J Indian Inst Sci., Jan.–Feb. 1989, **69**, 73–88.

Molecular and functional evidence for the presence of a tumor-suppressor gene on human chromosome 11

ERI S. SRIVATSAN*, BRIJESH C. MISRA,
Division of Ophthalmology, Childrens Hospital of Los Angeles, Los Angeles, CA 90027.

ASHUTOSH BANERJEE,
Center for Biotechnology, 4000, Research Forest Drive, The Woodlands, Texas 77381.

AND

SUBAL C. CHATTOPHADHYAY
Division of Medical Genetics, Mt. Sinal Medical Center, New York, New York 10029.

Received on September 5, 1988.

Abstract

Cytogenetic analysis of human intraspecific HeLa X fibroblast hybrids has implicated the loss of a single copy of chromosome 11 and 14 in the reexpression of tumorigenicity. Molecular analysis of the paired combination of non-tumorigenic and tumorigenic hybrids using chromosome 11- and 14-specific restriction fragment length polymorphic (RFLP) probes has identified the loss of a fibroblast chromosome 11 in the tumorigenic hybrid cells. There was no obvious correlation between the loss of normal chromosome 14 and the reexpression of the tumorigenic phenotype. The presence of the tumor-suppressor sequences on normal chromosome 11 was further confirmed by the derivation of non-tumorigenic cells with the introduction of a single copy of fibroblast chromosome 11 into the tumorigenic cells. The precise location of the tumor suppressor sequences was then determined by an extensive RFLP analysis of the HeLa X normal chromosome 11 hybrids using a large number of chromosome 11-specific probes. These results showed a perfect correlation between the presence of the long arm (q-arm) of chromosome 11 and the suppression of the tumorigenic phenotype. Also, one of the tumorigenic hybrids had lost q13-specific genetic markers while retaining other regions of the chromosome. We conclude therefore that the gene(s) involved in the suppression of the HeLa tumors is localized to the long arm of chromosome 11, most likely to the q13 region.

Key words: Tumorigenicity, tumor-suppression genes, HeLa cell tumors, long arm of chromosome 11, q13 region.

1. Introduction

Somatic cell hybrids derived by the fusion of two or more different cells of the same or different species have been extremely useful in the genetic analysis of malignancy. In these

* Author for correspondence.

studies the approach has been to fuse a cancer cell to a normal cell and determine whether or not the resulting hybrid cell is tumorigenic[1-5]. Earlier studies of Harris and coworkers[1-2] involving the fusion of highly tumorigenic mouse cells to mouse cells of low tumorigenicity or normal mouse cells resulted in hybrids with low tumorigenic potential. This led them to conclude that tumorigenicity behaves as a recessive trait. Similarly, studies carried out with the hybrids derived by the intraspecific rodent cell and interspecific rodent x human cell fusions indicated the recessive nature of the tumorigenic phenotype[3,4]. The initial hybrids from these fusions were non-tumorigenic and tumorigenic hybrids arose at regular intervals[3]. The tumorigenic hybrids contained fewer number of chromosomes in comparison to their non-tumorigenic counterpart. The appearance of the tumorigenic cells was therefore correlated with the loss of specific chromosomes.

The conclusion from the above studies is that the gene(s) responsible for the suppression of malignancy resides in normal cells and hence the fusion of the normal cell to a malignant cell results in the derivation of non-tumorigenic hybrids. The loss of specific chromosomes, those carrying the tumor suppressor gene(s) would then lead to the development of tumorigenic revertant cells. Identification of these specific chromosomes was difficult in the intraspecific rodent cell hybrid system due to the inadequacy of the karyotypic analysis to differentiate the parental origin of the chromosomes. In the interspecific human x rodent cell system where the parental chromosomes could be identified by the karyotypic analysis, few of the human chromosomes were indeed implicated to be involved in the suppression of tumorigenicity[3]. However, these studies were inconclusive due to the instability of the chromosomes in these interspecific hybrids. The tumorigenic suppression was only transient due to the continuous loss of human chromosomes from these hybrids. Chromosomally stable intraspecific human cell hybrid system generated by Stanbridge[5] was very helpful in the identification of chromosomes possibly involved in the suppression of the tumorigenic phenotype. The initial hybrids derived by the fusion of tumorigenic HeLa cells to the normal human cells were all non-tumorigenic[5,6]. After a prolonged passage in culture, rare tumorigenic segregants arose from these non-tumorigenic parental hybrids. Karyotypic analysis of the paired combination of non-tumorigenic and tumorigenic hybrids identified the loss of a single copy of chromosome 11 and other chromosomes, in particular, chromosomes 2 and 14, in the tumorigenic hybrids[7,8]. However, it was impossible to determine the parental origin of these specific chromosomes lost from the tumorigenic cells by the conventional cytogenetic analysis. In the present article, we show the use of molecular genetic techniques in identifying the missing chromosome to be the normal chromosome 11[9-11] and have extended the study to localize the tumor suppressor sequences to the long arm (q-arm) of chromosome 11.

2. Materials and methods

2.1. *Cell lines*

The parental cell lines and the non-tumorigenic and tumorigenic hybrids derived from the somatic cell and microcell fusion of the HeLa cells to a normal fibroblast or to a retinoblastoma cell line are presented in Table I. All the hybrid cell lines were tested for

Table I
Description of parental and hybrid cell lines

Parental cell lines Fibroblast X Hela	Hybrid cell lines	
	Non-tumorigenic	Tumorigenic
GM77 x D98/AH-2	CGL-1 CGL-2 ESH 541E	CGL-3 CGL-4 ESH 541L
IMR-90 x D98/AH-2	ESH 39E	ESH 39L
110.1[a] x ESH15[b]	110.1/ESH15.1	110.1/ESH15 6TG.1 110.1/ESH15 6TG.3 110.1/ESH15 6TG.5
Y79[c] x D98 OR[d]	HHY17p2c	HHY17p2cTuo

[a] Cell line 110.1 is a mouse (A9) cell line containing a translocated X:11 (11pter > 11q23::Xq26 > Xqter) chromosome as the only human chromosome.
[b] Cell line ESH15 is a tumorigenic revertant cell derived from the fusion of diploid fibroblast 75-55C and HeLa cells.
[c] Y79 is a retinoblastoma cell line.
[d] $D98^{OR}$ is a ouabain resistant clone of parental HeLa cells, D98/AH-2.

tumorigenicity by injection into *nude* mice. The cell lines were all grown in minimum essential medium containing non-essential amino acids and 10% fetal calf serum.

