Resistance to Chemotherapy: Patient Variability and Cellular Heterogeneity

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Abstract

The issue of resistance to targeted drug therapy is of pressing concern, as it constitutes a major barrier to progress in managing cancer. One important aspect is the role of stochasticity in determining the nature of the patient response. We examine two particular experiments. The first measured the maximal response of melanoma to targeted therapy before the resistance causes the tumor to progress. We analyze the data in the context of a Delbruck–Luria type scheme, wherein the continued growth of preexistent resistant cells are responsible for progression. We show that, aside from a finite fraction of resistant cell-free patients, the maximal response in such a scenario would be quite uniform. To achieve the measured variability, one is necessarily led to assume a wide variation from patient to patient of the sensitive cells’ response to the therapy. The second experiment is an in vitro system of multiple myeloma cells. When subject to a spatial gradient of a chemotherapeutic agent, the cells in the middle of the system acquire resistance on a rapid (two-week) timescale. This finding points to the potential important role of cell-to-cell differences, due to differing local environments, in addition to the patient-to-patient differences encountered in the first part.

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Cancer Res; 74(17); 4663–70. ©2014 AACR.

Introduction

One of the most important challenges facing the oncology community is moving toward a better understanding of the emergence of drug resistance in treated cancers. In a typical scenario, a targeted molecular therapy such as a BRAF-inhibitor (1) or an anti-EGFR antibody (2) is given to a patient. The patient experiences a transient reduction in tumor burden (as, e.g., measured by MRI), and this reduction can be quite extensive. Yet, all too often, after a few months, the tumor begins to grow back and soon the therapy fails as full resistance emerges.

An immediate consequence of resistance is that often one achieves only incremental improvements in the Kaplan–Meier curve when using a new chemotherapy agent or a new combination. As an explicit example, we present in Fig. I results for the ALMITA drug developed at Princeton, applied to patients with small-cell lung cancer (3). The tragedy of such results is that a drug can be highly successful from a financial perspective without having much of an overall effect on tumor progression and patient survival.

At the molecular and cellular levels, it is clear that there can be disparate mechanisms for resistance. The simplest possibility is that a mutational event has eliminated binding of the drug to its target (4), but there are many other possibilities. These often involve network "work-arounds"—what seemed at first glance to be an essential signaling pathway responsible for cancer growth turns out to be replaceable by other routes to the same downstream effect. Resistance to vemurafenib, a small molecule used to treat melanoma harboring V600 BRAF mutations, provides a good example of these disparate mechanisms (5–7). This multiplication of possibilities may greatly lower the mutation rate that would be needed to produce resistance if genomic changes are occurring at a fixed rate. The malignant state seems to be quite robust, notwithstanding the multitude of genomic abnormalities with which it has to contend.

Here, we touch on two aspects of the drug resistance problem, from a basic science perspective. First, we discuss the relative role of cellular stochasticity, that is, fluctuations arising from random events at the cellular level, in determining the observed patient variability. We will do this by using a simple model that was introduced by Luria and Delbruck (8) in the context of antibiotic resistance, which postulates that resistance is due to selection of preexisting, randomly generated clones. This example will be studied in some detail, showing how one might use patient data to back out important information about response variability.

On a more qualitative level, we will discuss the possibility that the classical approach may not capture other important aspects of the problem, possibly involving resistant subpopulations (9), stress-induced mutagenesis (10), and/or spatial variation due to microenvironmental heterogeneity (11). The possibility of the latter’s importance has been highlighted in a recent set of bacteria experiments using microengineered...
been directly adapted to the chemotherapy problem (14). Variability from colony to colony. Their quantitative ideas have discovered that the number of resistant cells exhibited huge variability (12). We present preliminary data indicating that habitats (12).

