Construction and analysis of painting probe for homogeneously staining regions in human neuroblastoma cell line IMR-32

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Neuroblastoma, a pediatric malignant neoplasm of neural crest origin, has a wide range of clinical virulence. The mechanisms contributing to the development of neuroblastomas are largely unclear, but non-random chromosomal changes identified over the past years suggest the involvement of genetic alterations. Amplification of the human N-*myc* proto-oncogene is frequently seen either in extrachromosomal double minutes or in homogeneously staining regions (HSRs) of aggressively growing neuroblastomas. N-*myc* maps to chromosome 2 band 24, but HSR have never been observed at this band, suggesting transposition of N-*myc* during amplification. We have constructed and analyzed the region-specific painting probe for HSR in neuroblastoma IMR-32 to determine the derivative chromosomes. Microdissection was performed on HSR using an inverted microscope with the help of microglass needles and an micromanipulator. We pretreated the microdissected fragments with Topoisomerase I which catalyzes the relaxation of supercoiled DNA, and performed two initial rounds of DNA synthesis with T7 DNA polymerase followed by conventional PCR to enable the reliable preparation of Fluorescent *in situ* hybridization probe from a single microdissected chromosome. With this method, it was possible to construct the region-specific painting probe for HSR. The probe hybridized specifically to the HSRs of IMR-32, and to 2p24, 2p13 of normal chromosome. Our results suggest there was coamplification of N-*myc* together with DNA of the chromosome 2p24 and 2p13. Moreover, the fluorescent signals for the amplified chromosomal regions in IMR-32 cells were also easily recognized at almost all the interphase nuclei. Thus this painting probe can be applied to detect the similar amplification of N-*myc* in neuroblastoma tissue, and the probe pool for HSR may be used to identify the cancer-relevant genes.

Keywords: 2p24, 2p13, HSRs, IMR-32, Microdissection, Painting probe

INTRODUCTION

The analysis of recurring chromosome aberrations has become an integral part of the diagnostic and prognostic workup of many human cancers (Seizinger *et al.*, 1991; Mitelman *et al.*, 1994; Heim and Mitelman, 1995), and their molecular analysis has facilitated the identification of genes related to the pathogenesis of cancer. But the technical limitation of conventional cytogenetic method is the inability to characterize all recognizable chromosome rearrangements. Especially in solid tumor the unidentifiable marker chromosomes or unbalanced translocations frequently prevent complete karyotypic analysis. At this point, chromosome microdissection which provides a direct method to identify the chromosomal composition of virtually any kind of cytologically visible chromosomal rearrangement is one of the best approach method.

Chromosome painting is a technique of Fluorescent *in situ* hybridization (FISH) combined with chromosome *in situ* suppression hybridization using a chromosome-specific DNA library as a probe pool, and depicts an entire chromosome or a chromosome region (Pinkel *et al.*, 1988; Deng *et al.*, 1992). The generation of chromosome band specific painting probes by PCR amplification of microdissected DNA has proven extremely useful in solving cytogenetic problems which are indeterminable by routine chromosome banding analysis (Kao *et al.*, 1991; Guan *et al.*, 1993; 1996; Zhuang *et al.*, 1996). Any abnormal chromosome segment can be microdissected and converted to a FISH probe for hybridization to normal metaphase chromosomes, resulting in a pattern of hybridization which reveals its chromosomal derivation (Park *et al.*, 1996).

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Neuroblastoma, a malignant neoplasm of neural crest origin, has a wide range of clinical virulence (Breslow *et al.*, 1971; Wilson *et al.*, 1974). It very often has a rapidly progressive clinical course and, although many advances has been made in the understanding of the genesis and biology of this tumor that have been translated into better clinical management, little improvement of survival rates have been achieved. The mechanisms contributing to the development of neuroblastomas are largely unclear, but non-random chromosomal changes identified over the past years suggest the involvement of genetic alterations (Tsuda *et al.*, 1987; Fong *et al.*, 1989; Savelyeva *et al.*, 1994).

