A novel human processed gene, DAD-R, maps to 12p12 and is expressed in several organs

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Abstract A cDNA of a processed gene of human DAD-1 (defender against apoptotic cell death) was cloned from the human neuroblastoma cell line SH-SY5Y. The genomic sequence of this novel processed gene, DAD-R, lacked introns and was flanked by 8 bp terminal repeats. RT-PCR showed that the transcript is expressed predominantly in testis, ovaries, pancreas, lung and skeletal muscle. DAD-R has several possible initiation codons, one of them producing an open reading frame comprising 75% of the DAD-1 gene. We determined the chromosomal localization of DAD-R as 12p11.2–12p12.1, an area linked to familial synpolydactyly and frequently amplified in a variety of cancers, including those of testis, ovaries, pancreas and lungs.

Key words: Processed gene; Apoptosis; *N*-Linked glycosylation; Human neuroblastoma; 12p12; Synpolydactyly

1. Introduction

Defender against apoptotic cell death (DAD-1) was first characterized as a ubiquitous protein necessary to prevent the initiation of the programmed cell death pathway [1]. A hamster BHK21-derived tsBN7 cell line containing a single mutation in DAD-1 undergoes apoptosis when shifted into a non-permissive temperature. Apoptosis under these conditions cannot be prevented by Bcl-2. This integral endoplasmic reticulum membrane protein, DAD-1, has been shown to be highly homologous to Ost2p, a yeast component of oligosaccharyltransferase (OST) complex, which is an enzyme carrying out the first step in the *N*-linked glycosylation pathway of proteins [2]. Later, the mammalian DAD-1 was confirmed to be a subunit of the mammalian OST complex [3], and shown to be required for *N*-glycosylation [4]. However, blocking of *N*-glycosylation with tunicamycin does not trigger apoptosis in mammalian cells. So eliminating DAD-1 has been hypothesized to trigger programmed cell death in some manner that does not arise directly from discontinuation of *N*-glycosylation [4]. The report that overexpression of DAD-1 in *Caenorhabditis elegans* suppresses developmentally regulated programmed cell death supports the view of DAD-1 as a suppressor of apoptosis [5].

Recently, transgenic mice overexpressing DAD-1 in the thymus and the peripheral immune system have been generated. While apoptosis of thymocytes remains unaffected, peripheral T-cells of these mice displayed hyperproliferation [6]. Another study prepared mice lacking the functional DAD-1 gene by gene targeting. Homozygous mutants died in utero displaying apoptotic features [7]. However, the OST activity was apparently retained even after the DAD-1-deficient cells were destined to die. Interestingly, heterozygous mice displayed mild thymic hypoplasia and soft tissue syndactyly [7]. These in vivo studies demonstrate the role of DAD-1 in the regulation of cell proliferation and apoptosis. One outcome of the disruption of developmentally regulated apoptosis is the syndactyly phenotype, a lack of separation of digits. A recent paper has mapped a translocation between chromosomes 12 and 22 in genomic samples of patients with a complex type of synpolydactyly [8].

Our group considered DAD-1 as one potential regulator for neuronal apoptosis. As a part of the work, we used PCR to sequence DAD-1-related sequences from neuroblastoma cell lines. As an embryonic malignancy of the sympathetic nervous system, neuroblastoma is a useful model for approaching neuronal apoptosis, especially since it has been proposed that a spontaneous regression of a certain form of neuroblastoma may involve programmed cell death [9]. We now report the cloning, initial characterization and chromosomal mapping of a human processed gene highly homologous to DAD-1. Processed genes are a result of reverse transcription of mRNA and subsequent integration into a genomic site different from that of the original gene. Consequently, they typically lack introns, possess direct repeats at either end and display mutations resulting in new start codons and a stop codon. Most of such genes are not expressed.

2. Materials and methods

2.1. Cloning and sequencing of DAD-R

For the PCR amplification of DAD-1, a related human sequence RNA from the human neuroblastoma cell line SH-5YSY was reverse-transcribed and amplified using 50 pmol DAD-1 primers published previously [10]: 5′-ATGTCGGCGTCGGTAGT-3′ and 5′-TCAGCCAACGAAGTTCAT-3′. PCR products were cloned into pGEM-T Vector System I (Promega) and sequenced. The full-length DAD-R cDNA sequence was then cloned using the 5′- and 3′-RACE technique (Marathon-Ready cDNA, Clontech) with DAD-R-specific primers DR1: 5′-AGTGTCAGGCACCCG-3′ and DR2: 5′-GTGCTAGCAAAGAGAAAG-3′. The same primers and pGEM-T vector were used to sequence the human genomic sequence of DAD-R employing the RACE technique (GenomeWalker, Clontech).

