The challenges posed by cancer heterogeneity

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A panel of five experts discuss the implications of cancer heterogeneity for diagnostics and therapy.

Heterogeneity in cancer cells has been noted since seminal studies carried out by the groups of Josh Fidler, Gloria Heppner and Harry Slocum in the seventies. In recent years, an increasing appreciation of genetic, epigenetic and phenotypic heterogeneity in cancer has renewed interest in the evolutionary dynamics and selective pressures that govern tumor initiation and progression. *Nature Biotechnology* brought together five investigators to discuss the current understanding of tumor heterogeneity and its implications for diagnostic and therapeutic approaches in the clinic.

What types of heterogeneity are seen in cancers?

Kornelia Polyak: You can view cancer heterogeneity on several levels. There is genetic heterogeneity—copy-number variations or point mutations and so on. So that's at the level of somatic cells. Then there is the issue of heterogeneity in the germline background, not only in the tumor but also in the tumor stroma. I think we are beginning to understand that the same tumor in two different individuals may not behave the same way. For certain *JAK2* [Janus kinase 2] mutations, for example, particular germline polymorphisms predispose for specific mutations. Then we see epigenetic heterogeneity. This can be associated with differentiation states, either stem cell–like or more differentiated properties, but also it might not necessarily be differentiation-related—for example, in cells that acquire the ability to be drug resistant. And then there is a phenotypic heterogeneity. And that's all just within one tumor. If you're talking about heterogeneity within a patient, then you can add the metastatic lesions as well.

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A. John Iafrate: As a pathologist, I spend my time looking at human tumors, most of which are heterogeneous at the morphological level and are a mix of different types of cells. Lung cancer is a great example. The most common histologic diagnosis for lung cancer is lung adenocarcinoma, mixed subtype. By mixed subtype, that means a pathologist will look at it and see at least two, and possibly three or four different morphologies in a single tumor. There is likely an underlying epigenetic cause for this morphologic heterogeneity. One region will look really distinct from the other, and when the tumor metastasizes/recurs, the morphology may look like one or another part of the tumor.

A. John Iafrate is an assistant pathologist at Massachusetts General Hospital and assistant professor of pathology at Harvard Medical School.

Acknowledging this common existence of morphological heterogeneity, from a genetic standpoint, for the majority of driver mutations, I would say we don't detect significant heterogeneity. For example, KRAS mutations in colon cancer are present in all the tumor cells, and when the tumors metastasize, they are present in all the tumor cells. We've analyzed in the molecular diagnostics lab many lung cancers over the past 5 years; we know EGFR [epidermal growth factor receptor] mutations or *ALK* [anaplastic lymphoma kinase] translocations are present in all of the tumor cells, both pre- and post-therapy. Even if a patient develops resistance to a targeted therapy, those drivers are still there. So in practice the important drivers are not heterogeneous. But recently I've started to change my mind.

Analysis of receptor tyrosine kinase gene amplifications in glioblastoma [*Cancer Cell*, **20**, 810–817, 2011] has really altered how I think about the genetics of tumors in general. We observed that there is substantial genetic heterogeneity at the copy number level from cell to cell. Within single tumors, we see intermixed populations of tumor cells with distinct genetics, one cell next to another next to another with three different genetic drivers; one cell with *MET* amplification, one with EGFR amplification, one with PDGFR [platelet-derived growth factor receptor α] amplification. These observations suggested to us that it is possible that tumor cell populations may subspecialize and begin to support each other.

Robert M. Hoffman: Another way to think about heterogeneity within a single tumor mass is heterogeneity in terms of cell division. For example, using the FUCCI system [Invitrogen, Carlsbad, CA, USA], which reports what phase of the cell cycle a cell may be in—resting cells express red fluorescent protein and dividing cells express a green fluorescent protein—[my] group has

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found some striking results. Within the surface of a tumor, about 150 micrometers or so, about 80% of the cells are green or yellow-green. That is, they're cycling. Deeper than that, though, approximately 90% of the cells are resting. They don't seem to get out of the cycle. They just remain resting, and as the cells at the surface grow, the cells in the center stay out of cycle.

