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Cancer Genetics and Cytogenetics 194 (2009) 96–110

Cancer Genetics and Cytogenetics

# On the karyotypic origin and evolution of cancer cells

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Received 16 May 2009; accepted 11 June 2009

**Abstract** Cancers have clonal, aneuploid karyotypes that evolve ever more malignant phenotypes spontaneously. Because these facts are hard to explain by conventional mutation theory, we propose here a karyotypic cancer theory. According to this theory, carcinogens initiate carcinogenesis by inducing random aneuploidy. Aneuploidy then catalyzes karyotypic evolutions, because it destabilizes the karyotype by unbalancing teams of proteins that segregate, synthesize, and repair chromosomes. Sporadically, such evolutions generate new cancer-causing karyotypes, which are stabilized within narrow limits against the inherent instability of aneuploidy by selection for oncogenic function. Here we have tested this theory prospectively by analyzing the karyotypes of distinct tumorigenic clones, which arose from mass cultures of human cells within a few months after transfection with artificially activated oncogenes. All clones from the same parental cells had individual, ''near-clonal'' karyotypes and phenotypes, although the parental oncogenes were identical. The karyotypes of distinct tumors formed by a given clone in immunodeficient mice were variants of those of the input clones. The karyotypes of tumorigenic clones also evolved on passages in vitro, in which they acquired either enhanced tumorigenicity spontaneously or resistance against methotrexate upon selection. We conclude that activated oncogenes initiate carcinogenesis indirectly by inducing random aneuploidy, much like conventional carcinogens, but more effectively because the oncogenes are integrated into the genome. Since aneuploidy destabilizes the karyotype, such cells evolve new, cancer-specific karyotypes spontaneously, much like new species. Because individual karyotypes of tumorigenic clones correlate and coevolve with individual phenotypes, we conclude that specific karyotypes as a whole are the genomes of cancer cells. Owing to the flexibility of their aneuploid karyotypes, cancers evolve at rates that are roughly proportional to their degrees of aneuploidy. In sum, genomes consisting of individual and flexible karyotypes explain the characteristic individuality, stability, and flexibility of cancers. 

## 1. Introduction

Cancers contain individual, clonal karyotypes, much like normal phylogenetic species [1–4]. Unlike the karyotypes of normal species, however, the karyotypes and phenotypes of cancers are flexible within certain limits, and thus are able to evolve ever more malignant phenotypes spontaneously [1–3,5–7]. As a result of this inherent flexibility, the karyotypes of cancers are heterogeneous [2,5,6,8,9]. It is still debated whether flexible, specific karyotypes or stable specific mutations cause cancer [10,11].

The somatic mutation theory holds that sets of three to six specific mutations of genes, termed activated oncogenes, transform human cells directly to cancer cells, independent of karyotypic alterations [12,13]: that is, ''without widespread genomic instability'' [14]. Nonetheless, experiments to confirm the mutation theory by transforming human cells to cancer cells with artificially activated genes have called the mutation theory in question:

1. Only 1 of $10^5$ human cells transfected with 6 activated oncogenes is transformed within 2 months after transfection [7,15]. Thus, oncogenes are not sufficient for transformation. Consistent with this view, tumorigenic cell lines, originating from cultures of transfected cells, are clonal, although integration of transfected oncogenes is random [16–18]. Mahale et al. [18] have proposed, therefore, that ''additional stochastic [or clonal] events'' are necessary for transformation by activated oncogenes. Likewise, transgenic oncogenes are insufficient to cause tumors in mice, because the resulting tumors are clonal for cell-specific markers, such as chromosomal

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0165-4608/09/$ – see front matter 
doi:10.1016/j.cancergencyto.2009.06.008

alterations, rather than systemic, involving whole animals or specific organs [19–22].
2. The oncogenes that induce transformation are not necessary to maintain transformation, because tumors and leukemias induced by oncogenes, such as the T-antigen of SV40, persist if artificially controlled oncogenes are turned off or lost [19,21–25].
3. All cells transformed by specific sets of oncogenes that have been tested are aneuploid and display genomic instability [16,18,19,25–29].

Thus, oncogenes are not sufficient to cause, and are not necessary to maintain, cancers cells. So what is the role of oncogenes in transformation?

Toward explication of the role of mutations and oncogenes in carcinogenesis, we have recently proposed a karyotypic theory of cancer [7,28]. According to this theory, a two-step-mechanism transforms normal cells to cancer cells. First, carcinogens, including conventional mutagenic and nonmutagenic carcinogens [28,30–33] and also activated oncogenes [7,17,25,26,28,34–36], destabilize the karyotype by inducing random aneuploidy. Aneuploidy destabilizes the karyotype by unbalancing teams of proteins that segregate, synthesize, and repair chromosomes—in proportion to the degree of aneuploidy [37–40]. Second, aneuploidy initiates and maintains karyotypic evolutions automatically because of the inherent instability of aneuploidy. Most of the newly evolving karyotypes are further, random aneuploidies that are functionally inferior to those of normal cells, or even lethal [41–45]. Occasionally, however, rare cancer-causing karyotypes evolve stochastically. These cancer-causing karyotypes are then stabilized against the inherent instability of aneuploidy by selection for transforming function within narrow clonal limits of variation [7,46]. The resulting stability within instability of cancer karyotypes thus explains exactly the flexibility and heterogeneity that are typical of a cancer genome. This flexibility and heterogeneity of cancer karyotypes is also the basis for the further, spontaneous evolutions that are known as tumor progression, such as metastasis and drug resistance [1,47,48]. The karyotypic theory thus postulates that individual clonal, yet flexible karyotypes are the genomes of cancers.

Now we have tested this theory prospectively by analyzing the karyotypes of clonal tumorigenic cell lines arising from mass cultures of human cells within months after transfection by artificially activated oncogenes [15,18,28]. We found (1) that different tumorigenic cell lines, arising from human cells transfected with the same set of oncogenes, had individual clonal karyotypes and phenotypes; (2) that the clonal karyotypes of such tumorigenic cell lines were basically stable, yet individually variable over 68 generations in vitro; (3) that the phenotypes and karyotypes of different tumors induced by these lines in different mice were karyotypic and phenotypic variants of the parental prototypes; and (4) that the karyotypes of drug-resistant derivatives of such lines were also specific variants of the parental prototypes.

Because individual karyotypes correlated with the individual phenotypes of clones derived from cells with the same oncogenes, and because these karyotypes co-evolved with new phenotypes during tumorigenesis and the acquisition of drug resistance, we conclude that individual "near-clonal" karyotypes are the genomes of individual tumorigenic clones. Furthermore, we deduce from our data that activated oncogenes are not sufficient for transformation, because they are present in mass cultures of transfected, pretumorigenic cells. We therefore conclude that activated oncogenes transform indirectly by inducing the evolution of new, cancer-causing karyotypes, much like conventional carcinogens [31,32,49].

## 2. Materials and methods

### 2.1. Cloning transformed cells in 0.3–0.4% agar gels

To obtain single-cell-derived clones of transformed human epithelial cells, we propagated these cells in agar gel suspensions, which discriminate against growth of normal cells [50]. For this purpose, ~$10^5$ to $10^6$ cells were suspended in 3 mL of 0.35–0.4% agar (A9915; Sigma–Aldrich, St. Louis, MO) in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum at 40°C. The suspension was layered on 2.5 mL of solidified 0.4% agar in the same medium in a 5 cm-culture dish. On the next day, the cultures were overlaid with 1 mL of RPMI 1640 medium supplemented with 5% fetal calf serum. This overlay was changed once or twice a week until colonies appeared in the agar suspension 1–2 months later. These colonies were picked with micropipettes and propagated for karyotype analyses.

