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Metastatic Potential and Spontaneous Mutation Rates: Studies with Two Murine Cell Lines and Their Recently Induced Metastatic Variants[1]

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ABSTRACT

To investigate the hypothesis that increased malignant potential correlates with increased genetic instability, we measured spontaneous mutation rates for the production of ouabain-resistant mutants in two benign (nonmetastatic) murine cell lines and their recently induced metastatic variants. Metastatic variants of the NIH 3T3 and CBA SP-1 cells were induced by transfection with the h-*ras* oncogene. Metastatic variants were also induced from the CBA SP-1 cell line by treatment with either 2'-deoxy-5-azacytidine or hydroxyurea. Mutation rates for the parent NIH 3T3 cells and their metastatic variants were less than 3×10^{-8} variants per cell per generation, with no significant differences between them. Rates for the CBA SP-1 line and its variants ranged from 9×10^{-9} to 8×10^{-8} variants per cell per generation, again without statistically significant differences. We conclude that in the cell lines studied the rate of spontaneous mutation for ouabain resistance was unrelated to the acquisition of the metastatic phenotype. This conclusion was based on the view that the generation of ouabain-resistant mutants is a reflection of the overall stability of the genome. Since the spontaneous mutation rate for ouabain resistance was unchanged in cells that had recently acquired the ability to metastasize, other genetic or epigenetic events were probably responsible for progression to the malignant (metastatic) phenotype.

INTRODUCTION

That tumors progress from benign to more malignant phenotypes is a fundamental assumption in cancer biology. While tumor progression is likely caused by the genetic instability of individual cells, the mechanisms of this instability remain unclear (1, 2). Point mutation, gene amplification, DNA rearrangements, and chromosomal alterations are some possible mechanisms. In addition, epigenetic mechanisms such as DNA hypomethylation may play a role (3). It is our view that it is unrealistic to expect any single mechanism to explain the complicated nature of tumor progression. However, an analysis of single mechanisms is a reasonable approach, considering the limitations of current experimental methods.

Central to the concept of tumor progression is the hypothesis that genetic instability increases as tumors evolve into more malignant phenotypes. This view derives primarily from the histopathological studies of Foulds (4) and the large body of evidence demonstrating that more aggressive tumors are associated with larger numbers of karyotypic abnormalities (5). Many researchers have proposed that the basis of tumor progression lies in an inherent genomic instability, which is presumed to result in some way from whatever initiated the transformation event. Nowell (1) proposed that the consequence of genomic instability is an increase in somatic mutation, and others have presented data supporting the view that spontaneous mutation rates are increased in highly metastatic cells when compared to less metastatic populations (6).

Our laboratory in collaboration with R. S. Kerbel has proposed that epigenetic mechanisms could also produce considerable changes in tumor cell phenotypes (3). Whereas we focused our attention on levels of DNA methylation, recent studies have provided several additional potential mechanisms for these alterations in phenotype. Gene amplification (7) and gene rearrangements (8) are but 2 such examples.

Our own interest in tumor progression led us to examine the relationship of the rate of spontaneous mutation to tumor progression by using murine cells with recently acquired metastatic potential. NIH 3T3 cells and the CBA SP-1 mammary carcinoma line were each transfected with the h-*ras* oncogene and became metastatic. In addition, we analyzed spontaneous mutation rates in murine cells that had been converted to the metastatic phenotype by 2 agents known to have different effects on cells. Metastatic ability was induced in the CBA SP-1 cell line by treatment with either 5-aza-dCyd[3] or hydroxyurea. These cell lines therefore provided a means for assessing differences in spontaneous mutation rates of cells with recently acquired phenotypic differences.

The conventional measurement of spontaneous mutation rates involves fluctuation analysis, a procedure designed by Luria and Delbruck (9) for the measurement of mutation rates in bacterial cell populations. Cifone and Fidler (6) applied this method to clones of 3 murine cell lines and demonstrated higher spontaneous mutation rates in the clones with greater metastatic potential. Later, Elmore *et al.* (10), through similar methods, showed no significant differences between spontaneous mutation rates of normal and transformed human fibroblasts. Recently, Yamashina and Heppner (11) found no correlation of spontaneous mutation rates with metastatic potential in a murine mammary carcinoma. These conflicting studies indicated the need for further investigation of the role of spontaneous mutation in tumor progression.