Sangeeta Bhatia: One thing I'd also add is the heterogeneity of the tumor microenvironment—the other cell populations, including stromal and immune cells, and the extracellular matrix. And then, besides the local microenvironment at the primary tumor, there are the disseminated cells. Many groups are now interested in accessing circulating tumor cells, but these are really very heterogeneous, and clearly a subset of cells at the primary tumor. We are still grasping to understand what they represent and which of these disseminated cells go on to form metastases.

Sangeeta Bhatia is the John J. and Dorothy Wilson Professor of Health Sciences and Technology & Electrical Engineering and Computer Science at the Massachusetts Institute of Technology, and a Howard Hughes Medical Institute Investigator.

How does heterogeneity relate to cancer stem cells and differentiation?

KP: With respect to cancer stem cells, I think people are discovering the reality. There was this boom in cancer stem cell research that promoted one particular perspective where cancer reflects differentiation hierarchies that are found in normal tissues. And I think people have realized it's more complex than that. John Dick, who published the very first paper on cancer stem cells, has written himself that it is more complicated and maybe, after all, you have clonal evolution in the tumor-initiating cells. So that kind of says it all. I think it's one layer of the complexity, but it's not the answer to everything. And very often we see tumor cells switching phenotypes, which means that even if you kill those cells, the other cells in the mass can switch.

There is also this debate about EMT [epithelial-mesenchymal transition]. In my experience one sees expression of some, but not all, markers characteristic of the transition, but not in every cell. I tend to think of cancer cell phenotypes being more like a continuum between epithelial and mesenchymal cells. And at what point you call it EMT is totally arbitrary. Now some people like defining things, but in my view it's never totally that you have this state and that state. You have a continuum of states.

AJI: Perhaps what we're seeing in EMT is a plasticity where cells move in and out of differentiation. That's the lowest common denominator—and the worst dedifferentiated state is this mesenchymal precursor. Or a tumor where you can't even establish what tissue the tumor came from. It's really common in sarcomas that we get these tumors that are just undifferentiated.

What kinds of technological challenges limit our ability to characterize tumors?

SB: Heterogeneity presents challenges in terms of targeting therapeutics or imaging agents, and for tumor characterization there remains a huge gap. We have imaging, which is improving. We have deep sequencing, which is largely whole-tumor sampling. We have biopsies, which is sub-sampling. And then we have some single-cell technologies emerging. The problem with the single-cell data is that we don't really know yet what they mean. Putting these together in a framework that allows us to understand what we all really care about clinically, which is resistance and recurrence, I think, is a challenge.

John V. Frangioni: Personally, I think we have the means and technologies to answer most of the important questions. But in my view, much more analysis should be done at a single-cell level, be it pathologies, histopathological specimens, or what have you. It's at the single-cell level that we're going to learn these things and understand them. And I think the technology exists now for almost every aspect of the phenotype, and for the phenotype to be characterized at the single-cell level. So I'm most excited about technologies that are addressing these questions at a single-cell level—in a collection of cells, but at a readout of the single cell. I think that's where the action's going to be.

John V. Frangioni is a professor of medicine and professor of radiology at Harvard Medical School.

KP: To us, one of the biggest questions is how heterogeneity is important for clinical behavior of the tumor in terms of progression, therapeutic responses, and also, trying to understand what sustains heterogeneity. I mean, what is the reason why you have particular combinations of clones or mutations and so on? So we have some data from experiments where we're putting in a heterogeneous mixture of tumor cells with a known driver, and it's not becoming homogeneous. So then the question we're asking ourselves is why is it that they don't have to become homogeneous to drive the tumor? Because that has been the accepted view—the driver mutation becomes the dominant clone; you don't have to care about the rest. And I think the experiments tell us many cancers don't conform to that.

To get back to the original question about technology, there are still things you cannot do on single cells. Like whole-genome sequencing; it's not there yet in terms of having mutation data. My dream experiment is to do whole-genome sequencing *in situ*, if we're going to get there, but I think the way to overcome it is to do whole-genome sequencing on the bulk and then have technologies that allow you to look back at the mutations in single cells. Because we have these studies coming out, they have hundreds of mutations per tumor. Are they in the same cell? Or what fraction of the mutations is? Because everybody assumes that you need five or six mutations to get a tumor. But then, why do we have so many mutations? And then, what is the composition in different cells and what are the different combinations?

To me, those are some of the big questions that we can address, technology-wise. We cannot fully do everything yet, and of course there's the cost of sequencing as well, which can still be high for single-cell work. But I think that's the way to go, to look at single cells.