### 2.2. Identification of metaphase chromosomes by in situ hybridization with chromosome-specific color-coded DNA probes (mFISH)

One to two days prior to karyotyping, cells were seeded at <50% confluency in RPMI 1640 medium containing 5% fetal calf serum. After reaching ~75% confluency, the medium was replaced by 3 mL of fresh culture medium per 5-cm culture dish. Cultures were then incubated either directly, or after replacing the culture medium, with 0.15 μg colcemid (KaryoMax Colcemid solution; GIBCO Invitrogen, Grand Island, NY) at 37°C for 1–4 hours. The medium was then collected, and the cells were washed with phosphate-buffered saline at pH 7, dissociated with 1 mL 0.5% trypsin–EDTA (GIBCO Invitrogen) for a few minutes at 37°C until they detached from the dish, combined with the removed growth medium, and centrifuged for 6 minutes at 175 g at room temperature. The cells were then resuspended in 10 mL hypotonic KCl at 0.075 mol/L (Sigma–Aldrich) and incubated at 37°C for

16 minutes. Thereafter, the cells were cooled in ice water for 3 minutes and 5% (v/v) of freshly prepared ice-cold fixative, a mixture of three volumes of methanol and one volume of acetic acid, was added [51,52]. This solution was then centrifuged for 6 minutes at 175 *g*. The cell pellet was resuspended in 0.3–0.5 mL supernatant and ~10 mL of ice-cold fixative was mixed in drop-wise. The solution was incubated at room temperature for 15 minutes, then centrifuged again at 175 *g* as above, and resuspended once more in fresh fixative. After 15 minutes at room temperature, the cells were again pelleted and resuspended in ~0.5 mL of the supernatant fixative. Ten-microliter aliquots were then placed with a micropipette onto glass microscope slides held at an angle and immediately inspected under a phase contrast microscope for the presence of metaphase chromosomes. Slides with suitable metaphase chromosomes were then processed for hybridization with chromosome-specific, color-coded DNA probes (mFISH) as described by the manufacturer (MetaSystems, Boston, MA; Altlussheim, Germany) and by us previously [7,53].

## 3. Results

### *3.1. Cells transfected with the same oncogenes generate tumorigenic cell lines with individual karyotypes and phenotypes*

To determine whether new individual karyotypes or specific sets of oncogenes are the genomes of new cancer cells, we have analyzed the karyotypes and phenotypes of four distinct tumorigenic cell lines that originated from normal human epithelial cells transfected with specific sets of oncogenes prepared by two different laboratories. Two of these lines had become the predominant clones within several months after transfection of mass cultures with oncogenes [18].

#### *3.1.1. Two distinct tumorigenic lines derived from human cells transfected with four oncogenes*

Mahale et al. [18] recently prepared two clonal lines of tumorigenic cells that originated from human epithelial cells transfected with the same four activated oncogenes. These oncogenes were derivatives of human cellular telomerase, cyclin-dependent kinase 4, the tumor suppressor protein p53, and H-RasV12, cloned into and activated by retrovirus vectors. These tumorigenic lines were termed here T-p39 (for TK4DnR-p39) and T-p33 (for TK4DnR-p33), respectively, because mass cultures of epithelial cells had turned into clonal lines of tumorigenic cells 39 or 33 passages after transfections with these four oncogenes [18]. The 39 passages correspond to ~78 cell generations, and the 33 passages to 66 cell generations [18]. These lines also formed colonies in agar gels—an established condition, which discriminates against the growth of normal cells [18,50].

As pointed out by Mahale et al. [18], both lines were found to be clonal, based on integration sites of transfected oncogenes, although activated oncogenes were introduced into epithelial cells in randomly integrating retrovirus vectors. In view of this, the authors concluded that the four transfected genes were not sufficient for transformation, and that transformation depended on a further clonal event [18].

T-p39 and T-p33 cells have distinct morphologies and sociologies (Figs. 1A and 2A), although containing the same four oncogenes. The T-p39 cells were refractile and spindle-shaped and grew three-dimensionally to high density, as described previously [18]. The T-p33 cells were also spindle-shaped, but thinner and less refractile, and did not grow three-dimensionally to high density under the same conditions at which T-p39 did.

Because the phenotypes of these tumorigenic lines were different, but their oncogenes were the same, it follows that these phenotypes were not encoded by the respective oncogenes. We therefore hypothesized that the distinct phenotypes of the two tumorigenic lines were generated de novo, possibly by the individual karyotypes, predicted by the karyotypic cancer theory, particularly because activated oncogenes are known to destabilize the karyotype (as discussed in the Introduction).

Alternatively, it could be argued that the individual phenotypes of the two different tumorigenic cell lines derived from unidentified cancer-causing mutations. Such mutations could transform either alone or in cooperation with the four transfected oncogenes that were already present in transfected nontumorigenic precursor cells. Lines derived from such cancer-causing mutations would then be clonal for these mutations, as well as for any preexisting aneuploidy. This hypothesis predicts, however, that the original aneuploidy of a mutationally transformed cell would not be relevant for transforming function and would thus randomize over time, owing to the inherent instability of aneuploidy, whereas the transforming mutations would be stabilized by selection for neoplastic transformation (see further in section 3.1.3.).

To test these hypotheses, we analyzed the karyotypes of the T-p33 and T-p39 lines by in situ hybridization of metaphase chromosomes with chromosome-specific colors (as described in section 2.2), following published procedures [7,28,53]. The results are given in Tables 1 and 2.

The T-p39 line had a near-tetraploid karyotype with an average chromosome number (± standard deviation [SD]) of 87 ± 5 (Table 1). The relatively narrow, ''near-clonal'' distribution of these chromosome numbers is typical of clonal populations of cancer cells (see the Introduction section, and also Li et al. [7]). The chromosome copy numbers were also ''near-clonal'', with a low of 45% to a high of 100% sharing the same peak or modal chromosome copy numbers (Table 1). The remaining percentages of cells had near-modal copy numbers, which oscillated between ±1 and, in a few cases, ±2 around the modal values (see examples in Fig. 3 and in Li et al. [7]). Such variability around the modal values is typical for aneuploid karyotypes. It reflects cancer karyotype-specific limits of

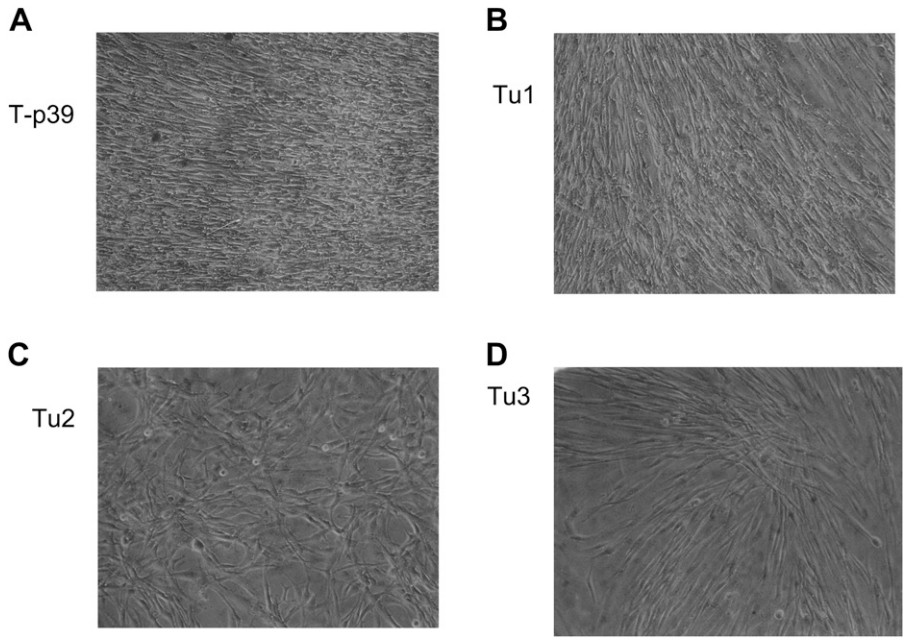


Fig. 1. (A) Cells from the tumorigenic clone T-p39, which predominated a stock of human epithelial cells 39 passages after transfection with four retrovirus-activated oncogenes as described in the text. The cells were refractile and spindle-shaped, and grew three-dimensionally. (B) Cells of Tu1, one of three tumors caused by injection of T-p39 cells into an immunodeficient mouse. These cells resembled the input line and also grew three-dimensionally. (C,D) Cells of a second and a third tumor, Tu2 and Tu3, caused by T-p39 cells in immunodeficient mice. The cells of Tu2 were either spindle-shaped or amorphous; and those of Tu3 were elongated, spindle-shaped cells. Both the Tu2 and Tu3 cells grew three-dimensionally, but markedly more slowly than those of the parental line or Tu1. All cells were photographed at ×120 magnification with a phase contrast microscope.

variation that are defined by selection for oncogenic function (for examples, see Li et al. [7]). In sum, T-p39 has a clonal, near-tetraploid karyotype with a narrow range of flexibility in chromosome copy numbers.

