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Chromosomal Analysis of a Diethylnitrosamine-induced Tumorigenic and a Nontumorigenic Rat Liver Cell Line[1]

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ABSTRACT

A chromosomal analysis was performed on two cell lines which were derived from the liver of two rats exposed to diethylnitrosamine *in vivo*. The cells were obtained by collagenase perfusion of the liver at an early stage of development of ATPase-deficient putative preneoplastic populations, and propagated from foci of epithelial cells which started growth *in vitro*. Cell line CL 38 proved to be tumorigenic after transplantation into nude mice, giving rise to hepatocellular carcinomas and metastases. Cell line CL 44 was nontumorigenic after transplantation into nude mice and was therefore considered preneoplastic. The diploid nontumorigenic line CL 44, with a modal number of 42 chromosomes, showed a deletion of chromosome 1 and a translocation of chromosomes 3 and 14 [t(3q12;14q21)]. The hyperdiploid neoplastic cell line CL 38 had a modal chromosome number of 52 and showed tri- or tetrasomy of chromosomes 3, 7, 9, 11, and 12 and a marker chromosome that might have originated from aberrant chromosome 1. One or two homologues of chromosome 3 showed terminal deletions (q42, q41, or q35). In both cell lines rearrangements of chromosome 11 were observed [rob(11q;?) or +11 or −11 or del(11)(q12)]. Some of these karyotype abnormalities are located on the same chromosome as described for transplantable hepatomas and for other chemically induced tumors of the rat.

INTRODUCTION

Karyotypes of hepatomas induced by various chemical carcinogens have been analyzed in the past, preferentially in solid transplantation tumors and cell lines derived thereof. The earliest reports date back to the time when banding techniques were not yet available (1). Later investigations were performed with hepatomas kept as transplantation tumors or *in vitro* for many years, thus representing the karyotype of a late stage of progression (2–6) or with manifest hepatocellular carcinomas (7). Despite this large amount of information on chromosomal aberrations of hepatomas, the question of specificity remains unsolved. The concept of this present study was to analyze rather early stages of hepatocarcinogenesis in order to obtain an insight into critical chromosomal aberrations involved in the neoplastic development, and to separate them from chromosomal deviations during progression and diversification of hepatomas.

A study of early stages of hepatocarcinogenesis became possible with the use of cell lines which were derived from rat liver after the exposure of rats to a carcinogen for a limited time interval (8). During the period of preneoplasia, characterized by the development of enzyme-aberrant altered hepatic foci (9–12), cells were obtained from rat liver by collagenase perfusion and propagated *in vitro* by an established selection method (8). Among the first cell lines which were obtained in these experiments, two cell lines were chosen for a detailed chromosomal analysis. One of these lines, CL 44, was nontumorigenic after transplantation into nude mice and thus had to be considered preneoplastic. The other line, CL 38, proved to be tumorigenic after transplantation and served as an example for a neoplastic cell line. Comparison of the karyotypes of preneoplastic and neoplastic cell lines disclosed differences which are discussed with regard to biological implications.

MATERIALS AND METHODS

Cell lines CL 44 and CL 38 were established after collagenase perfusion of the liver of male Wistar AF/Han rats which had been exposed to diethylnitrosamine (5 mg/kg/day) (11) for 64 or 47 days, respectively. The method of propagating these cells *in vitro* has been reported in detail (8). At the time of chromosomal analysis the cell lines were maintained in Earle's minimum essential medium, supplemented with 5% newborn calf serum. Metaphases were arrested by the addition of Colcemid (0.4 μg/ml, GIBCO, BRL) and centrifuged 60 min later (5 min at 40 × *g*). The sediment was resuspended in 0.075 M KCl and incubated at 37°C for 10 min. Cells were fixed 3 times in 4:1 freshly prepared ethanol/glacial acetic acid, and then dropped on cold wet slides. The G-banding technique was applied with slight modifications of the methods of Seabright (13) and Özkinay and Mitelman (14). The slides were air-dried at room temperature for 3 days, treated with 0.025% trypsin (Difco) in phosphate buffered saline (pH 7.2) at room temperature for 1–3 min and stained with 1.5% Giemsa (Merck, Darmstadt, FRG) solution in Sörensen's phosphate buffer (pH 6.8) for 15 min.

