Liquid Biopsies in Oncology Drug and Device Development Part 2
Transcript: Session I, Cancer Liquid Biopsies: State of the Science

Pasi Janne:	Get started. On behalf of the American Association for Cancer Research, I want to thank you for joining us today. My name is Pasi Janne, a member of the AACR's regulatory science and policy subcommittee. The AACR is proud to be cosponsoring this workshop for the second straight year with the US Food and Drug Administration. Along with my AACR co-chair, Dr. Carlos Arteaga, we're thrilled to co-chair this workshop with doctors Julia Beaver, Gideon Blumenthal, and Reena Philip, from the FDA.
[00:00:30]	Liquid biopsies are an exciting technology and hold much promise for improving cancer diagnosis and monitoring, as well as drug development. The technology has unique regulatory concerns, particularly in establishing analytic and clinical validity. The goal of this year's workshop is to explore the regulatory challenges in adopting this technology for early detection, disease monitoring, and potential use as surrogate endpoint markers for drug development.
[00:01:00]	The workshop will have four sessions. The first session will be the state of the art of the various technologies. Second session will explore using liquid biopsies for early diagnosis or residual disease detection. The third session will examine liquid biopsies in cancer drug development and clinical use. Finally, the fourth session will examine the regulatory considerations for development of these assays.
[00:01:30]	One of the hallmarks of the workshop is the interdisciplinary forum, with a wide range of perspectives from stakeholders, from academia, government, as well as industry. I'm happy to report that as of last night we have over 500 people registered for the workshop. In addition to the over 200 people registered to attend the workshop here in DC, there's almost 300 people watching this workshop. We have a live webcast. We'd like to express a note of appreciation to Amgen for their support of today's workshop, which is helping to defray some of the expenses associated with it, including allowing this workshop to be broadcast in real time. The AACR is continuously looking for opportunities to engage on important regulatory science issues, and the AACR looks forward to a continued, productive partnership with the FDA.
[00:02:00]	I want to again thank all of you for joining us today, and I will now turn it over to my fellow workshop co-chair, Dr. Gideon Blumenthal from the FDA, for additional opening remarks. Gideon.
Gideon Blumenthal:	Hey, thanks Pasi, and just wanted to piggyback on what you said. We're thrilled to be here for our second liquid biopsy and oncology workshop, back by popular demand. We had a lot of great interest from our first workshop, where we presented the case of the first approval of a liquid biopsy device, a plasma ctDNA EGFR test to detect EGFR mutations in metastatic non-small cell lung cancer. With this workshop we want to continue the discussion, also focusing on early
[00:02:30]	

[00:03:00] stage disease. We have a great session for that. We also want to talk about novel regulatory issues, including using these innovative technologies as potential surrogate endpoints as drug development tools for regulatory decision-making. This promises to be a great meeting. We want to keep this very interactive with the crowd, so hopefully there'll be a lot of time for robust discussion during the panel. For those of you listening via webcast, please send us your questions and we'll try to engage the panelists and speakers with your questions as well.

[00:03:30] With that, I'll turn it over to Julia Beaver for session one.

Julia Beaver: Thanks, Gideon. So, session one will be an update of the state of the science of liquid biopsies with talks on circulating cell-free tumor DNA, exosomes, and circulating tumor cells.

[00:00:30] These talks will go into updates on each biomarker, techniques to detect current uses and future directions. Each talk will be approximately 20 minutes. Followed by 5 minutes of questions from the audience to each of the consecutive speakers. We are lucky to have three expert speakers in their fields, who I will now introduce collectively.

[00:01:00] The first talk will be on new developments and analysis of circulating tumor DNA given my Dr. Max Diehn. Dr. Diehn is an Assistant Professor of Radiation Oncology at Stanford University, where his research is focused on thoracic malignancies and development and application of methods for detection of circulating tumor DNA.

The next talk will be on strategy to exploit the biology of exosomes for diagnosis and treatment of cancer; given by Dr. Raghu Kalluri. Dr. Kalluri is a Professor and Chairman of the Department of Cancer Biology and the Director of the Metastasis Research Center at MD Anderson Cancer Center. His research interests include the study of cell tissue micro-environment and it's impact on cancer progression and metastasis.

[00:01:30]

The last talk of the session will be given by Dr. Daniel Haber on new strategies for quantification of circulating tumor cells. Dr. Haber is Director of the Massachusets General Hospital Cancer Center, and the Kurt J. Isselbacher at Harvard Medical School Professor of Oncology at Harvard Medical School. His research has focused on the area of cancer genetics and development of novel technologies for quantifying and purifying circulating tumor cells.

