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NORMAL HUMAN CHROMOSOME 5, ON WHICH A FAMILIAL ADENOMATOUS POLYPOSIS GENE IS LOCATED, HAS TUMOR SUPPRESSIVE ACTIVITY

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The suppressive activity of normal human chromosome 5 was detected by means of the chromosomal transfer technique using DT cells as recipients. A hybrid clone, which exhibited reduced tumorigenicity, contained chromosomal regions such as 5pter-p15, q21 and q33-qter. Since a familial adenomatous polyposis gene has been reported to be located at 5q21-q22, the suppressive activity of chromosome 5 might be due to this gene.

Familial adenomatous polyposis (FAP) is an autosomally dominant genetic disease. Over a hundred colorectal polyps are induced in affected individuals and colorectal carcinomas arise in most patients. On linkage analysis with DNA probes showing RFLP, the gene for FAP was found to be located on chromosome 5q, especially q21-q22 (1). From the analogy with retinoblastomas (2), including the loss of heterozygosity in chromosome 5q in cases of sporadic and FAP-related colorectal carcinomas (3), the FAP gene is considered to be a tumor suppressor gene.

There are several methods for studying suppressor genes. Recently, the chromosomal transfer technique (4) has been used to detect the suppressive activity of a chromosome of interest (5, 6). To clarify the activity of the putative FAP gene and to clone it, we attempted to introduce human chromosome 5 into Kirsten sarcoma virus-transformed NIH/3T3 cells (DT cells) by means of the chromosomal transfer technique as the first step. We used DT cells as recipients because a high incidence of K-ras

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oncogene activation was found in colorectal carcinomas (7), and Noda et al. succeeded in cloning flat revertants by transfecting cDNA plasmid into DT cells (8). We have found that chromosome 5-transferred DT cells show flat reversion morphologically and exhibit reduced tumorigenicity in nude mice.

Materials and Methods

Cells: Mouse A9 cells containing human chromosome 5 tagged with a pSV2neo gene, A9(Neo-5)(9), were used as donors for microcell transfer. The DT cell line of Kirsten murine sarcoma virus-transformed cells (8) was used as microcell recipients.
Construction of DT-human chromosome 5 microcell hybrids: Microcell transfer was performed as described previously (6).
Analyses of nucleic acids: Extracted DNA from each clone was digested with EcoRI completely, electrophoresed on 1% agarose gels, and transferred to nitrocellulose filters (10). Hybridization was performed with ^{32}P-labeled chromosome 5 specific probes: L1.4 (D5S4, pter-p15)(11), C11p11 (D5S71, q14-q22)(1), π227 (D5S37, q21)(12), OB7 (GRL, q31-q32) (13), v-fms (CSF1R, q33-q35) (14), p12-65 (D5S2, q34-qter) (15), L1.7 (D5S1)(16) and a GTPase activating protein (GAP)(17); and a v-K-ras specific probe (18).
Tumorigenicity assay: Cells from each selected clone were inoculated subcutaneously at $5x10^5$ cells/site into athymic nude mice, which were periodically examined for tumor formation.

Results

Establishment of chromosome 5-introduced DT cells

We introduced human chromosome 5 into DT cells by means of microcell transfer and G418 selection. In total, 12 neo-resistant clones were obtained through four repeated experiments. The morphology of four clones (clones 3, 6, 7 and 12) was relatively flat, as compared to DT cells, and that of clones 1 and 2 was the same as that of transformed DT cells. The other 6 clones (clones 4, 5, 8, 10, 11 and 13) showed an intermediate morphology.

Presence of chromosome 5 specific probes in hybrid cell clones

High molecular weight DNAs extracted from individual clones were subjected to Southern blot analysis with eight chromosome 5 specific probes. Probe C11p11 and probe π227 among them have been reported to show close linkage to the FAP gene (1, 12). Clones 6, 7, 10, 11 and 12 gave bands corresponding to the same fragment length as normal human DNA with probe C11p11 (4.0 kilobase (kb)) and probe π227 (5.6 kb) (Fig. 1). Interestingly, clone 3 DNA hybridized with probe π227 (Fig. 1B) but not with probe C11p11 (Fig. 1A). Other clone DNAs did not hybridize with either probe.