In addition, the T-p39 line contained one subclonal marker chromosome, der(22;12;15), present in 40% of cells, and between 0 and 5 nonclonal marker chromosomes per cell (Table 1). Such subclonal or nonclonal aneusomies

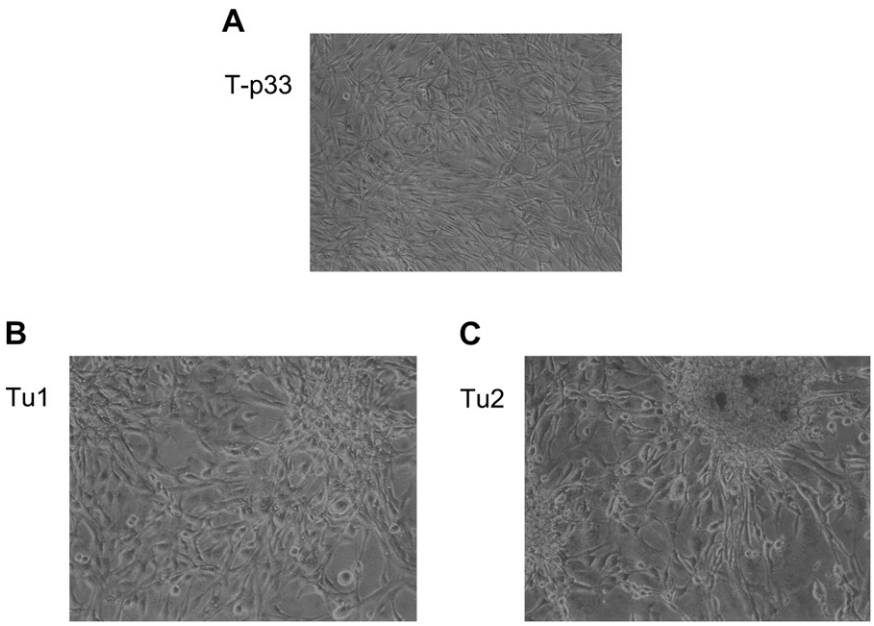


Fig. 2. (A) Cells from a second tumorigenic clone, T-p33, which predominated a culture of the same stock of human epithelial cells as described in Fig. 1, 33 passages after transfection with the same four retrovirus-activated oncogenes used to generate the T-p39 line (Fig. 1). The cells are spindle-shaped, and did not grow three-dimensionally to high density, in contrast to T-p39. (B,C) The cells of Tu1 and Tu2, two tumors caused by injection of T-p33 cells into immunodeficient mice. The cells of both tumors were either spindle-shaped or amorphous and grew three-dimensionally, forming foci of transformed cells among areas of low cell density. Both cell types were similar, but Tu2 cells seemed to grow to higher density than Tu1.

Table 1
Summary karyotypes of tumorigenic lines T-p39, T-p73, and T-p39 derived tumors Tu1, Tu2, and Tu3

| | Modal chromosome number (% of total) | | | | |
|---|---|---|---|---|---|
| Chromosome | T-p39 | T-p73 | Tu1 | Tu2 | Tu3 |
| 1 | 4 (65) | 4 (45) | 4 (55) | 3 (71) | 4 (53) |
| 2 | 4 (75) | 3 (75) | 3 (75) | 2 (68) | 2 (68) |
| 3 | 4 (85) | 4 (85) | 4 (80) | 3 (68) | 4 (47) |
| 4 | 4 (90) | 4 (70) | 4 (75) | 3 (66) | 3 (74) |
| 5 | 4 (90) | 4 (65) | 4 (90) | 4 (66) | 4 (68) |
| 6 | 4 (85) | 4 (70) | 4 (70) | 3 (68) | 3 (84) |
| 7 | 4 (90) | 3 (75) | 3 (85) | 3 (74) | 3 (53) |
| 8 | 4 (80) | 4 (60) | 3 (40) | 4 (55) | 4 (47) |
| 9 | 3 (55) | 3 (65) | 3 (75) | 3 (71) | 3 (42) |
| 10 | 4 (60) | 3 (60) | 4 (70) | 4 (47) | 3 (58) |
| 11 | 2 (75) | 2 (70) | 1 (70) | 4 (37) | 2 (47) |
| 12 | 4 (85) | 4 (70) | 4 (90) | 3 (58) | 4 (63) |
| 13 | 4 (70) | 3 (65) | 3 (75) | 3 (58) | 2 (68) |
| 14 | 4 (65) | 4 (65) | 4 (55) | 2 (68) | 3 (63) |
| 15 | 4 (45) | 3 (70) | 4 (50) | 4 (47) | 2 (47) |
| 16 | 4 (75) | 4 (80) | 4 (80) | 3 (61) | 3 (53) |
| 17 | 4 (85) | 3 (90) | 3 (85) | 3 (66) | 3 (68) |
| 18 | 4 (65) | 3 (70) | 3 (65) | 4 (63) | 3 (63) |
| 19 | 4 (85) | 4 (55) | 4 (60) | 3 (76) | 2 (47) |
| 20 | 6 (50) | 4 (75) | 4 (70) | 6 (55) | 4 (47) |
| 21 | 2 (65) | 3 (50) | 3 (65) | 2 (71) | 2 (68) |
| 22 | 2 (50) | 4 (60) | 4 (45) | 3 (50) | 2 (63) |
| X | 2 (100) | 2 (95) | 2 (85) | 2 (87) | 2 (79) |
| Y | 2 (85) | 2 (80) | 2 (70) | 2 (74) | 2 (47) |
| der(11;19) | 2 (85) | 2 (95) | 2 (90) | 1 (42) | 1 (32)/2 (16) |
| der(22;12;15) | 1 (5)/ 2 (35) | 2 (15) | — | 2 (8) | — |
| der(9;17) | — | 2 (20) | — | — | — |
| der(15;17) | — | 1 (35) | — | — | — |
| der(7;8) | — | — | 1 (70) | — | — |
| der(1) | — | — | 1 (20) | 1 (8) | 1 (16) |
| der(19) | — | — | 1 (15) | — | 3 (11) |
| der(8) | — | — | 1 (40) | — | 1 (11) |
| der(1;5) | — | — | — | 1 (68) | — |
| der(11;14) | — | — | — | 1 (71) | — |
| der(2) | — | — | — | 1 (71) | — |
| NCM | 0-5 | 0-3 | 0-4 | 0-4 | 0-5 |
| Metaphases, no. | 20 | 14 | 20 | 38 | 19 |
| Avg. chr. no. ± SD | 87 ± 5 | 82 ± 4 | 80 ± 5 | 75 ± 9 | 64 ± 9 |

*Abbreviations:* Avg, average; chr., chromosome; p, passage; NCM, nonclonal markers per cell; SD, standard deviation.

Table 2
Summary karyotypes of two stemlines of the T-p33 line and derivative tumors Tu1 and Tu2

| | Modal chromosome number (% of total) | | | |
|---|---|---|---|---|
| Chromosome | T-p33 sl 1 | T-p33 sl 2 | Tu1-p33 | Tu2-p33 |
| 1 | 2 (90) | 4 (100) | 4 (75) | 5 (50) |
| 2 | 2 (95) | 4 (100) | 4 (60) | 4 (70) |
| 3 | 2 (100) | 4 (100) | 2 (55) | 5 (55) |
| 4 | 1 (90) | 2 (100) | 2 (70) | 3 (70) |
| 5 | 2 (100) | 4 (100) | 4 (45) | 5 (40) |
| 6 | 2 (95) | 4 (100) | 4 (70) | 5 (60) |
| 7 | 2 (100) | 4 (100) | 4 (40) | 5 (50) |
| 8 | 2 (100) | 4 (100) | 4 (70) | 7 (50) |
| 9 | 2 (100) | 4 (67) | 4 (70) | 4 (35) |
| 10 | 2 (95) | 4 (100) | 4 (75) | 5 (60) |
| 11 | 2 (100) | 4 (100) | 4 (45) | 5 (40) |
| 12 | 2 (95) | 4 (100) | 4 (60) | 6 (55) |
| 13 | 2 (95) | 4 (67) | 4 (55) | 4 (35) |
| 14 | 1 (100) | 2 (67) | 2 (65) | 3 (70) |
| 15 | 1 (95) | 2 (67) | 2 (65) | 3 (80) |
| 16 | 1 (80) | 2 (67) | 2 (55) | 3 (50) |
| 17 | 2 (100) | 4 (100) | 4 (90) | 5 (65) |
| 18 | 2 (100) | 4 (100) | 4 (65) | 4 (35) |
| 19 | 2 (100) | 4 (100) | 2 (60) | 5 (60) |
| 20 | 2 (95) | 4 (100) | 4 (50) | 8 (40) |
| 21 | 2 (90) | 4 (100) | 3 (65) | 3 (55) |
| 22 | 2 (100) | 4 (100) | 4 (40) | 3 (65) |
| X | 1 (90) | 2 (100) | 2 (80) | 3 (90) |
| Y | 1 (100) | 2 (100) | 1 (45) | 2 (45) |
| der(14;17) | 1 (80) | 2 (67) | 2 (85) | 3 (80) |
| der(16;15) | 1 (75) | 2 (67) | 1 (70) | 1 (90) |
| der(4;7) | 1 (90) | 2 (100) | 2 (65) | 1 (50) |
| der(14;15) | 1 (20) | 1 (33) | — | — |
| der(3;22) | — | — | 1 (20) | 1 (85) |
| der(3)long | — | — | 1/2 (55/35) | — |
| der(3)short | — | — | 1 (20) | — |
| der(19;18) | — | — | 2 (40) | — |
| NCM | 0-1 | 0-1 | 0-5 | 0-8 |
| Metaphases, no. | 20 | 3 | 20 | 20 |
| Avg. chr. no. ± SD | 45 ± 1 | 90 ± 1 | 82 ± 6 | 111 ± 11 |