We note that the *in vitro* measurement of spontaneous mutation rates is fraught with difficulty (10, 12). Metabolic cooperation, selective growth advantage for variants, delayed phenotypic expression, alterations in chromosomal or gene copy number, and the concentration of selective agents could all influence these measurements. Furthermore, the mathematical methods used in measuring fluctuation analysis may themselves be inefficient estimators for mutation rates. Thus, if the mutation rates of 2 related cell lines are to be compared, it is essential that some estimate of the inherent statistical error be made. Given the inaccuracies of the experimental methods, measurements without these estimates are difficult to interpret. Such considerations are included in this report.

MATERIALS AND METHODS

Cell Lines. NIH 3T3 embryonal fibroblasts only rarely form tumors, and then only in nude mice. Variants of these cells, transfected with the h-*ras* oncogene and found to be metastatic in nude mice, were kindly supplied to us by Drs. Lance Liotta and Ruth Muschell of the Department of Pathology at the National Cancer Institute. The selec-

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[3] The abbreviations used are: 5-aza-dCyd, 2'-deoxy-5-azacytidine; oua[R], ouabain resistant; oua[S], ouabain sensitive.

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tion of h-*ras* transfected cells is described elsewhere (13).

CBA SP-1 is a spontaneous murine breast carcinoma (14) that does not metastasize when injected s.c. into mice. Transfected variants of the line were provided to us by Dr. Robert Kerbel of the Mt. Sinai Research Institute, Toronto, Canada. Additional metastatic variants were produced in our laboratory by the treatment of CBA SP-1 cell monolayers with either 3 μM 5-aza-dCyd or 1 mM hydroxyurea for 24 h prior to s.c. injection into CBA mice. The variants were recovered from micrometastases in the lungs after mechanical dissociation of these organs and the growth of derived tumor cells *in vitro*. The metastatic cells used in these experiments were passaged *in vivo* 3 times and retained their metastatic phenotype.

Culture Methods. All cell lines were grown in RPMI 1640 (Grand Island Biological Co., Grand Island, NY) supplemented with 50,000 units penicillin G, 50,000 units of streptomycin, 150 mg L-glutamine, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 375 mg sodium bicarbonate, and 10% fetal bovine serum in 500 ml of medium. Drug selection was done with 2 mM ouabain in culture medium. Cell monolayers were grown under constant humidity in a 5% CO_2 environment at 37°C. Cell harvesting was done with 0.05% trypsin and 0.02% EDTA in phosphate-buffered saline.

Animal Experiments. To confirm the metastatic behavior of the specific cell variants, each variant was injected s.c. into mice. The NIH 3T3 cell line and its transfected variants were injected into nude mice, whereas the CBA SP-1 cell line and variants were injected into syngeneic CBA mice. In the case of the CBA SP-1 tumor, the presence of metastasis was tested by visual inspection and organ culture, followed by the s.c. reinjection of cultured cells into CBA mice, so as to confirm the cells' tumorigenicity. On several occasions, cells that appeared morphologically as tumors *in vitro* were found not to be tumorigenic *in vivo*.

Drug Resistance. To determine the optimal concentration of ouabain for all of the cell lines used and the prevalence of oua^R mutants within the population of cells, 500 to 5×10^5 cells were seeded into 100-mm culture dishes containing media with ouabain at set concentrations (0 to 5 mM). The dishes were stained 14 days later with methylene blue and the numbers of colonies were counted.

Cell Density Experiments. To determine if metabolic cooperation between cells might influence the prevalence of oua^R mutants, 2 experiments were performed. In the first experiment, parallel cultures of CBA SP-1 cells were seeded with defined numbers of oua^R and oua^S CBA SP-1 cells and were then allowed to grow to different cell densities before the application of ouabain. (The oua^R CBA-SP1 cells were obtained after a single treatment of CBA-SP1 cells with 1-methyl-3-nitro-1-nitrosoguanidine and selection in 2 mM ouabain.) Once visible oua^R colonies had formed, the plates were stained with methylene blue and the number of oua^R colonies was counted.