[00:02:00]

Thank you for coming and I will turn the podium over to Dr. Diehn.

Max Diehn: Great. Well. Thank you very much, pleasure to be here today to speak to you about circulating tumor DNA and some of our work. It's a little bit early for a west coast person, it's about 5:45 in the morning, so if I fall asleep someone kick me please. Here are my disclosures. Today I'll cover a few items in this 20 minute talk. First is a brief background on circulating tumor DNA, which I assume the

[00:00:30] vast majority here and online are familiar with. Then, briefly talk about how we are already using this biomarker in the clinic currently, with a focus on lung cancer, which is what I specialize in clinically as well as what my research area is. Then kind of spend most time on three emerging applications or future directions related to detection of heterogeneity of tri-cul resistance mutations using circulating tumor DNA, minimal resistance disease detection, and early detection.

[00:01:00] Circling tumor DNA of course refers to small fragments of tumor DNA that originate in tumors that are found in the circulation of cancer patients. We all have circulating cell free DNA in our plasma, but in patients that don't have cancer that comes of course from the healthy cells that are turning over every day whereas in patients who have cancer, there is some DNA from the tumor as

[00:01:30] well as a large background of DNA from their healthy cells that are dying each day. We can isolate this anolyte through routine blood draws and then analyze it in a variety of ways. There's been a lot of interest in this area in the last five or so years. We've known about this biomarker for at least 60 years but the techniques needed to sensitively and specifically detect it have not been widely available until the last five or so years. That's really what's led to the explosion of interest in this field.

[00:02:00] There's a number of reasons why circulating tumor DNA is a very attractive biomarker. I list three main ones here. The first is that one I will be focusing on today is measuring mutations, somatic alterations, that we can find in circulating DNA that of course, come from the cancer genomes and that are very, very specific to those cancer cells. Really no other cancer cells in that patient's body will have those mutations and so we have a biomarker that's quite specific.

[00:02:30] Different than most protein-based biomarkers, which of course are also made by normal cells, usually. The second is, and very important from a biology standpoint, is that mutations are very important in the pathogenesis of the disease, if you were gonna design a biomarker from the ground up it makes sense to pick something that is really linked to the disease. Then, lastly and very importantly, some of the mutations are actionable and that means not only can we use this biomarker to track how much cancer a patient has in their body, but also potentially use the information we get from these assays to determine what the best treatment might be.

[00:03:00] One of the exciting aspects of circulating tumor DNA, but also one of the challenges is that it's really applicable to any part of a patient's disease trajectory. On this graph, here, I show on the Y axis tumor burden of a idealized patient and on the ... as the patient goes through time, the disease burden goes up or down, so in this example this patient has some sort of pre-diagnostic

[00:03:30] phase, then tumor grows, has some local treatment that has a response, recurs, gets systemic treatment and recurs again. We'll use this to just talk about where we might imagine using circulating tumor DNA in the clinic. The first and very active area of research and interest is, of course, early detection or screening where one might be able to use this biomarker to identify patients who are

asymptomatic or not known to have cancer, who then could have subsequent, other tests.

[00:04:00] The thing we're already doing routinely in the clinic, every day, is non-invasive tumor genotyping meaning identifying the genotype of the tumor from the plasma. I will talk about that. If a patient gets a local therapy we could imagine using this biomarker to attract response because if a treatment is working, it should decrease the amount of circulating tumor DNA. In a very exciting area of research is the idea of minimal residual diseases is trying to determine if patients have had local therapy, whether or not they still have microscopic disease in their body or not and we'll talk more about that. We could envision using it in

[00:04:30] surveillance, where we currently generally use imaging in most cancers but maybe someday we could do this all using circulating tumor DNA. Then if a patient recurs with systemic disease, one could again, use circulating tumor DNA to measure systemic treatment response. Then, very importantly, if one is on a targeted agent, we can try to look for resistance mutations in the plasma, which often can be found long before a patient actually has a clinical recurrence.

[00:05:00] That's all the exciting stuff, but of course, there remains significant challenges. One of the most important challenges is that there isn't very much of this DNA, total, meaning the combination of the germ-line DNA and the tumor DNA is the concentration of this is really, generally quite low. Usually only a few nanograms per milliliter in patients with early-stage disease or who are in remission. That means one needs assays that are very efficient, we can't be losing molecules and

[00:05:30] we need assays that work on nanogram input DNA amounts. Now, that's compounded by the fact that the fraction of this small amount of DNA that's from the tumor is usually very small.