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2.2. Chromosomal localization of the human DAD-R processed gene

The DAD-R gene was mapped by fluorescence in situ hybridization (FISH). A P1 clone used as a probe for FISH was obtained from Genome Systems Inc. For FISH, human peripheral blood lymphocytes were cultured according to standard protocols and cells were treated with 5-bromodeoxyuridine (BrdU) at early replicating phase to induce a banding pattern as described earlier [11,12]. An approximately 100 kb probe specific for the human DAD-R gene was labeled with biotin-11-dUTP (Sigma Chemicals) according to standard protocols (Nick Translation Kit, BRL). The FISH procedure was carried out in 50% formamide, 10% dextran sulfate in 2×SSC and the signals were detected according to conventional detection methods as described earlier [13,14]. A multicolor image analysis was used for acquisition, display and quantification of hybridization signals of metaphase chromosomes with a system described earlier [15]. To determine the assignment of the human DAD-R gene a specific probe was hybridized on metaphase chromosomes derived from normal human lymphocyte cell culture. The identification of the chromosomes was based on the DAPI banding pattern, which resembles G-bands after BrdU incorporation at the early replicating phase. Only double spot signals were taken into account as specific hybridization signals.

2.3. Semiquantitative RT-PCR

Total RNA of human tissues was obtained from Clontech and total RNA of neuroblastoma cell lines was extracted using the RNeasy Kit (Qiagen) according to the manufacturer's instructions. Reactions for first strand cDNA synthesis were carried out using 1.0 μg of total RNA in a total volume of 20 μl containing 150 ng random hexamer primers (Gibco BRL), 0.5 mM dNTPs (Gibco BRL), 10 mM dithiothreitol, 200 U SuperScriptII Reverse Transcriptase (RT) (Gibco BRL) and 2 U RNase H (Gibco BRL) in the reaction buffer consisting of 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$. Initially the total RNA was denatured at 70°C for 10 min and immediately chilled on ice. First strand cDNAs were obtained after 10 min at 23°C and 50 min at 42°C. The reaction was terminated at 70°C for 15 min. RNase H (2 U, Gibco BRL) was added to each RT reaction followed by incubation at 37°C for 20 min.

PCR was carried out in a final volume of 10 μl containing 0.5 U Taq Gold polymerase, 200 μM dNTPs, 0.4 μM of each primer, in a buffer consisting of 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 2.0 mM $MgCl_2$ and 1 μl of the RT product (reverse-transcribed total RNA). Specific PCR primers for DAD-R and human DAD-1 had the following sequences: for DAD-R: DR3 5′-GAA CAA AGT GGA CTT CGA AAG C-3′ and DR4 5′-GCC AAC AAA GTT CAT GAC GAT A-3′; for human DAD-1: 5′-ATG TCG GCG TCG GTA GT-3′ and 5′-TCA GCC AAC GAA GTT CAT-3′. The expected PCR product sizes were 300 and 342 bp, respectively. All PCR primers were biotinylated at their 5′ ends. PCR samples were overlaid with mineral oil and amplification was performed on a PTC-100 Programmable Thermal Controller (MJ Research). The samples were denatured initially at 95°C for 12 min, followed by 20 cycles with denaturation at 95°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 90 s. The final cycle was followed by a 5 min extension step at 72°C. The absence of contaminants was routinely checked by RT-PCR assays of negative control samples (H_2O control or no RT added). The housekeeping gene *GAPD* was coamplified as an internal standard control as previously described (Eggert et al., submitted to BioTechniques).