The simplest possibility is that selection of preexisting clones is the fundamental mechanism underlying the rapid emergence of drug resistance. A recent article by Fox and Loeb (13) states this quite explicitly: “Evolutionary changes are driven by selection of stochastically generated preexisting variants. The key processes of spontaneous mutation, competition and selection, which underlie adaptation and drive evolution, are evident throughout biology. Human cancers, for example, represent a microcosm of Darwinian evolution: tumor progression is a mutation-driven process that results from the adaptation of a heterogeneous cell population to different microenvironments through the preferential replication of the most suitable variants.”

Unfortunately, this notion has proven very difficult to test empirically and it is not the only logical possibility. In this section, we present some ideas for using this hypothesis to understand patient variability.

In 1943, Luria and Delbruck (LD) considered a similar question for the emergence of antibiotic resistance (8). They discovered that the number of resistant cells exhibited huge variability from colony to colony. Their quantitative ideas have been directly adapted to the chemotherapy problem (14–17) and here, we provide a brief summary. We assume that the untreated tumor population grows exponentially, with a birth rate, \( b \), and a negligible death rate. At every cell division (aka birth), there is a probability, \( \mu \), of irreversibly creating a resistant cell (i.e., acquiring a resistance mutation). Resistant cells then grow with (possibly) modified birth and death rates. These models lead to a distribution of the number of resistant cells in a tumor that is composed of some large number of sensitive cells (18). An example of such a distribution is presented in Fig. 2. See “Sampling from the Luria–Delbruck Distribution” box for more details.

To test such a model requires high-quality data about a tumor’s response to a therapy and also some simplifying assumptions. Let us imagine that we have a series of patients with measured data about tumor size over time. We note in passing that one will have to improve upon traditional apparent size measurements to be able to resolve or separate (deconvolve) the tumor cell number from any inflammatory process that may make an (active) tumor mass appear larger than its “actual” size and to account for necrotic areas that may arise as a tumor responds to therapy. One convenient way of representing these data is by means of a waterfall plot, which plots the maximal tumor shrinkage (i.e., the smallest tumor size) measured in each individual patient in size sequential order; an example of such a plot from a recent clinical trial is shown in Fig. 3 (1). [Note here that bars to the left represent patients in whom there was no tumor shrinkage but only growth or progression and that the values shown for all patients in whom there was no tumor shrinkage but only growth or progression and that the values shown for all patients are the smallest recorded while their tumors were being monitored, not the final value, and that in the majority, the tumor likely grew in size from this smallest recorded value before treatment was stopped]. To match this example from a clinical trial to the Luria–Delbruck model, we ignored
Sampling from the Luria–Delbruck Distribution

The mathematical details underlying calculations of the Luria–Delbruck distribution for specific values of the birth rate $b$ and the mutation probability (per birth) $\mu$ are given in ref. 18. One essential feature of this distribution is the presence of a “heavy tail.” This means that the probability of finding individuals with a large number of mutant (i.e., resistant) cells is very much larger than would be expected for a typical random process.

In our examples, an individual “patient” has a specific number of cells, $N_{mut}$, that are present before treatment and harbor a mutation that confers resistance. This number is a sample (one number) drawn from the Luria–Delbruck distribution. This distribution is a set of probabilities $P_{iD}$ ($N_{mut}$) that represents the fraction of occurrences or patients whose tumors harbor $N_{mut}$ cells, with $\sum P_{iD} (N_{mut}) = 1$. This drawing can be done with a simple computer algorithm. Draw a single random number (call it $x$) from a uniform distribution between zero and one, and determine $N_{mut}$ by the condition $P_{iD} (N_{mut}) < x < P_{iD} (N_{mut} + 1)$. If we repeat this procedure for some number of times by drawing successive samples from the Luria–Delbruck distribution, we generate a pool of synthetic data, with each data point representing a single patient.