Even in advanced patients, it may be in the single digit percents, but in patients who are responding to treatment or who are with early-stage disease, or in remission, the levels are often well below 1%, often even below .01%. We have a very small partial abundance that we have to be able to measure in a small

[00:06:00] number of molecules to begin with and that creates major analytical challenges. Lastly, and importantly, what we're tracking is mutations. Of course every patient has a unique set of mutations in their tumor. No two tumors are identical. Ideally, one would develop assays that off the shelf can work on any patient with a given disease but to do that you can't just track a single mutation because, let's say a K-ras mutation is only present in a sub-set of patients with lung cancer so you can't use one assay on all patients.

[00:06:30] Now, how do we measure this biomarker? There are a vast array of technologies that have been applied and I list some of the main ones, here, on the left hand side. On the right hand side is their approximate detection limit in a ten-milliliter blood draw. It's always very important to think about the sensitivities of the assays in the clinical context in a blood draw rather than in the idealized laboratory environment where one could have micrograms of DNA. In the early

[00:07:00] days, the field was trying to apply some of the existing methods like sangro-sequencing, or pyro-sequencing, which unfortunately were not sensitive enough

[00:07:30] since they can only get down to about 10% detection limits and as I already mentioned, that's actually well above what even many advanced patients have in their blood. So, initially, there was not that much progress in the field, but then really due in large part to a development of a variety of PCR-based approaches such as a LIA specific PCR and more recently, digital PCR. These PRC-based methods have sensitivities that are well within the range of what is clinically useful, say below .1%.

[00:08:00] More recently, there's been a growing interest in applying next generation sequencing to detection of circulating tumor DNA. That can be done in very broad ways, such as whole exome sequencing and whole genome sequencing, and these have been demonstrated to be useful, but in a research context, but their detection limits at current costs are not good enough to really make them useful, clinically. However, below this line were the clinically useful sensitivities are and that includes assays that would targeted next-gen sequencing where one tracks one mutation so this is sort of analogous to what one might do with a digital PCR assay and that can get down to the range of similar to digital PCR, .05%. Then, one of the advantages of [inaudible 00:08:12] sequencing is that we can track multiple mutations at a time.

[00:08:30] So, if one does target NGS, usually with a capture-based approach and tracks multiple mutations per patient, one can get to even lower sensitivities, .02%. Then if one actually personalizes a capture-based NGS assay to a patient's tumor and tracks hundreds or thousands of mutations, in parallel, one can get down to these very, very low percentages. We've shown as low as 2.5 in a million. There's this large menu of assays one could choose and in general, the more sensitive the better, however, of course cost is an issue and there are situations where one might chose a less expensive assay because if one only cares about, let's say, one mutation, then there's no need to sequence a large panel of genes.

[00:09:00] With that background, then, next we'll speak where circulating tumor DNA is in the clinic today, and we'll use non-small cell lung cancer as the example for this since this really is the poster child, I think, for how we are using and will be using liquid biopsies in the clinic. Circulating tumor DNA assays are being used routinely, clinically for non-small cell lung cancer patients. That includes both
[00:09:30] FDA approved assays, which I mentioned briefly, as well as a large number of clear laboratory based commercial assays that are on the market. Really, in lung cancer, sort of the standard of care, we're using it in two main applications. One could use it at diagnoses, in patients who can't have biopsies or where the biopsy tissue is used up. In order to try to identify advanced patients who have actionable mutations such as EGFR mutations. Secondly, and importantly, one could use it at the time of progression on target therapy to look for resistance
[00:10:00] mutations that could make patients candidates for additional tyrosine kinase inhibitors, such as the EGFR, T790m mutation.

The first FDA approved assay DNA test, which was discussed at last years' workshop, of course, was the Roche Cobas EGFR v2 assay, which is an assay for detecting EGFR mutations in the plasma for the PMA. The investigators analyzed

[00:10:30] samples from the insurer trial, which was a trial of EGFR-positive patients that had to have been biopsy proven positive to enter this trial by the tumor. Who are on either [alubnib 00:10:28] or chemotherapy. That trial found that patients who had EGFR activated mutations had significantly better progression-free survival than patients who got the chemotherapy. For the PMA, plasma samples for this trial were retrospectively analyzed and in 77% of them, EGFR activating mutations were detected, remember, they all had EGFR mutations found in the tumor.