Each PCR sample (10 μl+2 μl Ficoll Dye Reagent) was analyzed in parallel with a biotinylated molecular weight marker (Amersham) on a non-denaturing 6% polyacrylamide gel. DNA was electrotransferred to a nylon membrane (Hybond N^+, Amersham) and immobilized by UV crosslinking. Detection of biotin-labeled DNA was performed according to the 'Southern-Light protocol' (Tropix). Quantification of RNA transcript expression was performed by densitometric analysis on X-ray films using Scion Image 1.55 software. A modification of the *GAPD* primers (biotinylated: non-biotinylated at a ratio of 1:49) allowed accurate quantification within the linear range of detection of both the target transcript and *GAPD*. The expression of the target

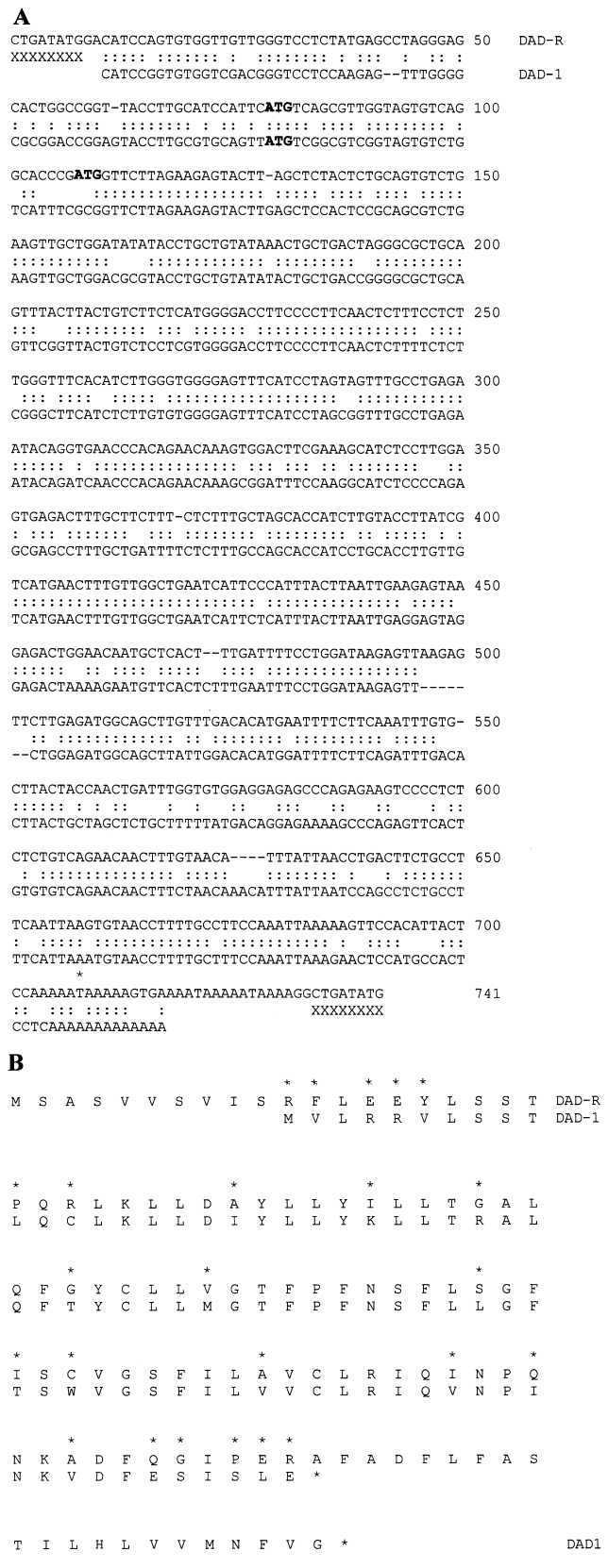

Fig. 1. Sequence analysis. A: Comparison of the human genomic sequence of DAD-R (top) with the cDNA of DAD-1 (bottom). The start of the 5′-UTR and the poly(A) tail in the DAD-R cDNA have been marked by arrowheads. The flanking 8 bp direct repeats are boxed. The initiation and termination codons of DAD-1 and putative initiation and termination codons of DAD-R are shown in bold. B: Amino acid sequence comparison of putative DAD-R and DAD-1 (translation frame of the DAD-R processed gene starting at nt 100). Thirteen amino acid substitutions are shown with asterisks.