In Fig. 2, we have plotted as circles the results of creating a patient pool of size 100; each circle is the number of individuals with a specific $N_{mut}$. Note the $y$-axis ranges from zero to fifteen. The circle on the $y$-axis itself indicates that there are eight occurrences (or 8 patients) whose tumors do not have any cells with mutations (the $x$-axis being number of mutant cells + 1), and the $y$-axis corresponding to a value of 1 on the $x$-axis identifies the circle on it as representing occurrences or patients with “zero mutant cells” in their tumors. Similarly, the two highest circles represent eleven patients whose tumors harbor one or two mutant cells and are plotted as 2 and 3 on the $y$-axis. The 8 patients represented by the circle directly on the $y$-axis whose tumors do not have mutant cells together with the 22 with one or two mutant cells account for 30 of the 100 patients. The ten circles each corresponding to one patient out of the pool are seen near the bottom of the graph and have a range of mutant cells from just under twenty to more than 300. As the pool size increases, the circles should increasingly lie precisely on the purple curve. For any finite-sized pool, there will be the type of data dispersion illustrated in Fig. 2.

any complications arising from variations in the drug concentrations reaching the individual cells and assume that the drug acts by inducing a uniform death rate, $d$, in the sensitive cells. We can then create a synthetic waterfall plot by sampling from the Luria–Delbruck distribution of the number of resistant cells. In detail, we create a sample of 100 “patients” from the distribution (see “Sampling from the Luria–Delbruck Distribution” box) to find the number of resistant cells in each of these patients; these resistant cells then continue to divide and the resistant subpopulation grows as the number of sensitive “wild-type” cells decays at net rate $d – b$; in other words, $d/b$ is the ratio of death rate, $d$, (due to treatment) to the cell birth rate, $b$, and this variable will affect the synthetic plots. The overall result is a tumor size that initially decays to a minimum and then begins to grow again, unless the number of mutant cells is strictly zero, in which case the tumor will disappear.

In Fig. 4, we plot two such synthetic plots for typical sets of parameters. We assume that the tumor has $10^6$ cells when treatment commences. The first point we wish to make is that the number of “cured” patients, that is, those whose tumor never grows or progresses, is effectively determined by the mutation rate. This is evident by the rightward shift in the plot as $\mu$ (the probability of irreversibly creating a resistant cell) is increased. There of course will be stochastic fluctuations in the precise number of such individuals for sample sizes such as 100, which is relatively small. In short, the mutation rate can be roughly determined by the fraction that does not progress.

Next, we show in Fig. 5 the effect of varying the death rate expressed as a multiple of the birth rate. In this waterfall plot, it is only the ratio of these two rates that matters, the probability, $\mu$, of irreversibly creating a resistant cell is held constant. Now, the maximal incomplete response decreases from about 80% to about 60% when the death rate is lowered. The depth of the minimum attained when the number of mutants is extremely small (the maximal incomplete response rate as described by the most negative $y$-axis value excluding -1) determines the right side of the plot. Thus, the (maximal; see later) death rate can be determined by the size of the maximal incomplete response. These synthetic datasets all show a striking qualitative departure from the patient data shown in Fig. 3. The synthetic curves are very flat, not varying much from the maximal incomplete response. Once the mutation rate and the death rate/birth rate ratio are fixed, giving the red (bottom) curve in Fig. 5 as the best parameter set, the left side of the plot only extends upward to 60% shrinkage. Note that patient data, such as that depicted in Fig. 3, extend all the way to zero and even higher, as some patients progress immediately without any regression of the tumor. This is impossible to match with our simulation, showing that there must be something beyond the Luria–Delbruck model controlling patient-to-patient variability.

Phenotypic Variability

Although we have not directly computed a best-fit $P$ value, it is clear that fluctuations inherent in the pure Luria–Delbruck model will not be able to fit typical patient data. Also, there is no allowance in this model for individuals that show no response, that is, progress almost immediately. In this section, we explore a more complex model involving phenotypic variability that could account for more diverse behavior in the emergence of drug resistance.

Our basic notion concerns the role of phenotypic fluctuations. It is certainly the case that even cells with identical genomes will exhibit different responses to drug treatments. It is therefore reasonable to assume that individual patients with
different genetic background and different tumors will respond differently. We will therefore postulate that the death rate of the wild-type cells that do not harbor a mutation is not a fixed number, but instead varies significantly from individual to individual; although it will still be taken to be the same for all cells in the tumor. This approach of course implies that efforts to pool patient data and derive a mean death rate may be misguided.