2.2. *Plasmid and phage DNAs*

Chromosome 11-, 13- and 14-specific RFLP probes used in the present analysis were obtained from a number of laboratories. Plasmids were grown in *Escherichia coli* strain HB101 and purified by the ethidium-bromide-CsCl density gradient centrifugation[12]. The phage particles and the phage DNAs were prepared by the method of Lawn *et al*[13]. The insert RFLP probes were cut out from the plasmid and phage DNAs by digestion with appropriate restriction enzymes and isolated by separation on 0.8–1.0% low-melting agarose gels. Nick translation of the probe DNAs was performed according to Feinberg and Vogelstein[14]. Genomic DNAs were prepared by the method of Jolly *et al*[15].

2.3. *Blot hybridization analysis*

Genomic DNAs were digested with restriction enzymes appropriate for the different RFLP probes. The information about the restriction enzymes used, size of the polymorphic alleles, and the chromosomal location of the informative probes is presented in Table II. DNA samples were digested in a buffer containing 33-mm Tris-Hcl (pH 7.8), 60 mm potassium acetate, 10 mM $Mgcl_2$ and 1 mM DTT at 37°C overnight for all the enzymes except Taq 1. The Taq 1 digestion was performed in the same manner at 65°C overnight. Digested DNAs were subjected to the Southern hybridization analysis as mentioned earlier[9].

Table II
Chromosome 11-, 13- and 14-specific RFLPs

Probe	Locus	Restriction enzyme	Constant fragments (kb)	Polymorphic fragments (kb)	Chromosomal location
6.6 kb	c-Ha-ras	Taq I	2.3	VNTR 2.5 to 4.5	11p15
phins310	Insulin	Pvu II	None	VNTR 0.75 to 2.3	11p15
APO-AI	APO-lipo protein	Apa I	1.5	2.5, 4.0	11q13
p2-7-1D6	D11S84	Taq I	None	4.5, 6.7	11q22-23
ϕ6-3	D11S85	MSP I	1.7, 2.5	3.85, 9.50 2.50, 2.75	11q22-23
pTH162	D13S39	Bgl II	Many	VNTR 5.0 to 8.2	13q14
pAW101	D14S1	EcoR I	None	17.0, 20.0	14q23

VNTR—Variable number of tandem repeats.

3. Results

3.1. *Implication of normal chromosome 11 in tumor suppression*

Genomic DNA isolated from the parental cell lines and the non-tumorigenic and tumorigenic hybrids (Table I) were subjected to the RFLP analysis using the different chromosomes 11- and 14-specific probes listed in Table II. The Southern blot hybridization revealed unique polymorphic fragments in the fibroblast and HeLa DNAs with the different probes. c-Ha-ras probe was a useful chromosome 11- RFLP probe[16]. Fragment lengths of 4.0 and 2.5 kb were detected in the fibroblast GM77-DNA, 3.0 and 2.5 kb in the fibroblast IMR-90 DNA and 3.0 kb in the HeLa DNA (fig. 1). All the DNAs contained a common fragment of 2.3 kb. Thus the fibroblast cell lines were found to be heterozygous and the HeLa cell line homozygous for the c-Ha-ras probe.

The hybridization analysis of the non-tumorigenic cell lines CGL-2, ESH 541E and ESH 39E revealed the presence of both the fibroblast- and HeLa-specific fragments (fig. 1). The cell lines CGL-2 and ESH 541E derived from the HeLa x GM-77 fusion contained the GM-77 specific 4.0 and 2.5 kb fragments and the HeLa-specific 3.0 kb fragment. The corresponding tumorigenic segrements CGL-4 and ESH 541L had lost the fibroblast-specific fragments of 2.5 and 4.0 kb, respectively. It is interesting to note that the loss of either of the fibroblast chromosome 11s has led to the development of the tumorigenic phenotype.

The non-tumorigenic hybrid cell line ESH 39E derived from the HeLa x IMR-90 fusion contained the fibroblast-specific 2.5 kb fragment in addition to the 3.0 kb fragment common to both the fibroblast and HeLa DNAs. The tumorigenic revertant ESH39L had lost the fibroblast-specific 2.5 kb fragment. Thus the loss of fibroblast chromosome 11 was again found to be responsible for the development of the tumorigenic revertants.

