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Subchromosomal Mapping of a Putative Transformation Suppressor Gene on Human Chromosome 1

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We previously reported that the introduction of a normal human chromosome 1 via microcell-mediated chromosome transfer suppressed the transformed phenotypes, including anchorage-independent growth, of Kirsten murine sarcoma virus-transformed NIH3T3 (DT) cells. Soft-agar clones derived from DT-#1 cells (DT cells with an intact transferred human chromosome 1) exclusively failed to retain an intact form of this chromosome. Thus, a gene(s) with a suppressive activity on this chromosome had probably been lost. We therefore attempted to identify a commonly deleted region on human chromosome 1 in these soft-agar clones. Although eight of the 9 soft-agar clones examined still contained regions on this chromosome, to a greater or lesser degree, four loci on 1q21 and 1q23-q24 were commonly lost in all of them. Furthermore, the soft-agar clones had growth properties similar to those of DT cells. Thus, chromosome and DNA analyses suggested that human 1q21 and/or 1q23-q24 carries a transformation suppressor gene(s) which controls the transformed phenotypes of DT cells.

Key words: Human chromosome 1 — Chromosome transfer — Microcell-hybrid — Deletion mapping — Tumor suppressor gene

A considerable number of tumor suppressor genes have been isolated and characterized.[1–6] However, more tumor suppressor genes remain to be identified and isolated. The most typical approach to isolating a tumor suppressor gene is so-called "positional cloning" based on the precise mapping of the gene,[2,4–6] which is accomplished by detecting a loss of heterozygosity (LOH) in tumor tissues.[7] Chromosome transfer experiments via microcell fusion[8] have not only confirmed that candidate chromosomes indicated by LOH analyses actually carry a suppressive activity on tumor cells,[9–15] but have also independently identified chromosomes which can suppress the transformed phenotypes of some tumor cells, even when the candidate chromosomes have not yet been identified by other approaches.[16–18] Moreover, the altered phenotypes in microcell-hybrids with a specific transferred chromosome can provide a basis for speculation upon the cellular functions of a gene(s) on the chromosome prior to cloning it.[13,15–17]

By means of microcell-mediated chromosome transfer, we found that the normal human chromosome 1 suppressed various transformed phenotypes of Kirsten murine sarcoma virus-transformed mouse NIH3T3 (DT) cells.[18] The interspecies microcell-hybrids (DT-#1 cells: mouse DT cells with a human chromosome 1) regained the transformed phenotypes, accompanied with the disappearance of intact human chromosome 1, indicating that the spontaneous chromosomal deletions/rearrangements could lead to the loss of a suppressor gene(s) on this chromosome. Thus, in the present study, we examined the presence or absence of human chromosome 1-derived fragments in the revertants (the cells which proliferated in soft-agar media) derived from DT-#1 cells, enabling us to map a putative suppressor gene(s) to human chromosome 1.

MATERIALS AND METHODS

Cells and culture conditions DT cells (Kirsten murine sarcoma virus-transformed NIH3T3 cells)[19] were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) on collagen-coated dishes. DT-#1 cells (DT cells containing a human chromosome 1 introduced via microcell fusion)[18] were maintained in DMEM containing 10% FCS and 800 μg/ml G418. To obtain the soft-agar clones, DT-#1 cells (#1-2 or #1-3 in ref. 18) were plated in semisolid (soft-agar) media containing 10% FCS without G418, as previously described.[18] Macroscopic colonies were isolated 3 weeks later, and expanded in DMEM containing 10% FCS without G418 for DNA and RNA extractions, chromosome analyses and assays of *in vitro* growth properties.

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Chromosome analyses Quinacrine plus Hoechst 33258 staining[20] and fluorescence chromosomal *in situ* hybridization (FISH)[21, 22] were performed as previously described, to confirm the presence or absence of the transferred human chromosome 1 (or the chromosomal fragment) in microcell-hybrids and soft-agar clones.

Southern blots High-molecular-weight DNAs were isolated from cultured cells according to the standard procedures. The DNAs were completely digested with appropriate restriction enzymes, separated by electrophoresis through a 0.8% agarose gel, and transferred to a charged nylon membrane (Hybond N+, Amersham) as recommended by the supplier. Probe DNA was radiolabeled with [α-^{32}P]dCTP by random-priming.[23] The membrane was hybridized and washed under highly stringent conditions.