In Fig. 6, each data point (i.e., patient) has been sampled not just from the Luria–Delbruck distribution of the initial number of resistant cells but also from a uniform distribution of death rates. In detail, a given patient from our synthetic pool now is assigned a number of mutant cells from the Luria–Delbruck distribution (as before) and in addition a specific value for the \(d/b\) ratio. We argue that the maximal death rate is still fixed by the maximal incomplete response at the right side of the plot (the deepest minimum for any noncured patient) but that individuals with the smallest death rate now control the left side. In fact, if the death rates were to include some individuals with \(d/b < 1\), then we will also see a fraction of cases where there is immediate progression or growth (data not shown); we have chosen not to focus on this possibility and thus our distribution starts at \(d/b > 1\). This approach yields a qualitatively satisfactory match to our experimental dataset. For comparison, we have also plotted what would have been obtained by a fixed death rate simulation, with the single fixed \(d\) value equal to the mean of the uniform death rate distribution.

From a physical science perspective, the Luria–Delbruck distribution is very heavy tailed (18, 19), with many more patients with large number of mutants than would be naively expected. But, this is not enough to give a broad waterfall plot—the strong variation in the population prefactor (due to the distribution of the number of resistant cells before treatment) cannot compensate for a fixed exponential death rate. The same reasoning explains why a different choice of the initial population size (at treatment) would not change our conclusions. Future efforts should attempt to discern the underlying reasons why there should exist a broad distribution of death rates.

More Complex Approaches

We have directly shown the crucial importance of phenotypic variability at the patient level. But, there can also be phenotypic variation at the cellular level, which could affect the emergence kinetics. One version of the appeal to cellular variability relates not to continuous phenotypic variation, but instead to the existence of a specific subpopulation of resistant cells. Evidence for such a population was provided in a recent study on EGFR inhibitors in a non–small cell lung cancer (9), and it was argued there that drug tolerance can be connected to modified chromatin structure. In other cases, “stem-like” subpopulations seem to be tolerant due to high levels of expression of efflux pumps (20). In general, this notion is quite analogous to the well-established phenomenon of bacterial persistence (21), known since the early days of antibiotics (22).

In the usual tolerance scenario, genetic mutations that confer permanent resistance arise after treatment only in the tolerant subpopulation (23). In order for this idea to be consistent, something must compensate for the much smaller cell numbers and much reduced time interval compared with the traditional Luria–Delbruck assumption. Thus, this hypothesis requires something akin to stress-induced mutagenesis to endow the surviving cells with much higher evolvability. There is evidence that stresses such as hypoxia can lead to error-prone replication (24), and again there is direct evidence and established mechanisms for the occurrence of this effect in bacteria (25). This assumption should be testable if the genotype of circulating tumor cells after the treatment can be compared with the before treatment situation.

A simpler possibility is that the drug resistance is truly a consequence of a heritable epigenetic change, with no relevant
enables the population diversity. This is so becoming in turn require understanding the genetic circuitry that equivalent of the Luria to know the distribution of tolerant cell numbers (i.e., the patients that eventually exhibited tumor progression.

place where the lines hit the data seems at each patient by allowing the cell population to advance with a fixed birth death (all cells) and a fixed death rate (wild-type nonresistant cells). The place where the lines hit the x-axis corresponds to the total number of patients that eventually exhibited tumor progression.