The human neuroblastoma cell line IMR-32, which was established from tissue of a patient who had not been exposed to any chemotherapy (Tumilowicz *et al.*, 1970), constitutes an excellent system for analysis of HSR-specific sequences (Kanda *et al.*, 1983). IMR-32 cells, which exhibit some karyotypic heterogeneity, possess, in addition to a normal chromosome 1, two abnormal chromosomes 1 in which an HSR is inserted in the short arm (Brodeur *et al.*, 1981).

To determine the chromosomal derivation for HSR in neuroblastoma IMR-32 we have constructed and analyzed the region-specific painting probe for HSR of IMR-32. The protocol presented here is based on direct PCR amplification of the microdissected chromosome fragment using priming sites naturally present in the chromosome (Meltzer *et al.*, 1992). We microdissected HSR of IMR-32 cell, and pretreated the microdissected fragments with Topoisomerase I which catalyzes the relaxation of supercoiled DNA, and performed two initial rounds of DNA synthesis with T7 DNA polymerase followed by conventional PCR to enable the reliable preparation of Fluorescent in situ hybridization probe (Park *et al.*, 1996). We constructed the painting probe for normal chromosome band 2p24 with same method and compared the signal intensity of the painting probe between HSR and 2p24.

MATERIALS AND METHODS

Chromosome preparation

IMR-32 cells, originally obtained from the American Type Tissue Culture Collection and maintained in culture for several months, were used. Cells were cultured in RPMI medium supplemented with 10% fetal bovine serum. For cytogenetic analyses the cells were treated with colcemid, harvested and fixed according to routine procedures.

Chromosome microdissection

The microdissection was performed with a microglass needles controlled by a micromanipulator attached to an inverted microscope (magnification 1000 ×). Metaphase spreads from peripheral blood lymphocytes and IMR-32 cells were prepared on 24 × 50 mm coverslips and stained by trypsin Giemsa banding. One copy of a targeted chromosomal region was microdissected.

Amplification of dissected DNA

Each dissected fragment was transferred into a 5 μl collection drop [containing 40 mM tris-HCl, pH 7.5, 20 mM $MgCl_2$, 50 mM NaCl, 200 μM dNTP, 1 unit Topo I (Promega), and 5 pM universal primer (CCGACTCGAGNNNNNNATGTGG)]. The collection drop was covered with a drop of mineral oil and incubated at 37°C for 30 min, followed by incubation at 96°C for 10 min.

The DNA was denatured at 97°C for 2 min and cooled to 4°C to let the primer anneal at random sites. One unit of T7 DNA polymerase (USB, Sequenase Version 2.0) was added, and the temperature was ramped to 37°C over an 8 min interval and kept at 37°C for 8 min. After denaturation and annealing, the synthesis step with T7 was repeated once more by adding fresh enzyme. Following this pre-amplification step, a conventional PCR reaction catalyzed by Taq DNA polymerase was performed in the same tube. Fifty μl PCR reaction mixture [10 mM tris-HCl pH 8.4, 2 mM $MgCl_2$, 50 mM KCl, 0.1 mg/ml gelatin, 200 μM each of dNTP, 50 pM universal primer, 2.5 unit Taq DNA polymerase] was heated to 95°C for 5 min followed by 35 cycles at 94°C for 45 s, 56°C for 45 s, 1 min at 72°C, with a 5 min final extension at 72°C.

Fluorescent *in situ* hybridization

To prepare a painting probe, 2 μl of the PCR product were labelled with biotin 16-dUTP in a secondary PCR reaction identical to that described above except for the addition of 40 μM biotin 16-dUTP. The reaction was continued for 12 cycles of 45 s at 94°C, 45 s for 56°C, 1 min at 72°C, with a 5 min final extension at 72°C. Metaphase spreads from normal human lymphocytes were prepared according to the method of Dutrillaux and Viegas-Pequignot (1981).