*Abbreviations:* Avg, average; chr., chromosome; p, passage; NCM, nonclonal markers per cell; SD, standard deviation; sl, stemline.

indicate ongoing chromosomal instability [7]. The rates at which such nonclonal marker chromosomes are generated and lost per cell are directly proportional to the degree of aneuploidy [7,37,38].

The predominant karyotype of T-p33 was hypodiploid, with an average total chromosome number (± SD) of 45 ± 1 (Table 2). It lacked one copy of the four intact chromosomes 4, 14, 15, and 16, and contained 3 clonal marker chromosomes (Table 2, Fig. 3B). In addition, the line contained one subclonal marker chromosome der(14;15) and between zero and one nonclonal marker per cell (Table 2). There was also a minor, near-tetraploid T-p33 sideline, which is almost an exact chromosomal duplication of the predominant line (Table 2) (see also section 3.3.2). As expected from the low degree of aneuploidy of the hypodiploid and near-tetraploid lines, the T-p33 line was more clonal and thus more stable than the T-p39 line described above (compare Tables 1 and 2). Nevertheless, the presence of a few nonclonal markers and of a minor ploidy-variant again indicates ongoing instability.

To give a visual impression of the individuality, clonality, and flexibility of the karyotypes of the two tumorigenic clones T-p39 and T-p33, and to facilitate visual comparisons between tumorigenic and normal karyotypes, we generated three-dimensional karyographs of T-p39 and T-p33 and of normal human male cells (Figs. 3A, 3B, and 3F). Such three-dimensional karyographs connect in parallel tracks the copy numbers of the intact and clonal marker chromosomes of individual metaphases. Chromosome numbers are plotted on the *x*-axis (dimension 1), chromosome copy numbers on the *y*-axis (dimension 2), and metaphase numbers on the *z*-axis (dimension 3). In

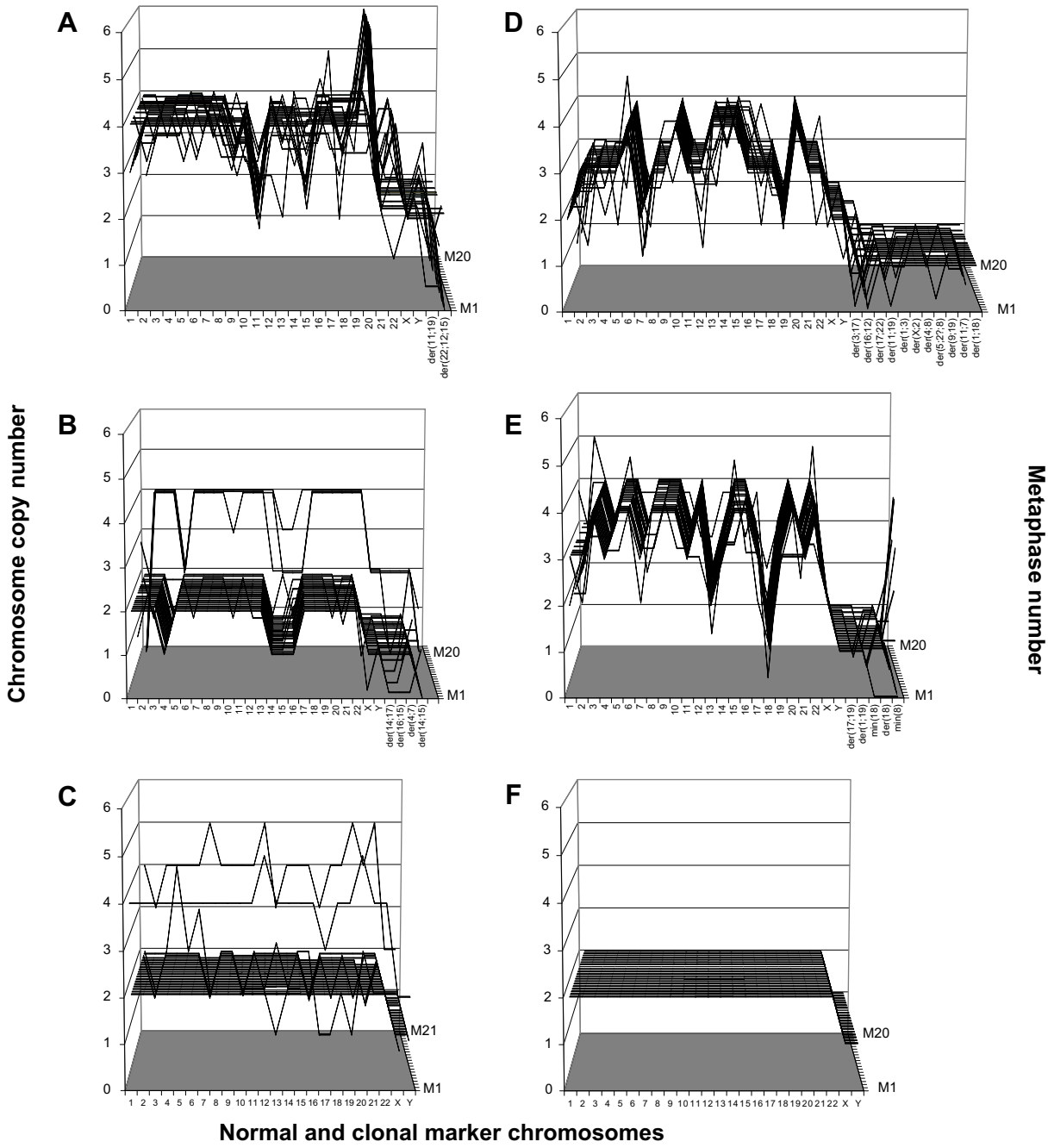


Fig. 3. (A,B) Karyographs of the clonal, tumorigenic lines T-p39 (A) and T-p33 (B), which arose from normal human cells transfected with the same set of four virus-activated oncogenes. (C) Karyograph of a basically diploid and partially tetraploid culture transfected with three of the four oncogenes used to generate T-p39 and T-p33. (D,E) Karyographs of the lines BJ (D) and HA1 (E), which arose from human cells transfected with a set of three virus activated oncogenes. (F) Karyograph of a normal human male. These three-dimensional karyographs connect in parallel tracks the copy numbers of the intact and clonal marker chromosomes of individual metaphases. Chromosome numbers are on the *x*-axis; chromosome copy numbers are on the *y*-axis; and metaphase numbers are on the *z*-axis.

normal diploid human cells, 20 metaphases generate 20 identical parallels (Fig. 2F), consistent with the stable copy numbers of normal cells. As shown in Fig. 3, the tumorigenic T-p39 and T-p33 cell lines consist of predominantly clonal but karyotypically distinct cells with minorities of nonmodal chromosome copy numbers reflecting the inherent chromosomal instability of aneuploid karyotypes (summarized in Tables 1 and 2). These karyographs show directly that the karyotypes of the two tumorigenic lines T-p39 and T-p33 are markedly different from each other (Fig. 3), although both derived from the same parental cells transfected with the same four oncogenes.

These results support the karyotypic theory in two ways:

1. The individual clonal karyotypes of the two lines correspond with their individual phenotypes, rather

than with the four oncogenes that they share with each other and with the nontumorigenic precursor cells.