genetic alteration. The same lung cancer experiment detected resistant cells that were able to begin regrowing at almost normal rates and that retained their phenotype for many generations; these are apparently induced to emerge from tolerant cells that are initially quiescent. A key indicator of this type of adaptation would be reacquired sensitivity (26). In other words, stopping and then restarting treatment should yield renewed response, as the epigenetic change retreats when the drug is not being administered. Another key indication would be the response to multiple agents. Phenotypically tolerant strains are typically tolerant of many drugs, whereas the likelihood of randomly finding all the independent genetic changes needed to counteract independent therapeutic agents is much smaller. However, trying to use this model to compare with direct patient response data seems at first to be rather problematic. We would need to know the distribution of tolerant cell numbers (i.e., the equivalent of the Luria–Delbruck distribution), which would in turn require understanding the genetic circuitry that enables the population diversity. This is only becoming available for the much simpler bacterial systems (27, 28) and is well beyond the state of our knowledge in complex mammalian cells. But, as we have already pointed out, this distribution may not be the most important one in generating the waterfall plot, as even a very broad distribution of initial numbers of tolerant states is insufficient to match the typical dataset from a study of patients without variability at the patient level in the death rate of the nontolerant (sensitive) population. The converse is also true, namely that the broad waterfall plot can be reasonably fit by models assuming a population with a relatively fixed fraction of phenotypically resistant cells as long as we still have a broad distribution of death rates. Distinguishing these two possibilities thus requires new experimental data, possibly about reacquired sensitivity as discussed above.

The Failings of In Vitro Experimental Models

One of the fundamental problems in cancer research is the need for biologically relevant but relatively simple experimental models that accurately reproduce the emergence and spread of cancer cells often in concert with a population of normal differentiated cells. Even more challenging is the development of biologically relevant models that: (i) include the influence of the immune system on the development of cancers; (ii) can model the phenomena of metastasis that is responsible for 90% of cancer deaths; and (iii) accurately reproduce the effects of therapies.

Although most research has relied on in vitro experiments, the primary cancer cells derived from tissue biopsies are adapted to extraordinarily complex environments that cannot be reproduced in vitro. Even the use of cancer cell lines is questionable, since by definition these lines can be passaged in a clonal way over many generations with little genomic or phenotype change unlike cancers that are characterized by constant change and growth. Furthermore, growth as pure cultures of cells ignores the fact that tumors exist surrounded
by stromal cells, not cancerous in that they do not exhibit constant cell growth, but that can provide nutrients and additional signals needed for cell growth. This strong coupling between stromal cells and cancer cells means that even an \textit{in vitro} system with stromal cells but without the attendant microecology that nurtures this complex, heterogeneous community will fail to adequately capture what is occurring in an actual tumor. The difficulties of traditional experimental approaches to cancer present a challenge for those biologists and physicists who would like to approach the fundamental foundations of this subject at a quantitative level. But, we have already seen that new experiments are crucial in distinguishing different mechanisms of the emergence of drug resistance. One idea, to be discussed next, is to use modern microfluidics technology to mimic at least some of the microenvironmental heterogeneity.

\textbf{Emergent In Vitro Models}

Because cancers emerge and spread within extraordinarily complex ecologies with many players, it is necessary to try to develop technologies that at least attempt to reproduce some of the main complexities of the tumor microenvironment so that we might hopefully develop drugs more rationally. Five main ingredients are needed:

1. Microfluidic heterogeneity. Cancer cells do not grow \textit{in vivo} in a well-stirred test tube or on a homogeneous cell culture plate, but rather within a complex of vessels supplying and removing fluids with many different parameters of flow, pressure, and nutrient levels.

2. Cellular heterogeneity. Culturing clonal cancer cell lines without some attempt to include stromal cells greatly reduces the relevance of the results for \textit{in vivo} studies.

3. Ecological heterogeneity. Cancer is typically not a homogeneous cell mass but consists of many different cells (different both in their genome and phenotype) distributed over space. The impact of this ecological heterogeneity (microhabitats) can be quite profound in terms of cancer evolution.

4. Spatial and temporal variations in external drivers of cancer evolution and growth. "Spatial variation" refers predominantly to the existence of gradients in applied drugs and nutrients (29, 30). In such an environment, natural selection (possibly utilizing stress-induced mutations and cell motility) can more quickly generate drug-resistant progeny. "Temporal variation" includes

![Figure 6. Waterfall plot with assumed uniform variation in individual patient death rate, ranging from $d = 1.01$ to 1.6. For comparison, we show the results for the mean value of $d = 1.305$. Clearly, the extra variability changes the nature of the waterfall plot to be more in line with typical clinical data](image)

![Figure 7. Microhabitat design, courtesy of Amy Wu and James C. Sturm (Princeton University, Princeton, NJ).](image)
the possible role of the time dependence of cancer drug dose level on the evolution of resistance.