Northern blots Total cellular RNAs were prepared from exponentially proliferating cells by acid guanidinium thiocyanate-phenol-chloroform extraction.[24] Electrophoresis, Northern transfer, hybridization to the ^{32}P-labeled probe, and washing of the membrane were conducted as previously described.[18]

DNA probes The DNA probes and their localization on human chromosome 1 are shown in Table I and Fig. 2. The pSV2*neo*, with which a transferred normal human chromosome 1 was tagged, was previously shown to be integrated into 1p34-p36 by chromosomal *in situ* hybridization.[8] Cosmid clones on human chromosome 1 (cYS1-X) were isolated and mapped by FISH in our laboratory.[22] A 618-base pair *Sst*II/*Hin*cII fragment of the v-Ki-*ras* gene (Oncor), a full-length human *Krev*-1 cDNA[25] and a mouse α-tubulin cDNA were used for Northern blot hybridization. The human chromosome 1-specific painting probe, pBS1,[26] was used for FISH.

Polymerase chain reaction (PCR) PCR proceeded in a total volume of 20 μl containing 100 ng of genomic DNA, 1 μM of each primer, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.001% gelatin, 200 μM dNTPs and 0.5 unit of Taq DNA polymerase (AmpliTaq, Perkin Elmer Cetus). The oligonucleotide primer sequences and PCR conditions to amplify the APOA2 locus,[27] CRP locus,[28] D1S104 locus,[29] AT3 locus[30] and D1S102 locus[31] on human chromosome 1 were as described by the original developers. The localizations of these loci are shown in Fig. 2. The PCR products were analyzed by electrophoresis through a 5% non-denaturing polyacrylamide gel.

Analyses of *in vitro* growth properties The anchorage-independent growth of cells was indicated by the SAE/PE value (SAE, colony-forming efficiency in soft-agar media; PE, colony-forming efficiency on plastic plates).[18] To determine the SAE, 2×10^2 cells were seeded in semi-solid (soft-agar) media containing 10% FCS, and macroscopic colonies were counted 3 weeks later. To determine the PE, 2×10^2 cells were plated on 60 mm collagen-coated dishes with media containing 10% FCS, and macroscopic colonies were counted 10 days later. To examine the population doubling time of cells, cells (1×10^5) were plated on 35 mm collagen-coated dishes with media containing 10% FCS, and the cell numbers were counted periodically.[18] The index of serum-independent growth was expressed as the cell numbers in 1% FCS/cell numbers in 10% FCS.[18] Cells (1×10^4) were plated on 35 mm collagen-coated dishes in media containing 10% FCS. This was replaced with media containing 1 or 10% FCS the following day, and the cells were counted 7 days later. All experiments were performed in triplicate.

RESULTS

Chromosome analyses of soft-agar clones derived from DT-#1 cells Nine soft-agar clones were isolated from DT-#1 cells. The Quinacrine plus Hoechst 33258 stainings of the metaphase spreads revealed that none of these

Table I. First Screening of Soft-agar Clones by Southern Blot Analyses with DNA Probes on Human Chromosome 1

Probes			Cells										
					Soft-agar clones								
Name	Locus	Location	DT	DT-#1	1	2	3	4	5	6	7	8	9
pMCT58	D1S77	p36	−	+	−	−	+	−	+	+	−	+	+
pSV2*neo*		p34-p36	−	+	+	+	+	−	+	+	+	+	+
pYNZ2	D1S57	p34-p36	−	+	+	−	+	−	+	+	−	+	+
pEFZ13	D1S64	p22-cen	−	+	−	−	+	−	−	+	−	+	+
pMCR3	NRAS	p13	−	+	−	−	+	−	+	+	−	+	+
pSD1-1	D1Z5	cen	−	+	−	−	+	−	+	+	−	+	+
3021E1	SPTA1	q21	−	+	−	−	−	−	+	−	−	−	−
pEKH7.4	D1S65	q25-q32	−	+	−	−	+	−	−	−	−	−	−
pYNZ23	D1S58	q32	−	+	−	−	+	−	−	−	−	−	−
pTHH33	D1S81	q42-q43	−	+	−	−	+	−	−	−	−	+	−

+, Retention; −, loss.

clones had an intact human chromosome 1 (data not shown), whereas the original DT-#1 cells did in more than 90% of the cells. This was consistent with our previous results.[18] Some clones however, seemed to retain the human chromosome-derived fragment, which was suggested by the presence of a chromosome without the strong, centromeric fluorescence characteristic of mouse chromosomes (for example, clone 3 in Fig. 1b). Using one of these clones (clone 3) as well as original DT-#1 cells, we performed FISH analysis with the human chromosome 1-specific whole painting probe.[26] As shown in Fig. 1a, clone 3 contained two human chromosome 1-derived fragments. Re-staining of the same metaphase with Quinacrine plus Hoechst 33258 revealed that one was the entire short arm of human chromosome 1, and the other was the distal half of the long arm of human chromosome 1 (1q25-qter) which was translocated to the mouse chromosome (Fig. 1b).