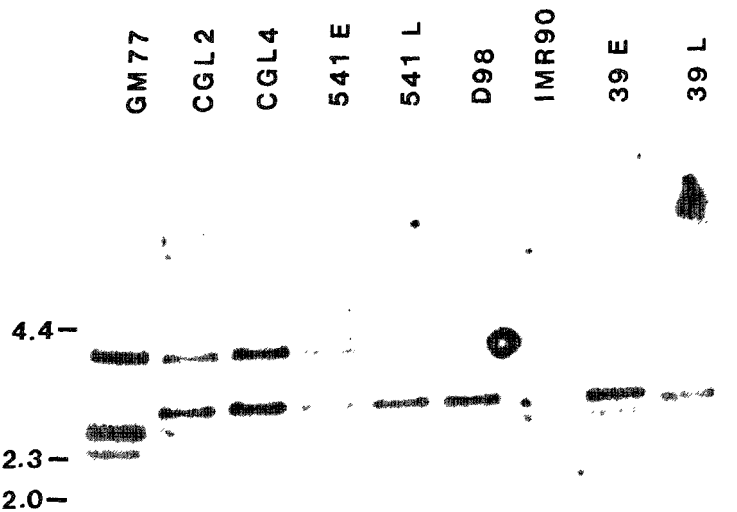

FIG 1 RFLP analysis of the HeLa x fibroblast hybrid DNAs with the chromosome 11-specific c-Ha-ras probe (11p15). DNA samples digested with Taq I were analyzed on a 0 8% agarose gel. The fibroblast-specific polymorphic alleles are present in the non-tumorigenic hybrids, the 2.5 and 4.0 kb alleles of the GM77 parent in CGL-2 and ESH 541E hybrids and 2.5 and 3.0 kb alleles of the IMR-90 parent in the ESH39E hybrid. These hybrids also contain the 3.0 kb allelic fragment of the HeLa cell line. The tumorigenic cell lines have lost a copy of the fibroblast chromosome 11. The cell line CGL-4 has lost the 2.5 kb and ESH 541L the 4.0 kb fragments of the GM77 parent and the cell line ESH 39L has lost the 2.5 kb fragment of the IMR-90 parent.

The detailed RFLP analysis thus identified the loss of a normal chromosome 11 in four of the five tumorigenic hybrids. The variant tumorigenic cell line, CGL-3, showed the loss of a HeLa-specific chromosome 11, at least the p-arm, by the RFLP analysis (fig. 2). The loss of a single copy of the HeLa chromosome 11 from this hybrid cell line was identified by the densitometric analysis of the intensities of the fibroblast and HeLa-specific fragments (data not shown).

Analysis of the different cell lines with the chromosome 14-specific probe, pAW101[17] indicated the loss of the fibroblast-specific allele only in the tumorigenic hybrid CGL-3 (fig. 3). There was no obvious loss of the HeLa- or the fibroblast-specific chromosome 14 alleles in the other tumorigenic cell lines.

Thus the molecular genetic analysis of the various non-tumorigenic and tumorigenic hybrid cell lines using the chromosome-specific RFLP probes clearly implicated the

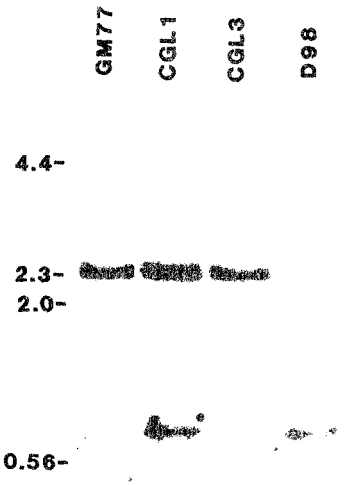

FIG. 2. Analysis of the hybrid cell DNAs with the chromosome 11-specific phins 310 probe (11p15). The tumorigenic hybrid CGL-3 contains both the 0.75 kb fragment of the HeLa parent and 2.3 kb fragment of the GM77 parent like its non-tumorigenic counterpart, CGL-1. However, the intensity of the two fragments indicates the loss of one of the HeLa alleles, and a copy of the HeLa chromosome 11 in this tumorigenic cell line.

involvement of a normal chromosome 11 in tumor suppression. It is not obvious at the present time why the tumorigenic cell line CGL-3 had lost the HeLa-specific chromosome 11. It is possible that a homologous recombination might have taken place between the HeLa and fibroblast chromosome 11s resulting in the transfer of the fibroblast chromosomal 11 region containing the 'tumor suppressor' sequences to the HeLa chromosome 11.

3.2. *Confirmation of the presence of the tumor-suppressor sequences on chromosome 11*

The conclusive evidence for the presence of the tumor suppressor sequences on chromosome11 was provided by Saxon *et al*[11]. They were able to convert HeLa-derived tumorigenic cells

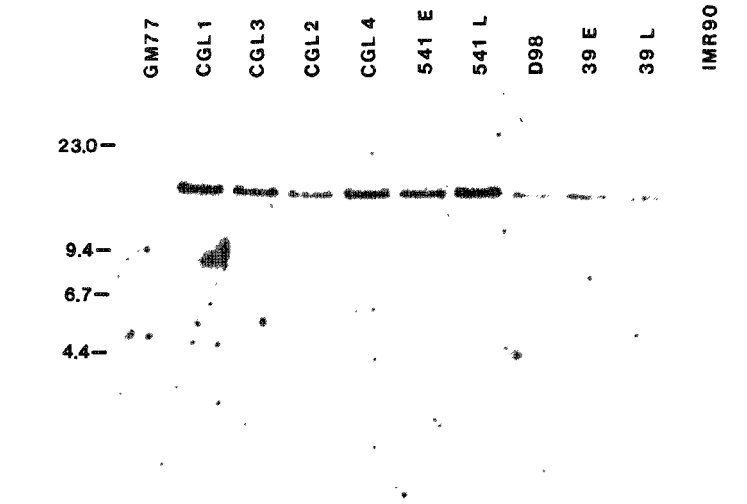

FIG. 3. RFLP analysis of the hybrid cell DNAs with the chromosome 14-specific pAW101 probe (14q23). Tumorigenic hybrid CGL-3 is the only cell line that has lost the fibroblast-GM77, specific 20 kb allele. None of the other hybrids shows the loss of either HeLa or fibroblast alleles.

into non-tumorigenic cells with the introduction of a normal chromosome 11 by the microcell-mediated chromosome transfer[18]. The donor chromosome was a t(X; 11) translocation chromosome found in the human fibroblast cell line GM3552. The translocation break points of this chromosome are 11pter $>$ 11q23::$X_q26 > X_q$ter. Transfer of this chromosome enables the recipient cells to grow in a HAT(Hypoxanthine-aminopterine-thymidine)-selective medium. The t(X; 11) chromosome was introduced into a 6-TG (6-thioguanine)-resistant tumorigenic hybrid cell line, ESH 15 (T1), which was derived by the fusion of a normal fibroblast (75-55C) to HeLa cells[19]. Five HAT-resistant colonies were isolated and assayed for tumorigenicity in *nude* mice. One of the clones that had been converted into a non-tumorigenic cell was reselected in a 6-TG medium in order to select clones that had now lost the t(X; 11) chromosome. This 6-TG-resistant clone was found to be tumorigenic like the original parent indicating thereby the ability of the t(X; 11) chromosome to suppress the tumorigenic phenotype and the loss of which leads to the reappearance of the tumorigenic cells. The possibility still existed that there were other chromosomes besides t(X: 11) that were essential for tumor suppression and the loss of these chromosomes by the 6-TG back selection was responsible for the reexpression of the tumorigenicity. This possibility was eliminated by the conversion of the tumor cells back into non-tumorigenic cells by the

reintroduction of the t(X; 11) chromosome. Thus the presence of the tumor suppressor sequences on normal chromosome 11 was confirmed by a functional assay involving the introduction of the chromosome into the tumorigenic cells.

