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# Characterization of $\text{psu dic}(6;5)(\text{p}21.3;\text{q}13)$ with reverse chromosome painting in a patient with secondary myelodysplastic syndrome following treatment for multiple myeloma

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## Abstract

We report a case of a  $\text{psu dic}(6;5)(\text{p}21.3;\text{q}13)$  in a patient with secondary myelodysplastic syndrome (sMDS) following treatment for multiple myeloma. The abnormal chromosome was isolated by flow karyotyping and initially identified by reverse chromosome painting. The findings were then confirmed by forward painting. The value of flow karyotyping as a diagnostic technique in hematologic malignancies is discussed.

## 1. Introduction

Chromosomal studies are well established as having an important role in both the diagnosis and prognosis of patients with hematologic malignancies. An increasing awareness of the significance of such studies, and improvements in cytogenetic techniques in recent years, has resulted in the identification of more than 100 consistent chromosomal abnormalities in leukemia and lymphoma [1–4]. Molecular analysis of such rearrangements has led to the isolation and characterization of genes involved in the neoplastic process [5,6] and has led to the development of treatments which are targeted to the genetic aberration present.

The introduction of fluorescence in situ hybridization (FISH) as a routine cytogenetic technique has proven to be of great benefit in the study of malignancy [7,8]. The FISH technique has also helped in the interpretation of rearrangements too complex to be characterized by conventional cytogenetics. The identification of such abnormalities is important, as they may benefit patient management and could also reveal novel genomic regions involved in tumorigenesis and increase our understanding of the neoplastic

process. Conventional FISH studies using whole-chromosome paints (WCPs) and centromeric probes on abnormal chromosomes can be time-consuming and expensive, and may be limited by the amount of chromosome material available. An alternative approach is to use flow karyotyping to sort the marker chromosome, which is subsequently amplified by degenerate oligonucleotide primed polymerase chain reaction (DOP-PCR), and reverse-painted onto normal metaphase spreads [9–11]. Using this technique, all the chromosomes and the chromosomal regions involved in the rearrangement can be identified in a single hybridization, as can the breakpoint regions.

We report a case in which we have isolated an  $\text{add}(6)(\text{p}23)$  chromosome in a patient with secondary myelodysplastic syndrome (sMDS) following treatment for multiple myeloma. We used a combination of reverse and forward chromosome painting to characterize it.

## 2. Case report

A 45-year-old female presented in March 1991 with IgA- $\kappa$  myeloma, with 32% bone marrow plasma cells. She received melphalan and prednisolone as induction chemotherapy. After six courses of treatment, IgA paraprotein disappeared and bone marrow plasma cells were less than 1%.

Maintenance treatment with  $\alpha$ -interferon, 3 MU for 3 d/wk, was administered. She relapsed in April 1993, with 44% marrow plasma cells, but no evidence of the IgA paraprotein. She received a further 10 courses of melphalan and prednisolone, which resulted in a reduction of bone marrow plasma cells to 1.2% (October 1993).

She was treated with cyclophosphamide (4 g/m<sup>2</sup>), and then 300  $\mu$ g granulocyte colony-stimulating factor for the following 8 days, which resulted in an adequate peripheral-blood stem-cell harvest in April 1994. In July of the same year the patient was admitted for high-dose melphalan treatment and peripheral-blood stem-cell transplant. The following month, she was discharged after completion of the treatment without any complications. An uneventful recovery ensued, and in December 1994 her blood showed hemoglobin 11.1 g/dL (normal range 11.5–15.5 g/dL), white blood cell count  $4.7 \times 10^9/L$  (normal range  $4–11 \times 10^9/L$ ), platelets  $61 \times 10^9/L$  (normal range  $150–400 \times 10^9/L$ ), and normal protein electrophoresis.

During the subsequent 6 months, persistent macrocytosis was observed in her blood; a bone marrow aspirate taken in July 1995 displayed mild dyserythropoiesis, with less than 1% of nucleated cells being plasma cells. At this time, an abnormal karyotype was first detected.