Identification of the commonly deleted region on human chromosome 1 To determine roughly the human chromosome 1-derived materials in the soft-agar clones, ten DNA probes on human chromosome 1 were first used for Southern blotting (Table I). DNA from clone 4 did not hybridize with any of these probes, suggesting that this particular clone might have lost the whole human chromosome 1 through chromosomal non-disjunction. The remainder (eight of 9 clones) contained, to a greater or lesser degree, regions on the transferred human chromosome 1. In this primary examination, relatively low incidence of retention at the long arm (1q) loci was observed, although the commonly deleted region was not identified. FISH results also suggested that the region lies in the proximal half of human 1q.

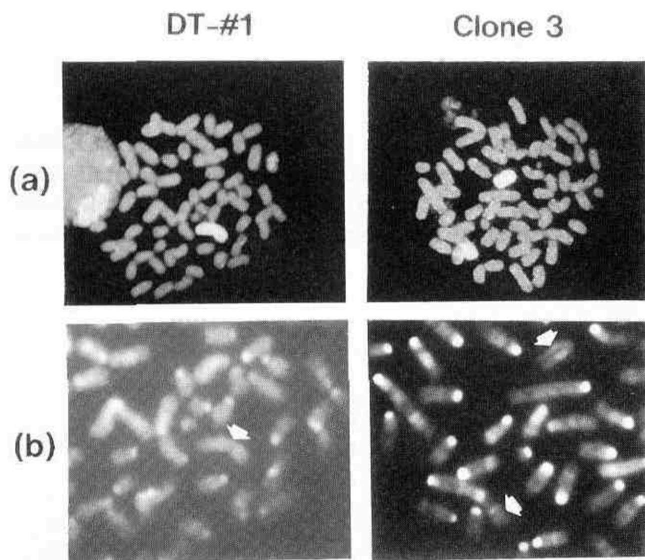

Fig. 1. Painting of human chromosome 1 by fluorescence chromosomal *in situ* hybridization (FISH) (a) and Quinacrine plus Hoechst 33258 staining of the same metaphase spread (b). Arrowheads in (b) indicate an intact human chromosome 1 in DT-#1 cells, and two human chromosome 1-derived fragments (entire 1p and 1q25-qter translocated to the mouse chromosome) in clone 3.

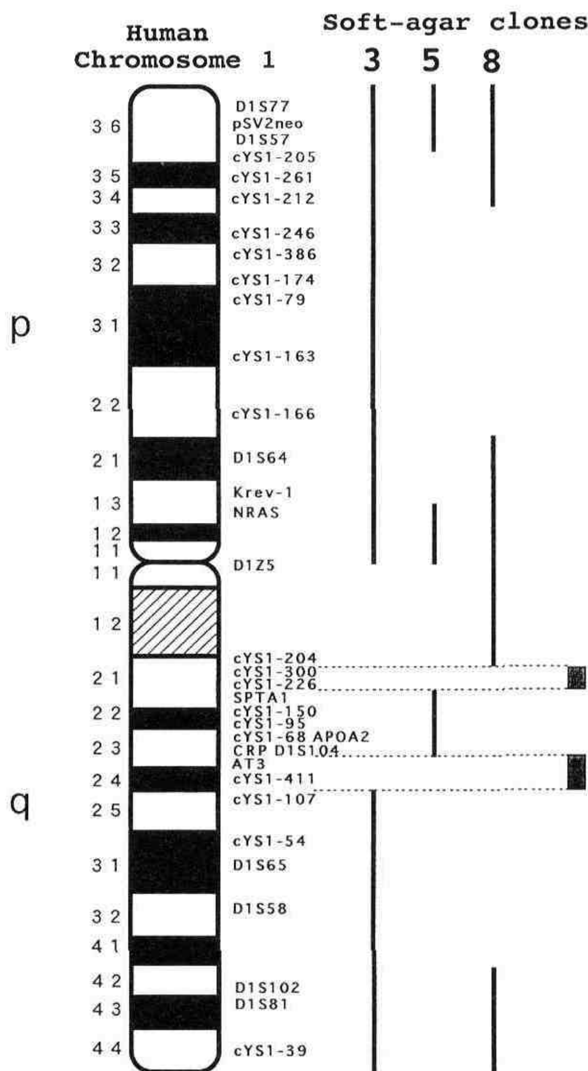

Fig. 2. Identification of the commonly deleted region on human chromosome 1 in DT-#1-derived soft-agar clones. The results of Southern blots with 19 cosmid probes (cYS1-X) and the *Krev*-1 cDNA, and the results of PCR analyses at five loci (APOA2, CRP, D1S104, AT3 and D1S102) are presented together with the data in Table I. The vertical lines indicate the presence of the corresponding locus in each clone. The commonly deleted region is shown as the hatched boxes.