2. The limited variability of the chromosome copy numbers of T-p39 and T-p33 around modal numbers, despite the inherent instability of aneuploidy, are consistent with the view that these karyotypes are selected for the oncogenic phenotypes of these lines (see the Introduction section).

We conclude, therefore, that the individual, flexible karyotypes as a whole encode the complex individual phenotypes of T-p39 and T-p33, rather than their common, inflexible oncogenes. (Further evidence against the argument that unknown individual mutations cause the individual phenotypes of these lines is discussed in section 3.2.)

Mahale et al. [18] questioned this conclusion, however, because of a reportedly highly aneuploid but nontumorigenic line, TK4DnV, which was generated with three of the four oncogenes (omitting H-RasV12) used to obtain T-p39 and T-p33. We have therefore reexamined the TK4DnV line and found it diploid, although the ploidy and chromosome copy numbers were less stable than those of normal human diploid cells. Approximately 10% of the TK4DnV chromosome copy numbers were nonmodal, above or below 2, and ~2 out of 21 metaphases were near-tetraploid (Fig. 3C), in contrast to the normal diploid male karyograph (Fig. 3F). So, in view of its quasi-diploid karyotype, the TK4DnV line does not challenge the karyotypic cancer theory. Instead, it would be expected to be nontumorigenic, exactly as observed by Mahale et al. [18]. The relative instability of this line probably reflects the destabilizing effect of the three transfected oncogenes.

### *3.1.2. Three other distinct tumorigenic lines derived from human cells transfected with three oncogenes*

Next, we analyzed the karyotypes of a second pair of phenotypically distinct tumorigenic lines, derived from human epithelial cells transfected with a set of three oncogenes by Hahn et al. [13]. In contrast to the four oncogenes described above, these three oncogenes were said to be sufficient for transformation [13]. These oncogenes were retrovirus-activated human cellular telomerase and mutated H-RasV12 genes, and the T-antigen gene of the tumor virus SV40, which is thought to be sufficient to transform cells by itself [23]. Thus, two of the oncogenes studied by Hahn et al. [13] were the same as two of the four studied above (namely, activated telomerase and activated H-RasV12) (see section 3.1.1.).

The sufficiency of these three genes for transformation is uncertain, however, in view of the following observations. Hahn et al. [13] obtained these lines from human epithelial cells (the HA1 line from kidney cells and the BJ line from skin fibroblasts) only after at least 60 population doublings since the introduction of the three activated oncogenes. Moreover, at least one of the two lines, HA1, had a long prior history in cell culture [34,54]. This strategy thus allowed ample time for the evolution and selection of a cancer-causing karyotype, predicted to be the cause of transformation by the karyotypic theory. Furthermore, both lines were found to be clonal, based on the integration sites of transfected retrovirus-activated oncogenes, although retrovirus integration is random [16,17]. Moreover, both lines were found to be highly aneuploid and to contain clonal marker chromosomes [16,17]. Since this all suggests that the three transfected putative oncogenes were not sufficient for transforming function, we have reanalyzed these lines.

The cells of both lines grew three-dimensionally in culture (Figs. 4A and 4B). Nonetheless, despite common oncogenes, the cells of the two lines had different phenotypes. The HA1 cells were polygonal and refractile, whereas BJ cells were elliptical and much less refractile than HA1 cells.

Analyses of the karyotypes of these two tumorigenic lines are presented in Table 3 and in Figures 3D and 3E. The BJ line had a hypotetraploid, ‘‘near-clonal’’ karyotype with an average chromosome number (± SD) of 82 ± 5. Between 75 to 100% of the BJ cells shared the same

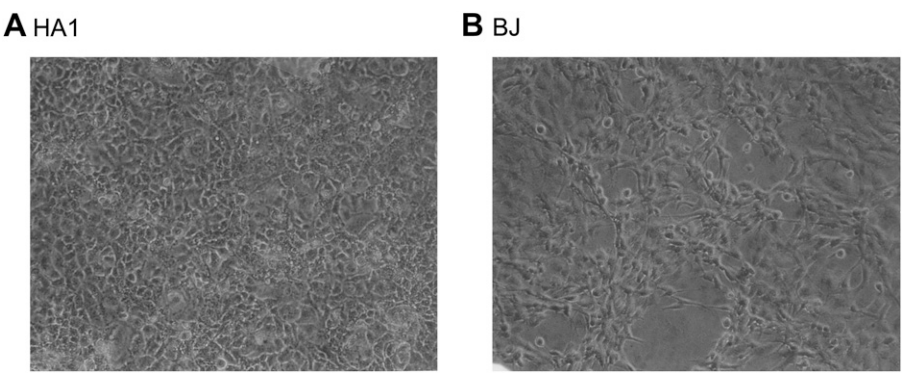


Fig. 4. (A) Cells from the tumorigenic clone HA1, which predominated a culture of human epithelial kidney cells over 60 generations after transfection with three virus-activated oncogenes. The cells had polygonal morphologies and grew to high densities three-dimensionally. (B) Cells from a tumorigenic clone, BJ, which predominated a culture of human epithelial skin cells over 60 generations after transfection with the same three virus-activated oncogenes as for HA1. The cells had elliptical morphologies and grew to high densities three-dimensionally.

Table 3
Summary karyotypes of the BJ line, two stemlines of HA1, and of a methotrexate-resistant subclone

| Chromosome | Modal chromosome number (% of total) | | | |
|---|---|---|---|---|
| | BJ | HA1 sl 2 | HA1 sl 1 | HA1 MTX |
| 1 | 2 (85) | 2 (94) | 3 (50) | 3 (90) |
| 2 | 3 (75) | 2 (100) | 3 (80) | 3 (95) |
| 3 | 3 (90) | 2 (100) | 4 (95) | 4 (85) |
| 4 | 3 (90) | 2 (100) | 3 (100) | 3 (95) |
| 5 | 3 (95) | 2 (100) | 4 (95) | 4 (85) |
| 6 | 4 (80) | 2 (94) | 4 (95) | 3 (90) |
| 7 | 2 (85) | 2 (100) | 3 (85) | 4 (100) |
| 8 | 3 (95) | 2 (89) | 4 (85) | 4 (95) |
| 9 | 3 (90) | 2 (94) | 4 (100) | 3 (90) |
| 10 | 4 (95) | 2 (100) | 4 (95) | 3 (50) |
| 11 | 3 (95) | 2 (89) | 3 (95) | 3 (70) |
| 12 | 3 (80) | 2 (94) | 4 (90) | 3 (95) |
| 13 | 4 (75) | 2 (94) | 2 (90) | 2 (95) |
| 14 | 4 (85) | 2 (89) | 3 (95) | 2 (80) |
| 15 | 4 (90) | 2 (100) | 4 (85) | 3 (90) |
| 16 | 3 (80) | 2 (94) | 4 (90) | 2 (85) |
| 17 | 3 (85) | 2 (100) | 3 (90) | 3 (85) |
| 18 | 3 (90) | 2 (100) | 1 (85) | 1 (95) |
| 19 | 2 (90) | 2 (100) | 3 (100) | 2 (90) |
| 20 | 4 (95) | 1 (94) | 4 (90) | 4 (95) |
| 21 | 3 (100) | 2 (94) | 3 (95) | 2 (75) |
| 22 | 3 (90) | 2 (100) | 4 (90) | 3 (100) |
| X | 2 (95) | 1 (100) | 2 (100) | 2 (95) |
| Y | 2 (95) | 0 (83) | 1 (100) | 2 (85) |
| der(17;19) | — | — | 1 (100) | 1 (90) |
| der(18) | — | — | 1 (95) | 1 (95) |
| der(1;19) | — | — | 1 (90) | 1 (100) |
| min(18) | — | — | 1 (80) | — |
| min(8) | — | — | 1/2/3/4 (40) | — |
| der(13;20) | — | 1 (100) | — | — |
| der(1;3) | 1 (65) | — | — | — |
| der(X;2) | 1 (95) | — | — | — |
| der(5;2?;8) | 1 (90) | — | — | — |
| der(9;19) | 1 (100) | — | — | — |
| der(11;7) | 1 (95) | — | — | — |
| der(1;18) | 1 (80) | — | — | — |
| der(3;17) | 1 (50) | — | — | — |
| der(12;16) | 1 (65) | — | — | — |
| der(17;22) | 1 (100) | — | — | — |
| der(11;19) | 1 (80) | — | — | — |
| der(4;8) | 1 (100) | — | — | — |
| NCM | 0-4 | 0-1 | 0-4 | 0-3 |
| Metaphases, no. | 20 | 20 | 20 | 20 |
| Avg. chr. no. ± SD | 82 ± 5 | 45 ± 1 | 82 ± 1 | 71 ± 3 |

*Abbreviations:* Avg, average; chr., chromosome; p, passage; MTX, methotrexate resistant; NCM, nonclonal markers per cell; SD, standard deviation; sl, stemline.

modal chromosome copy numbers, including those of the 11 clonal monosomic marker chromosomes of BJ (Table 1). Thus, the BJ line has a unique, ''near-clonal'' karyotype, extending a preliminary analysis reported previously from our research group [17]. The high clonality of the BJ line is also evident from the parallelisms of the 20 chromosome number tracks (Fig. 3D). This high clonality, and particularly the large number of clonal marker chromosomes of the BJ line, despite a high degree of destabilizing aneuploidy, indicates an advanced age since the origin of this clone, which is consistent with its long passage history of possibly a year or more in culture [13,34,54].

