Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 6574–6578, November 1982
Cell Biology

Evolution of tumor cell heterogeneity during progressive growth of individual lung metastases

(cancer/phenotypic stability)

GEORGE POSTE*†, JAMES TZENG†, JOHN DOLL*, RUSSELL GREIG*, DAVID RIEMAN*, AND IRVING ZEIDMAN†

*Smith Kline & French Laboratories, Philadelphia, Pennsylvania 19101; and †Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104

Communicated by Sidney Weinhouse, August 4, 1982

ABSTRACT **The metastatic properties of tumor cell clones isolated from individual lesions of B16 melanoma metastatic to lung have been examined at different stages in the evolution of metastasis. Clonal analysis of metastatic lesions produced by B16 melanoma populations containing clones with identifiable, stable drug-resistance markers revealed that the majority (>80%) of experimental metastases produced by intravenous injection of tumor cells are of unicellular origin. During the early stages of their growth (<25 days after initial tumor cell arrest), the majority of metastatic lesions contain cells with indistinguishable metastatic phenotypes (intralesional clonal homogeneity) although different clonally homogeneous lesions from the same host contain tumor cells with different metastatic phenotypes (interlesional clonal heterogeneity). Progressive growth of metastatic lesions is accompanied by emergence, within originally clonally homogeneous lesions, of variant tumor cells with altered metastatic properties (intralesional clonal heterogeneity). By 40–45 days after initial arrest of injected tumor cells in the lung, 90% of the metastatic lesions are populated by cells with heterogeneous metastatic phenotypes.**

Studies in several laboratories have shown that malignant tumors contain subpopulations of cells that differ widely in their metastatic abilities (1–14). This cellular heterogeneity is believed to result from the formation of variant subpopulations of tumor cells with altered metastatic properties during progressive tumor growth (15). The factors that influence the genesis and regulation of cellular diversity within malignant tumors are poorly understood. Recent studies (16–19) suggest that the rate at which tumor cell variants with altered metastatic properties are generated *in vitro* is influenced by the extent of subpopulation diversity within the overall cell population. We have shown that the rate of formation of metastatic variants is significantly higher in populations containing a limited number of tumor cell subpopulations than in highly heterogeneous, polyclonal populations containing multiple cellular subpopulations (16, 17). We have also shown that the majority of lung metastatic lesions produced by the murine B16 melanoma arise from the single cells (17). Formation of such lesions thus represents a situation in which subpopulation diversity is restricted. It was therefore considered of interest to determine whether this situation would stimulate rapid formation of new tumor cell variants to generate phenotypically diverse subpopulations of tumor cells within individual metastases.

MATERIALS AND METHODS

Animals. C57BL/6 mice were obtained from The Jackson Laboratory and from the Laboratory Animal Services Division of Smith Kline & French Laboratories (Philadelphia). Animals were age- and sex-matched within each experiment.

Cells. The origin and properties of the B16 melanoma B16-F10 subline have been described (14, 20). Drug-resistant (D^r) variants were selected from the B16-F10 subline by treatment with the chemical mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine as described (16, 17). Variants were selected for resistance to trifluorothymidine (2 μg/ml; TFT^r), diaminopurine (47 μM; DAP^r), vincristine (20 ng/ml; VCR^r), and adriamycin (50 ng/ml; ADR^r) at concentrations that are toxic to wild-type (wt) B16-F10 cells as described (17). wt B16-F10 cells and D^r variants were cloned by replica plating as described (17, 21). Although these D^r phenotypes are stable during serial passage of clones *in vitro* and *in vivo* (16, 17), the metastatic properties of B16 clones (wt and D^r) are unstable during passage (16, 17). The metastatic phenotype can be stabilized, however, by cocultivating the clones with other B16 clones. As described (17), to generate adequate numbers of cloned D^r cells with stable metastatic phenotypes, D^r clones were passaged in Dulbecco's modified Eagle's minimum essential medium containing 10% fetal calf serum (GIBCO; CMEM medium) in the presence of wt B16-F10 cells (wt/D^r cells, 1:10) and then recovered by treating the mixed culture with the appropriate cytotoxic drug to kill wt cells. The surviving D^r cells were harvested and, after confirmation of their D^r phenotype, were either used in experiments or frozen for future use. All cell cultures were grown in CMEM medium without antibiotics in humidified 5% CO_2/95% air as described (14) and were free of contamination by reovirus type 3, pneumonia virus of mice, K virus, Theiler virus, Sendai virus, minute virus of mice, adenovirus, mouse hepatitis virus, LCM virus, ectomelia, and LDH virus (14). Cell cultures were also found to be free of *Mycoplasma* sp. prior to animal injection as determined by staining with Hoechst 33258 (22).