AJI: I think we have to develop a few additional tools, especially the ability to analyze whole genome sequences from single cells to fully understand genetic heterogeneity. Emerging techniques, such as immunohistochemical analysis with mutation-specific antibodies, will allow us to perform in situ studies of heterogeneity of common driver mutations. We also need multiple sets of

antibodies or genetic in situ tools that can sample things other than copy number (e.g., *HER2* or EGFR amplifications). That type of technology I think will be critical.

KP: I would also argue that we need to exploit more testing at autopsy. For example, we have analyzed all the metastases in the same patient using a rapid autopsy. And when we did the mutational analysis, the metastases had different variants. When we compared metastases in the lung and the liver and some other organs using FISH [fluorescence *in situ* hybridization], they were not the same. This raises the question, do we really understand a patient's cancer if we just analyze the primary tumor? Because at the moment this is what we are doing to diagnose the patient and then design the treatment. Instead, one would like to sample metastatic lesions or develop models that predict what kind of metastatic lesions may develop from a primary tumor.

RMH: I think one of the most useful additions to our present diagnostic tools would be to have a marker—especially a fluorescent marker—that would work in the clinic that tells us whether a cancer cell is dividing or not. That would help guide what kind of drug to use.

How does tumor heterogeneity impact diagnostic approaches?

JVF: In the imaging community, the [US] National Institutes of Health's Gary Kelloff has written about this extensively. He asks, do we really want to have imaging agents specific for every type of tumor, and every phenotype of tumor … every classification of every tumor? Or do we really just want a proliferation agent, a metabolic agent—you know, group them in large functional classes and call it a day. And he makes the argument that with a combination of ^{18}fluorodeoxyglucose [FdG] for glycolytic tumors, maybe ^{11}C-methionine, maybe ^{18}F-fluorothymidine [FLT] in some cases—overall, a very small number of general agents—maybe that's the best approach. Certainly, the heterogeneity will make a lot of academic careers, but in the end we may find out that a lot of these mutations are red herrings and there are only a few fundamental changes to the tumor that matter. Then we've wasted a lot of time and money.

AJI: From a clinical laboratory standpoint, diagnostics are still rather rudimentary at present. We're just not capable of providing meaningful analysis of heterogeneity with the present assays. Just to give one example where we do report heterogeneity, it is not uncommon for breast cancer FISH tests to show that tumors carry both *HER2*-amplified and *HER2* non-amplified populations in our lab reports; and we can be descriptive, but we can't provide real insight into their clinical importance.

JVF: HER2 immunodiagnostics are also a good example. I am aware of a set of papers where three different groups around the world studied three different patient populations and looked at radiolabeled Herceptin [trastuzumab; Genentech, S. San Francisco, CA, USA] antibody. Only about 45% of patients were positive when a radiolabeled HER2 antibody was used. Yet these were patients that all had 3+, 4+ scores by pathological criteria. So, clearly, in those breast tumors, either HER2/neu isn't accessible on the surface or the heterogeneity is so high that Herceptin binding is low enough to return [a false negative]. This should have been a best-case scenario: you have a pathologist telling you that the tumor has a 3+/4+ [strongly immunoreactive] score; you have an antibody [Herceptin] that you know has high affinity; you radiolabel it, you have high specific activity and you inject it. And yet these three different patient groups failed to show up positive. That's a problem. That's a real problem. That's when you know you have a long way to go.

To what extent do current treatments address heterogeneity?

KP: I don't think intratumor heterogeneity has been that well-incorporated into clinical practice yet. I think we're still in the phase of trying to understand it. Resistance and recurrence happens because of heterogeneity. In hematopoietic malignancies, you can take blood and sequence the tumor cells, and you see a clone that seems to be dominant. But then after treatment, in a recurrence, sometimes you have a clone that was not even really present very much in the original diagnostic sample but is some kind of earlier-stage clone. And so, during treatment, that clone must have expanded and changed. Using deep sequencing, you can see much more of this heterogeneity. And of course you have translocations that you can follow at the single-cell level.