Hybridization of the FISH probes followed our procedure described previously (Park *et al.*, 1996). Approximately 100 ng of labeled probe was added to 10 μl hybridization mixture [containing 50% formamide, 2 × SSC, and 2.5 μg *Cot* I DNA, 5 μg salmon sperm DNA] and denatured at 75°C for 5 min, preannealing of repetitive DNA sequences was allowed for 2 h at 37°C before application to separately denatured chromosome specimens. The painting probe from 2p24 and HSR were hybridized to IMR-32 and normal metaphase cells.

Hybridization signals were detected by two layers of

fluorescein isothiocyanate-conjugated avidin (Vector) and amplified with one layer of anti-avidin antibody (Vector). The slides were counterstained with propidium iodide (1.0 μg/ml) in antifade solution and examined by Zeiss Axioscope fluorescence microscope. The microphotographs were taken with Ektachrome film (Kodak, ASA 400) and then the same metaphase spreads were sequentially stained using Giemsa to confirm the precise band.

RESULTS AND DISCUSSION

The IMR-32 cells used for the present experiments contain a modal number of 49 chromosomes. Approximately 70% of cells have only one normal chromosome 1 plus two apparently identical abnormal chromosomes 1 (Fig. 1). These abnormal chromosomes result from insertion of a segment of HSR into the distal portion of the chromosome 1 short arm. The HSR-containing chromosome 1 was 50% larger than the normal chromosome 1 and hence was easily recognized.

The HSR of IMR-32 was microdissected (Fig. 2). Amplified PCR products from microdissected fragment appeared as a smear ranging from 200-800 base pairs. There was no apparent DNA amplification in the negative control lane.

FISH was conducted with probe pools generated from PCR products to confirm the construction of the painting probe and to determine the derivation of the HSR. Figure 3 shows painting probe hybridized to the metaphase chromosomes of IMR-32 and normal lymphocyte. The painting probe for HSR was hybridized to the HSR of IMR-32 (Fig. 3b) and was mapped to 2p24 and 2p13 of normal metaphase cells (Fig. 3a). We could deduce that the HSR included amplified materials from both chromosome segment. To confirm the mixed chromosome derivation for HSR, we constructed the painting probe for 2p24, and compared the intensity of hybridization signal (Fig. 4a). The painting probe for 2p24 hybridized to HSR of IMR-32, but the intensity of signals was reduced to half (Fig. 4b). Therefore HSRs were considered to have originated from coamplification of N-*myc* together with DNA of the chromosome 2p24 and 2p13.

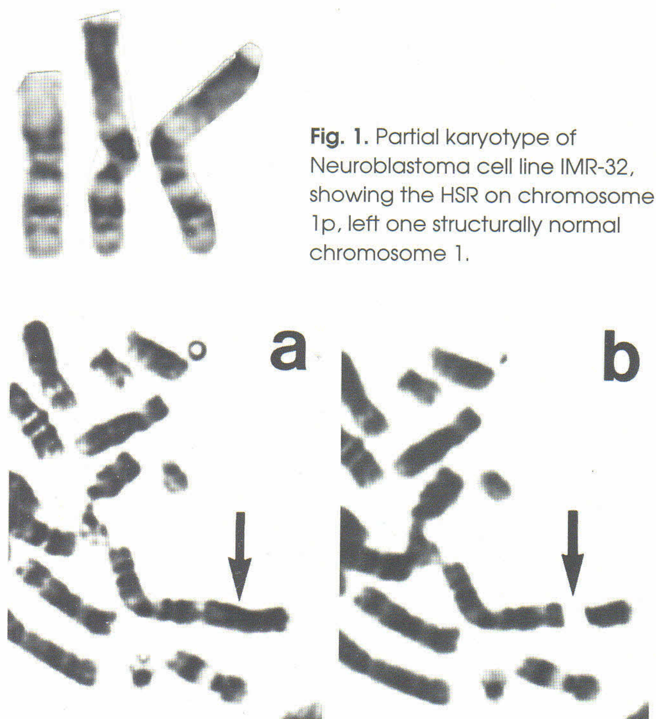

Fig. 1. Partial karyotype of Neuroblastoma cell line IMR-32, showing the HSR on chromosome 1p, left one structurally normal chromosome 1.