Metaphases from the 5th, 18th, and 39th passages of cell line CL 44 and from the 1st, 24th, and 36th passages of cell line CL 38 were used for the determination of modal chromosome numbers. Tetraploid metaphases were not considered. Thirty karyotypes of cell line CL 44 of the 18th passage and 20 karyotypes of cell line CL 38 of the 1st, 12th, 15th, 24th, and 36th passages were photographed and analyzed for structural aberrations. Chromosomes were arranged according to the recommendations of the Committee for a Standardized Karyotype of Rattus norvegicus (15) and to the recommendations of Levan (16).

Tumorigenicity of both cell lines was tested in nude mice (Swiss Nunu, a gift of MPI Martinsried, FRG), by an i.p. injection of 1–4 × 10^6 cells. The karyotype of a resulting tumor derived from cell line CL 38, 25th passage, was compared with the original cell line CL 38. For this purpose, a tumor developing from CL 38 cells in a nude mouse was finely chopped with scissors and cultivated *in vitro*. The culture conditions were as described for the cell lines. Chromosome analysis was performed from the 2nd passage as described above.

RESULTS

Nontumorigenic, "Preneoplastic" Cell Line CL 44. In the 18th passage of cell line CL 44, the chromosome numbers had an approximate modal value of 42 (52%). Cells with this normal number of 42 chromosomes were more frequent in the 5th passage (76%) and were reduced in the 39th passage (32%) (Fig. 1).

Three aberrations were found in 100% ($N = 30$) of the analyzed karyotypes in the 18th passage (Fig. 2): (*a*) interstitial deletion of chromosome 1, with breakpoints at 1q42 and 1q36; (*b*) trisomy of chromosome 2, with a possible insertion at q24; (*c*) translocation of a part of one homologue of chromosome 14 to chromosome 3, with breakpoints at 14q21 and 3q12. In some cases the other homologue of chromosome 14 was lost.

A schematic presentation of aberrations is given in Fig. 3. In 77% of the analyzed metaphases, chromosome 11 was involved,

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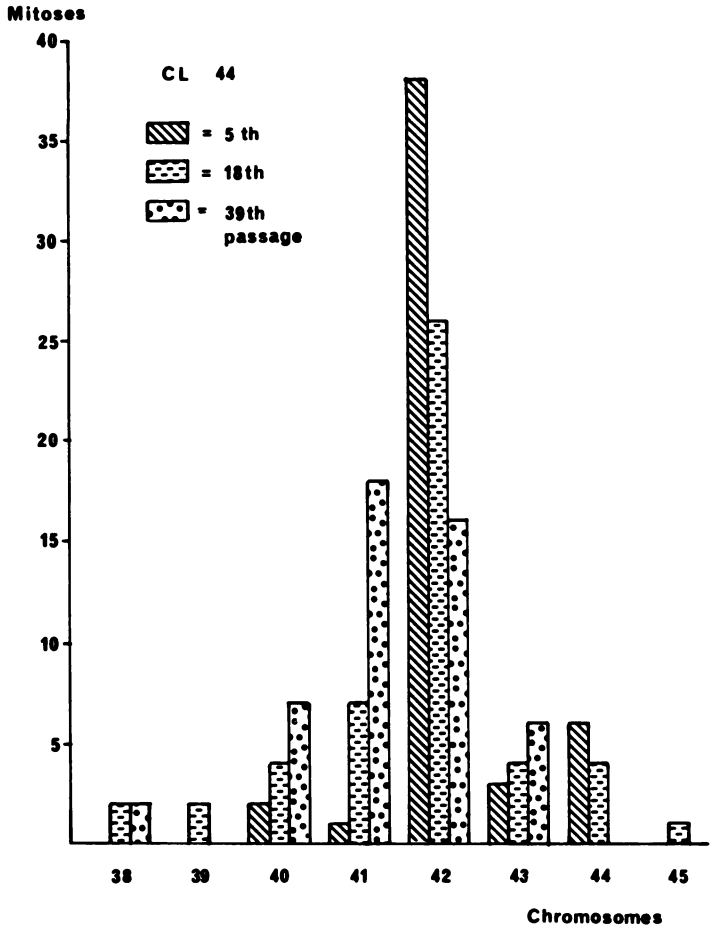

Fig. 1. Cell line CL 44: Number of chromosomes in metaphases of the 5th, 18th, and 39th passages ($N = 50$) (Tetraploid metaphases were not included).