For the following 18 months, she remained mildly anemic and moderately thrombocytopenic; however, her peripheral blood counts then deteriorated, with worsening thrombocytopenia (platelet count  $20 \times 10^9/L$ ). In October 1996, a bone marrow aspirate revealed progression of MDS. Erythropoiesis and myelopoiesis were markedly dysplastic and megakaryocytes were absent. Myeloblasts constituted 20% of the nucleated cells within the marrow, and erythroid, myeloid, and lymphoid cells constituted 55%, 12%, and 13% of the nucleated cells, respectively (French–American–British type classification: refractory anemia with excess blasts in transformation [RAEB-t]). Nonmyeloablative therapy with all-trans-retinoic acid (ATRA) and low-dose cytosine arabinoside was commenced in an attempt to improve the peripheral blood cytopenias by encouraging differentiation of dysplastic hematopoietic precursors. After several months, however, her blood and bone marrow did not show any improvement. At the end of 1996, she became transfusion-dependent and slowly deteriorated as a result of anemia and recurrent chest infection associated with her progressive neutropenia.

Serial bone marrow examinations in February and April of 1997 showed 16% and 26% plasma cells, respectively, indicating that, in addition to persistent MDS, the myeloma had relapsed. In April 1997, the patient was admitted with septicemia, failed to respond to intravenous antibiotics, and died several days after admission.

### 3. Materials and methods

#### 3.1. Cytogenetic analysis

Cytogenetic studies were performed on a series of bone marrow aspirate samples from the patient. The samples

were cultured at 37°C in McCoy's 5A medium (Invitrogen Life Technologies, Carlsbad, CA), supplemented with 20% vol/vol fetal calf serum, 100 U/mL penicillin, 100  $\mu$ g/mL streptomycin, 2 mmol/L L-glutamine, with deacetyl methylcolchicine (Colcemid; Ciba-Geigy, Research Triangle Park, NC) being added for a final concentration of 0.1  $\mu$ g/mL. Three cultures were established for analysis: one culture underwent a 17-hour exposure to colchicine; another culture was incubated for 24 hours prior to the addition of colchicine for 1 hour; and a further 24-hour culture was blocked with methotrexate ( $10^{-7}$  mol/L) and released with thymidine ( $10^{-5}$  mol/L) for the final 5 hours of culture before a 15-minute exposure to colchicine. Standard cytogenetic techniques were employed in harvesting and slide making.

#### 3.2. Flow karyotyping

Mononuclear cells were isolated from a bone marrow specimen using Ficoll density-separation medium (Nycomed Pharma AS, Asker, Norway). Cultures were established in Iscove's modified Dulbecco's medium (Sigma-Aldrich, St. Louis, MO), supplemented with 20% vol/vol fetal calf serum, 100 U/mL penicillin, 100  $\mu$ g/mL streptomycin, 2 mmol/L L-glutamine, and a variety of growth factors (unpublished data). The cells were cultured for 48 hours at 37°C, with colchicine added for the final 16 hours at a concentration of 0.1  $\mu$ g/mL. The chromosomes were isolated for flow karyotyping using the digitonin–polyamine method [12]. They were stained with bizbenzimidazole (Hoechst 33258, Hounslow, UK) and chromomycin A3, and analyzed with a fluorescence-activated cell sorter (FACS 440, Becton Dickinson, Franklin Lakes, NJ).

#### 3.3. Probe preparation for reverse chromosome painting

Approximately 500 copies of sorted chromosomes were amplified by DOP-PCR [13]. The probe was labeled by incorporation of biotin-16-dUTP by reamplification of the primary PCR product: 400 ng of the labeled PCR product and 2.5  $\mu$ g of Cot-1 DNA (Invitrogen Life Technologies) were mixed in hybridization buffer (50% vol/vol deionized formamide, 10% wt./vol. dextran sulfate, 2 $\times$  standard saline citrate, or SSC).