[00:11:00] Based on that, the analysis could be done retrospectively, again, to compare outcome of patients who got alubnib versus who got the chemotherapy. You see that it shows very, very similar results of tumor biopsy. This was one of the main pieces of evidence used to get approval of this marker as allowing a plasma-based test to select patients for therapy. Importantly, the sensitivity is not 100%. In general, we always still prefer biopsy first approach, of course in the diagnostic setting where also we need to make the diagnosis histologically.

[00:11:30] The second approved FDA test also was in the EGFR space for lung cancer and this was not focusing on what happens when patients have received their first generation tyrosine kinase inhibitor and progress. About 56% of the time, those patients of course, get another mutation of EGFR at position T-790 that causes resistance to the first generation inhibitors, the first-line agents, and recently

[00:12:00] then, another inhibitor that targets T790m was FDA approved and this drug for the initial approval required the patient to have evidence of T790m. Was approved with a companion diagnostic, again Cobas, to look for T790m in the plasma. The availability of this assay has led to a paradigm, that was as represented by a number of investigators including [Jeff Oxnard 00:12:21] and

[00:12:30] [Dr. Yanni 00:12:23], where if patients with EGFR mutations acquire resistance to their first line TKI, one can first do a blood test, and look for this T790m mutation. If that's positive, then skip biopsy and go directly to Osimertinib.

However, because of the imperfect sensitivity of theses assay, if T790m is negative in the plasma, then one should try to pursue a biopsy to give patients still a chance to potentially get this drug. That is how we are currently using CT DNA, every day in the clinic and advanced non-small cell lung cancer patients

[00:13:00] already and I think this is a paradigm that we will use in many other tumors as well.

In the meantime, I'll focus on some new directions. The first is detection of heterogeneity of treatment resistance mechanisms, still in this EGFR mutant lung cancer space. As I already mentioned, about half the time when patients become resistant to the first line agents, they get this T790m mutation, but the other half of the time they don't have it. There's a small scattering of patients who have

[00:13:30] multiple resistance mechanisms, meaning, T790m plus something else that were found in studies using biopsies of patients at progression. Now, because biopsies can only look at a single lesion, we hypothesize that it's likely that actually more patients that have multiple resistance mechanisms at the time of progression but

that this was under-recognized because the fact that we were looking at biopsies previously.

[00:14:00]
[00:14:30]
So, therefore, we hypothesized that if we were to look at blood, we could see more multiple resistance mechanisms in the same patient, so intra-patient heterogeneity of resistance mechanisms and very importantly, from a clinical standpoint that if patients have multiple resistance mechanisms, they shouldn't respond as well to an inhibitor of T790m, because you're not blocking the other resistance mechanisms. To this, we applied the next-generation sequencing based in the 80% gene method that we developed called [Capsink 00:14:18] which is a capture based sequencing approach. Which we specialize to the disease of interest, we cover usually about 300 hundred genes, ultra-deep sequencing, we can discover all the major classes of mutations, and we have a high analytic sensitivity where we care about a single position, similar to digital PCR, but then because we can track multiple mutations or personalize, we can get these very high sensitivities for the presence of circulating tumor DNA. The assay is sensitive such that we can use it directly on the plasma in advanced patients and don't have to use tumor tissue.

[00:15:00]
[00:15:30]
We applied this method to a core of 43 patients who had progressed on first line EGFR TKI's and who had T790m detected by biopsy in their tumors. These patients were enrolled on a clinical trial that required that. These patients then went on the be treated with a third generation inhibitor called rocelitinib, which did not end up going forward for FDA approval, but, which had a similar approach as the approved osimertinib. Then we analyzed the baseline samples. The samp;es before they got this third generation inhibitor. And, found that about half of the patients did indeed have multiple resistance mechanism present in their blood, either T790m plus another point mutation or T790m plus a copy number alteration. These included things like mutation of the [PIK3CA 00:15:30], or copy number alterations of other genes like [Her2 00:15:35], EGFR, and MET.