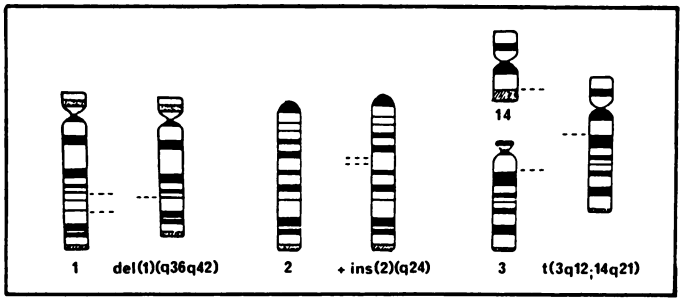

Fig. 3. Schematic presentation of the most frequent rearrangements in cell line CL 44. G-bands exactly according to Levan 1974 (16).

Table 1 *Frequency (%) of chromosomal aberrations in CL 44 (18th passage, N = 30) and CL 38 (24th passage, N = 20)*

CL 44		CL 38	
Aberration	%	Aberration	%
del(1) (q36q42)	100	mar	100
+ins(2) (q24)	100	+(+)12	100
t(3q12;14q21)	100	++3	100
−11	30	del(3)	100
del(11) (q12)	27	(q42 or q41 or q35)	
−7	23	rob(11q;?)	95
rob(11;?)	20	+(+)7	85
− in group 16–20	20	+11	65
−9	17	+9	55
−10	17	−5	35
+ in group 16–20	17	−10	10
−4	13	rob(10;?)	10
−X	13	del(4)	10
−5	7		
13	7		
t(4;15)	7		
−8	7		

being either translocated [rob(11q;?)] (20%), deleted [del(11)(q12)] (27%), or missing (−11) (30%). A summary of aberrations including less frequent abnormalities is given in Table 1.

Tumorigenic Cell Line CL 38. Cell line CL 38 was hyperdiploid. In the 1st passage the majority of the metaphase cells had 52 chromosomes (64%). This preferential number became less predominant in the 24th passage (54%) and even less in the 36th passage (36%) (Fig. 4).

The most obvious difference from CL 44 was the polysomy of several chromosomes in the 24th passage of CL 38 ($N = 20$) (Fig. 5): (*a*) tetrasomy of chromosome 3. One or two of these chromosomes showed a terminal deletion with breakpoints at 3q42, 3q41 or 3a35 (100%); (*b*) trisomy on tetrasomy of chromosome 7 (85%); (*c*) trisomy of chromosome 9 (55%); (*d*) trisomy or tetrasomy of chromosome 12 (100%); (*e*) trisomy of chromosome 11 (65%), two of them presenting a Robertsonian translocation with two unidentified fragments of different lengths (95%).