#### 3.4. FISH studies

The FISH studies were performed using the reverse chromosome paint, WCP5 (Cambio, Cambridge, UK), and an  $\alpha$ -satellite probe for chromosomes 1, 5, and 19 (Qbiogene, Carlsbad, CA). Both paints were denatured at 72°C for 10 minutes and preannealed at 37°C for 1 hour; the  $\alpha$ -satellite probe was denatured for 10 minutes only. Slides were denatured in 70% formamide–2 $\times$  SSC at 72°C for 2 minutes and hybridized overnight at 37°C in a humidified chamber. The commercial probes were washed according to the manufacturer's instructions. The reverse paint was washed in 0.5 $\times$  SSC at 72°C for 5 minutes. Detection was achieved

by successive applications of fluorescein isothiocyanate (FITC)-conjugated avidin, biotinylated antiavidin, and FITC-conjugated avidin again (Vector Laboratories, Burlingame, CA). The preparations were counterstained with propidium iodide and 4',6-diamidino-2-phenylindole (DAPI). The slides were examined using a Zeiss Axioplan epifluorescence microscope equipped for viewing FITC, DAPI, and propidium iodide and photographed using Kodak Ektachrome HC film.

## 4. Results

### 4.1. Cytogenetic studies

An abnormal karyotype was first found in July 1995, when, in addition to a normal female cell line, two unrelated abnormal clones were present. The mainline clone had monosomy of chromosome 5 and additional material on the short arm of chromosome 6 that could not be characterized by G-banding: 45,XX,-5,add(6)(p23). The abnormal chromosome was similar in size to chromosome 3. The other clone contained a derived chromosome 7: 46,XX,der(7)t(1;7)(q21;q22). The karyotype was 45,XX,-5,add(6)(p23)[22]/46,XX,der(7)t(1;7)(q21;q22)[7]/46,XX[1].

Fifteen months later, every cell analyzed had a hyperdiploid karyotype displaying the abnormalities seen in the previous mainline population, in addition to extra copies of chromosomes 1, 2, 8, 10, 11, 13, 19, 20, 21, and 22, an abnormal chromosome 15 with additional material of unknown origin attached to the short arm, and two copies of the add(6). The karyotype was 57,XX,+1,+2,-5,+add(6)(p23)×2,+8,+10,+11,+13,add(15)(p11),+19,+20,+21,+22.

A further 6 months later, all cells analyzed showed a different hyperdiploid karyotype that had monosomy of chromosome 5, two copies of the add(6) chromosome, and trisomy of chromosomes 8 and 11: 48,XX,-5,add(6)(p23),+add(6)(p23),+8,+11 (Fig. 1). A bone marrow sample taken at this time was used for reverse chromosome painting.

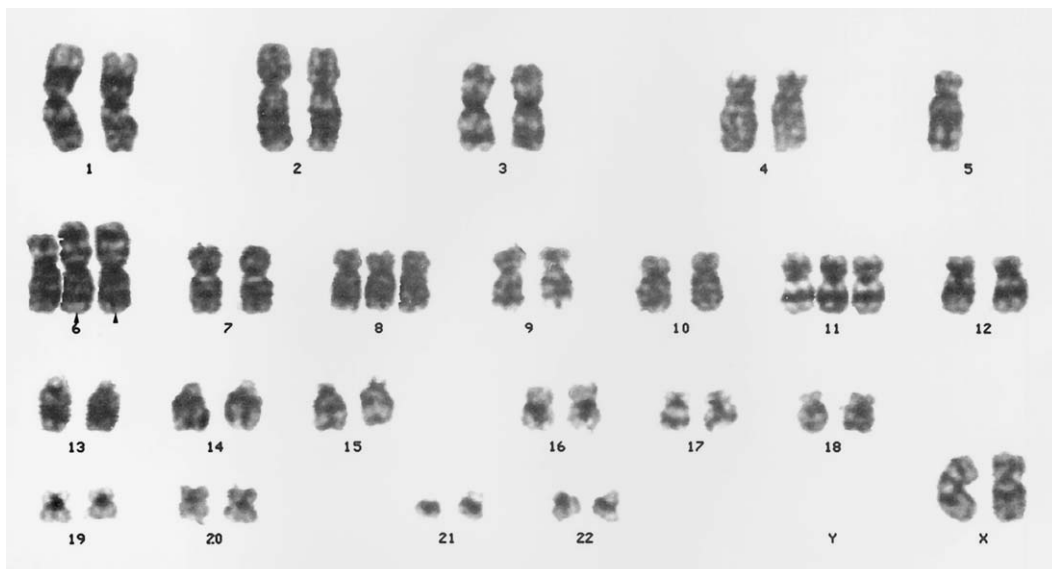


Fig. 1. Karyotype of sMDS case: 48,XX,-5,add(6)(p23),+add(6)(p23),+8,+11. The add(6)(p23) are indicated by arrowheads.