Metastasis Formation and Cloning of Cells from Metastatic Lesions. Experimental lung metastasis was produced by injection of 5×10^4 or 1×10^5 tumor cells into the tail vein of unanesthetized mice in 0.2 ml of Hanks balanced salt solution as described (14, 17). The number of lung lesions was then determined at intervals (17). The size of individual lesions was measured before removal by using a micrometer scale in the eyepiece of a Wild stereozoom dissecting microscope. Individual metastatic lesions were then excised by sterile technique. Tumor cells were recovered from individual lesions as described (17). Clones were isolated from the recovered tumor cells by using the plating method described above and their metastatic properties were assayed within no more than eight passage generations after their initial isolation (17). The statistical signifi-

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Abbreviations: ADR^r, adriamycin resistant; CMEM medium, Dulbecco's modified Eagle's minimum essential medium plus 10% fetal calf serum; D^r, drug-resistant cells; DAP^r, diaminopurine resistant; TFT^r, trifluorothymidine resistant; VCR^r, vincristine resistant; wt, wild type.

cance of differences in the number of lung metastatic lesions produced by different tumor cell populations was determined by the Mann–Whitney *U* test (23).

RESULTS

The metastatic properties and D^r phenotypes of tumor cell clones developed from individual lung lesions produced by intravenous injection of a mixed cell population containing wt and D^r variant B16 melanoma clones were examined at different stages in metastatic growth.

Metastatic lesions were classified as clonally homogeneous if all clones developed from the same lesion had identical metastatic phenotypes and the same D^r phenotype. Individual lesions that yielded clones with the same D^r phenotype but significantly different metastatic properties were classified as clonally heterogeneous, as were lesions yielding clones with different D^r phenotypes.

The results of clonal analysis of individual B16 melanoma metastases at different stages of their growth are summarized in Fig. 1 and clonal analyses of "early" (16 days) and "advanced" (45 days) metastatic lesions are shown in detail in Fig. 2.

The results indicate that the cell composition of individual lesions changes during progressive growth. The majority

