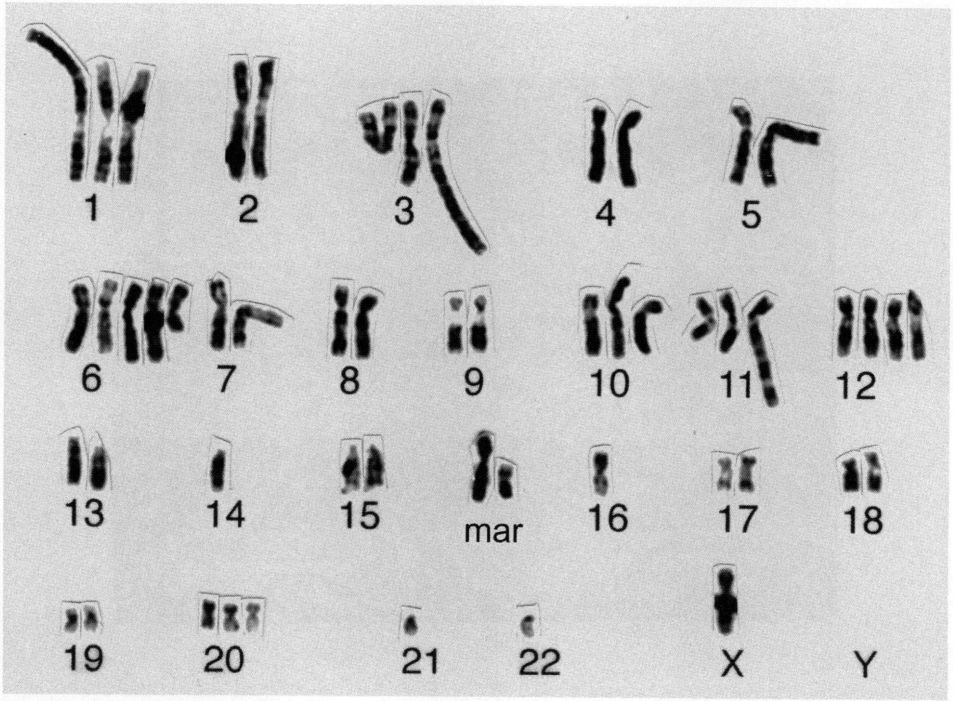
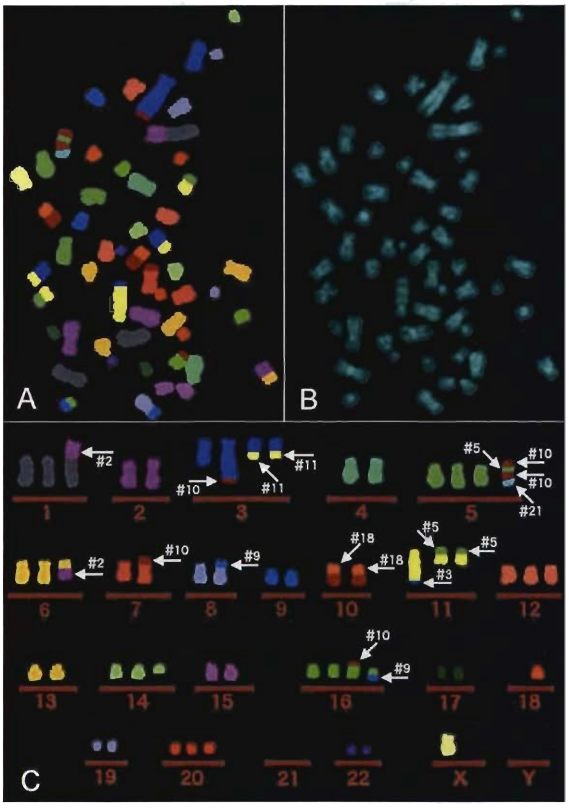


Fig. 2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#11
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#10
#21
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4
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#2
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#18
#18
#5
#5
#3
6
7
8
9
10
11
12
#10
#9
13
14
15
16
17
18
C
19
20
21
22
X
Y