In the second experiment oua^R and oua^S cells were mixed in varying proportions and plated directly into culture medium containing 2 mM ouabain. The number of oua^R colonies was determined 2 weeks later.

Fluctuation Analysis. Less than 300 viable cells per well were seeded in 2 ml of medium in 24-well flat-bottom tissue culture plates. For each experiment 48 to 120 such wells were seeded. The cells were incubated for 1 to 2 weeks until 10^4 to 10^5 cells/well were present. The cells were then trypsinized, and each well was individually harvested and its contents plated in individual 100-mm plastic tissue culture dishes containing 10 ml of medium. These cultures were incubated until each dish contained the number of cells required for each experiment. This varied from one-half million to 3 million cells per dish. Four culture dishes were then selected at random, and the number of viable cells in each was counted. The medium from the remaining tissue culture dishes was decanted and replaced with 10 ml of fresh medium containing 2 mM ouabain without dispersion of the cells. The plates were incubated for an additional 2 to 3 weeks. At weekly intervals, the cultures were refed with fresh medium containing 2 mM ouabain. After this final incubation, the culture dishes were stained with methylene blue and the proportion of cultures without any oua^R colonies was recorded.

To determine if omission of cell dispersion in our fluctuation analysis had any influence on the measurement of mutation rates we performed a control fluctuation analysis that did use cell dispersion. Twenty parallel cultures were seeded in a 24-well tissue culture plate with 500 CBA SP-1 cells in 2 ml RPMI 1640 for each well. After 6 days of incubation each well was trypsinized, harvested, and the cells were transferred to T-75 flasks with 15 ml RPMI 1640. After an additional 7 days of incubation the cells of each flask were trypsinized, harvested, and counted. For each parallel culture eight 100-mm culture dishes were seeded with 1×10^6 cells per dish in 10 ml of 2 mM ouabain medium. Two of the parallel cultures were selected at random and additional cells from these cultures were seeded into 5 ml RPMI 1640 at a density of 200 cells/60-mm tissue culture dish. The plates were incubated for 2 weeks and the colonies were stained with methylene blue prior to counting.

Calculations. For most of our experiments the mutation rate, a, was calculated using the P_0 method (9). Given the fraction of culture plates with no resistant colonies, P_0, and the average number of cells per plate at the time of ouabain application, N, the following equation was used:

$$a = -\ln(2)\ \ln(P_0)/N.$$

Two sources of error were accounted for: first, the error arising from the estimate of P_0, and, second, the error arising from the estimate of N. The error from the first source was estimated by the method of Li *et al.* (12). The error from the second source was estimated using a SD calculated from each set of 4 plates counted at the time of ouabain application. As a first approximation, P_0, and N can be treated as independent quantities. The contribution of error from each may then be calculated using a Taylor series expansion:

$$\mathrm{var}(a) = (1 - P_0)\ \ln(2)^2/(CN^2P_0) + [\ln(P_0)/N^2]^2\ \ln(2)^2\ \mathrm{var}(N).$$

Here the variance of the mutation rate is var(a). C is the number of parallel cultures treated with ouabain, and var(N) is the variance of N. All error bounds in this paper represent 1 SD about the mean.

For the control fluctuation analysis using cell dispersion and the method of Luria and Delbruck (9) based on the mean number of drug-resistant colonies per parallel culture, we used the equation

$$r = aN\ \ln[NCa/\ln(2)]/\ln(2)$$

to estimate the mutation rate. Here r is the mean number of drug-resistant colonies. The number, ln(2), has been inserted to convert the measurement units from the natural logarithmic base to base 2.

RESULTS

Animal Experiments. NIH 3T3 cells and their transfected variants were injected s.c. into groups of 3 nude mice, at a dose of 1×10^6 cells/mouse. As found by other investigators (13), the nontransfected cells were nontumorigenic and nonmetastatic, whereas the transfected cells were tumorigenic and metastatic.

Parental CBA SP-1 cells and their transfected variants were injected s.c. into groups of 10 mice. Neither the parental cells nor the cells transfected with inactive h-*ras* oncogenes produced metastases. However, the cells transfected with the neomycin-resistant gene alone and those transfected with active h-*ras* formed recoverable lung metastases.