The tumorigenic HA1 line contained two different stemlines that were subcloned in agar gels prior to karyotype analyses (Table 3). The major stemline1 (~2/3 of the original HA1 cells) had a hypotetraploid karyotype with an average near-clonal chromosome number (± SD) distribution of 82 ± 1, which included four clonal marker chromosomes (Fig. 3E; Table 3). Approximately 80–100% of the cells of the hypotetraploid stemline1 of HA1 (subclone 4) shared the same modal chromosome copy numbers (except for one outlier, chromosome 1, shared by only 50%) (Table 3). The high clonality of the HA1 stemline1, despite its high degree of aneuploidy, again suggests an advanced age of this clone since its origin in culture, compared with that of T-p39 (Table 1; Fig. 3A). In addition, 40% of the cells of stemline1 of HA1 also contained a marker with different ploidies per cells (i.e., 1, 2, 3, and 4) and contained between zero and four nonclonal markers per average cell.

The minor HA1 stemline2 had a hypodiploid karyotype with an average ''near-clonal'' chromosome number (± SD) of 45 ± 1, which included one unique marker chromosome (Table 3). Approximately 80–100% of the hypodiploid stemline had the same modal chromosome copy numbers, including that of a minor-stemline-specific marker chromosome, der(20;13). This same marker chromosome has been previously observed by our research group in HA1 [16]. In addition, this minor HA1 stemline contained zero to one nonclonal marker per average cell.

A comparison of the karyotypes of the two HA1 stemlines clearly indicates that the copy numbers of their chromosomes are not related by a common factor, and that both lines had unique marker chromosomes. It follows that the two lines are not related to each other and thus represent a mixture of two independently arisen clones. In subcloning the two stemlines of HA1, we found that both stemlines formed colonies in soft agar gels—a condition that discriminates against the growth of normal cells [18,50]. It is likely, therefore, that both lines are tumorigenic.

A comparison of the karyographs of the predominant HA1 stemline and of the BJ line shows very distinct, highly clonal karyotypes, even though the lines carry the same three activated oncogenes (Table 3; Figs. 3D and 3E).

We conclude, as before, that individual clonal karyotypes are the genomes of the tumorigenic BJ and HA1 lines, which encode their complex individual phenotypes, rather than the three oncogenes that they share with each other and with the nontumorigenic precursor cells from which they arose.

### *3.2. Tumorigenic cells with clonal karyotypes and clonal mutations: which is causal?*

As pointed out in section 3.1.1, it could be argued that the individual clonal karyotypes of the four different

tumorigenic cell lines studied here were transformed by unidentified cancer-causing mutations and had picked up aneuploid karyotypes accidentally from randomly aneuploid precursor cells. Such mutations could transform either alone or in cooperation with the common sets of transfected oncogenes. This hypothesis predicts, however, that such accidental aneuploidies would randomize over time, owing to the inherent instability of aneuploidy, because they would not be selected for transforming function.

To test the prediction that the aneuploid karyotype of cells transformed by mutation would randomize over time, we compared the karyotypes of T-p39 cells before and after 68 further cell generations (i.e., 34 further passages in vitro). The karyotype of the resulting derivative line, termed T-p73, is given in Table 1, next to that of the parental T-39. The karyographs of the precursor T-p39 and its descendent T-p73 are shown in Figure 5.

The basic hypotetraploid, ''near-clonal'' karyotype of T-p39 was conserved in T-p73, including the highly clonal marker der(11;19) of T-39. At the same time, the karyotype of T-p39 had evolved as follows. The average chromosome number (± standard deviation [SD]) had dropped to 82 ± 4 from 87 ± 5 of the parental line T-p39. Accordingly, the modal copy numbers of 8 of 24 chromosomes of T-p39 had decreased by one or two copies, whereas the copy numbers of two chromosomes had increased by one and two, respectively. This karyotype evolution is compatible with the observation by Mahale et al. [18] that the tumorigenicity of the T-p73 line was enhanced, relative to that of the parental T-p39 line. In addition, T-p73 had acquired two new subclonal marker chromosomes. Thus, our experiment shows that the basic, clonal karyotype of the T-p39 line was conserved, yet evolved over time together with the evolution of its tumorigenicity.

Because the basic karyotype of T-p39 retained a constant level of instability over 68 generations (i.e., very similar percentages of nonmodal chromosome numbers) but was not randomized, we conclude that it was stabilized and consolidated by selection for transforming function. Thus, the aneuploid karyotype of T-p39 was not accidental.

By contrast to the aneuploid karyotypes of cancer cells, which are stabilized by selection for transforming function, the aneuploid karyotypes of nontumorigenic cell hybrids, formed by fusions of cancer cells with normal cells, are rapidly lost because of the absence of selection for transforming function. They are, however, stabilized once the original or a variant selectable tumorigenic karyotype reemerges [55,56].

We conclude that the basic clonal, yet flexible T-p39 karyotype is the genome of the tumorigenic T-p39 line (and the T-p73 line), because it is stabilized by selection for oncogenic function despite its inherent instability and evolution.

Next, we analyze examples of karyotypic evolutions of T-p39 and T-p33 during tumorigenesis in mice, which is a model for natural tumor progression [1,57].

### *3.3. The karyotypes of different tumors, induced by the same tumorigenic cells, are variants of the parental prototype*

To test the prediction of the karyotypic cancer theory that, owing to the inherent instability of aneuploidy, the specific karyotypes and phenotypes of neoplastic cells evolve spontaneously, particularly during tumorigenesis, we analyzed several tumors that were caused by inoculation of T-p39 cells and T-p33 cells into immunodeficient mice [18]. Three T-p39-derived tumors were termed Tu1-p39, Tu2-p39, and Tu3-p39; two T-p33-derived tumors were termed Tu1-p33 and Tu2-p33.

#### *3.3.1. The phenotypes and karyotypes of three tumors derived from line T-p39*

We found that the cells of the three T-p39-derived tumors differed from those of the parental T-p39 line and from each other in cell morphologies and in their growth rates. The Tu1-p39 cells were elliptical and largely aligned in parallel (Fig. 1B). They grew three-dimensionally at high densities, much like the parental T-p39 cells (compare Figs. 1A and 1B). The Tu2-p39 cells were partially spindle-shaped and partially amorphous. Tu2-p39 cells grew three-dimensionally, arranged in random crisscross orientation, but considerably more slowly than those of Tu1-p39 and T-p39 (Fig. 1C). The Tu3-p39 cells were spindle-shaped, arranged partially in parallel and partially in random orientation (Fig. 1D). They also grew slowly, like Tu2-p39. Thus, each of the three tumor progeny of T-p39 had individual nonparental phenotypes, although they all shared the same four oncogenes.

The karyotypes of each of the three tumors had individually evolved from the parental T-p39 karyotype as follows (see Table 1):

1. The copy numbers of approximately one half of the T-p39 chromosomes had evolved individually in the three progeny tumors (e.g., 10 of 25 in Tu1-p39, 15 out of 25 in Tu2-p39, and 14 out of 25 in Tu3-p39) (Table 1). Nearly all tumor-associated chromosome copy number changes were losses of one or, rarely, two copies, relative to those of the parental line. There were also a few gains of one or two chromosome copies (e.g., two copies of chromosome 11 in Tu2, one copy of chromosome 21 in Tu1 and Tu2, and one copy of chromosome 22 in Tu1 and Tu2) (Table 3). As a net result, the average total chromosome numbers (± SD) of each of the three tumors had declined modestly from 87 ± 5 in T-p39 to 80 ± 5 in Tu1, 75 ± 9 in Tu2, and 64 ± 9 in Tu3. Such consolidations of the average chromosome number of a new neoplastic karyotype during carcinogenesis are consistent with selection for increased fitness in tumor progression [58]. (Karyotype compression on evolution in vitro is discussed in section 3.2.).