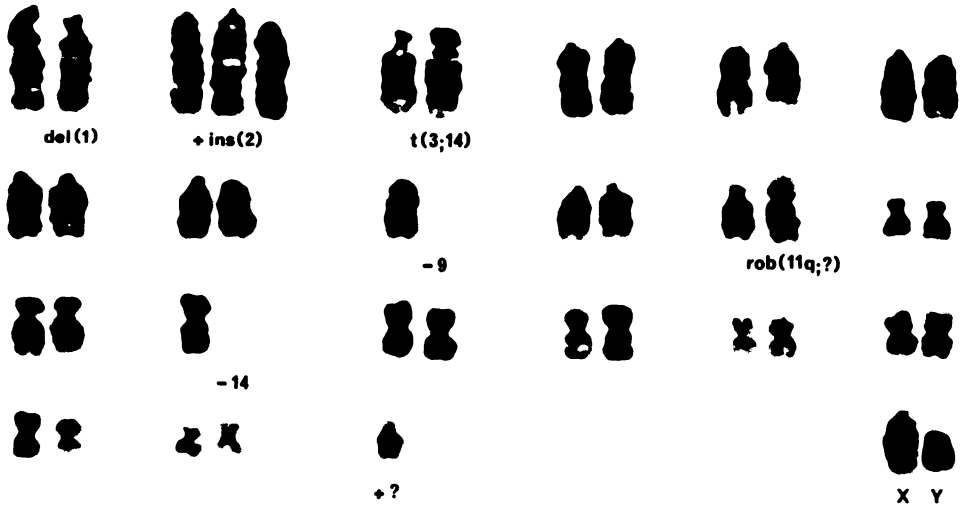

Fig. 2. GTG-banded karyotype of cell line CL 44, 18th passage. The short system designations are given below the rearranged chromosomes. According to the detailed system the aberrations are: del(1)(pter → q36::q42 → qter), +ins(2)(q24) or q24+, t(3;14)(14pter → 14q21::3q12 → 3qter), and t(11;?)(? → cen → 11qter). *+?(bottom line)*, unidentified chromosome.

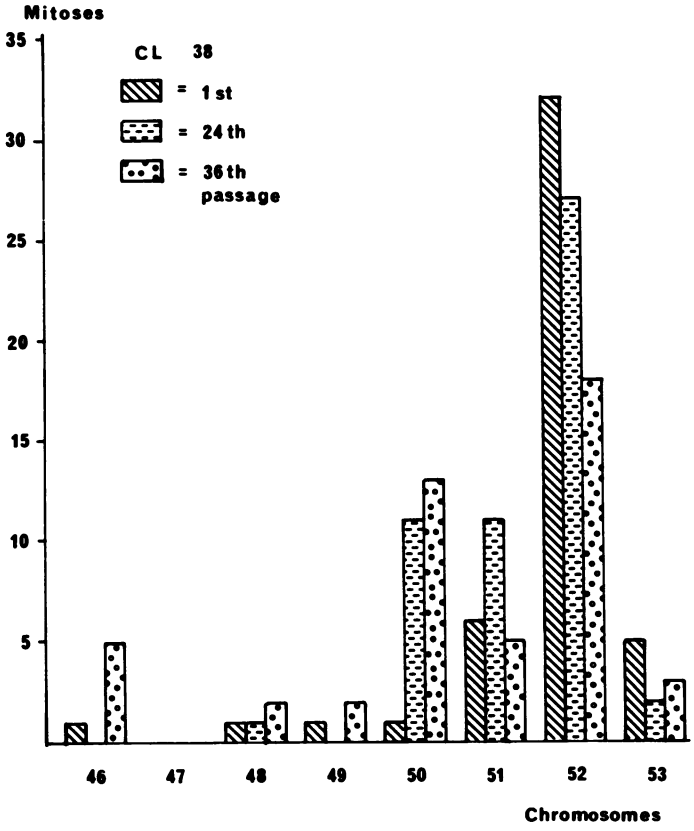

Fig. 4. Cell line CL 38: number of chromosomes in metaphases of the 1st, 24th, and 36th passages ($N = 50$), (Tetraploid metaphases not included).

Besides this, a marker chromosome was found (100%). Chromosomes that could not be identified are arranged at the end in Fig. 5.

A schematic presentation of aberrations is given in Fig. 6. These and other more infrequent aberrations are listed in Table 1.

Karyograms of cells from the first passage showed already tetrasomy and deletions of chromosome 3, tri- or tetrasomy of chromosome 7 and 12, in a few examples a Robertsonian translocation of chromosome 11, partly combined with trisomy, and in one out of five cases trisomy of chromosome 9.

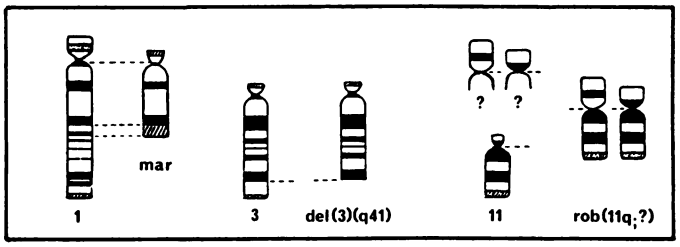

Fig. 6. Schematic presentation of the most frequent rearrangements and the assumed origin of the marker chromosome in cell line CL 38. G-bands exactly according to Ref. 16.

Tumorigenicity in nude mice was three of three (animals with hepatocellular carcinoma/animals injected) after transplantation of passage 2 to 3; two of two for passage 12 to 15; and three of three for passage 25.