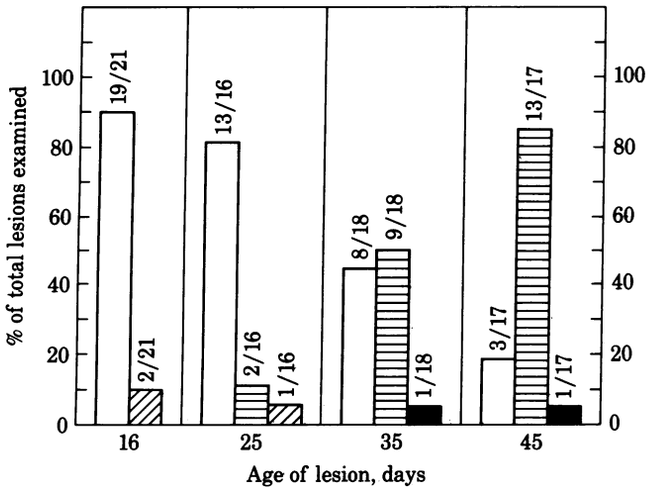

FIG. 1. Frequency of clonally homogeneous and heterogeneous metastatic lesions at different stages in the growth of individual B16 melanoma lesions metastatic to the lung. Aliquots (2×10^4 cells) of cloned B16-F10 variants resistant to either ADR, DAP, TFT, or VCR were mixed with uncloned wt B16-F10 cells (2×10^4) and the mixed cell suspension was injected intraveneously into C57BL/6 mice. Individual metastatic lung lesions were excised at the indicated times after injection. At least six clones were isolated from the cells recovered from single lesions and assayed for their metastatic properties and drug sensitivities. Lesions were classified as clonally homogeneous (open bar) if all of the recovered clones had the same metastatic phenotypes and the same D^r phenotype. Clonally heterogeneous lesions were identified by the isolation, from the same lesion, of clones with the following phenotypes: diagonal hatching, different D^r phenotypes (i.e., lesion is of multicellular origin) but metastatic properties do not differ from those of parent cells with the same D^r phenotype (i.e., new metastatic variants have not been formed); horizontal hatching, significantly different metastatic properties but the same D^r phenotype (i.e., lesions of unicellular origin in which new metastatic variants have been formed); solid bars, different D^r phenotypes and metastatic phenotypes differ significantly from those of the parent clones with the same D^r phenotype (i.e., lesion is of multicellular origin and new metastatic variants have also been formed). The numbers above each bar refer to the number of lesions exhibiting the indicated phenotype(s) and the total number of lesions examined. At least three lesions were sampled at each time interval from animals injected with the same batch of tumor cells. At least six clones were developed from each lesion.

(>80%) of individual lesions sampled at 16 or 25 days after injection of tumor cells contained cells with the same D^r phenotype and uniform metastatic properties (i.e., intralesional clonal homogeneity). This suggests that these lesions are of unicellular origin. These data are consistent with published results (17) on the clonal composition of individual B16 melanoma metastatic lesions of the same age produced by different D^r B16 melanoma clones. In the two cases in which intralesional clonal heterogeneity was detected in 16-day-old metastatic lesions, the recovered clones exhibited different D^r phenotypes (nos. 6 and 14, Fig. 2 *Left*), indicating that they were of multicellular origin. No examples were found of 16-day-old lesions containing clones with diverse metastatic phenotypes sharing the same D^r marker.

As reported (17), although the majority of individual lesions in early growth exhibited intralesional clonal homogeneity, the metastatic capacities of clones from different clonally homogeneous lesions differed significantly (i.e., interlesional clonal heterogeneity) (Fig. 2).

Progressive growth of lesions was accompanied by the development of significant intralesional clonal diversity (Figs. 1 and 2 *Right*). This process resulted from the formation of new variant cells within individual lesions. This is indicated by the finding that clones with different metastatic phenotypes that were recovered from the same lesion exhibited the same D^r phenotype. This interpretation is also supported by the finding that, in both early and advanced lesions produced by injection of the same preparation of tumor cells, only advanced lesions showed a high frequency of intralesional clonal heterogeneity (Figs. 1 and 2 *Right*). Instability of the metastatic phenotype and rapid generation of intralesional clonal metastatic heterogeneity was not confined to metastatic lesions of unicellular origin. Two examples were identified (Fig. 1) of lesions which yielded clones with different D^r phenotypes (see lesion 12 in Fig. 2 *Right*), indicating that the lesion was of multicellular origin, but significant variation in metastatic properties was also found among clones bearing the same phenotype. This indicates that the development of two clones from a lesion from the outset is not sufficient to "quench" rapid emergence of variants with new metastatic phenotypes.

Development of intralesional clonal heterogeneity was not related to size of metastatic lesions. There was significant size variation in the 45-day lesions that yielded clones with heterogeneous metastatic properties (Table 1). In addition, certain 45-day clonally heterogeneous lesions (lesion 7, Table 1) were smaller than the majority of the clonally homogeneous 16-day lesions. Also, the few 45-day lesions that remained clonally homogeneous (nos. 1–3) were larger than several clonally heterogeneous lesions (nos. 4, 5, and 7 in Table 1).

The possibility that the instability of the metastatic phenotype during progressive growth of metastatic lesions might reflect a generalized phenotypic "destabilization" caused by the use of mutagens to induce D^r phenotypes in the tumor cells used in these experiments is considered to be unlikely for several reasons.

First, the various D^r phenotypes are stable under conditions such that new metastatic phenotypes are generated (17).

Second, the clones used in the present experiments generate a similar spectrum of metastatic variants when grown in isolation from other clones *in vitro* but fail to do so when cocultivated *in vitro* with other clones (results not shown). This indicates that the (in)stability of the metastatic phenotype is not influenced by exposure to mutagens but instead is regulated, albeit via an unknown mechanism(s), by "interactions" occurring between tumor cell subpopulations.