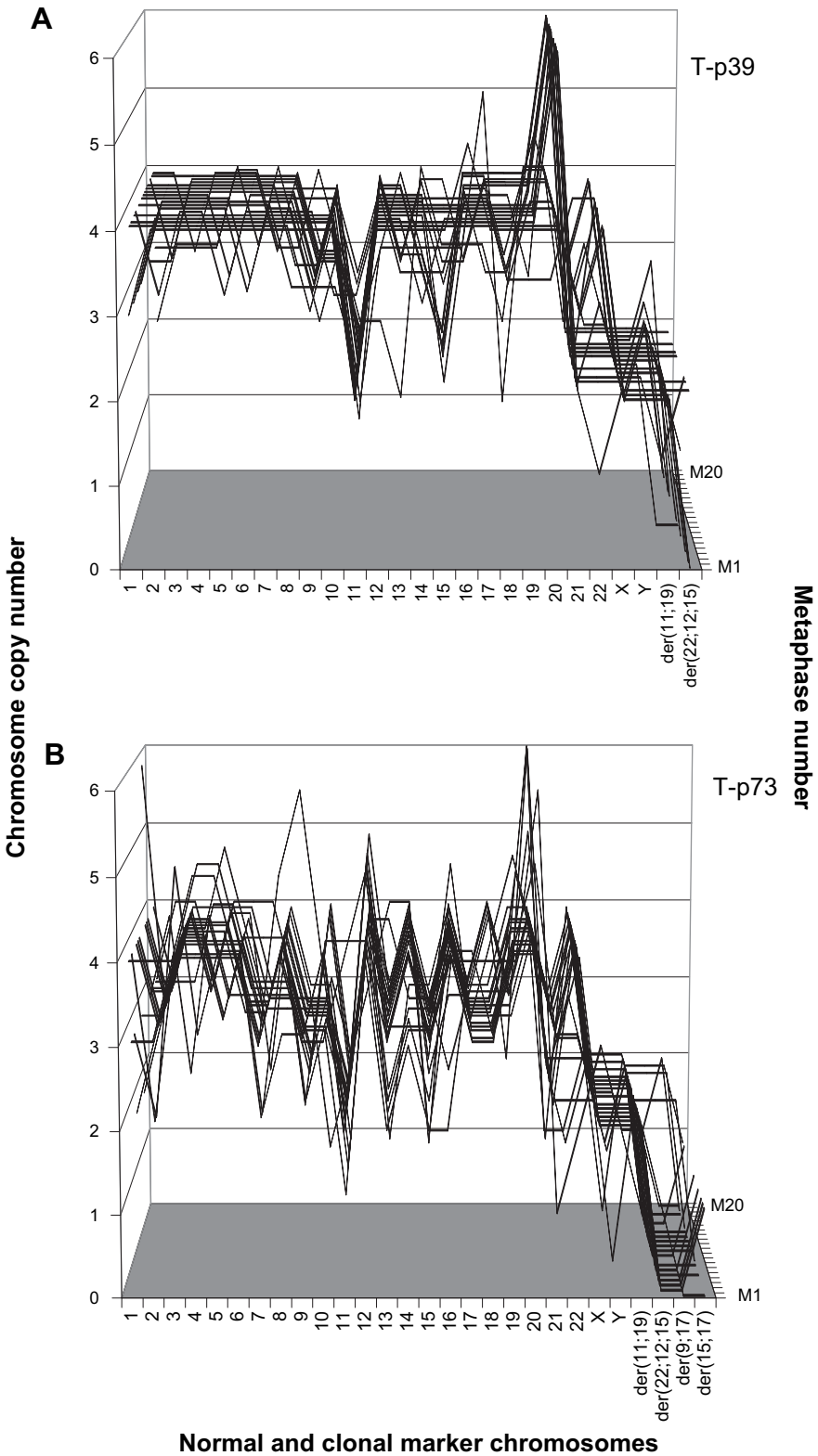


Fig. 5. Three-dimensional karyographs of the tumorigenic line T-p39 (A) before and (B) after 34 unselected passages in vitro, generating T-p73. These karyographs were prepared from the primary cytogenetic data summarized in Table 1, as described in Figure 3.

2. All three tumor progeny of T-p39 have retained the highly clonal marker chromosome of T-p39, der(11;19), although at a lower clonal percentage level in the slow-growing tumors, Tu2 and Tu3, than in the parental line and in Tu1. The conservation of this marker in three tumors derived from T-p39 signals that

it is relevant for transforming function in these karyotypes (as was suggested in section 3.1.1.).

3. Each tumor progeny of T-p39 had picked up new, line-specific marker chromosomes. One of these, der(7;8), was highly clonal in Tu1, and three were highly clonal in Tu2 (Table 1). The high clonality of these markers again suggests that they are relevant for transforming function. Each tumor also contained several new, subclonal and nonclonal markers, which, owing to their low clonality, are not relevant for the transforming function; these do, however, signal, inherent karyotypic instability and, in turn, flexibility.

These results confirm the view that, because of the inherent instability of aneuploidy, the karyotypes of neoplastic cells evolve individually, irrespective of shared oncogenes [59].

We conclude that the individual, nonparental elements of the karyotypes of Tu1, Tu2, and Tu3 are responsible for their individual phenotypes, such as cell morphologies and proliferative characters. Their four common oncogenes cannot be directly relevant for the tumorigenicity of these tumors, because these were shared with the nontumorigenic precursor cells.

### *3.3.2. The phenotypes and karyotypes of two tumors derived from line T-p33*

Next, we analyzed two tumors, Tu1-p33 and Tu2-p33, which were caused in mice by injection of T-p33 cells [18]. The morphologies of the Tu1-p33 and Tu2-p33 cells differed markedly from those of the parental T-p33 line, although they all carried the same four oncogenes (Figs. 2B and 2C). The Tu1-cells were either spindle-shaped or amorphous, growing in irregular patterns. In contrast to the parental line, these cells rendered the culture medium highly viscous, probably by the production of hyaluronic acid, which is considered a tumor marker in natural cancers [60]. The Tu1-cells also grew faster than the parental line, and some Tu1 cells grew into mountains that detached from the dish and continued to grow as spheres in suspension. The Tu2 cells resembled those of Tu1, including the production of hyaluronic acid. They grew into even higher mountains of cells, but these did not detach from the dish (Fig. 3C). Thus, the two T-p33-derived tumors differed markedly from the parental line, from each other, and also from the three T-p39-derived tumors, even though all contained the same four oncogenes.

The karyotypes of Tu1-p33 and Tu2-p33 differed from the parental T-p33 most strikingly in their ploidies (Table 2). Based on the copy numbers of the chromosomes, the karyotype of Tu1 was near-tetraploid, almost an exact duplication of the near-diploid parental T-p33 line; however, the copy numbers of five Tu1 chromosomes—namely chromosomes 3, 19, 21, Y, and der(15;16)—were not duplicated based on T-p33. Tu1 also shared three highly clonal marker chromosomes with the parental T-p33. Furthermore, Tu1 contained one highly clonal individual marker that was partially monosomic and partially disomic, der(3)-long (which probably compensated for the nonamplified chromosome 3), and three new subclonal markers. In addition, the tumor contained between zero and five nonclonal marker chromosomes per cell, indicating ongoing chromosomal instability.

The karyotype of Tu2-p33 was hypertetraploid (Table 2). Based on the copy numbers of its chromosomes, it was between a duplicate and a triplicate of the parental T-p33 line. However, the copy numbers of two Tu2 chromosomes, 8 and 20, were amplified more than threefold; the copy numbers of two other chromosomes, 21 and 22, were amplified less than twofold; and those of two marker chromosomes were not amplified. Tu2 also contained one highly clonal individual marker chromosome, der(3;22). In addition, the tumor contained zero to eight nonclonal markers per cell (not shown individually in Table 2). The higher rates of nonlinear chromosomal polyploidization of Tu2 compared to Tu1, signal that the karyotype of Tu2 is less stable than that of Tu1.