The two chemically treated CBA SP-1 cells were: (*a*) cells treated with 1 mM hydroxyurea and then injected s.c. into 20 mice, the cells later recovered from lung metastases; and (*b*) cells treated with 5-aza-dCyd, injected into 20 mice, and recovered from lung metastases. On 2 additional occasions, 5×10^5 of both the hydroxyurea- and 5-aza-dCyd-treated cells were reinjected s.c. into groups of 5 mice. These cells retained their metastatic potential, as demonstrated by the presence of lung tumors in all animals.

Drug Resistance. The drug sensitivity experiments detailed in Table 1 showed that the relative plating efficiencies of all the cell lines used were essentially zero at ouabain concentrations

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Table 1 *Relative plating efficiencies for cell lines at different ouabain concentrations*

Cells from each of the cell lines used in this study were seeded into 100-mm tissue culture dishes in 10 ml medium containing ouabain at fixed concentrations. The dishes were stained with methylene blue 14 days later and the plating efficiencies were assessed.

Ouabain concentration (mM)	NIH 3T3	NIH 3T3 h-*ras*	CBA SP-1	CBA SP-1 h-*ras*	CBA SP-1 inactive h-*ras*	CBA SP-1 neomycin gene	CBA SP-1 5-aza-dCyd	CBA SP-1 hydroxyurea
0	1	1	1	1	1	1	1	1
0.5	$>4 \times 10^{-3}$	$>6 \times 10^{-2}$	1×10^{-2}	1×10^{-2}	3×10^{-2}	6×10^{-6}	1×10^{-2}	4×10^{-2}
1	0	7×10^{-6}	1×10^{-6}	1×10^{-6}	0	6×10^{-6}	7×10^{-7}	0
2	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	7×10^{-7}	0
5	0	0	0	0	0	0	0	0

of 2 mM or greater. Only variant cells were able to survive at these concentrations. We thus chose 2 mM as the optimal ouabain concentration for our further experiments. The prevalence of oua^R variants within the NIH 3T3 lines was less than 1/million viable cells at this concentration. Similarly for the CBA SP-1 lines, the prevalence was from 1 to 3 variants/million viable cells.

Cell Density Experiments. Cell density experiments were performed so as to determine whether metabolic cooperation was important in enhancing the recovery of oua^R mutants. In addition, we were concerned that the death of a large number of the cells when ouabain was added could impair the growth of the remaining oua^R cells. The latter issue was resolved by decanting on a weekly basis the spent medium containing cellular debris. Resolution of the former issue required a series of more detailed experiments.

The cell density experiment detailed in Table 2 revealed no significant change in the plating efficiency of oua^R cells when they were plated in ouabain with increasing numbers of oua^S cells. These data do not demonstrate any detectable effect of metabolic cooperation on plating efficiency in ouabain.

In addition to this standard analysis of the effect of cell density on mutant selection, we performed an additional study that more closely simulated the assays used in our experimental protocols. In the method we used, cells were plated and allowed to multiply prior to the application of ouabain. To simulate this aspect in a cell density experiment, we plated fixed numbers of

Table 2 *Failure to demonstrate metabolic cooperation*

Increasing numbers of oua^S CBA SP-1 cells were seeded into 100-mm tissue culture dishes with 200 oua^R cells in 15 ml of medium containing 2 mM ouabain. After 1 week, the dishes were stained with methylene blue and the number of oua^R colonies was counted.

Total no. of cells plated		No. of oua^R colonies
oua^R	oua^S	
200	1×10^4	73 ± 5[a]
200	1×10^5	81 ± 6
200	5×10^5	82 ± 10
200	1×10^6	71 ± 10
200	2×10^6	79 ± 5

[a] Mean ± SD.

Table 3 *Effect of cell density on the survival of oua^R cells*

Plastic tissue culture dishes (100 mm) were seeded with 100 oua^R cells and 1.0×10^4 oua^S cells in 10 ml RPMI 1640. At daily intervals thereafter, 2 mM ouabain was applied to a subgroup of dishes, an additional plate was trypsinized, and the number of viable cells was counted. After visible oua^R colonies had formed, the dishes were stained with methylene blue and the colonies were counted.