Karyotype of Transplantable Tumors Obtained from Cell Line CL 38. Cell line CL 44 did not induce tumor growth after an i.p. injection in thymus-aplastic nude mice. In contrast, nude mice injected with CL 38 developed hepatocellular carcinomas within 2 weeks. A cytogenetic evaluation of cells of one of these tumors revealed almost exactly the same karyotype as described in the cells of the original cell line CL 38 (Fig. 7).

DISCUSSION

Development of cancer is a multistep process. Chromosomal aberrations are likely to be involved in tumor progression and diversification (17), but it is difficult to characterize aberrations crucial for initiation and early steps of tumor development. The normal diploid number in cell line CL 44 suggests that a minimal deviation of chromosome number might correlate with an earlier preneoplastic stage. However, transplantable hepatomas with a diploid or near-diploid karyotype have also been reported (4). This illustrates that not so much the chromosome number, but the structural variation may contribute to tumorigenicity.

Cell line CL 44 is unable to induce tumors in thymus-aplastic nude mice and is therefore considered to represent the preneoplastic state. Nevertheless, constant chromosomal aberrations were found, *i.e.*, deletion at chromosome 1, translocation involving chromosome 3 and 14, and trisomy of chromosome 2.

Fig. 5. GTG-banded karyotype of cell line CL38, 24th passage. *++3* and *++7*, tetrasomy of chromosomes 3 and 7. The short system designations are given below the rearranged chromosomes. Aberrations according to the detailed system are: del(3)(pter → q41:), rob(11q;?)(? → cen → 11qter). *+?* (*bottom line*), unidentified chromosomes.

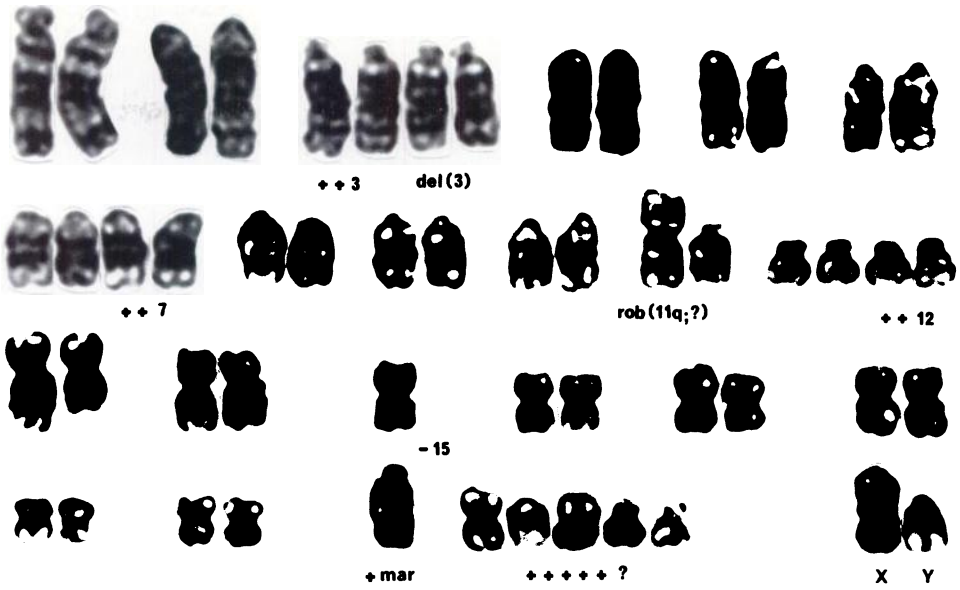

Fig. 7. GTG-banded karyotype of cell line CL 38 after transplantation into a nude mouse, 2nd passage *in vitro*. Similar aberrations as observed in the original culture before transplantation (see Fig. 5).

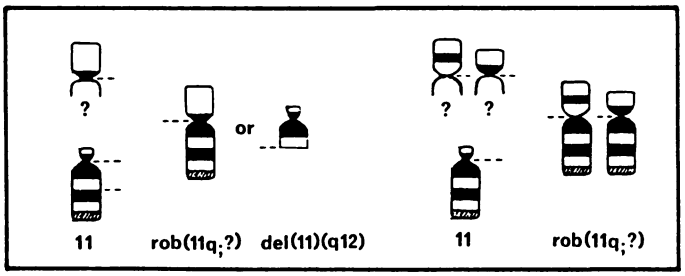

Fig. 8. Similarities of rearrangements of chromosome 11 in cell line CL 44 (*left*) and CL 38 (*right*). G-bands exactly according to Ref. 16.