RFLP analysis was performed on the t(X; 11) x ESH 15 hybrids with the chromosome-11-specific c-Ha-ras probe in order to conclusively show the involvement of the t(X; 11) in tumor suppression. The data indicated the presence of the t(X; 11) chromosome-specific 2.5 kb fragment in addition to the 3.0 and 3.5 kb fragments of the tumorigenic ESH15 cell line in the non-tumorigenic hybrid 110.1/ESH15.1 (fig. 4). The 6-TG back-selected tumorigenic clones had lost this 2.5 kb fragment implicating t(X; 11) chromosome in tumor suppression. The resuppressed clones again contained the 2.5 kb fragment of the t(X; 11) chromosome (data not shown). These results thus confirmed the presence of the tumor suppressor sequences on chromosome 11 of normal cells.

3.3. *Localization of the tumor-suppressor sequences to the long arm (q-arm) of chromosome 11*

It is important to localize the gene(s) to a specific region of the chromosome in order to attempt the isolation and cloning of the gene(s). One of the somatic cell hybrids derived by the fusion of HeLa (D98OR) x retinoblastoma (Y79) was very useful in the present analysis. Development of retinoblastoma has been correlated with the loss of genetic information on chromosome 13[20–24] and we have shown the involvement of gene(s) on chromosome 11 in the suppression of the HeLa cell tumors[9,11]. Hybrid clones isolated from the fusion of retinoblastoma cells to the HeLa cells have been shown to be non-tumorigenic upon injection into *nude* mice[25]. A tumorigenic segregant was isolated from one of the non-tumorigenic hybrids. The non-tumorigenic mass culture (HHY17p2c) and the tumorigenic revertant cell line (HHy17p2c Tuo) were subjected to the RFLP analysis with the chromosome 13- and 11-specific probes in order to determine the genetic locus involved in the derivation of the tumorigenic revertant cells.

Of the various chromosome 13-specific probes used in the analysis, one of the probes, pTH162[26], was informative. Results presented in fig. 5 showed the presence of polymorphic alleles for both Y79 and HeLa cell lines. The Y79 cell line contained the 8.2 and 6.1 kb alleles and the HeLa cells contained the 7.7 and 5.0 kb alleles. The non-tumorigenic hybrid cell line, 17p2c, and its tumorigenic revertant cell line, 17p2c Tuo, contained the allelic fragments of both the parental cell lines, namely, the 8.2, 7.7, 6.1 and 5.0 kb fragments. Thus the presence of HeLa chromosome 13 in both the non-tumorigenic and the tumorigenic hybrid cells indicated that at least chromosome 13 was not involved in the reappearance of the tumorigenic phenotype.

Figures 6a and b contain the results obtained by the analysis of the non-tumorigenic (17p2c) and tumorigenic (17p2c Tuo) DNAs with the chromosome 11-specific ras-(11p15) and APOA1 (11q13) probes[16,27]. The parental cell lines, HeLa and Y79, were polymorphic for the ras probe containing the 3.0 and 2.5 kb alleles respectively (fig. 6a). Both the non-tumorigenic and tumorigenic hybrids contained the Y79-specific 2.5 kb allele in addition to the 3.0 kb HeLa allele. Hence, the p-arm of Y79 chromosome 11 was not found to be involved

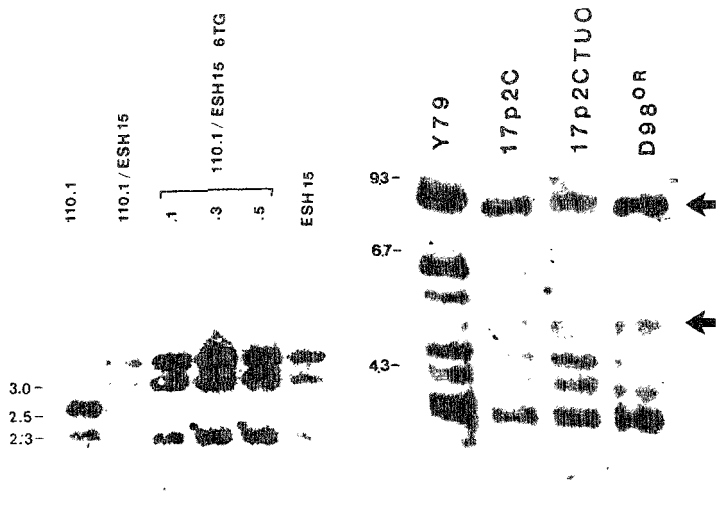

FIG. 4. RFLP to detect the presence or absence of the t(X, 11) chromosome in the 110.1 x ESH15 microcell hybrids. Hybridization was performed on Taq I-digested DNAs with the c-Ha-*ras* probe (11p15). The cell line 110.1 is a mouse cell line (A9) containing t(X; 11) chromosome as the only human chromosome. The cell line ESH15 is a tumorigenic HeLa-derived hybrid cell line[19] The non-tumorigenic hybrid 110 1/ESH15.1 contains the 2.5 kb allelic fragment of the t(X; 11) chromosome in addition to the 3 0 and 3.5 kb fragments of the ESH 15 DNA. The tumorigenic 6-Thioguanine (6-TG) resistant revertant cell lines have lost this t(X; 11)-chromosome-specific fragment. These results confirmed the involvement of a normal chromosome 11 in tumor suppression.