Total no. of cells at time of ouabain application	No. of surviving oua^R cells
2×10^4	27 ± 6[a]
1.5×10^5	17 ± 4
5.2×10^5	32 ± 32
2.5×10^6	28 ± 7
7.7×10^6	42 ± 8

[a] Mean ± SD.

oua^R and oua^S cells and allowed them to propagate before application of ouabain. These results are presented in Table 3 with no significant cell-to-cell interaction shown.

Fluctuation Analyses. Spontaneous mutation rates for the generation of oua^R mutants and the relationship of these rates to the malignant potential of each cell line were analyzed and are shown in Table 4. For NIH 3T3 cells, no significant difference was observed between the spontaneous mutation rates of the parental and metastatic cells. In general, the mutation rates must have been extremely low, since no mutations were observed under these experimental conditions.

Similar results were obtained when analyzing the CBA SP-1 line and its metastatic variants. No significant differences in spontaneous mutation rates were observed. There were no differences for the cells transfected with either the active or inactive h-*ras* oncogene, or the gene for neomycin resistance. In addition, the metastatic CBA SP-1 cells developed after treatment with 5-aza-dCyd or hydroxyurea had spontaneous mutation rates similar to those of the parental cell line.

We did not find any major difference in mutation rates obtained by the method of fluctuation analysis used here and the method based on Luria and Delbruck's method of means. As an example we present data obtained from the latter method using parental CBA SP-1 cells. For the 20 parallel cultures grown to $1.7 \times 10^7 \pm 0.4 \times 10^7$ (SD) cells and then seeded into 2 mM ouabain we found a mean number of 2.5 ± 8.8 oua^R colonies per parallel culture. The plating efficiency in ouabain-free medium was 0.43. The mutation rate (corrected for the plating efficiency) was thus 7×10^{-8} variants/cell/generation.

DISCUSSION

We have demonstrated that despite the recent acquisition of a more malignant phenotype, NIH 3T3 and CBA SP-1 cells did not manifest an increase in spontaneous mutation rate when compared to their less malignant counterparts. This was true for both oncogene-transfected metastatic variants as well as metastatic cells derived after treatment with 2 unrelated drugs. These findings imply that, for the cell lines analyzed, the acquisition of a more malignant phenotype probably results from mechanisms other than an increase in spontaneous mutation rate.

We recognize that the use of oua^R as a measure of spontaneous mutation rates has its limitations. There is no reason to presume that oua^R *per se* has any relation to metastatic potential, but it is currently impossible to assess spontaneous mutation rates at loci important for the metastatic phenotype, since the gene or genes exclusively responsible for this phenotype are unknown. The basic assumption of these (and other) studies (7, 10, 11) is that a change in spontaneous mutation rate is a general genomic event and that detection of a rate change in any one locus is a reflection of general genomic instability.

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Table 4 *Spontaneous mutation rates for cell lines with different malignant potentials*

Fluctuation analyses were performed using the P_o method (9) comparing NIH 3T3 and CBA SP-1 cell lines with their more malignant variants. These variants were induced by transfection with the active or inactive h-*ras* oncogene, the gene for neomycin resistance alone, or treatment of the parent cells with either 5-aza-dCyd or hydroxyurea.

	NIH 3T3	NIH 3T3	NIH 3T3 h-*ras*	NIH 3T3 h-*ras*	CBA SP-1	CBA SP-1	CBA SP-1	CBA SP-1 h-*ras*
Malignant potential	None	None	Tumorigenic	Tumorigenic	Nonmetastatic	Nonmetastatic	Nonmetastatic	Metastatic
No. of cultures	50	56	51	60	86	62	67	89
Final no. of cells ($\times 10^6$)	1.9 ± 0.5	2.9 ± 0.3	0.43 ± 0.05	2.7 ± 0.2	1.6 ± 1.6	2.0 ± 0.6	1.2 ± 0.2	0.5 ± 0.1
No. of cultures with oua^R colonies	0	0	0	0	15	10	16	1
P_o	1	1	1	1	0.83	0.84	0.76	0.99
Mutation rate ($\times 10^8$) variants/cell/division	<0.7 ± 0.7	<0.4 ± 0.4	<3 ± 3	<0.4 ± 0.4	8 ± 9	6 ± 3	16 ± 3	2 ± 2