JVF: From a clinical perspective, cancer is a few hundred different diseases. But the key question is what the genetic and epigenetic underpinnings are. Certain cancers begin with a single type of aberration like a *BCR-ABL* [breakpoint cluster region, c-abl oncogene] translocation in CML [chronic myelogenous leukemia]. In those cases, we're finding that even a single drug can be effective. But in other carcinomas, the majority of tumors are evolving over time, after multiple mutations, from environmental hits in a particular germline background, and these are so different that the chance of having single-drug treatments or even two- or three-drug treatments, I think, is low.

For the majority of tumors, we're already thinking combinatorial treatments only because it matches the heterogeneity of the tumor. But the successes to date are with single drug agents like Gleevec [imatinib; Novartis, Basel]. But CML and retinoblastoma are special cases and rare tumors where you have a predefined genetic abnormality and [in the case of CML] it happens to be druggable. The majority of tumors are much too heterogeneous, and something I worry about a lot is whether the tumors in a patient we're treating even matter. And what I mean by that is, if you take a small-cell carcinoma, they'll melt away with conventional chemotherapy, but a few months later, they'll all come back and be deadly. We can treat the bulk of the tumor, but clearly, there's something else underlying that. And its not clear whether these are stem cells or we're selecting for *de novo* resistant clones. I think that's an unanswered question that needs to be answered. But if it's stem cells, then we can more or less ignore most of the tumor we see by imaging or anything else; it's the cells we can't see yet that are the problem.

So the question in my mind is, what is the heterogeneity at that level? Because that's probably all that matters. I think a lot of what we do in oncology, we treat bulk disease, but it's not clear to me that the bulk of disease we're treating, when there's a response, whether it matters.

RMH: To me, the most important heterogeneity is between dividing cells and non-dividing cells because most drugs target only dividing cells. So I think this maybe could explain why we get variable results with current chemotherapy treatments: when it works, you're killing off the dividing cells. But then the non-dividing cells are relatively resistant to the chemotherapy and eventually they begin to cycle, especially as they get near the surface of the tumor as the other dividing cells are killed by the chemotherapy. The tumor starts growing again. So to me, the most important heterogeneity is between dividing and non-dividing cells in a tumor. I think the goal is to find drugs that target non-dividing cells.

What about the role of heterogeneity in drug resistance?

AJI: There are multiple anatomic patterns of resistance to targeted therapies; some patients recur with only one predominant lesion for example. Other patients will have multiple lesions appear simultaneously, at exactly the same time, often at the site of a previously treated lesion. About the most logical explanation for this second pattern is that there is genetic heterogeneity before therapy is initiated, with rare pre-existing resistant clones. The most logical approach in the era of targeted therapy is once we find a driver mutation, and understand the common mechanisms for the developing resistant clones (e.g., 'gatekeeper' mutations) that we have to be able to find drugs hit that inhibit target genes even with gatekeeper mutation with a second class of drug.

If you could use combination therapy, where you hit tumors with inhibitors against the primary driver, and then with inhibitors that worked in in vitro models of resistance at the same time, you may prevent recurrence. I do not think that the problem of heterogeneity is insurmountable. If we can uncover the most common mechanism of resistance and treat appropriately, then we may get another year of survival—maybe not a cure, but another year. If you had a third drug, maybe you could add another year.

At MGH, our thoracic oncology team has performed numerous rebiopsies of lung cancer patients at recurrence post-targeted therapy. We have analyzed over 100 patients at the time of relapse. Interestingly, all tumors have maintained the primary driver mutation at relapse that had been identified in the original tumor. Half of the EGFR-mutant lung cancers develop a 'gatekeeper' mutation T790M that prevents the drug from accessing the EGFR kinase domain (there are drugs in development that can effectively target T790M). Ten percent of recurrent EGFR-mutant tumors have a fascinating phenotype of histology transformation, where a tumor that presented as an adenocarcinoma, recurs as a small cell carcinoma. These tumors can then be treated with small cell carcinoma regimens and so far they have responded. Importantly, they still have the original EGFR mutation. So the tumor cells have an ability to change from one morphology to another; it is unclear if there is an underlying genetic or epigenetic mechanism.