FIG. 5. Hybridization analysis of the HeLa ($D98^{OR}$) x retinoblastoma (Y79) hybrids with chromosome 13-specific RFLP probe, pTH162 (13q14). BglII-digested DNAs were hybridized to the probe as described in the text. The results showed the presence of Y79-specific 8.2 and 6.1 kb and HeLa-specific 7 7 and 5.0 kb fragments in both the non-tumorigenic and tumorigenic cell lines indicating the non-involvement of chromosome 13 in the suppression of the tumorigenic phenotype.

in the derivation of the tumorigenic revertant cell line. However, the result was different for the q-arm-specific APO AI probe. The results presented in fig. 6b strongly suggested the involvement of q-arm in tumor suppression. The retinoblastoma cell line (Y79) was polymorphic containing the 4.0 and 2.5 kb allelic fragments. HeLa cell line was homozygous containing the 2.5 kb allele. Both the cell lines contained the 1.5 kb common fragment. The Y79-specific 4.0 kb allelic fragment was observed only in the non-tumorigenic hybrid cell line. It was absent in the tumorigenic revertant cells. Both the hybrid cell lines contained the 2.5 kb polymorphic allele, possibly derived from the HeLa parent. Thus, the loss of the q-arm of

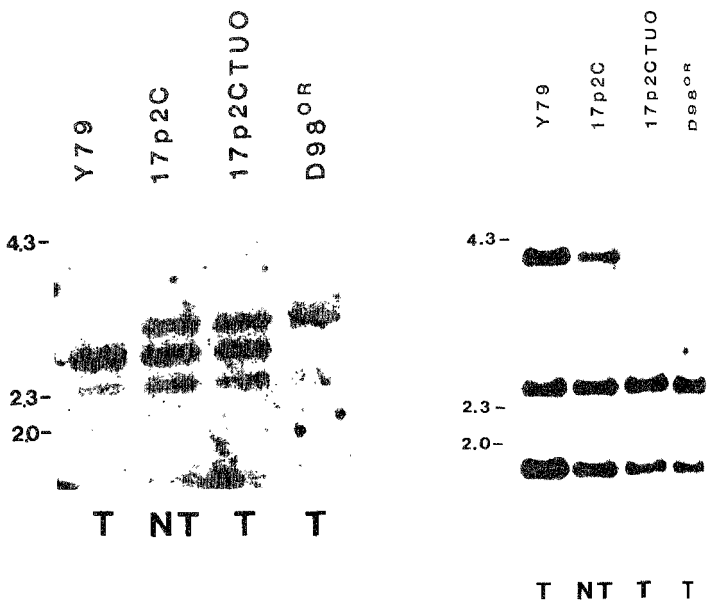

FIG. 6. RFLP analysis of the HeLa x retinoblastoma hybrids with chromosome 11-specific probes. (a) Taq-I digested DNAs were hybridized to the p-arm specific chromosome 11 probe, c-Ha-ras (11p15). The observation Y79-specific 2.5 kb fragment in the tumorigenic cell line indicated the non-involvement of p-arm of chromosome 11 in tumor suppression. (b) Apa I-digested DNAs were hybridized to the q-arm specific chromosome 11 probe, 1.4 kb genomic fragment of the APOAI gene (11q13). The tumorigenic cell line contains the 2.5 kb genomic fragment, the fragment common to both the Y79 and HeLa cell lines, but has lost the Y79-specific 4.0 kb allelic fragment, present in its non-tumorigenic counterpart. The results therefore indicate a perfect correlation between the loss of the q-arm of chromosome 11 and reversion to the tumorigenic phenotype.

chromosome 11 derived from Y79 correlated with the reappearance of the tumorigenic phenotype.

Additional evidence for the absence of the q-arm of Y79 chromosome 11 in the tumorigenic cells was provided by analysis with other q-arm-specific probes. Results are presented for the analysis with the p2-7-1D6 ($11q^{22,23}$) probe[28]. The HeLa cell line contained heterozygous allelic fragments of 6.7 and 4.5 kb and chromosome 11 of the Y79 cell line contained homozygous allelic fragment of 4.5 kb (fig. 7a). The tumorigenic cell line had retained the HeLa-specific 6.7 kb allelic fragment but had lost the 4.5 kb fragment that was present in the non-tumorigenic cells. This 4.5 kb fragment was derived from the Y79 chromosome since the non-tumorigenic cells contained the q-arm of Y79 chromosome 11 (fig. 6b). These results