Third, clonal analysis of individual lesions produced by non-

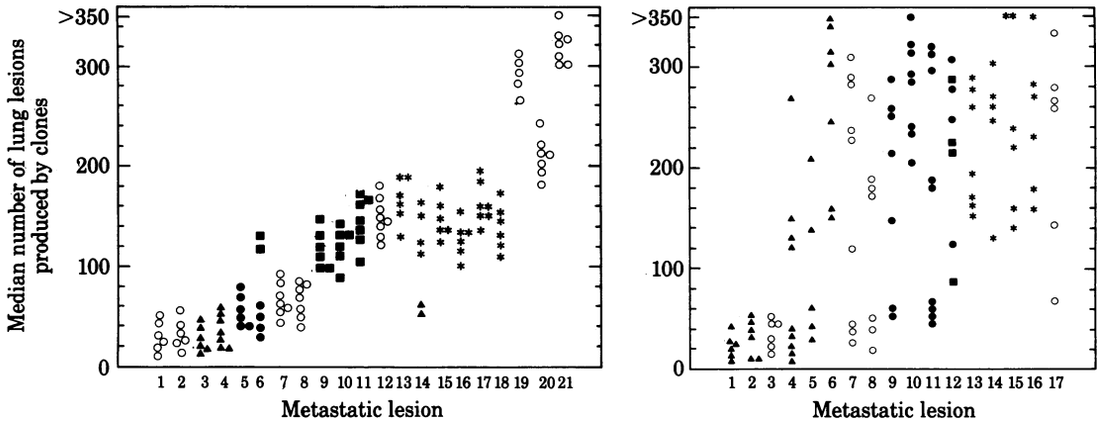

FIG. 2. D^r phenotypes and metastatic properties of clones isolated from individual B16 melanoma lung metastases 16 (*Left*) and 45 (*Right*) days after intravenous injection of tumor cells. Protocol: C57BL/6 mice were injected with a mixture of uncloned wt B16-F10 cells and B16-F10 clones resistant to ADR, DAP, TFT, or VCR as in Fig. 1. Individual metastatic lesions were excised 16 or 45 days later. Clones were developed from the recovered cells and assayed for their metastatic properties and for resistance to ADR (*) at 50 ng/ml, 47 μM DAP (●), TFT (▲) at 2 μg/ml, and VCR (■) at 20 ng/ml. wt cells (○) were identified by their susceptibility to killing by all of these drugs. Wherever possible, at least six clones were developed from each lesion. The metastatic properties of individual clones were assayed by intravenous injection of 5×10^4 cells and enumeration of lung lesions after 21 days. The results represent median values derived from measurements on five mice per clone. (*Left*) The following groups of metastatic lesions were produced by injection of different tumor cell preparations: nos. 1, 3, 13, 18, and 19; 4, 5, 7, 8, 12, and 16; 2, 10, and 17; 6, 11, and 20; 9, 14, 15, and 21. The following were excised from different mice: nos. 1, 3; 13, 18, and 19; 4, 7, and 8; 5 and 12; 16; 2, 10, and 17; 6, 11, and 20; 9 and 21; 14 and 15. (*Right*) The following metastatic lesions were produced by injection of different tumor cell preparations: nos. 1, 7, 13, and 16; 2, 4, 6, and 10; 3, 9, 11, 12, and 15; 5, 8, and 14 (these preparations correspond to four of the five batches of tumor cells used in the experiments shown in *Left*). The following were excised from different mice: nos. 1 and 7; 13 and 16; 2, 4, and 6; 10; 3, 9, and 11; 12 and 15; 5 and 8; 14.

mutagenized wt cells reveals similar progression of clonally homogeneous "early" lesions to "advanced" lesions that exhibit intralesional clonal heterogeneity (Fig. 3). In the absence of identifiable cell markers it is impossible to exclude the possibility that the intralesional clonal heterogeneity in 45-day lesions was not present from the outset. However, the conspicuous absence of intralesional clonal heterogeneity in early lesions produced by the same tumor cell inocula suggests that intralesional clonal heterogeneity in advanced lesions results from the emergence of variants with altered metastatic properties during the life-time of individual lesions.