### 4.2. Flow karyotyping studies

The flow karyotype of the patient is shown in Fig. 2A. Despite a low mitotic index, resulting in a flow karyotype of reduced resolution, it was evident that the peak for chromosome 3 was enlarged. Using WinMDI software (Joe Trotter, Scripps Research Institute, CA), gating analysis revealed the number of events in the peak representing chromosome 3 to be approximately double that of the peak for chromosome 4 (data not shown). The gating data, together with the cytogenetic findings, suggested that the enlarged peak represented both the add(6) chromosome and the normal chromosome 3. Chromosomes from this peak were used to produce a probe specific for both the add(6) and normal chromosome 3. The flow karyotype also displayed diminished peaks for chromosomes 5 and 6, as a result of only one normal homolog of these chromosomes.

### 4.3. FISH studies

Reverse chromosome painting of the probe representing the enlarged peak hybridized to chromosome 6 from p21.3 to qter, chromosome 5 from pter to q13, and the entire length of chromosome 3 (Fig. 2B). Subsequent forward chromosome painting of a WCP5 onto metaphases from the bone marrow revealed hybridization to the entirety of normal chromosome 5 and to the region from the short-arm telomere to 6p21.3 in both copies of the add(6) (Fig. 2C). The  $\alpha$ -satellite probe for chromosomes 1, 5, and 19 showed that the add(6) contained an inactive chromosome 5 centromere and was thus dicentric (Fig. 2D).

## 5. Discussion

Our study shows the potential of using reverse chromosome painting on flow-sorted chromosomes from patients