[00:16:00]
[00:16:30]
We could then ask, if patients have multiple resistance mechanisms, how do they do compared to patients who only have T790m? What we found is that patients who had multiple mechanisms here on the red, this being a co-hort that had both T790m and MET alterations, had significantly less tumor shrinkage than patients who had only T790m. This also translated to a significant difference in progression based survival with patients who had multiple resistance mechanisms having more CPFS. Rocelitinib is not as active a drug as osimertinib, and so we would anticipate that the split between these [inaudible 00:16:13] would be larger potentially in patients receiving osimertinib. I think it's a nice example of how one can use circulating tumor DNA and the ability to interrogate multiple [inaudible 00:16:25] deposits to identify what could potentially be clinically relevant heterogeneity in treatment resistance mechanisms. This of course, leads to the question of whether personalizing treatment for patients of multiple resistance mechanisms potentially treating them with multiple inhibitors for both of their resistance mechanisms and if they have two, could potentially improve outcomes. That's something that is being tested in trials.

[00:17:00]

[00:17:30]

Next, I want to talk about minimal residual disease detection. Minimal residual disease of course refers to a state in patients who have localized disease and who have been treated with curative intent. Where we can't see any disease left on scans but there's still disease left in the body. Microscopic disease. There are no reliable MRD assays for most solid tumors, there are some for hemophalgic malignancies and there they are very useful and integrated into treatment paradigms for some of the liquid tumors. In the last couple of years there's been several papers suggesting that minimal residual disease can be detected using circulating tumor DNA, it was initially demonstrated in breast cancer by [Nick Turner's 00:17:31] group who you'll be hearing from later. As well as [Ben Rogstens 00:17:36] group for colon cancer. In both cases, patients who are CT DNA negative had significantly better outcomes than patients who are CT DNA positive.

[00:18:00]

[00:18:30]

We set out to test this for lung cancer in a prospective study where we enrolled 41 patients who were treated either with radiation or surgery. All stage one through three disease, blood was collected before treatment, as well as at the first follow-up time point, which was about three months after treatment. Circulating tumor DNA was analyzed using Capsink. Here are just the treatment characteristics, about 2/3 of the patients were stage three, but a 1/3 of them were stage one, too. Then we could ask how do patients do if we can detect circulating tumor DNA after completion of their local therapy, and what we found is that patients who had detectable circulating tumor DNA were at very high risk of failure. Every patient where we could detect circulating tumor DNA after treatment ultimately reoccurred. Whereas only one patient where we did not detect circulating tumor DNA after treatment recurred. This was true for freedom from progression, but also translated to his disease-specific survival and overall survival.

[00:19:00]

[00:19:30]

We could then also look at this in a sub-set analysis of just patients with stage one, two diseases, these are the known negative patients. The earliest stage patients. Here also, the results were similar where patients in this co-hort who had no detectable circulating tumor DNA had very good outcomes, whereas patients who had detectable circulating tumor DNA had ... basically all recurred. This suggests, I think, adds to the growing body of evidence that using circulating tumor DNA for minimal residual disease detection is going to be likely to be able to show clinical utility if we can show that we can personalize treatment by, for example, avoiding [adumen 00:19:21] therapy in these patients and giving adumen therapy or even escalating adumen therapy in these patients and maybe even tracking how that adumen therapy is working by looking at what it's doing to the levels of residual circulating tumor DNA. These ideas will need to be tested in prospective trials.

Lastly, I'll just say a few things about early detection. Early detection, of course, is a very exciting area for application of circulating tumor DNA, the reason is obvious if we can detect tumors earlier, they can be cured by surgery or radiation. This is true ergo likely across cancers where patients with early stage

[00:20:00]   treatment have much better survival than patients with late stage disease and circulating tumor DNA is attractive for potential application in this area because of course, it's non-invasive. It has this very high specificity, which is required for good screening assays, and it doesn't require ionizing radiation from scans, which is also attractive.

[00:20:30]   There is a growing body of evidence in the literature that this is something that might be feasible. It's clear that early stage tumors can shed circulating tumor DNA, it's been shown in a number of studies. However, the levels are extremely low, the median in stage one patients when we look across studies is generally below .01%, and much less than one copy of one cancer genome per milliliter and if you look at sensitivities of assays that have been applied to this question, they generally range from less than 10% to about 50% for stage one tumors. Here I show some data from the Hopkins group looking at stage one patients where they had about a 40% sensitivity with single mutation tracking by next-gen sequencing.