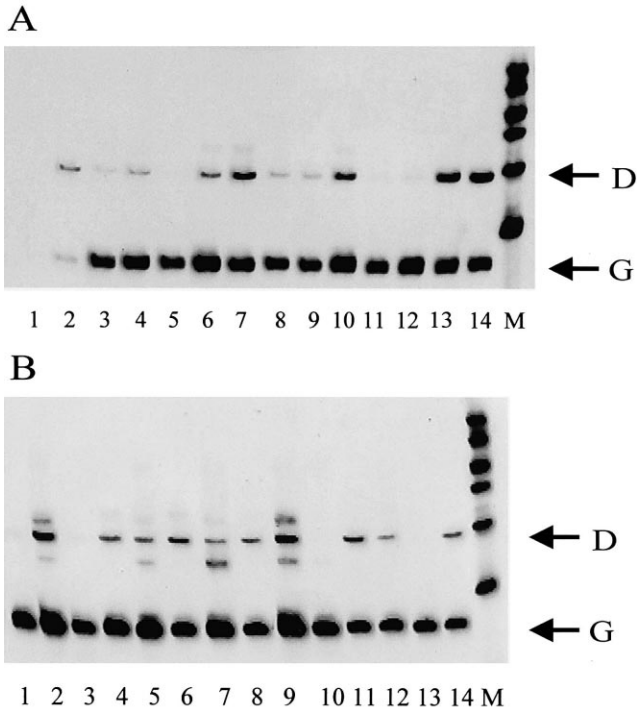

Fig. 2. Expression of DAD-R in various human tissues tested by RT-PCR. Reverse-transcribed RNA was amplified by PCR with primers DR3 and DR4 (see Section 2). A: DAD-R (D) in human organs. RNA control (no RT, testis) (lane 1), pancreas (2), adrenal gland (3), thymus (4), brain (5), skeletal muscle (6), small intestine (7), spleen (8), placenta (9), testis (10), liver (11), kidney (12), ovary (13), lung (14). M: molecular weight markers. GAPDH (G) was used as an internal control for RNA quantification in all RT-PCR reactions. B: DAD-R (D) in human neuroblastoma cell lines. NBL-wN (lane 1), CHP903 (2), CHP901 (3), KAN (4), NLF (5), NGP (6), KCNR (7), IMA5 (8), CHP 134 (9), NBL-S (10), SK-N-AS (11), LAN6 (12), NB69 (13), SY5Y (14). M: molecular weight markers. GAPDH (G) was used as an internal control for RNA quantification in all RT-PCR reactions.

transcript was normalized by taking the ratio between the densitometric unit of the transcript and that of the internal control, *GAPD*.

3. Results and discussion

Processed genes that are expressed as mRNA are sometimes called retrogenes [16]. We decided to call our novel processed gene DAD-R to make a distinction with other DAD-1-related genes, of which DAD-2 has been recently cloned from *Arabidopsis thaliana* (GenBank accession number AF030172). Some reported processed genes that are expressed at the mRNA level also retain the functionality of the 'parental gene'. The two best known examples in humans, PGK2 and PDHA2, are expressed only during spermatogenesis and were derived from X-linked genes [17,18]. These genes are expressed exclusively in the testis and they have retained the full-length open reading frame (ORF) of their parental genes. For these reasons, we decided to study the structure of DAD-R mRNA and its expression in several tissues. Since we discovered a novel, DAD-1-related gene, and since DAD-1 itself has been mapped to chromosome 14, we decided to map the novel gene.

DAD-R genomic gene has several common characteristics of a retrogene: lack of introns; direct 8 nt long repeats; nucleotide sequence homology to DAD-1 both within and outside the translated region; and novel start and stop codons (Fig. 1A). There are several possible initiation codons in DAD-R mRNA, one of which matches relatively well with the Kozak consensus sequence for mammalian protein biosynthesis and is followed by an ORF corresponding to 75% of the DAD-1 ORF (Fig. 1B).

We found expression of DAD-R mRNA in several organs. Strongest expression was seen in lung, ovaries, testis and small intestine (Fig. 2A). Pancreas, skeletal muscle, spleen, placenta and thymus also showed some expression. The RT-PCR reaction was performed twice independently, resulting in identical expression patterns. Expression of a human processed gene in several organs has not been reported before, though there are several reports on human processed genes having single organ expression. To ensure that the PCR products seen in the RT-PCR panels were derived from DAD-R cDNA, each product was cut with the restriction enzyme