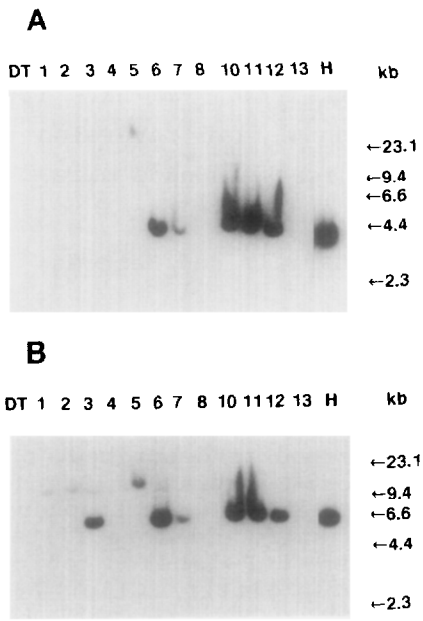

Fig. 1. Southern blot analysis with human chromosome 5 specific probes. DNAs (15 μg) were extracted from the indicated cell lines, digested with EcoRI and then analyzed as described under Materials and Methods with probes (A) C11p11 and (B) π227. Faint bands at about 13 kb are pSV2neo DNA which hybridized with the contaminating vector plasmid DNA in the probes. The numbers, 1 to 8 and 10 to 13, indicate the individual hybrid clones; DT, DT cell clone; H, human DNA.

The results of Southern blot hybridization with other probes are summarized in Table 1. Five clones (clones 6, 7, 10, 11 and 12) were positive with all eight human chromosome 5 specific probes examined. Another five clones (clones 2, 4, 5, 8 and 13) were negative with all eight probes. Clone 3 was positive with probes L1.4, π227, v-fms, p12-65 and L1.7, but negative with GAP, C11p11 and OB7. Clone 1 was positive with L1.7 and GAP. In this microcell transfer experiment, human chromosome 5 was transferred to heterogeneous mouse cells. Therefore, parts or most of human chromosome 5 might be eliminated during cell growth in seven hybrid cell clones. Two copies of the v-K-ras gene and pSV2neo DNA were retained in all clones (Table 1).

Tumorigenicity of hybrid cell clones

DT-microcell hybrids with human chromosome 5 were inoculated subcutaneously at 5×10^5 cells/site into nude mice. All 12 clones formed tumors at all inoculation sites (Table 1). However, the latency periods for tumor formation differed. All clones, which showed a flat morphology, except clone 3, hybridized with every chromosome 5 specific probe. Clone 3 hybridized with L1.4,

Table 1. Hybridization of DT-microcell hybrid cell DNAs with chromosome 5 specific probes

Probes in chromosome 5	DT#5 clone												
	6	7	10	11	12	3	1	2	4	5	8	13	DT
L1.4 (pter-p15)	+[a]	+	+	+	+	+	-	-	-	-	-	-	-
GAP (q13.3)	+	+	+	+	+	-	+	-	-	-	-	-	-
C11p11 (q14-q22)	+	+	+	+	+	-	-	-	-	-	-	-	-
π227 (q21)	+	+	+	+	+	+	-	-	-	-	-	-	-
OB7 (q31-q32)	+	+	+	+	+	-	-	-	-	-	-	-	-
fms (q33-q35)	+	+	+	+	+	+	-	-	-	-	-	-	-
p12-65 (q34-qter)	+	+	+	+	+	+	-	-	-	-	-	-	-
L1.7 (?)	+	+	+	+	+	+	+	-	-	-	-	-	-
v-K-ras	+	+	+	+	+	+	+	+	+	+	+	+	+
pSV2neo	+	+	+	+	+	+	+	+	+	+	+	+	-
Tumorigenicity													
No.tumors/ no.sites injected	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	8/8
Latency period in days	7.8	11.0	9.3	12.0	13.8	9.3	7.0	8.5	7.8	8.5	7.0	7.8	7.8

[a] + and - indicate cell clones which hybridized and did not hybridize with the probe in question, respectively.