Table II. *In vitro* Growth Properties of DT, DT-#1 Cells and DT-#1-derived Soft-agar Clones

Cells	Colony-forming ability in soft agar (%)[a]	Doubling time (h)	Index of serum-independent growth[b]
DT	86.3	11.9	0.26
DT-#1-2[c]	8.1	21.3	0.04
DT-#1-3[c]	9.9	25.1	0.004
Clone 3	75.8	14.0	0.29
Clone 5	72.6	12.0	0.21

a) SAE/PE. See "Materials and Methods."
b) 1%FCS/10%FCS. See "Materials and Methods."
c) DT-#1 cells from which soft-agar clones were isolated.

Thus, we further analyzed clones 3, 5 and 8. These three clones were selected because they appeared to contain a relatively large quantity of human-derived materials (Table I). The results of Southern blots with 19 cosmid clones (cYS1-X)[22] and the human *Krev*-1 cDNA, and of PCR analyses at five loci on human 1q are schematically represented in Fig. 2, together with the results shown in Table I. Clone 3 retained all loci on human 1p and 1q25-qter, in agreement with the FISH results. Clones 5 and 8 appeared to have a more complicated profile of human-derived regions, which were all different. It should be noted that in clones 3 and 8, the *Krev*-1 cDNA mapped to 1p13.3,[32] the enforced expression of which exhibits a tumor suppressor activity for DT cells,[25] detected human-specific bands in addition to mouse-derived endogenous bands (autoradiograph not shown). Most importantly, the loci defined by cYS1-300 (1q21), cYS1-226 (1q21) and cYS1-411 (1q24) as well as the AT3 locus (1q23) were commonly lost in clones 3, 5 and 8 (Fig. 2). Furthermore, these four loci were also absent in the other six clones and in ten additional clones independently isolated (data not shown). Thus, we concluded that there was a commonly deleted region on 1q21 and 1q23-q24.

Growth properties of soft-agar clones The *in vitro* growth properties of soft-agar clones (clones 3 and 5) were examined and compared with those of DT and DT-#1 cells (Table II). The soft-agar clones again proliferated in soft-agar media at a similar efficiency to that of DT cells. The population doubling time and the index of the serum-independent growth of the soft-agar clones were also similar to those of DT cells. Thus, the suppressive activity observed in DT-#1 cells was abrogated in the soft-agar clones.

Northern blot analyses Differences in v-Ki-*ras* mRNA expression between DT-#1 and parental DT cells have not been observed.[18] Also in the soft-agar clones derived from DT-#1 cells, a similar amount of v-Ki-*ras* mRNA was expressed (Fig. 3a). When the human *Krev*-1 cDNA[25] was used as a probe (Fig. 3b), endogenous mouse *Krev*-1 mRNA was detected in NIH3T3 cells and DT cells. The soft-agar clones 3 and 8 which had the human genomic DNA corresponding to this cDNA (Fig. 2), as well as DT-#1 cells, expressed a slightly increased level of *Krev*-1 mRNA compared with NIH3T3 and DT cells (Fig. 3b), probably due to mRNA transcribed from the human *Krev*-1 gene.