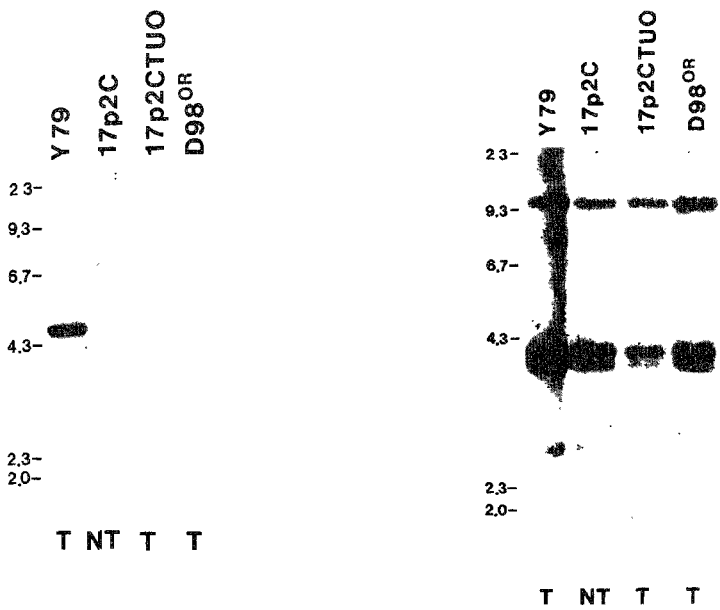

FIG 7. Confirmation of the deletion of q13-22 region of normal chromosome 11 in the tumorigenic cells. (a) Taq I-digested genomic DNAs were hybridized to the q-arm specific p2-7-1D6 probe ($11q^{22,23}$) The tumorigenic cells (17p2cTuo) have retained the HeLa-specific 6.7 kb allelic fragment, but have lost the Y79-specific 4.5 kb allelic fragment present in its non-tumorigenic counterpart (17p2c). (b) MSP I-digested DNAs were hybridized to another of the q-arm-specific probes, ϕ6-3 ($11q^{22,23}$). Both the non-tumorigenic and the tumorigenic cell lines contain a copy each of the polymorphic 9.50 and 3.85 kb fragments, a copy each of the ϕ6-3 region of the Y79 and HeLa chromosome 11s. These results indicate the retention of the part of the long arm of Y79 chromosome 11 in the tumorigenic cell line that has lost at least the q13-22 region of the chromosome.

supported the concept that tumorigenicity was associated with a loss of sequences in the long arm of Y79 chromosome 11. The loss of the genetic material within the q13-23 region in the development of the tumorigenic hybrid 17p2cTuo was confirmed by the RFLP analysis with other q23-specific probes. One of the probes ϕ6-3 was informative[28]. The parental HeLa and retinoblastoma DNAs contained the heterozygous allelic fragments of 9.50 and 3.85 kb for this probe (fig. 7b). A homozygous fragment of 3.0 kb and a common fragment of 2.5 kb were also present in the two DNAs. Both the non-tumorigenic (17p2c) and the tumorigenic (17p2cTuo) hybrid DNAs contained the polymorphic 9.50 and 3.85 kb fragments. Since, it has already been shown that the non-tumorigenic cell line contained the p- and q-arms of the Y79 and HeLa chromosome 11s (figs 6a and b) and the tumorigenic cells had lost at least the

part of the $q^{22,23}$ region of Y79 chromosome 11 (fig. 7a), the results obtained with the ϕ6-3 probe would indicate the presence of the Y79 chromosome 11 in addition to the HeLa chromosome at this probe locus of the 11q22-23 region. Thus the hemizygous deletion of sequences in the q13-q23 region of chromosome 11 correlated well with the reexpression of the tumorigenic phenotype. Additional studies with the APO Al and other q-arm-specific probes using several restriction enzymes indicated the presence of a single copy of the HeLa and Y79 chromosome 11s in the non-tumorigenic hybrid (data not shown). These results indicated that the tumorigenic hybrid had retained a complete copy of the HeLa chromosome 11 but had lost at least the q^{13-23} segment of the Y79 chromosome 11. Cytogenetic studies carried out at the 450 band level also showed the presence of two normal appearing chromosome 11s in both the non-tumorigenic and tumorigenic hybrid cell lines (data not shown). Additionally, gene dosage analysis confirmed the presence of two copies of the p-arm and q23 region of chromosome 11 but only a single copy of the q13 region in the tumorigenic hybrid cell line[29]. Hence, the reversion to the tumorigenic phenotype seems to be due to the selective loss of genes in the q^{13-23} (submicroscopic deletion) region of the Y79 chromosome 11.

4. Discussion

Previous cytogenetic analysis of HeLa x human fibroblast hybrids has implicated the loss of a single copy each of chromosome 11 and 14 in the reexpression of the tumorigenic phenotype. The tentative implication of these two chromosomes in the control of tumorigenic expression of human cell hybrids was based upon the number of copies of the chromosomes in a given metaphase spread using trypsin-Glemsa banding techniques. The loss of specific chromosomes was observed, but it was impossible to determine the parental origin of the chromosomes using karyotypic analysis of the intraspecific human cell hybrids.

Molecular genetic studies involving the use of chromosome-specific RFLP probes have been successfully used in differentiating the chromosomes of the parental cell lines. The RFLP analysis has clearly identified the loss of a copy of normal chromosome 11 in most of the tumorigenic hybrid cells. There was no obvious loss of chromosome 14 in these tumorigenic cell lines. The single tumorigenic cell line that has lost a copy of the HeLa chromosome 11, at least the p-arm, showed the loss of a copy of normal chromosome 14. It remains to be seen whether there is any relationship between the two chromosomes in tumorigenic suppression. Also, the loss of the HeLa p-arm could be due to a homologous recombination involving the transfer of the fibroblast chromosomal 11 region containing the tumor-suppressor sequences to the HeLa chromosome. Identification of this recombination event might require the use of probes that detect highly variable tandem repeat 'mini satellite regions'[30].

The presence of the tumor-suppressor sequences on normal chromosome 11 as identified by the RFLP analysis was confirmed by the introduction of a copy of fibroblast chromosome11 into the tumorigenic cells[11]. The functional assay clearly indicated a perfect correlation between the presence of a normal chromosome 11 and the suppression of the tumorigenic phenotype (fig. 4). Introduction of a copy of fibroblast chromosome 14 or another

chromosome, chromosome x, into the tumorigenic cells did not reverse the tumorigenic phenotype confirming the specific effect of normal chromosome 11 in tumor suppression[11] (and Saxon, personal communication). The functional analysis performed by Saxon *et al*[11] indicated yet another phenomenon, the transfer of a single chromosome 11 into the HeLa cells was sufficient for the suppression of tumorigenicity. This result is in contrast to the finding that two normal chromosome 11s are needed for tumor suppression in the tumorigenic HeLa x fibroblast hybrid cell lines. The gene dosage effect observed with the hybrids could be due to the presence of transacting factors on other fibroblast chromosomes that are present in the hybrids but are absent in the HeLa cells. This phenomenon of gene dosage could only be verified with the introduction of the tumor suppressor gene, when isolated.