We've done a lot of re-biopsying of lung cancer patients, post–EGFR inhibitors. We probably looked at 50 to 100 patients that have gotten biopsies at the time of relapse. They all maintained the primary driver mutation at relapse that they originally had. You talk about a fossil mutation—that thing is still there, and it's still driving the tumor. Half of them develop a gatekeeper mutation that prevents the kinase domain from being accessible. Ten percent have this fascinating phenotype where they go from being a lung adenocarcinoma, histologically, to being a small-cell carcinoma ... at the time of recurrence. When we get a biopsy, it's small-cell carcinoma. They can then be treated with small-cell carcinoma therapy and respond to that. They still have the mutation. When they relapse following small cell, they recur as an adenocarcinoma, the original morphology. So the tumor cells have an ability to go from one morphology to another, which is probably at the epigenetic level—we don't know—but it seems to be able to mediate some cellular process, whether it's their ability to divide or something else that correlates with drug response.

SB: This is taking things in a different direction, but I thought I would mention the analogy with microbiology and how we treat infections and deal with drug resistance. So, for highly mutable viruses like HIV and HCV [hepatitis C virus], it's accepted: there are quasi-species. There are many. There will be. And you don't know them all. You won't know them all. You can learn about them, and we can understand viral evolution, but in a given patient, you just accept that they're there.

And you have to do combination therapy. So you study the three main pathways of resistance, and you apply drug pressure, simultaneously, to all of them. I think we really have to think about this paradigm because I don't think just classifying and subclassifying and subclassifying and trying to find single agents is the way to go.

AJI: I would agree. I think it is informative that even with a thousand or a million quasi-species in every patient with HIV, you can come at the virus with perhaps five or six drugs simultaneously and corner all of the quasi-species at the same time. This approach is naturally being thought about in cancer treatment.

SB: Another thing that clinical microbiology has that we don't have in oncology is the ability to sample cells, study drug resistance rapidly in a clinical lab and prescribe the right drug in very short order. I think this is something the oncology community has tried. We've made some progress in models where we grow tumors orthotopically in mice—so-called 'biobanking'. We have to ask ourselves, are there tests we can do to empirically predict drug sensitivity? Is there a set of single-cell proliferation tests that we could do using cells from a fine-needle aspirate or circulating tumor cells? It's time to revisit some of these ideas now that we can access patients' samples in many different ways.

AJI: The trouble is, I don't think the models and assay systems for prediction are good enough yet—there are companies that offer such testing, but to date there is questionable utility. I think you're right that if it is done carefully and correctly it could be very meaningful.

JVF: Every time this comes up in one of my faculty meetings, it is just shot down. And for good reason, because as soon as you take the tumor out of the microenvironment, it's not the tumor anymore.

SB: But maybe that's an engineering problem. Maybe we can make smarter synthetic scaffolds and niches, or maybe we can build better mouse models?

KP: People have made progress building scaffolds that mimic hypoxic environments. It has the advantage of avoiding the problem in mice that if you inject tumors into the animal, you're selecting for tumor cells that will grow in mice. And I can tell you, when we compare the primary tumor with a xenograft, it's never going to be 100% identical because you're selecting for a clone or a mixture of clones.

Is the way forward combinations of molecularly targeted drugs addressing different signal transduction pathways?

JVF: I'm a bit of a heretic on that. I trained with Lew Cantley [at Harvard], so I should be the one championing signal transduction pathways. But Lew and I differ on this. I'm not a believer that knowing every signal transduction pathway and targeting every signal transduction pathway is going to treat metastatic cancer, because of heterogeneity and because of the fact that these are redundant pathways. We're not going to kill the cancer from the inside out by knowing every signal transduction pathway because it's essentially a cell-phone network. It's distributed. Knocking out a tower or two isn't going to do it. Instead we need to find therapies that differentiate more specifically between normal and cancerous cells.

AJI: I am more optimistic for the promise of targeted therapy because I don't think we've

finished exhausting all the avenues. We still have more targets and more drugs to try, as well as combinations. For example, we don't have a drug against mutant KRAS, despite years of hard work. And it's not a problem with genotyping or of heterogeneity. It's a problem of chemistry and drug development. No one has developed a KRAS inhibitor because thus far it has been difficult to drug. If a KRAS drug was developed, the outlook for many cancers, including most pancreatic, and many lung and colon cancers, would be greatly improved.

What other approaches to treatment are on the horizon?