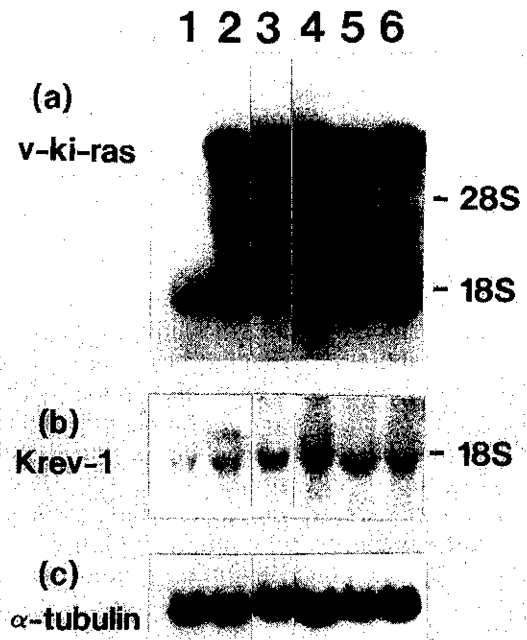

Fig. 3. Northern blot analyses of v-Ki-*ras* mRNA (a) and *Krev*-1 mRNA (b). The migrations of ribosomal RNAs are indicated on the right. The most rapidly migrating species in (a) is probably the cross-hybridization to c-Ki-*ras* mRNA. The same blot was rehybridized with the mouse α-tubulin cDNA as an internal control (c). Lane 1, NIH3T3; lane 2, DT; lane 3, DT-#1-2; lane 4, DT-#1-3; lane 5, soft-agar clone 3; lane 6, soft-agar clone 8.

DISCUSSION

Microcell-mediated chromosome transfer has facilitated the identification of human chromosomes carrying a putative tumor suppressor gene in various tumor cells.[9-18] However, this technique has not satisfactorily allowed the mapping of the gene to a limited chromosomal region, unless the candidate gene is near the integration site of a selectable marker gene on the transferred chromosome.[33, 34] To map the gene to a specific region, chromosome fragmentation by X-irradiation has been adopted as a step in microcell-mediated chromosome

transfer.[35,36] In contrast, we attempted regional mapping based on spontaneous chromosome fragmentation in interspecies (mouse × human) microcell-hybrids.

None of the soft-agar clones derived from DT-#1 cells retained an intact human chromosome 1. However, the majority of clones still contained a human-derived chromosomal region(s) (Table I and Fig. 2), confirming that the spontaneous chromosomal deletions/rearrangements were not a rare event, at least under our experimental conditions. This result probably reflects the finding that donor chromosomes in foreign, recipient cells can be stabilized by spontaneous translocation to a recipient chromosome in whole cell hybrids.[37] Thus, in this study, the regional mapping of a putative suppressor gene was accomplished by "deletion mapping" (Fig. 2). Two loci on 1q21 and two loci on 1q23-q24 were commonly lost in all the soft-agar clones examined. Although we can not completely rule out the presence of other common deletions which might have been overlooked even by the 35 markers, the entire 1p and 1q25-qter in clone 3 had no gross deletion/rearrangement, at least by chromosome analysis. Therefore, it is most likely that the loss of a gene(s) on the identified region was responsible for the re-acquisition of anchorage-independent growth. Moreover, the soft-agar clones not only grew again in soft-agar media, but also in adherent cultures exhibited transformed properties comparable to those of DT cells (Table II), indicating that the loss of the identified region is not casual, but is a significant event. However, at present, it is not clear whether a single gene or multiple genes on this region are involved in the suppression of various transformed phenotypes.

The human *Krev*-1 cDNA, the enforced overexpression of which can suppress the transformed phenotypes of DT cells,[25] has been assigned to 1p13.3.[32] Two soft-agar clones (clones 3 and 8) contained a human *Krev*-1 gene, and probably expressed its transcript, as did the original DT-#1 cells (Figs. 2 and 3). This suggests that the human *Krev*-1 gene cannot play a crucial role in the suppression when it is introduced into DT cells under the control of its own enhancer/promoter by chromosome transfer. The v-Ki-*ras* oncogene of Kirsten murine sarcoma virus was comparably expressed in DT cells, DT-#1 cells and in soft-agar clones (Fig. 3), indicating that the suppression and re-acquisition of the transformed phenotypes were not controlled by the level of v-Ki-*ras* expression.

Thus, the results presented here indicate that human 1q21 and/or 1q23-q24 most likely carries a transformation suppressor gene(s) which controls the transformed phenotypes of DT cells. Although LOHs on chromosome 1q have been reported in breast carcinomas[38] and gastric carcinomas,[39] no candidate suppressor gene on 1q has so far been isolated. It has also been reported that human chromosome 1q carries a gene inducing cellular senescence in some immortal cells.[40,41] At present, it is unknown whether the suppressor gene for DT cells is identical with any of the putative tumor suppressor genes in human cancers and the senescence-inducing gene on 1q. To clarify this, each gene must be cloned.

The use of interspecies microcell-hybrids may also be helpful in cloning a candidate gene. In our system, the isolation of human-derived transcribed sequences[42,43] is equivalent to the isolation of sequences transcribed from the transferred chromosome. As an approach to identifying a candidate suppressor gene, we are now isolating a large number of human-derived transcribed sequences by the use of human-specific interspersed repeats,[44,45] and selecting those which exist on the region determined in this study.

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