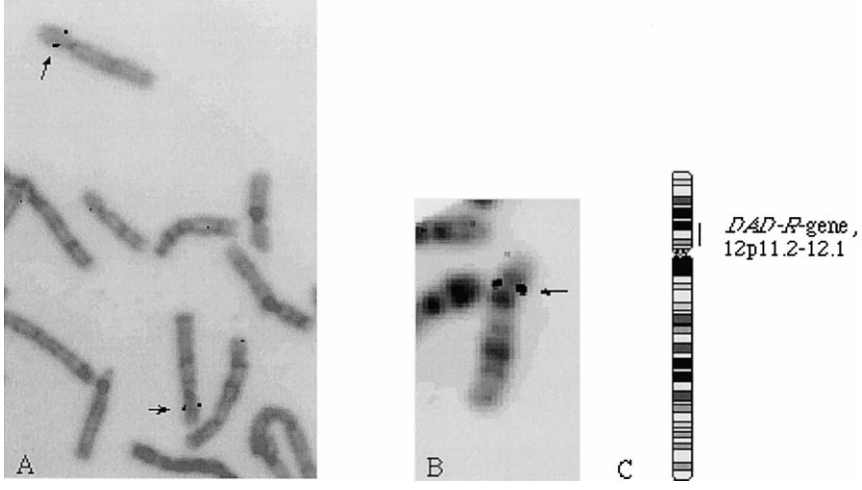

Fig. 3. Chromosomal localization of DAD-R. A: Grayscale image of partial metaphase spread showing specific hybridization signals on chromosomes 12. A grayscale image (B) and an ideogram (C) of chromosome 12 demonstrating the specific assignment of the human DAD-R gene on band 12p11.2–12.1.

*Pst*I, which cuts DAD-R but not DAD-1. This resulted in the disappearance of the 300 bp PCR product from the RT-PCR panel. Conversely, treatment with the restriction enzyme *Ava*I, which cuts DAD-1 but not DAD-R, had no effect on the 300 bp bands in the panel.

A panel of 14 neuroblastoma cell lines revealed DAD-R mRNA expression in 10 of them (Fig. 2B). We will follow this by expanded expression studies of primary neuroblastoma tumors and a wider selection of neuroblastoma cell lines.

Most processed genes are transcriptionally inactive and contain a number of mutations that render them non-functional. However, there are some functional processed genes. As mentioned previously, PGK2 and PDHA2 are expressed only during spermatogenesis and were formed from X-linked genes [17,18]. Atypically, though DAD-R is expressed in several organs, it contains mutations truncating the length of the ORF of the original gene, and the parental gene, DAD-1, is not X-linked. Apparently DAD-R is not expressed at high levels. Whether possible translation products have any biological activity remains to be determined.

Human DAD-R gene hybridizations showed specific hybridization signals on chromosome 12p11.2–12p12.1 (Fig. 3). It is interesting to note that DAD-R is localized at 12p11.2–p12.1, an area amplified in several human tumors. Recent data suggest that there are more than one putative oncogenes in the vicinity of this area [19]. DAD-R is expressed predominantly in testis, ovaries, pancreas, lung and skeletal muscle; high level gains of 12p or 12p12 have been characterized in tumors associated with testicular germ cell tumors (GCT), ovarian GCTs, pancreatic cancer and lung adenocarcinoma [20]. Gains have also been reported in other tumors, such as gliomas [21] and secondary myeloid leukemia [22]. The highest prevalence of 12p12 amplifications has been reported in testicular GCTs, 80% of which display a high copy number of 12p or 12p12 regions. Our FISH mapping places DAD-R at 12p11.2–p12.1. CGH results from secondary myeloid leukemia implicate a small amplicon localized in the 12p11.2–p12 subregion. A recent study was able to determine a 300 kb long shortest region of amplification common to all GCTs [23].

A paper describing the chromosomal translocation cloned from patients with familial synpolydactyly pinpointed two regions involved in the event: 12p11.3 and 22q13.3 [8]. Thus, 12p11.2–p12.1, where DAD-R is located, seems to be a region linked to both synpolydactyly and chromosomal amplification associated with certain neoplasms – a common feature of both being the control of cell proliferation. In vivo studies on the DAD-1 gene, mapped to chromosome 14, clearly demonstrate that DAD-1 is involved in the regulation of apoptosis and cell proliferation in certain tissues [6,7]. Surprisingly, DAD-1 heterozygous mice have the polydactyly phenotype. This poses an interesting dilemma about the possible biological activity of DAD-R and indicates that it could be a candidate gene for these pathologies.

Presumably the predicted oncogene or oncogenes residing at 12p12 confer on certain neoplasms some type of advantage that led to the amplification of this chromosomal region. Based on ubiquitous amplification of several 12p bands in testicular GCT samples it has been hypothesized that more than one gene contributing to oncogenesis may be located within the 12p area [19]. Our work on tumor sample Southern blots shows rearrangement of DAD-R in some samples; we are currently expanding these studies and investigating elevated expression levels of DAD-R in certain tumor samples (Kuittinen et al., in preparation). Our low cycle number PCR gives an accurate comparison of mRNA copy numbers and DAD-R expression in tumor samples is higher than the organ expression. In light of the reported role of DAD-1 in the regulation of apoptosis, the multiple organ expression of its processed gene DAD-R and the chromosomal localization of the latter, the possibility of some type of biological activity of DAD-R should be investigated further.