	CBA SP-1 h-*ras*	CBA SP-1 inactive *h-ras*	CBA SP-1 neomycin gene	CBA SP-1 neomycin gene	CBA SP-1 5-aza-dCyd	CBA SP-1 5-aza-dCyd	CBA SP-1 hydroxyurea	CBA SP-1 hydroxyurea
Malignant potential	Metastatic	Metastatic	Metastatic	Metastatic	Metastatic	Metastatic	Metastatic	Metastatic
No. of cultures	45	88	86	91	67	88	63	91
Final no. of cells ($\times 10^6$)	0.8 ± 0.5	1.5 ± 0.5	2.0 ± 0.9	1.4 ± 0.3	0.7 ± 0.4	1.1 ± 0.6	2.4 ± 1.4	0.5 ± 0.1
No. of cultures with oua^R colonies	3	1	5	5	3	5	2	5
P_o	0.93	0.99	0.94	0.95	0.96	0.94	0.97	0.95
Mutation rate ($\times 10^8$) variants/cell/division	6 ± 5	0.5 ± 0.6	2 ± 1	3 ± 2	5 ± 4	4 ± 3	0.9 ± 0.9	8 ± 4

While this may be an imperfect assumption, it is a reasonable one given the experimental circumstances.

Besides these theoretical considerations, there are practical problems in measuring mutation rates. For example, rates measured by different investigators on the same cell lines may differ by orders of magnitude (15). Li *et al.* (12) noted that differences in tissue culture techniques, cell density, culture medium, or the concentration of selective agents may all influence rate measurements. Perhaps more importantly, the error that arises from the statistical methods of rate calculation may be manyfold larger than the rate itself. Thus, a severalfold difference in mutation rates between parental and variant cell lines may have no statistical significance.

The problem of cell density as a factor in the estimation of mutation rates also deserves consideration. We chose to minimize this effect by using a dominant marker, namely, oua^R. Although we performed standard assays for the effect of cell density, we also devised experiments that did not require cell dispersion, so as to reflect more closely the actual experimental methods we used. We observed no significant decrease in number of oua^R colonies for cell densities up to seven million cells per tissue culture dish. Moreover, repeated fluctuation analyses, differing only in the density of cells at the time of ouabain application, failed to show any significant influence of cell density on the measurement of mutation rates. For these reasons, we did not consider cell density as a significant factor in our experiments.

Our inability to correlate mutation rates with malignant potential is different from the results published by Cifone and Fidler (6). This may be due to differences between the respective methods, including cell dispersion prior to ouabain application, different concentrations of ouabain, the use of Luria and Delbruck's method of means, different cell lines, and possibly the use of uncloned rather than cloned populations. This latter issue is currently being addressed.

Our work is of added significance in that it is the first to use cell lines that recently acquired their metastatic potential either through transfection or through chemical induction. The advantage of using cells with a recently acquired phenotype is that the mechanisms responsible for the phenotypic change may still be operative. The fact that we could not demonstrate any change in spontaneous mutation rates in these cell lines implies that the number of spontaneous mutations was probably not biologically significant.

These findings also demonstrate that transfection itself does not result in an increase in spontaneous mutation rates. However, our analyses do not address the issue of insertional mutagenesis.

These data do not rule out a role for spontaneous mutation at some point in tumor progression. This mechanism may still be important in the early stages of tumor development, whereas other host or environmental factors may be more important in later stages of tumor progression. Also, other mechanisms for genetic instability, not detectable by the induction of ouabain resistance, may have a predominant role. In addition, findings from a few murine cell lines should not be assumed to apply to all tumor cells.

It is our view that the rate of spontaneous mutation, while it may contribute to tumor progression, does so at low frequency or at early stages in tumor development, which are not easily measured. This implies that other mechanisms of genomic "deregulation" could play a major role in affecting tumor progression. The evidence presented herein of drug-induced metastatic potential by agents that appear to have different effects on the genome, indicates that tumors may progress by a plethora of means. Single, mechanistic explanations of tumor cell genomic changes are unlikely to be true.

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