SB: As a technologist thinking beyond the existing drugs in our armamentarium, one question to ask is, how can we take advantage of the heterogeneity? If it's true that we don't have to worry about the bulk of the tumor, and perhaps there are distinct tumor-initiating cells, how can we get to those? What would we deliver and how? Should it be a targeted therapy or a cytotoxic therapy? Nanotechnologies that can localize a therapeutic payload to cell populations of interest are showing promise in preclinical models and entering clinical trials. This type of selective homing is another kind of 'targeting'. There are nice examples of targeting just the immune cells, just the endothelium or just marker-positive tumor cells. The payloads can be small-molecule drugs—including those that may have been sidelined due to unacceptable systemic toxicity—interfering RNAs, or even combinations of therapeutics and diagnostics, so-called 'theranostics'. In addition, there is heterogeneity in the microarchitecture of tumors. Tumor parenchymal cells near the endothelium can be accessed preferentially through leaky vasculature; however, we still can't deliver any cargo homogenously throughout solid tumors, as far as I can see. There are technologies being developed to improve tumor penetration and vascular leakiness by exploiting natural trafficking mechanisms. People are also now making measurements to show that the mechanics of the tumor microenvironment are different than healthy tissues. Whether you can exploit the differences in mechanics or not remains to be seen. Thus, there are all kinds of physicochemical properties of tumor cells and their microenvironment that differ from healthy cells and tissues that are poised to be exploited.

AJI: We were talking about tumor-specific, unusual microenvironments, and I think a major component of the tumor microenvironment that continues to receive intense study is hypoxia. The degree of hypoxia present in many malignancies is not something that most normal cells are exposed to commonly, and so this could be exploited therapeutically. Many tumors have substantial necrosis, the end result of severe tissue hypoxia. If you follow markers of hypoxia, such as *GLUT_1* expression, one observes high level expression around the areas of necrosis and little to no expression in oxygenated areas (e.g., around the blood vessels). So within a tumor there's an incredible heterogeneity of hypoxia-related genes at the expression level, some of which are good drug targets. One challenge with this is how do you deliver agents into the hypoxic region, which is difficult to perfuse.

RMH: I agree. We need to be able to target cells that are hypoxic and non-dividing; most of these cells are not susceptible to our present drug arsenal that act against dividing cells. For me, the main heterogeneity you see in the tumor is whether a cell is cycling or not. And we need to find agents that target the non-cycling cells, especially the hypoxic ones.

KP: Another general property we've known for a long time is that DNA is always globally hypomethylated in tumor cells compared with normal cells. So maybe that DNA hypomethylation releases a lot of steps genetically. It may then continue to generate instability. If you hypermethylate the genome, the prediction is that you start expressing things that you would normally not express. There's been a fair amount of work developing DNA methyltransferase inhibitors [5-azacytidine and 5-aza-2′-deoxycytidine are approved by the US Food and Drug Administration (FDA)], as well as agents that target histone-modifying enzymes, so I think there is more to be done there.

RH: I think every cancer cell that we've ever looked at has altered methylation. What happens in the cancer cell is that they all seem to require excess methionine, they're methionine dependent, and if you remove methionine they arrest in S, or late S, or G2. This may be a universal, and probably if you use a methionine-degrading enzyme, you can arrest the cancer cells in S or late S. I think that we have to find features that are general to cancer. That's the way to overcome heterogeneity. One such feature might be methionine dependence for therapy, or excess glycolysis may be another general feature of cancer cells.

What about the nightmare scenario where tumor heterogeneity in a patient will necessitate a battery of personalized therapies?

JVF: From my standpoint, every organ, every tissue, every microenvironment has a different selection pressure. So maybe all this heterogeneity we're seeing is a red herring. Maybe we're seeing it because we have all of these microselection pressures, so naturally, even in a single tumor in a single location, we're going to see heterogeneity, and maybe none of it matters.

Some people have suggested you can target a small number of cancer stem cells. If it's true that it's really that small number of cells that matter and everything else is just a reflection of local environment, then we're in trouble, because we're not going to treat that and we're not going to image that. The best imaging technology we have today for whole-body imaging is not going to get us to that level, which is going to be on the order of maybe 1,000 cells per cubic centimeter, which is beyond any technology we have now or on the horizon. So it's going to make that very difficult.

I worry because we already have learned things from the clinic like the fact that almost every carcinoma has a doubling time of 30 to 45 days. So what that means is that 99.99% of the cells are dying off. So it's one big selection pressure to double the volume of a tumor, you're talking about 30–45 days, and that's telling us that the bulk of the tumor, most of it, is sick. Most of it is undergoing selection pressure. And I still wonder whether all this heterogeneity is a big red herring and we're missing the small number of cells that really matter.