The results thus suggested the presence of specific genes on normal chromosomes whose function is essential for normal cellular growth and the loss of which leads to the onset of neoplastic transformation. This type of tumor suppressor gene or recessive cancer gene observed on chromosome 11 for the control of HeLa cell tumors has also been observed on other chromosomes, specifically on chromosomes 13 (13q14) and 11 (11p12). These two chromosomes have been implicated in the development of retinoblastoma and Wilm's tumor, respectively[31–33]. Knudson[34,35] has proposed a 'two-hit' model for the development of these tumors. He proposed that the first hit can be a germ line mutation (in hereditary bilateral cases) or a somatic mutation (in sporadic unilateral cases). The second hit will be a somatic mutation in both the hereditary and sporadic forms of the disease. The molecular genetic studies of retinoblastoma by the RFLP analysis with chromosome 13-specific probes as well as by the structural analysis with the cDNA probe have provided considerable proof for Knudson's hypothesis. It remains to be seen whether a similar molecular mechanism is operative in the development of HeLa cell tumors.

Finally, we have localized the HeLa tumor-suppressor gene(s) to the long arm of chromosome 11, possibly to the q13-23 region of the chromosome. It is interesting to note that one of the probes localized to the q22-23 region is present within the deletion (fig. 7a) and another probe localized to the same region is present outside of the deleted segment of the normal chromosome 11 (fig. 7b). Hence, one end of the deleted segment, possibly a part of the tumor-suppressor gene(s), could be identified by the isolation of DNA sequences in between the two probes, p2-7-1D6 and ϕ 6-3. The recently developed techniques of pulsed field gel electrophoresis[36–37] and cloning with the YAC-yeast artificial chromosome system[38] can be very useful in the identification and isolation of the structural sequences of the tumor-suppressor gene.

Acknowledgement

We wish to thank Drs William F. Benedict, Eric J. Stanbridge, Bernard Weissman and A. Linn Murphree for the donation of different cell lines and for helpful discussions. Thanks are also due to Drs Graham Bell, Graham Casey, Webster Cavenee, Channing Der, P. F. Frossard, R. A. Grevel, David Housman, Mark Hughes, Sotirios Karathanasis, Samuel Latt, Mike Litt, A. E. Retief, Thomas Taggart, Cox Terhorst and Ray White for the gift of RFLP

probes. We also wish to thank Carolyn Cook and Helen Kim for technical assistance, K. C. Chen for photographic assistance and Claire Sunga Berriman for typing the manuscript. This research was supported by The Margaret Bundy Scott Research Foundation, Childrens Hospital of Los Angeles Research Program Funds, and the American Cancer Society Institutional Research Grant IN-21-28 to the USC Comprehensive Cancer Center.

References

1. HARRIS, H., MILLER, O. J., KLEIN, G., WORST, O. AND TACHIBANA, T. — Suppression of malignancy by cell fusion, *Nature* (Lond.), 1969, **223**, 363–368.

2. HARRIS, H. — Cell fusion and the analysis of malignancy, *Proc. R. Soc Lond. B.*, 1971, **179**, 1–20.

3. KLINGER, H. P., BAIM, A. S., EUN, C. K., SHOWS, T. B. AND RUDDLE, F. H. — Human chromosomes which affect tumorigenicity in hybrids of diploid human with heteroploid human or rodent cells, *Cytogenet. Cell Genet.*, 1978, **22**, 245–249.

4. KUCHERLAPATI, R. AND SHIN, S — Genetic control of tumorigenicity in interspecific mammalian cell hybrids, *Cell*, 1979, **16**, 639–648.

5. STANBRIDGE, E. J. — Suppression of malignancy in human cells, *Nature* (Lond.), 1976, **260**, 17–20.

6. KLINGER, H. P. — Suppression of tumorigenicity in somatic cell hybrids. I. Suppression and reexpression of tumorigenicity in diploid human x D98AH2 hybrids and independent segregation of tumorigenicity from other cell phenotypes, *Cytogenet. Cell Genet.*, 1980, **27**, 254–266.

7. STANBRIDGE, E. J., FLANDERMEYER, R. R., DANIELS, D. W. AND NELSON-REES, W. A. — Specific chromosome loss associated with the expression of tumorigenicity in human cell hybrids, *Somat. Cell Genet.*, 1981, **7**, 699–712.

8. KLINGER, H. P. — Suppression of tumorigenicity, *Cytogenet. Cell Genet.*, 1982, **32**, 68–84.

9. SRIVATSAN, E. S., BENEDICT, W. F. AND STANBRIDGE, E. J. — Implication of chromosome 11 in the suppression of neoplastic expression in human cell hybrids, *Cancer Res.*, 1986, **46**, 6174–6179.

10. KAELBLING, M. AND KLINGER, H. P. — Suppression of tumorigenicity in somatic cell hybrids. III. Cosegregation of human chromosome 11 of a normal cell and suppression of tumorigenicity in intraspecies hybrids of normal diploid x malignant cells, *Cytogenet. Cell Genet.*, 1986, **41**, 65–70.

11. SAXON, P. J., SRIVATSAN, E. S. AND STANBRIDGE, E. J. — Introduction of human chromosome 11 via microcell transfer controls tumorigenic expression of HeLa cells, *EMBO J.*, 1986, **5**, 3461–3466.

12. CLEWELL, D. B. AND HELINSKI, D. R. — Supercoiled circular DNA-protein complex in *E. coli*: purification and induced conversion to an open circular DNA form, *Proc. Natn. Acad. Sci. USA*, 1969, **62**, 1159–1166.

13. LAWN, R. M., FRITSCH, E. F., PARKER, R. C., BLAKE, G. AND MANIATIS, T. — The isolation and characterization of linked α- and β-globin genes from a cloned library of human DNA, *Cell*, 1978, **15**, 1157–1174.

14. FEINBERG, A. P. AND VOGELSTEIN, B. — A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity, *Anal. Biochem.*, 1983, **137**, 266–267.

15. JOLLY, D. J., ESTY, A. C., BBERNARD, H. U. AND FRIEDMANN, T. — Isolation of a genomic clone partially encoding human hypoxanthine phosphoribosyl transferase, *Proc. Natn. Acad. Sci. USA*, 1982, **79**, 5038–5041.