Fig. 3

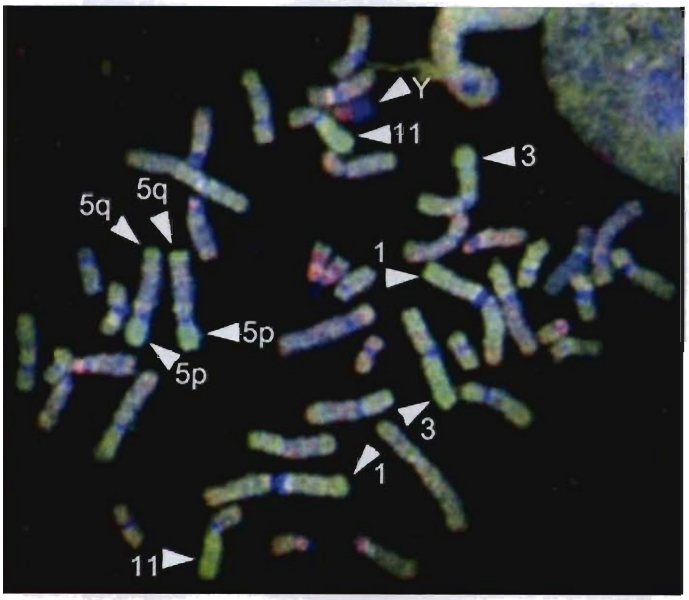
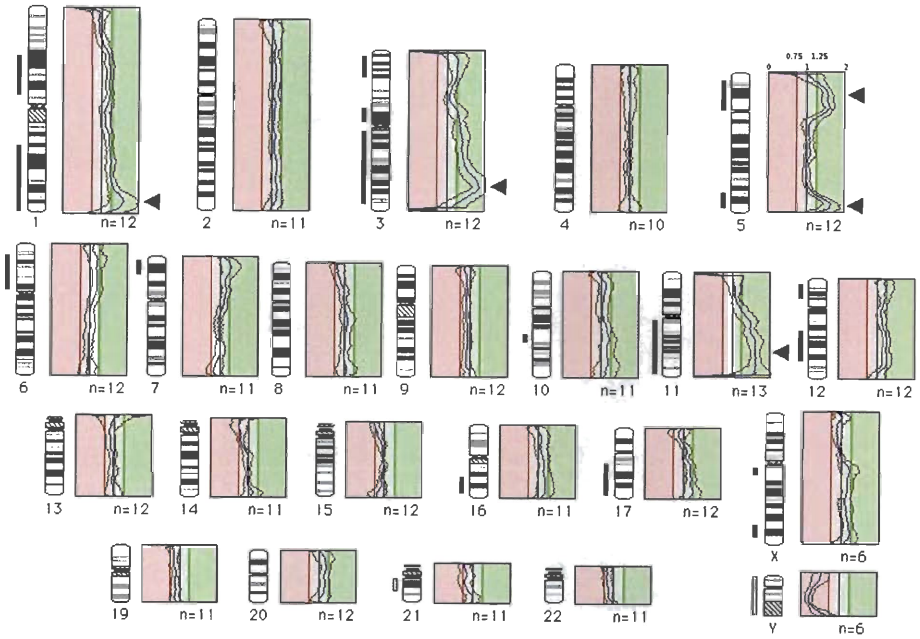


Fig. 4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3 n=12
4 n=10
5 n=12
6 n=12
7 n=11
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10 n=11
11 n=13
12 n=12
13 n=12
14 n=11
15 n=12
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17 n=12
X n=6
19 n=11
20 n=12
21 n=11
22 n=11
Y n=6

Fig. 5

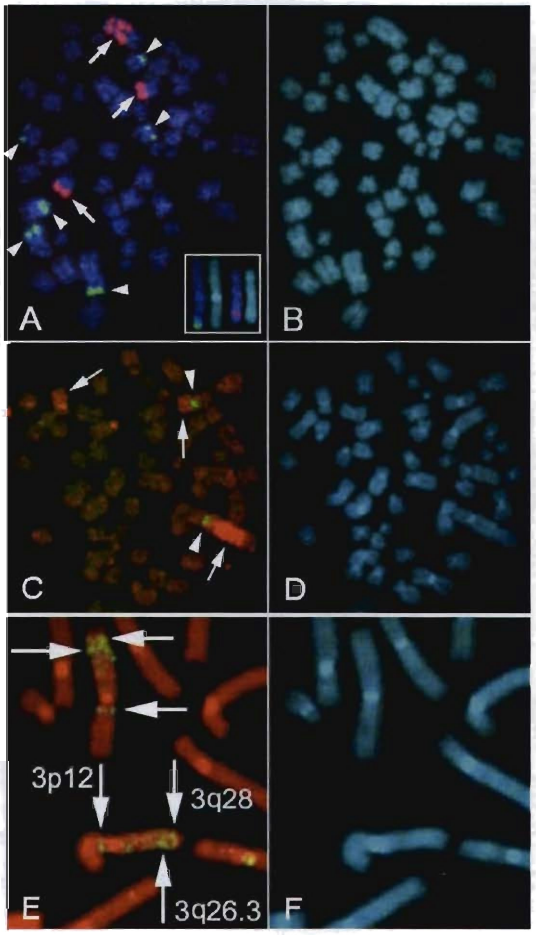

Fig. 6