π227, v-fms, p12-65 and L1.7. Since probe L1.7 hybridized with clone 1, which resembled to DT cells in morphology, the tumor suppressor gene seemed to be located around the locus of L1.4, π227, v-fms or p12-65. Hence, we classified the 12 clones as to these four probes. Clones 3, 6, 7, 10, 11 and 12 were positive clones (group A), and clones 1, 2, 4, 5, 8 and 13 were negative ones (group B). The latency periods were 10.5 $\pm$ 3.1 days and 7.8 $\pm$ 1.3 days (mean $\pm$ SD) for groups A and B, respectively, i.e. the latency period was significantly long in group A ($p < 0.001$, Wilcoxon's test).

To confirm this weak suppressive activity indicated by the long latency period for tumorigenicity, we again inoculated the 12 hybrid clones into nude mice. Although we could not obtain consistent results for a few clones, the latency period of clone 3 among them was 10.6 $\pm$ 2.6 days (mean $\pm$ SD, n=7. No tumor was formed at another site.) and it remained long.

Discussion

FAP is a rare human disease. Nevertheless, the elucidation of the steps involved in the colorectal cancer formation in FAP and nonFAP has allowed much and will facilitate futher progress in the study of tumor suppressor genes. Loss of heterozygosity in chromosomes 5, 17 and 18 is involved in these steps (19). Putative suppressor genes, p53 on chromosome 17 and DCC on chromosome 18, have already been cloned (20, 21). However, the FAP gene, the putative suppressor gene on chromosome 5, has not been cloned yet, but the location of this gene was determined to be at 5q21-q22 on linkage analysis (1).

Loss of chromosome 5 has been frequently observed in therapy-related acute nonlymphocytic leukemia cells and myeloid cells of patients with refractory anemia. The 5q31 region has been commonly found to be deleted in these cells on cytogenetic evaluation. It is, therefore, likely that another tumor suppressor gene is located at 5q31 (22).

GTPase activating protein (GAP) is thought to be an effector of ras protein, and to facilitate the change from the ras-GTP binding (activated) form to the ras-GDP binding (inactivated) form (23). Recently, GAP has been deduced to exhibit a tumor suppressive activity from the following results. Firstly, GAP suppresses the transformation of NIH/3T3 cells induced by normal H-ras, but not that by activated v-H-ras (24). Secondly, neurofibromatosis type 1 (NF1) is an autosomally dominant genetic disease. The NF1 gene is considered to be a tumor suppressor gene. The NF1 gene has been isolated and the NF1 peptide showed similarity to GAP (25). Since the human GAP gene was reported to be located at 5q13.3 (26), it seems possible that GAP induces the reversion of DT cells.

There are at least three candidates, as noted above, for the suppressor gene on chromosome 5. We showed that DNA from clone 3, which showed a stable reverted phenotype, hybridized with probes L1.4 (pter-p15), π227 (q21), v-fms (q33-q35) and p12-65 (q34-qter), but not with probe GAP,C11p11 or OB7 (q31-q32). Thus, the suppressor gene examined in this study is located in a region such as 5pter-p15, q21 or q33-qter, and appears to be the FAP gene.

One reason why we used DT cells, which contain two copies of the v-K-ras gene, is that mutations of the c-K-ras gene are frequently observed in colorectal carcinomas (7). Since two copies

of the v-K-ras gene were retained in the hybrid cell clones, and the v-K-ras expression was not reduced in clone 3 (data not shown), a possible mechanism by which the suppressor gene acts is changes in v-K-ras gene translation, its downstream or other possible pathways independent of the K-ras protein.

We showed here that the activity of the tumor suppressor gene on chromosome 5 can be detected using DT cells as recipients. By transfecting human chromosome 5 DNA into DT cells, we are now trying to identify the tumor suppressor gene itself.

Yamada et al. recently reported that introduction of human chromosome 1 into DT cells suppresses their transformed phenotypes but introduction of chromosome 11 or 12 into DT cells does not (27).

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