DISCUSSION

We have identified significant changes in the clonal composition of individual B16 melanoma lung metastatic lesions developing within 45 days after initial metastasis. By using B16 melanoma cell clones bearing identifiable D^r markers we have shown that the majority (>80%) of lung lesions produced by intravenous injection of tumor cell suspensions containing several different clones arise from single tumor cells. This is consistent with our earlier observations on the formation of B16 melanoma lung lesions obtained with a different series of D^r clones (17).

As a consequence of their unicellular origin, the tumor cells populating early metastases (<25 days old) displayed homogeneous metastatic phenotypes (the metastatic phenotypes of cells in different clonally homogeneous metastases within the same host do differ, however). This initial intralesional clonal homogeneity was short-lived. Progressive growth of metastatic lesions was accompanied by rapid emergence of tumor cell variants with altered metastatic properties. Within as little as 45

Table 1. Size of individual B16 melanoma lung metastatic lesions containing tumor cell clones with homogeneous and heterogeneous metastatic phenotypes

16-day lesions			45-day lesions		
Lesion no.*	Size, mm†	Clonal metastatic phenotype‡	Lesion no.*	Size, mm†	Clonal metastatic phenotype‡
1	1.1 × 0.9	Homo	1	3.6 × 3.8	Homo
2	0.6 × 0.8	Homo	2	2.3 × 2.4	Homo
3	1.5 × 1.4	Homo	3	3.1 × 5.0	Homo
4	2.3 × 1.6	Homo	4	2.6 × 4.1	Hetero
6	1.4 × 1.2	Hetero§	5	1.9 × 3.2	Hetero
8	0.6 × 1.1	Homo	7	1.4 × 1.6	Hetero
9	0.8 × 0.8	Homo	10	4.1 × 4.3	Hetero
10	0.5 × 0.8	Homo	11	3.0 × 5.1	Hetero
14	2.0 × 1.5	Hetero§	12	4.0 × 4.0	Hetero§

* Lesions correspond to those with the same number in Fig. 2. For brevity, not all of the lesions in Fig. 2 are included. The sizes of the lesions not included in this table did not differ significantly from the range of lesion sizes shown.

† Measured *in situ* before excision; a micrometer scale in the eyepiece of a Wild stereozoom dissecting microscope was used.

‡ Clones isolated from the same lesion exhibited uniform (homo) or significantly different (hetero) metastatic properties as derived from the data shown in Fig. 2.

§ Metastatic lesion of multicellular origin containing cells with different D^r phenotypes.

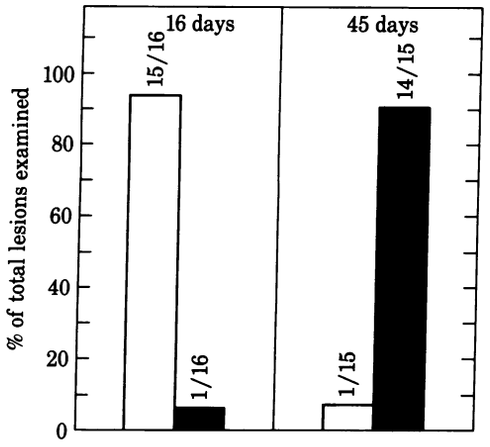

FIG. 3. Frequency of metastatic homogeneity (□) and heterogeneity (■) in clones developed from individual lung metastatic lesions of different ages produced by intravenous injection of 1×10^5 uncloned B16-F10 cells. Individual lesions were excised at the indicated times. The metastatic properties of clones from individual lesions were assayed as in Fig. 2. The numbers above each bar refer to the number of lesions tested that exhibit the indicated phenotype and the total number of lesions examined. At least three lesions were sampled at each time interval, from animals injected with the same batch of tumor cells.

days the overwhelming majority of metastatic lesions examined in the present study (>85%) yielded clones with heterogeneous metastatic phenotypes.

The drug-resistant B16 clones used in the present study were isolated after treatment of B16 cell populations with a chemical mutagen. The possibility that the instability of the metastatic phenotype in these clones *in vivo* might reflect a generalized phenotypic instability imposed by mutagen-induced genomic alterations is considered to be unlikely. We have shown that similar rapid evolution of intralesional clonal metastatic heterogeneity occurs in metastatic lesions produced by nonmutagenized B16 cells. In addition, as discussed in more detail below, instability of metastatic properties and formation of variants with altered metastatic properties in B16 clones can be decreased significantly simply by cocultivating them with other clonal subpopulations (D^r or wt subpopulations) (16, 17). Finally, if mutagen treatment predisposed to metastatic instability, this effect would appear to be selective for the metastatic phenotype because the D^r phenotypes in the clones used in the present experiments remain stable under conditions such that new metastatic variants are formed at a high frequency (16, 17).