Fig. 2. Chromosome microdissection. a, Giemsa banded chromosome spread before cutting and removing chromosome fragments. Arrow indicate the HSR on 1p3. b, Same chromosome spread after cutting at HSR.

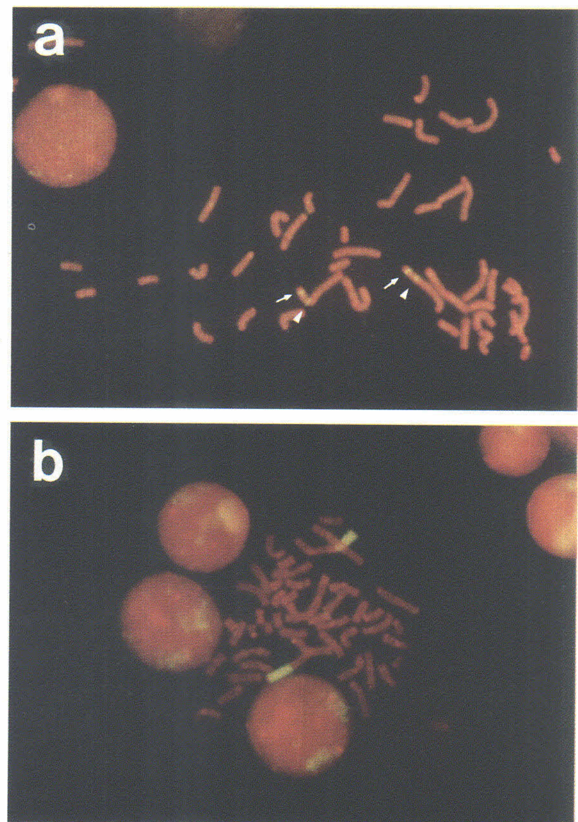

Fig. 3. FISH with painting probe for HSR in IMR-32 cells. Hybridization was performed on normal lymphocyte (a) and IMR-32 cells (b). The painting probe was hybridized specifically to the HSRs of IMR-32, and to derivative chromosome segments 2p24 (arrow) and 2p13 (arrowhead) of normal metaphase chromosome. The fluorescent signal from the amplified chromosomal region in IMR-32 cells was also easily recognized at almost all the interphase nuclei.