Once again we conclude that the individual tumor karyotypes are responsible for the individual phenotypes of the Tu1-p33 and Tu2-p33 tumors. It appears that the near-tetraploidizations of the parental karyotype have primary responsibility for their distinct tumor-specific phenotypes, because tetraploidizations have increased the dosages of nearly all of the 25,000 human genes of Tu1-p33 and Tu2-p33 by $2 \pm 1$-fold, relative to the parental cells. Individual, discordantly polyploidized and nonpolyploidized chromosomes and markers of Tu1-p33 and Tu2-p33 further differentiate the karyotypes of Tu1 and Tu2. That the two distinct tumors, the parental T-p33 line, and the nontumorigenic precursor cells all shared the same four transfected oncogenes once again rules out a direct genomic role of these genes in tumor-specific phenotypes.

A minor sideline (~1 in 50 cells) of T-p33 was already near-tetraploid prior to tumorigenesis (Table 2). This suggests that the near-tetraploid karyotypes of T-p33 were selected by the tumorigenic karyotypic evolutions that generated Tu1 and Tu2. The karyotypic tetraploid karyotypes of T-p33 are thus probably the most tumorigenic karyotypes of T-p33, if not the only ones.

In sum, we found that the same four oncogenes have induced in the same parental human cell line two very different tumorigenic cell lines, namely T-p39 and T-p33, and that each of these tumorigenic lines has generated distinct, but related, tumors. Because the individual phenotypes of these tumors coevolved with their individual karyotypes, we conclude that these individual karyotypes are the genomes of these tumors. Further work is necessary to correlate specific karyotypic alterations with specific phenotypic alterations, but one such example is considered next.

### 3.4. Karyotypic alterations associated with the evolution of drug resistance of the HA1 line

To obtain an independent example of coevolving cancer phenotypes and karyotypes, we asked whether the acquisition of drug resistance by one of the tumorigenic lines studied here could be linked with a specific karyotypic alteration. For this purpose, the tumorigenic HA1 line was exposed to increasing doses of methotrexate, ranging from 0.1 to 1 μg per 5 cm-culture dish as described previously [53,61,62]. Within 1–2 months after the first addition of methotrexate, resistant colonies evolved that were grown up and karyotyped. The karyotype of a methotrexate-resistant HA1 colony differed from the parental line in the copy numbers of 13 of 28 chromosomes (Table 3). Among the methotrexate-induced chromosome copy number changes, 11 HA1-specific copy numbers had decreased by one and two copies and 2 HA1-specific copy numbers had increased by one copy. The average chromosome number (± SD) was thus reduced from 82 ± 1 to 71 ± 3 (Table 3). A loss of chromosome copy numbers is common in the acquisition of drug resistance by cancer cells, possibly because of the loss of drug receptors and the loss of drug metabolizing enzymes [53].

We conclude that the karyotypic alterations associated with the acquisition of drug resistance are those that convert the HA1 genome to drug resistance.

## 4. Discussion

Despite more than a century of cancer research findings, it is still debated whether the cancer genome is a new, cancer-specific karyotype or a new, cancer-specific set of mutations [10,11]. The answer to this question is complicated by the exceedingly long latent periods between the initiation of carcinogenesis by carcinogens and the subsequent, stochastic appearance of cancers among carcinogen-treated cells [33,63,64], and also by the complexity of the cytogenetic and genetic alterations of natural cancers [11,33,58,65,66].

In the present prospective study of carcinogenesis, designed to investigate the theory that cancer cells originate with cancer-specific karyotypes, we have analyzed the karyotypes of nascent clones of tumorigenic cells arising stochastically from normal human cells within a few months after transfection with artificially activated oncogenes. Such relatively short time intervals virtually exclude new sets of specific mutations as primary causes of carcinogenesis [10,15,67]. Accordingly, normal human cells remain untransformed in cell culture in the absence of activated oncogenes for at least 6 to 12 months [68,69]. The cancer cells obtained in this system would thus either be caused by the added artificially activated oncogenes or by another nonmutational event, such as the evolution of a new karyotype. Furthermore, we have analyzed the karyotypes of new tumors caused by inoculation of the newly generated tumorigenic lines into immunodeficient mice, and in progeny of tumorigenic lines that acquired resistance against cytotoxic drugs within a few months after selection in vitro.

Because all tumorigenic lines derived stochastically from cells transfected with the same sets of oncogenes had individual, clonal, and flexible karyotypes and phenotypes, but the same oncogenes, we conclude that these clonal karyotypes are the genomes of these tumorigenic cells. Moreover, because all individual tumors caused by the same tumorigenic cell line in different mice had evolved individual variants of parental karyotypes and phenotypes, we draw the conclusion that their genomes are their flexible and individual karyotypes, rather than common, inflexible oncogenes. A flexible, clonal karyotype also explains the acquisition of drug resistance by one tumorigenic line within months after drug challenge, at rates that far exceed conventional rates of mutation [10,53]. Thus, all experimental observations described here support the karyotypic cancer theory outlined in the Introduction and in two recent studies of ours [7, 28].

In contrast to the karyotypes and phenotypes of individual tumorigenic clones, transfected oncogenes do not specifically correlate with carcinogenesis. Instead, these oncogenes are nonspecifically shared by the nontumorigenic precursor cells from which rare tumorigenic cells arise, by different tumorigenic lines arisen stochastically from transfected human cells, and by different tumors evolved from the same primary tumorigenic cell lines. In view of this, we propose that activated oncogenes induce neoplastic transformation indirectly, much like conventional carcinogens, but more effectively because they are integrated into the genome. By inducing random aneuploidy (as discussed in the Introduction), activated oncogenes destabilize the karyotype and thus catalyze karyotypic evolutions that eventually generate cancer-causing karyotypes.

The karyotypic cancer theory thus explains why, according to the many studies described in the Introduction, activated oncogenes are not sufficient for neoplastic transformation. According to this theory, the critical stochastic event in carcinogenesis beyond the addition of oncogenes [18], or beyond conventional carcinogens, is the origin of a cancer-causing karyotype. The theory also explains the spontaneous evolution of tumors (also known as tumor progression) and of drug resistance by spontaneous karyotypic evolutions—based on the inherent flexibility of aneuploid karyotypes (discussed in the Introduction). In contrast, conventional genetic mutations (e.g., hemophilia) are stable for the life of an individual and are inherited unchanged by progeny.

The evidence we have presented here that cancers have a karyotypic rather than mutation-based genome is independently supported by the following points.

1. Analyses of transcription profiles of cancer cells show that nearly all cellular genes are expressed in

proportion to the dosage of the corresponding chromosomes [46,47,70,71]. This supports the view that cancer karyotypes as a whole, rather than specific oncogenes, encode cancer-specific phenotypes.

2. The karyotypes of most cancers from the same tissue of origin are closely related [72–74] because the number of cancer-causing reassortments of karyotypes are restricted by developmentally fixed tissue-specific transcriptomes of normal precursors [75]. The case would be analogous to restrictions on the phenotypes of new species by the transcriptomes of parental species [76].

In sum, genomes consisting of aneuploid individual karyotypes explain the characteristic combination of individuality, stability, and flexibility of cancers [1].

## Acknowledgments

We thank Alka Mahale, Makoto Igarashi, and Stuart Aaronson for critical discussions and advice (Mount Sinai School of Medicine, New York, NY). Further, we acknowledge their generous gifts of different clonal tumorigenic human cell lines, which were derived from the same human cells transfected with the same oncogenes, and for different tumors caused by these lines in mice (see Mahale et al. [18]). We also thank Robert Weinberg and William Hahn for providing HA1 and BJ cells (Massachusetts Institute of Technology, Cambridge, Boston MA). Amanda McCormack is gratefully acknowledged for diligent assistance. For support of this work we are especially grateful to the Abraham J. and Phyllis Katz Foundation (Newnan, GA); the Foundation for Advancement of Cancer Therapy (New York); the philanthropists Robert Leppo (San Francisco), Dr. Christian Fiala (Vienna, Austria), Peter Rozsa (Los Angeles), and Stefan Ebeling (Duesseldorf, Germany); other private sources; and the Forschungsfonds der Fakultät für Klinische Medizin Mannheim. Further, we are obliged to David Rasnick (Berkeley), Bill Redfearn (Berkeley), Rainer Sachs (UC Berkeley) and Reinhard Stindl (Vienna, Austria) for critical discussions and information. Last, we dedicate this article to Kathy Cerami, a cancer patient who recently passed and was an inspiration, particularly to one of us (J. N.).

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