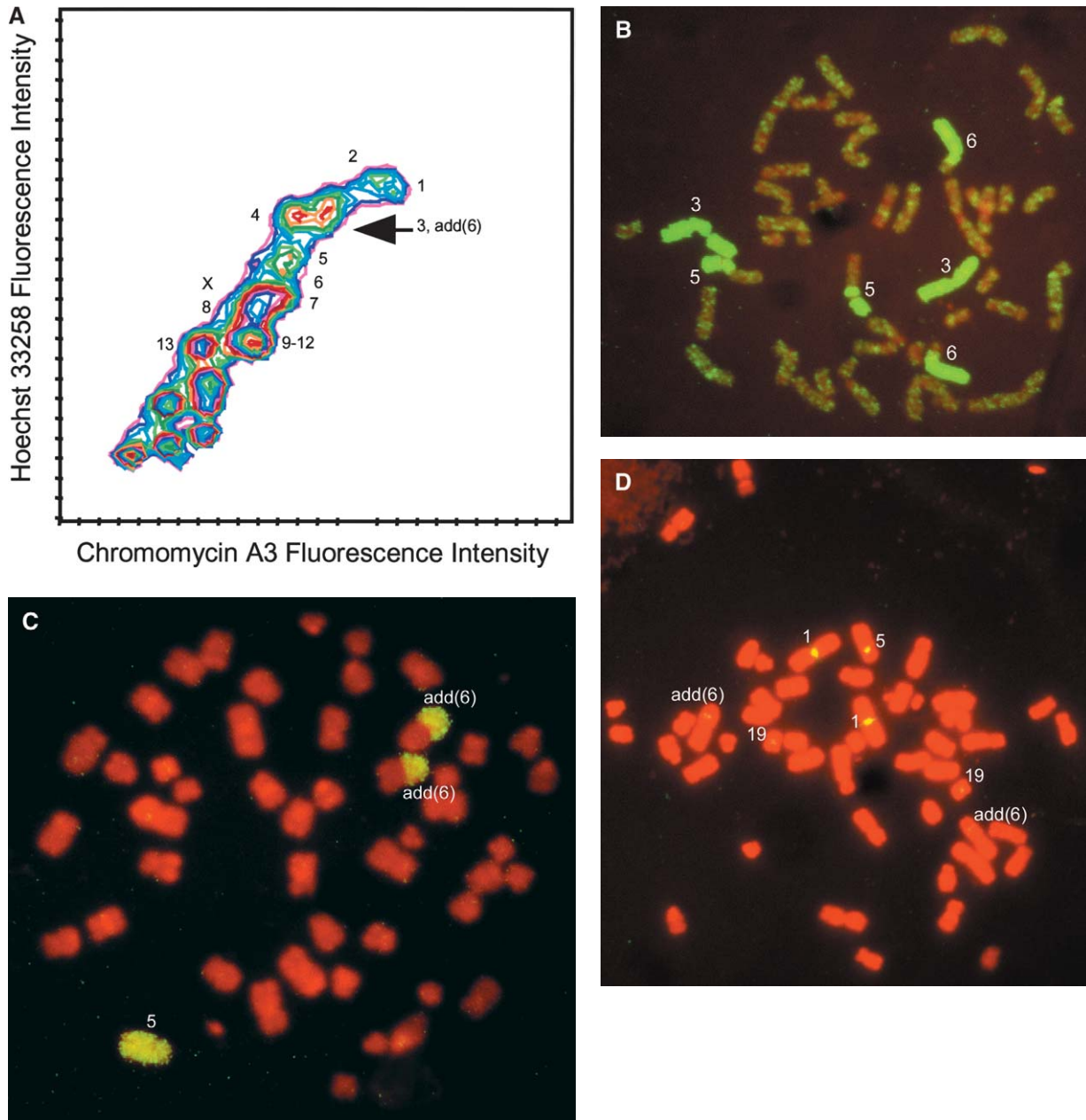


Fig. 2. (A) Bivariate flow karyotype of the patient; the peak containing both chromosome 3 and add(6) is indicated by an arrow. The axes show fluorescence intensities of Hoechst 33258 and chromomycin A3 as an arbitrary scale. (B) Reverse chromosome paint of flow-sorted peak containing add(6) and chromosome 3 onto a normal metaphase. (C) Forward paint using WCP5 onto a metaphase from the patient's bone marrow, chromosome 5 homolog (indicated by 5), and the region 5pter→5qter13 in both copies of the add(6), indicated by add(6). (D) FISH using a  $\alpha$ -satellite probe for chromosomes 1, 5, and 19. A signal is present on the normal chromosome 5 homolog and on each copy of the add(6) at an unconstricted region of the chromosome. Signals are also detected on both copies of chromosomes 1 and 19. The numbers and add(6) represent the chromosomes showing hybridization to the probe.

with hematologic malignancies. G-banding studies on an abnormal chromosome 6 from this patient with sMDS could only characterize the chromosome as an add(6)(p23) (Fig. 1). Reverse chromosome painting, however, allowed an exact determination of its composition. Furthermore, it reassigned the breakpoint on chromosome 6. The chromosome was shown to be derived from an unbalanced rearrangement involving chromosomes 6 and 5, with breakpoints at p21.3

and q13, respectively (Fig. 2B). Subsequent forward chromosome painting onto metaphase spreads from the patient with a WCP5 (Fig. 2C) and a centromeric probe for chromosome 5 confirmed these findings and showed that the abnormal chromosome 6 contained an inactive 5 centromere (Fig. 2D). The karyotype was thus redefined, in accordance with the International System for Human Cytogenetic Nomenclature (ISCN 1995) [14], as 48,XX,-5,add(6)

(p23),+add(6)(p23),+8,+11.rev ish psu dic(6;5)(p21.3;q13),+psu dic(6;5)(p21.3;q13)(WCP5+,D5Z1+).