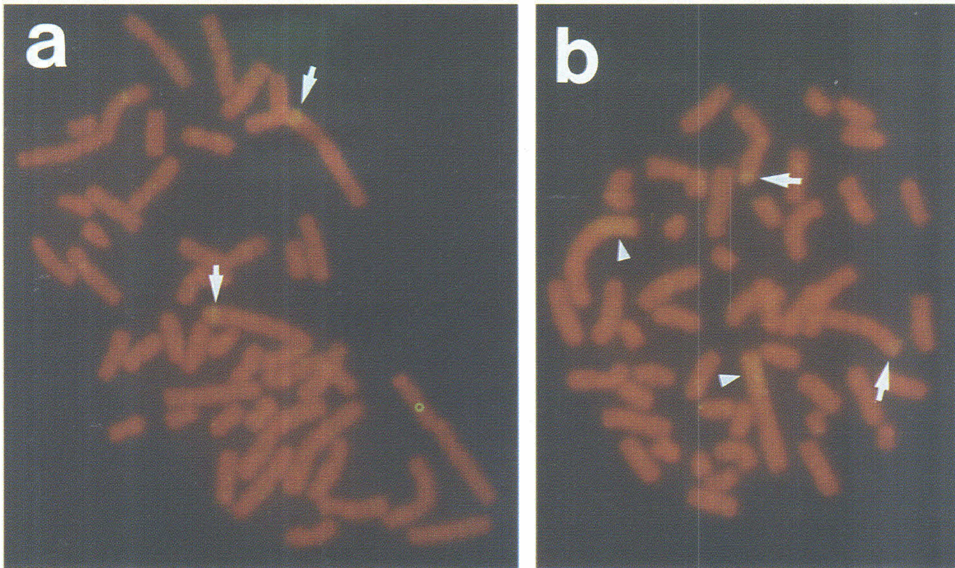

Fig. 4. FISH with painting probe of 2p24. Hybridization was performed on normal lymphocyte(a) and IMR-32 cell(b). The painting probe was hybridized to 2p24(arrows) and the HSRs of IMR-32(arrowheads), but the intensity of signals in HSRs was reduced to half.

In neuroblastoma, cytogenetic studies have revealed two types of obviously non-random chromosomal alterations: HSRs and double minutes (DMs): and alterations in the distal portion of the chromosome 1 short arm (Hayashi *et al.*, 1989; Schwab *et al.*, 1992). N-*myc* is thought to play an important role in determining the biological behavior of neuroblastoma, as genomic amplification of the N-*myc* proto-oncogenes correlates with both advanced disease stage and rapid tumor progression (Brodeur *et al.*, 1984; Seeger *et al.*, 1985). Multiple copies of the N-*myc* oncogene are only rarely detected in patients with localized neuroblastoma, and in most of these reported cases, the presence of N-*myc* amplification is associated with poor outcome (Cohn *et al.*, 1995). N-*myc* maps to chromosome 2 band p24 (Schwab *et al.*, 1984), amplified copies of N-*myc* localized to HSRs and DMs. Neither the resident site of N-*myc* or other regions of the short arm of chromosome 2 have been found to harbor amplified N-*myc* (Corvi *et al.*, 1994). This implies that the evolution of HSR involves the transpositon of N-*myc* from its original location to distant chromosomal sites.

Amplification is one of the mechanisms by which cellular oncogenes can be activated to express abnormally high levels of protein and to participate in tumorigenesis, but little is known about the molecular mechanism involved. In Neuroblastoma cell line LS, amplified N-*myc* was detected in two HSRs on 12q, and the HSR in line LS involves coamplification of N-*myc* together with host-chromosome DNA (Corvi *et al.*, 1994). It is possible that HSRs in other cell lines also contain amplified host chromosome sequences.

Amplification of 2p24 has detected in rhabdomyosarcoma, small-cell lung carcinoma cell line, retinoblastoma cell line, medulloblastoma (Weber *et al.*,1996; Okazaki *et al.*, 1996; Bayani *et al.*, 1995). Novel DDX1 gene containing a DEAD box motif maps to the same chromosome band as N-*myc* at 2p24 and is co-amplified with the N-*myc* gene in neuroblastoma cell lines (Squire *et al.*, 1995).

Chromosome band 2p13 is fragile site and involved in recurrent translocations in lymphoid leukemia. Previous studies suggested that GFA (glutamine fructose 6 phosphate amidotransferase), TGFA (transforming growth factor alpha), pAT133 (zinc-finger gene), NGFI-C (nerve growth factor-induced clone C), MAD, human homeobox containing genes OTX1, EMX1 and proto-oncogene REL all map to chromosome region 2p13 (Zhou *et al.*, 1995; Field *et al.*, 1994; Holst *et al.*, 1993; Crosby *et al.*, 1992; Edelhoff *et al.*, 1994; Kastury *et al.*, 1994; Houldsworth *et al.*, 1996). REL proto-oncogene is frequently amplified in extranodal diffuse large cell lymphoma. At this time we could not say the meaning of the coamplification in chromosome 2p13 and 2p24,

Thus this painting probe can be applied to detect the similar amplification of N-*myc* in neuroblastoma tissue, and the probe pool for HSR may be used to identify the cancer-relevant genes.

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