KP: If you have to develop drugs for every single mutation, that would be a nightmare. It's not going to be feasible, especially if every patient has a different combination of mutant clones. But I think if we understand which mutations track together, maybe we don't have to target every tumor clone because they may have codependencies on each other. For example, we know ER [estrogen receptor] is heterogeneous in breast cancer, and pathologists classify a tumor as ER positive if the fraction of cells expressing ER is above about 10%. And most breast tumors that are ER positive respond well to endocrine therapy. So this could mean that the cells positive and negative for ER have some codependency or that in reality all cells are dependent on estrogens but the expression of ER fluctuates. Similarly, HER2 positive tumors have heterogeneity, even for HER2 copy number, and they generally still respond well to HER2-targeted therapy such as trastuzumab [Herceptin]. So my idea is to develop

drug combinations that target the key mutant clones together with their so-called community. I feel that current cancer treatments frequently drive selection for the most aggressive variants when you're hitting these tumors with these very strong pharmacological pressures.

I think what really matters depends on the context, because as you said, you have continuous selection. So whichever are the cells with the highest fitness levels recorded could depend on the selection at a particular stage. When in leukemia you follow the clones, you're interested in knowing which one is the subtype with the highest percentage of cells. It varies depending on your treatment. In contrast, when we study a solid tumor, we don't sample serially, we don't biopsy frequently. So I think heterogeneity matters in the sense that you have to look at a tumor from an evolutionary perspective. You will have continuous selection. And you know the doubling time—you're talking about doubling time from when the tumor was diagnosed, which is usually in people over 50. But probably the tumor started 10 or 20 years earlier. Like in breast cancer, we know that early life events such as exposure to radiation and full-term pregnancy influence the risk of postmenopausal breast cancer decades later. So there can be a very long latency. It's clear that there is a very good tumor-suppression mechanism in our body, because otherwise we would be seeing cancer much more frequently. So it works pretty well. And when it doesn't work, it's still—the tumor cells, initially those mutant cells, they're probably at a disadvantage compared with the normal cells because they don't have the highest fitness level in their microenvironment.

SB: I am also more positive. Sure, we're not going to have a drug for every target. There are undruggable targets, and there will continue to be undruggable targets. But we also have the cancer genome atlas, and that's giving us lists of genes that are interesting to prioritize. Bill Hahn's group at the Broad Institute has identified 54 genes that were amplified in ovarian tumors and essential for proliferation in a panel of ovarian cancer cell lines. So, OK, now you have 54 genes. The next logical thing to do for a given candidate is to put an inducible short hairpin RNA in a cell line and make a mouse model and see if it's important. But to do that 54 times for every single cancer cell subtype is untenable. In collaboration with Bill, the technology approach we took was to make an siRNA [short interfering RNA] delivery technology that was very modular—it allowed them to silence candidate oncogenes in mouse models without having to make a new cell line or mouse model for every target. Thus, I believe there are avenues developing where one can go from single-cell data or lists of genes through an *in vivo* 'filter' to prioritize which are the most important targets to develop drugs against.

What's the best approach for combination therapies?

RMH: I think we're going to place much more emphasis on trying new combination of drugs that are already out there. I'd like to see combinations where one drug targets the dividing cells and another will target non-dividing cells, and perhaps a third can push some of the non-dividing cells to start dividing and be sensitive to the drugs that target dividing cells. I think there's a lot of drugs out there, and maybe we just try a different kind of strategy and see if we can find some combinations that work better than existing ones.

KP: One problem is that we're still working out the best way to come up with combinations. A lot of drug combinations are simply put together through trial and error, or on intuition of the investigator, or because they make sense.

SB: In that respect, I think systems biology can help. You can now take a reasonably complete pathway model and try and find the key nodes for therapeutic intervention.

KP: Right, but that's still very early. Another issue is how to test combinations. Clinical trial design is very complicated because you don't know, when you start combining therapies, especially two or three drugs, whether there will be additive or synergistic toxicities. I think, going forward, clinical trial design will have to change because the standard approach of phase 1, phase 2 and phase 3 will not work. This is particularly true if we continue to develop drugs that are less toxic—things like immunotherapies. We should be able to skip some of those steps, because by the time you finish the trial you may lose most of the patients in some particularly aggressive fast-course diseases like glioblastoma and pancreatic cancer. I think that the whole concept of how oncology drugs are approved and how trials are run will have to change.