5. Complex materials that can replicate or mimic the structural aspects of the matrix underlying the tumor's microenvironment.

This is a formidable list, and we remain far from being able to generate an in vitro analog of the tumor microenvironment. Figure 7 shows an example of a microfluidic chip, a metapopulation evolution chip, that has some, but by no means all, of the necessary ingredients for a complete in vitro model. This design addresses or can be easily adjusted to cover ingredients 1, 3, and 4 discussed above. At present, the ecologies are sealed with glass slides that are impermeable to air, and the structures are etched out of equally impermeable silicon. This means the gas environment of the inner parts of the device has gradients that are coupled with nutrients and metabolism, and that pH and other variables are also not under our control to the extent we would like them to be. Oxygen tension and CO_2 levels are highly variable within the tumor microenvironment and we can have the ability to control these variables, while still maintaining the high spatial imaging that a microscope cover slip provides. As designed, the chip allows for long-term gradient and dose-dependent studies of the emergence of drug resistance.

This rather simple device enables the reproduction of at least one aspect of cancer that makes it so formidable, namely the emergence of resistance to chemotherapy on relatively short-time scales. In Fig. 8, we plot data showing how placing cells in a gradient of doxorubicin (0–200 nmol/L across the device) allows for a 16-fold increase in viability over a 2-week period. Placing the same cells in a device with a fixed concentration (200 nmol/L) did not lead to any resistance, consistent with the concept that resistance is more likely to emerge when at least some part of the population is exposed to lower concentrations that are sufficient to affect selection but not kill the resistant cells. More details and more data will be published elsewhere. These experiments could easily be extended to study the effects of targeted agents rather than more general chemotherapeutic agents. These results imply that the emergence is not just due to the selection of preexisting tolerant cells but instead reflects the appearance of novel cellular “solutions” to the imposed stress, helped along by the spatial diversity created by the gradient. Understanding how this occurs and the extent to which this will cause us to rethink our analysis of patient data are challenges that must be faced as we try to develop a more accurate and more global picture of drug resistance during therapy.

**Discussion**

In this short article, we have shown how one can begin to unravel some of the mechanisms governing drug resistance by using the techniques of both theoretical modeling and experimental physics. As already mentioned, we are hardly in a position to offer a comprehensive theoretical approach for all cases and of course we are hardly the first group to attempt to fit data by evolutionary models. Nonetheless, we feel that it is worth going through a calculation that explicitly demonstrated how one can use these models to draw inferences from clinical data. We suspect that some situations will prove in the end to be consistent with simple evolutionary models whereas others will require rethinking of some of the basic assumptions, especially related to whether resistant cells always predate the treatment.

In the former cases, the key will be to understand why the sensitive cells of different patients exhibit rather variable (mean) behavior. This is a deterministic effect whose cause lies in the complex relationship between the tumor genome, the physiology of the tumor cell, and the mode of attack being used by the drug. In the latter situation, stochastic evolution of the tumor population is the dominant aspect that needs modeling, and experiments that can test specific scenarios (such as the critical role of heterogeneity) are of the utmost importance. We have described some initial attempts at doing the right type of experiments, and we are already seeing a need to expand our modeling repertoire to encompass the surprising data.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Grant Support**

This work was supported by National Science Foundation (NSF) Center for Theoretical Biological Physics Grant NSF PHY-1308264, the Cancer Prevention and Research Institute of Texas Scholar Program of the State of Texas at Rice University, the NCI through the Physical Science-Oncology Center program, and the Israel Science Foundation.

Received January 14, 2014; revised February 21, 2014; accepted June 5, 2014; published online September 2, 2014.
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