The factors that affect the stability of the metastatic phenotype in malignant cells are poorly understood. Recent studies in our laboratory indicate that stability of the metastatic phenotype in B16 melanoma clones cultured *in vitro* is influenced by the extent of clonal diversity in the total cell population (16–19, 24). Cloned B16 cells of defined metastatic potential were found to generate variants with altered metastatic properties at a much faster rate when grown in isolation as single clones than when cocultivated with a series of clones (16, 17, 24). Conversely, treatment of polyclonal cultures with cytotoxic drugs to destroy all but one or two D^r subpopulations stimulated rapid formation of metastatic variants from the surviving subpopulations (16, 24). The rapid evolution of metastatic variants within individual metastatic lesions reported here may thus represent an analogous expression of the influence of tumor cell subpopulation diversity on variant formation.

The tumor cell suspensions used to produce metastatic lesions in the present experiments contained multiple subpopulations of cells with different metastatic properties. When maintained *in vitro* for 45 days as a polyclonal population, the formation of new variants with altered metastatic phenotypes from the constituent clones was not detectable (16, 17). This indicates that "interactions" occurring between the various subpopulations somehow stabilize the metastatic phenotype. However, when the same polyclonal cell population was injected into animals, the majority of the resulting lesions arose from single cells (17, 25). Any stabilizing effect on the metastatic phenotype operating in the original parent cell population as a result of polyclonal interactions will thus be lost. In undergoing conversion from their initially clonally homogeneous status to heterogeneous lesions containing clones with diverse metastatic properties, metastatic lesions of unicellular origin may be behaving in a fashion analogous to that of B16 clones *in vitro*, in which marked instability of the metastatic properties develops when individual clones are cultured in the absence of other B16 subpopulations. Support for the concept that clonal subpopulation diversity may influence the rate at which metastatic variants are formed comes from other studies showing that tumor cells isolated from clonally homogeneous early metastatic lesions quickly generate metastatic variants when cultivated *in vitro* but fail to do so when cocultivated *in vitro* with polyclonal B16 melanoma cell populations (unpublished data). Perhaps of even more interest is that the formation of metastatic variants in clonally homogeneous B16 cell populations isolated from early metastatic lesions (16 days) can be prevented by cocultivating them with clonally heterogeneous B16 cell populations recovered from advanced (45 days) lesions (unpublished data).

Studies in several laboratories have shown that malignant animal neoplasms contain multiple clonal subpopulations with widely different metastatic abilities (1–14). Data presented here, and elsewhere (1, 17, 24), indicate that formation of individual metastatic lesions from such heterogeneous cell populations involves selection of individual subpopulations of metastatic tumor cells, with different subpopulations giving rise to different lesions in the same host. The present data add a new dimension of complexity to the problem of tumor cell heterogeneity in malignant neoplasms by showing that individual metastatic lesions also quickly convert to phenotypically heterogeneous tumor cell subpopulations. Data to be presented elsewhere indicate that the evolution of phenotypic diversity among tumor cells within individual metastatic lesions is not limited to changes in metastatic properties. Conversion of initially clonally homogeneous lesions to clonally heterogeneous lesions is accompanied by the formation of cell variants that are more resistant to destruction by anticancer drugs (unpublished data). In addition, evolution of heterogeneous metastatic phenotypes in clonal subpopulations within a single metastatic lesion is not a peculiarity of the B16-F10 cell line. The same phenomenon has been demonstrated in spontaneous metastatic lesions arising from a murine fibrosarcoma and in experimental lesions produced by intravenous injection of human malignant melanoma cells into *nude* mice (unpublished data).

If the process described here is valid for other metastatic tumors, then successful therapy of metastatic disease will require the development of treatment modalities that not only circumvent variation in the therapeutic sensitivities of the different metastatic cell subpopulations but also can overcome differences in the therapeutic responses of heterogeneous tumor cell subpopulations that may be formed within individual metastatic lesions.

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