Some aberrations have also been reported to occur in the transplantable hepatomas H-35tcl, 7316A, and 8994, as described by Kovi *et al.* (5). They observed a chromosome 1 with a duplication segment on its long arm at breakpoint 1q54 in hepatomas 7316A and 8994, translocations involving chromosome 3 in three hepatomas and a trisomic chromosome 2 in hepatoma 8994. In an earlier paper, the same group (4) had reported similar changes in other Morris hepatomas. Wolman *et al.* (18) have observed breakage of chromosome 1 in primary hepatocellular carcinomas of rats.

Chromosomal aberrations are combined with alterations at the molecular level. In liver cell line CL 44 neither deletion of chromosome 1, nor translocation of the chromosomes 3 and 14 or trisomy of chromosome 2 appear connected with tumorigenicity. The best known example of relations between translocation, molecular alteration, and tumorigenicity in human cancer is Burkitt's lymphoma (19), with translocation of chromosomes 8 and 14 accompanied with an increased expression of c-*myc* (20). Nothing is yet known in the rat about altered expression of transformation-related genes after the 3;14 translocation. However, chromosome 14 carries the albumin and the α-fetoprotein gene in the rat (21). Translocations and rearrangements involving this chromosome as observed in CL 44 may bring critical parts of the genome under the influence of the liver-specific, strong promoter of the albumin gene.

It is interesting in this context that the c-Ha-*ras* gene has been located on rat chromosome 1 (22), which shows a deletion in CL 44 and seems to be the origin of the marker chromosome (Fig. 6) in CL 38. These deletions might interfere with the regular expression of c-Ha-*ras* (23).

Chromosome 11 is involved in rearrangements in the preneoplastic cell line CL 44, as well as in the tumorigenic cell line CL 38 (Fig. 8). In cell line CL 38 it is 65% trisomic, with two homologues showing a Robertsonian translocation. In cell line CL 44 it is diploid, but in 20% of 30 karyotyped metaphases it also shows a Robertsonian translocation (or p+). This finding is in agreement with observations made by Kovi *et al.* (5). They describe a chromosome 11 with a small translocated short arm, that was detected in a homologue of the diploid chromosome 11 in the hepatoma H-35tcl, and in two members of the trisomic chromosome 11 in the hepatoma 8994.

A high fragibility in the centromeric region of chromosome 11 might render this position prone to translocations. Such fragibility has been reported for 11q11 in the rat (24). Another explanation would be a carcinogen-sensitive site on this chromosome. Marker chromosomes involving chromosome 11 have been observed in rat sarcomas induced by 3,4-benzo(*a*)pyrene and 20-methylcholanthrene (25, 26).

Progression may be accompanied by amplification of critical genes as shown for instance in N-*myc* in neuroblastoma cells (27). Similar gene dosage effects could be exerted by multiplication of the gene copy number by polysomy as observed in the tumorigenic cell line CL 38 for chromosomes 3, 7, 11, and 12. Kovi *et al.* (5), also described a nearly tetraploid complement in hepatoma cell line 7316A. Only chromosome 9 was missing, as well as one homologue of chromosomes 4, 8, and 16. Other missing chromosomes were identified in rearranged chromosomes. In an early passage of line 7316A they had found a nearly diploid chromosome number with only a few tetraploid metaphase cells. Tetrasomy of several chromosomes in cell line CL 38 will cause an imbalance of genetic expression. It is remarkable that c-*myc* is located on chromosome 7 (28), which is trisomic or tetrasomic in this neoplastic cell line CL 38. An overexpression and amplification of c-*myc* was observed in RNA and DNA, respectively, of cell line CL 38.[3]

Further studies with other neoplastic and preneoplastic liver cell lines now available in our laboratory will show whether rearrangement of chromosome 11 could be interpreted merely as a side-effect of *in vitro* propagation of liver cells or as a marker for initiation of liver carcinogenesis in the rat, and might help to characterize more precisely the extent of gene

[3] Suchy, Sarafoff, Kerler, and Rabes, manuscript in preparation.

activation and amplification during the acquisition of tumorigenicity.

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