JVF: There's another problem with combination therapy, and that is the IP [intellectual property] legal problem. You have different therapies owned by different companies, and up until recently, nobody's been willing to work together. I think we could make enormous immediate progress if we could do more clinical trials with drugs from different companies. Until now, it's hardly been tried. There are very few examples because there are few market reasons why companies would put effort into letting this happen.

AJI: Keep in mind that numerous combination trials with targeted agents are underway and showing some success. One example is the BOLERO-2 [Breast Cancer Trials of Oral Everolimus 2] phase 3 study recently published by Jose Baselga at MGH [Massachusetts General Hospital]. That trial showed dramatic improvement in progression-free survival in women with metastatic breast cancer through a combination of the mTOR [mechanistic target of rapamycin] inhibitor everolimus [Afinitor; Novartis] with the hormonal therapy exemestane [Aromastin; Pfizer].

KP: There are also trials underway by Geoffrey Shapiro of experimental drugs like the PARP [poly(ADP-ribose) polymerase] inhibitor [veliparib; Abbott, Deerfield, IL, USA] combined with a CDK [cyclin-dependent kinase] inhibitor [dinaciclib; Merck, Whitehouse Station, NJ, USA] on patients with triple-negative cancers that retain BRCA activity. By depleting cells of CDK activity, BRCA function can be disrupted and the tumors sensitized to the PARP inhibitor. And there are other experimental therapies being combined [e.g., see *Nat. Biotechnol.* **28**, 765–766, 2010]. I think this is starting to happen.

JVF: The commercial reality is that somebody's going to have to realize that you can make a lot more money keeping people alive than letting them die. After all, that's what happened with Gleevec. Novartis tried to kill Gleevec several times because they weren't going to make money on a disease with 5,000 patients a year. And then, all of a sudden, they realized that they can make more money if the patients live.

And that's the only thing that's going to get companies to work together. It's going to have to be a market decision. They're going to have to say, "We can get orphan drug status for this in combination, and therefore we'll try it." Or they have to say, "Our other drugs are coming off patent. We need something. We better work together." I think it's going to be a profit-driven equation, and if the profits aren't there, it's not going to happen. I think there are probably enough drugs already in existence to find some effective regimens just by doing combinations. If we could magically have all these companies work together on all these different small-volume diseases they don't care about because market size isn't big enough, I think we would find something. But

at the same time, I don't think that the number of drugs hitting targets is nearly enough to do what we want to do.

Going forward, where do you see the biggest changes and challenges in cancer diagnostics and therapy?

KP: Counterintuitively, I think we'll see more progress in the worst types of tumors, like glioblastoma and pancreatic cancers. These are where I think we have the highest chance of doing trials, and of attracting companies, and of finding a smoother path through the regulatory agencies. You have to go after diseases that currently are not treated well. And I think for glioblastoma there is a lot of interest now in combining multiple receptor tyrosine kinase inhibitors, combining transforming growth factor–β inhibitors. Nothing really works in these cancers at the moment, and I think many drug companies are focusing on these diseases now.

JF: Cancer trials are incredibly expensive because of the effect size. When you have a 15% response rate, it's going to be a very expensive trial to show any benefit. At the same time, if you think about the approval of Gleevec, it was done with 78 patients in only about a couple of months. The total cost was just roughly about a couple million bucks. So I think that if you can get a higher effect size, the FDA can give approval and the trials cost less money. That is one reason why companies will be targeting cancers that currently have no effective treatments.

SB: I'd like to finish by emphasizing that cancer is not only a disease associated with advanced economies—it's a global disease. Seventy percent of new cases by 2020 are going to be in the developing world. Put that together with a healthcare and regulatory setting that may be wildly different in those settings. So we have to think about what that means for our current therapies and what that means for diagnostics. We have to consider how to monitor cancer without sophisticated imaging equipment and how to treat cancer without a sterile bed for chemotherapy infusion. Maybe the diagnostics need to be breath tests or urine tests. Maybe the drugs need to be oral. I think, then, one needs to think about these factors as drivers for future innovation. We have to start to think about how this is going to change the face of cancer treatment and diagnosis.