16. DER, C. J., KRONTIRIS, T. D. AND COOPER, G. M. — Transforming genes of human bladder and lung carcinoma cell lines are homologous to the ras genes of Harvey and Kirsten sarcoma viruses, *Proc. Natn. Acad. Sci. USA*, 1982, **79**, 3637–3640.

17. WYMAN, A. R. AND WHITE, R. A. — A highly polymorphic locus in human DNA, *Proc. Natn. Acad. Sci. USA*, 1980, **77**, 6754–6758.

18. SAXON, P. J., SRIVATSAN, E. S., LEIPZIG, G. V., SAMESHIMA, J. H. AND STANBRIDGE, E. J. — Selective transfer of individual human chromosomes to recipient cells, *Mol. Cell. Biol.*, 1985, **5**, 140–146.

19. DER, C. J. AND STANBRIDGE, E. J. — Lack of correlation between the decreased expression of cell surface LETS protein and tumorigenicity in human cell hybrids, *Cell*, 1978, **15**, 1241–1251.

20. CAVENEE, W. K., DRYJA, T. P., PHILIPS, R. A., BENEDICT, W. F., GODBOUT, R., GALLIE, B. L., MURPHREE, A. L., STRONG, L. C. AND WHITE, R. L. — Expression of recessive alleles by chromosomal mechanisms in retinoblastoma, *Nature*, 1983, **305**, 779–784.

21. BENEDICT, W. F., SRIVATSAN, E. S., MARK, C., BANERJEE, A. SPARKES, R S. AND MURPHREE, A. L. — Complete or partial homozygosity of chromosome 13 in primary retinoblastoma, *Cancer Res.*, 1987, **47**, 4189–4191.

22. FRIEND, S. H., BERNARDS, R., ROGELJ, S., WEINBERG, R. A., RAPAPORT, J. M., ALBERT, D. M. AND DRYJA, T. P. — A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma, *Nature*, 1986, **323**, 643–646.

23. LEE, W. H., BOOKSTEIN, R., HONG, G., YOUNG, L. J., SHEW, J. Y. AND LEE, E. Y. H. — Human retinoblastoma susceptibility gene: cloning, identification and sequence, *Science*, 1987, **235**, 1394–1399.

24. FUNG, Y. K. T., MURPHREE, A. L., T'ANG, A., QIAN, J., HINRICHS, S. H. AND BENEDICT, W. F. — Structural evidence for the authenticity of the human retinoblastoma gene, *Science*, 1987, **236**, 1657–1661.

25. PASQUALE, S. R., JONES, G. R., DOERSEN, C.-J. AND WEISSMAN, B. E. — Tumorigenicity and oncogene expression in pediatric cancers, *Cancer Res.*, 1988, **48**, 2715–2719.

26. NAKAMURA, Y., LEPPERT, M., O'CONNELL, P., WOLFF, R., HOLM, T., CULVER, M, MARTIN, C., FUJIMOTO, E., HOFF, M., KUMLIN, E. AND WHITE, R. — Variable number of tandem repeat (VNTR) markers for human gene mapping, *Science*, 1987, **235**, 1616–1622.

27. FROSSARD, P. M., COLEMAN, R., FUNKE, H. AND ASSMAN, G. — Apa I RFLP 5.4 kb 5′ to the human apoliprotein Al (APO Al) gene, *Nucleic Acids Res. RFLP Rep.*, 1986, **14**, 1922.

28. MASLEN, C. L., JONES, C., GLASER, T., MAGENIS, R. E., SHEEHY, R., KELLOGG, J AND LITT, M. Seven polymorphic loci mapping to human chromosomal region 11q22-qter, *Genomics*, 1988, **2**, 66–75.

29. MISRA, B. C. AND SRIVATSAN, E. S. Localization of a human tumor suppressor gene to the long arm (q-arm) of chromosome 11, *Am. J. Human Genet*, 1988 (submitted).

30. JEFFREYS, A. J., WILSON, V. AND THEIN, S. L Hyper-variable 'minisatellite' regions in human DNA, *Nature* (Lond.), 1985, **314**, 67–73.

31. BENEDICT, W. F., MURPHREE, A. L., BANERJEE, A., SPINA, C A., SPARKES, M. C. AND SPARKES, R. S. Patient with 13 chromosome deletion: evidence that the retinoblastoma gene is a recessive cancer gene, *Science*, 1983, **219**, 973–975.

32. SPARKES, R. S., SPARKES, M. C., WILSON, M. G., TOWNER, J. W., BENEDICT, W., MURPHREE, A. L. AND YUNIS, J. J. Regional assignment of genes for human esterase D and retinoblastoma to chromosome band 13q14, *Science*, 1980, **208**, 1042–1044.

33. RICCARDI, V. M., HITTNER, H. M., FRANCKE, U., YUNIS, J. J., LEDBETTER, D. AND BORGES, W. The aniridia-Wilms' tumor association: the clinical role of chromosome band 11p13, *Cancer Genet. Cytogenet.*, 1980, **2**, 131–137.

34. KNUDSON, A. G. (JR) Mutation and cancer: statistical study of retinoblastoma, *Proc. Natn. Acad. Sci., USA*, 1971, **68**, 820–823.

35. KNUDSON, A. G. (JR) Hereditary cancer, oncogenes and antioncogenes, *Cancer Res.*, 1985, **45**, 1437–1443.

36. SCHWARTZ, D. C. AND CANTOR, C. R. Separation of yeast chromosome-sized DNAs by pulsed field gradient gel electrophoresis, *Cell*, 1984, **37**, 67–75.

37. CARLE, G. F., FRANK, M AND OLSON, M. V. Electrophoretic separation of large DNA molecules by periodic inversion of the electric field, *Science*, 1986, **232**, 65–68.

38. BURKE, D. T., CARLE, G. F. AND OLSON, M. V. Cloning of large segments of exogenous DNA into yeast by means of artificial chromosome vectors, *Science*, 1987, **236**, 806–812.