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References

[1] Nakashima, T., Sekiguchi, T., Kuraoka, A., Fukushima, K., Shibata, Y., Komiyama, S. and Nishimoto, T. (1993) Mol. Cell. Biol. 13, 6367–6374.
[2] Silberstein, S., Collins, P.G., Kelleher, D.J. and Gilmore, R. (1995) J. Cell Biol. 131, 371–383.
[3] Kelleher, D.J. and Gilmore, R. (1997) Proc. Natl. Acad. Sci. USA 94, 4994–4999.
[4] Makishima, T., Nakashima, T., Nagata-Kuno, K., Fukushima, K., Iida, H., Sakaguchi, M., Ikehara, Y., Komiyama, S. and Nishimoto, T. (1997) Genes Cells 2, 129–141.
[5] Sugimoto, A., Hozak, R.R., Nakashima, T., Nishimoto, T. and Rothman, J.H. (1995) EMBO J. 14, 4434–4441.
[6] Hong, N.A., Kabra, N.H., Hsieh, S.N., Cado, D. and Winoto, A. (1999) J. Immunol. 163, 1888–1893.
[7] Nishii, K., Tsuzuki, T., Kumai, M., Takeda, N., Koga, H., Aizawa, S., Nishimoto, T. and Shibata, Y. (1999) Genes Cells 4, 243–452.
[8] Debeer, P., Schoenmakers, E.F., Thoelen, R., Fryns, J.P. and Van de Ven, W.J. (1998) Cytogenet. Cell Genet. 81, 229–234.
[9] Maris, J.M. and Matthay, K.K. (1999) J. Clin. Oncol. 17, 2264–2280.
[10] Apte, S.S., Mattei, M.G., Seldin, M.F. and Olsen, B.R. (1995) FEBS Lett. 363, 304–306.
[11] Lemieux, N.M., Dutrillaux, B.E. and Viegas-Pequiqnot, E. (1992) Cytogenet. Cell Genet. 59, 311–312.
[12] Tenhunen, K., Laan, M., Manninen, T., Palotie, A., Peltonen, L. and Jalanko, A. (1995) Genomics 30, 244–250.
[13] Pinkel, D., Straume, T. and Gray, J. (1986) Proc. Natl. Acad. Sci. USA 83, 2934–2938.
[14] Lichter, P., Cremer, T., Chang Tang, C.J., Watkins, P., Manuelis, L. and Ward, D.C. (1988) Proc. Natl. Acad. Sci. USA 85, 9664–9668.
[15] Heiskanen, M., Peltonen, L. and Palotie, A. (1996) Trends Genet. 12, 379–382.
[16] Dahl, H.H., Brown, R.M., Hutchison, W.M., Maragos, C. and Brown, G.K. (1990) Genomics 8, 225–232.
[17] McCarrey, J.R. and Thomas, K. (1987) Nature 326, 501–505.
[18] Gebara, M.M. and McCarrey, J.R. (1992) Mol. Cell. Biol. 12, 1422–1431.
[19] Henegariu, O., Vance, G.H., Heiber, D. and Pera, M. (1998) J. Mol. Med. 76, 648–655.
[20] Mahlamäki, E.H., Hoglund, M., Gorunova, L., Karhu, R., Dawiskiba, S., Andren-Sandberg, A., Kallioniemi, O.-P. and Johansson, B. (1997) Genes Chromosomes Cancer 20, 383–391.
[21] Patel, A., van Meyel, D.J., Mohapatra, G., Bollen, A., Wrensch, M., Cairncross, J.G. and Feuerstein, B.G. (1998) Cancer Genet. Cytogenet. 100, 77–83.
[22] Willem, P. and Mendelow, B. (1997) Cancer Genet. Cytogenet. 99, 30–37.
[23] Mostert, M.C., Verkerk, A.J., van de Pol, M., Heighway, J., Marynen, P., Rosenberg, C., van Kessel, A.G., van Echten, J., de Jong, B., Oosterhuis, J.W. and Looijenga, L.H. (1998) Oncogene 16, 2617–2627.