Previous groups have produced paints for reverse chromosome painting from established cell lines [15–22] and phytohemagglutinin (PHA)-stimulated lymphocytes [9,23]. To our knowledge, there has been only one other report of the use of this technique to characterize a rearranged chromosome from primary neoplastic patient material [24]. The present study demonstrates that flow karyotyping and reverse chromosome painting have been achieved on samples previously reported to be difficult, due to insufficient metaphase cells being obtained for flow karyotyping [21]. Additionally, the interphase nuclei obtained from bone marrow are more fragile than those of fibroblasts. This contributes to the debris continuum, adversely affecting the resolution obtained [25].

The development of sMDS subsequent to treatment for multiple myeloma with alkylating agents is well documented [26,27]. The chromosomal abnormalities found in the patient appear to be consistent with the diagnosis of sMDS. Partial loss of long-arm material from chromosome 5 is one of the most frequent changes reported, and dicentric chromosomes are also occasionally observed [28,29]. Interestingly, another case also showed a partial deletion of the long arm of chromosome 5 together with an acquired dicentric chromosome [30]. Trisomy 8 is a common finding in myeloid malignancies, including sMDS [29]. The der(1;7)(q10;p10) translocation is not uncommon in sMDS [29]. It is particularly associated with cytotoxic therapy or occupational exposure to toxic substances [31,32]. It is usually unbalanced, which effectively results in trisomy of 1q and monosomy of 7q [33]. Our case has a der(1;7)(q21;q22), with different breakpoints to the standard der(1;7)(q10;p10), and results in monosomy of distal 7q; the significance of which is unknown. Increased plasma cell numbers or hypergammaglobulinemia as well as trilineage dysplasia have been reported in patients with der(1;7)(q10;p10) and MDS [34].

Reverse chromosome painting was used to characterize the marker chromosome because this method uses metaphase spreads from phytohemagglutinin (PHA)-stimulated lymphocytes. These are generally of far superior quality than those from neoplastic tissue, making the interpretation of results easier and allowing a more exact determination of any breakpoints involved. Spectral karyotyping FISH (SKY-FISH) and multicolor FISH (M-FISH) are performed on chromosomes obtained from the neoplastic material, and consequently the resolution of the analysis possible with these techniques is limited by the quality and quantity of available material. Additionally, reverse chromosome painting provides information regarding the chromosomal regions and origins of the extra material. The M-FISH and SKY approaches would have identified only the chromosome of origin of the extra material, as this was an unbalanced chromosomal rearrangement.

In addition to reverse chromosome painting, a number of FISH techniques have recently been developed that may

facilitate the characterization of complex cytogenetic abnormalities found in neoplastic tissue. These include comparative genomic hybridization (CGH) [35], M-FISH [36], SKY-FISH [37], cross-species hybridization (RxFISH) [38], and micro-FISH [39]. Each technique has its own strengths and weaknesses, and not all are universally available in all diagnostic cytogenetic laboratories.

On our patient material, CGH and micro-FISH would have given the same result as reverse chromosome painting, whereas M-FISH and SKY-FISH would have identified the additional material as being of chromosome 5 origin, but not its dicentric nature. A major advantage of flow karyotyping and micro-FISH is that any probes generated can be used for general FISH purposes. A further advantage of reverse chromosome painting, as compared with conventional FISH, is that no prior knowledge of the composition of aberrant chromosomes is required. Conventional FISH can be potentially time-consuming, as numerous hybridizations may be performed prior to the appropriate paint being used and there may be limited patient material available precluding a complete analysis. These disadvantages can be overcome by reverse chromosome painting. Reverse chromosome painting provides information on the chromosomal origin and the subchromosomal regions involved. Reverse chromosome painting has an advantage over CGH in its ability to characterize structural rearrangements, whereas CGH identifies only imbalances within the genome. Reverse chromosome painting can identify subtle deletions that remain undetected by M-FISH [40]. Finally, reverse chromosome painting provides the result in duplicate, which is advantageous when there is nonspecific background signal [11].

We believe that the identification of structural rearrangements that cannot be resolved by G-banding is an important role for the cytogeneticist, first because it may affect patient management, and second because it may identify potential genomic regions involved in tumorigenesis. The methods used by individual laboratories should depend on local facilities and expertise. In our hands, flow karyotyping followed by reverse chromosome painting has proven to be a reliable and robust technique.

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