[00:21:00]   Those are the positives, there are some major limitations of the existing data in this area, however. I list some key ones here. First of all, mostly groups have analyzed larger early stage tumors so they're the very, very biggest tumors that one might classify as early stage. Also, generally these papers don't give all the clinical information you might want on disease burden and volume to really get a sense for that, but that's one of the biases of literature. The other and another important one is that controls are generally not matched for risk factors. So, for
[00:21:30]   example, for lung cancer, you'd really want to look at controls that are heavy smokers and that generally has not been done. Also, the vast majority of studies have done analysis with prior knowledge of mutations, so they already had the tumor, they knew what mutations to look for, and went looking for those mutations. That, of course, is not how you could do screening. It's more of really for proof of principal.

[00:22:00]   Then, and this is a very important technical thing to think about when you're evaluating studies in this area is, how is the controls and the number of studies were not analyzed similarly as the cases, for example, they were not sequenced as deeply sometimes, which of course, then biases the result in favor of detecting mutations in cancer patients. Lastly and very importantly, these are all still proof of concepts studies. Likely are overfit because they don't have true validation sets and I list a number of publications here including ones from our lab and so this is still a general issue in this field that really, we're still at the proof of concept stage and need larger studies.

[00:22:30]   There is some data that this could work. This is data from pre-natal testing analyses where women were screened for a down's syndrome and other chromosomal abnormalities in their fetus and in a small number of patients, cancer was found. Meaning, based on these whole genome sequencing approaches, the cancers that were found are listed here and I apologize they're too small to see, but basically, it's stage three and four cancers. A few solid tumors and then mostly, tumors that are in the blood, like Hodgkin's, non-

[00:23:00] Hodgkin's Lymphoma and Leukemia. This is an example where actually it was done prospectively, the controls and the patients are the same, this would suggest this could be done, the screening, but with this approach, we're catching just advanced patients and that is not what we want ultimately.

[00:23:30] Let me just finish up here by saying that circulating tumor DNA analysis has numerous potential clinical applications. It's already being used clinically in many situations. I think the use will just continue to increase. As there are many potential future applications, I highlighted three ones that are currently being heavily investigated but there are of course others. Very importantly, what we really need now is perspective clinical trials that really get at the question of clinical utility. Do these assays help us improve outcomes for patients in a way that is cost effective? With that, I'd like to acknowledge this is the work of a large team and thank our sources and out patients. Thank you.

Julia Beaver: [00:24:00] Okay, so we'll open it up to audience questions. If there are ... we'll ask you to use the microphones for the webcast listeners. We go to podium two.

Speaker 1: Thank you. For the [Capsink 00:24:09] what was the concordance of the mutation detection that you have seen in the liquid biopsies compared to the solid biopsies? 70%, 80%?

Max Diehn: In the tumors, yes. In concordance, we've looked at, which has basically been in mostly advanced patients, it's well above those and above in the 90% plus range. That concordance, and that's consistent in the size of the field, it's ranged from 70 to 90 plus percent. There's several things that affect that. One is the sensitivity of the assay, the more sensitive, likely the higher the concordance. The other is the disease burden, so very early stage patients, in a given tube of blood, you won't expect to see every mutation of that patient's cancer because you just don't have enough DNA to see it. Whereas in advanced patients, generally, you do have high enough levels that you would expect to see every mutation.

[00:24:30]

Speaker 1: [00:25:00] The [capsink 00:24:55] method that you have used, how similar it is to the [inaudible 00:24:58] method that was published by John Hopkins group earlier this year?

Max Diehn: Right. That approach is also a capture-based bar-coded approach, which is exactly what Capsink is, so it is similar in that regard. The details of how the bar coding is done and the informatics are different, slightly, so its they're the same category. I would say they're the same overall category.

Speaker 1: Would you say [inaudible 00:25:17]?

Max Diehn: [00:25:30] We are doing some experiments to analyze that. It looks like [Capsink 00:25:25] is more sensitive when we apply it similarly, but we are generating data on that exact question.

Speaker 1:   Thank you.

Julia Beaver: We will take question, podium one?

Speaker 2:   Max [inaudible 00:25:37], I'm sorry. Help me get around this. Am I right now, in view of the [inaudible 00:25:47] data, the T-790, we're being misled that it's not

[00:26:00]   a specific target. It really, what it's done, is enrich the patient's who haven't really gotten resistance to a better drug. What we may have been doing by this approach you put up is that 30% that you had in your circle of unknown mechanisms, because we know ... we knew from the very get-go ... the recurrent

[00:26:30]   patients, about T-790 also responded. We may have been denying those patients the opportunity to see if they respond? The ones who don't have MET, don't have small cell, don't have amplification.

Max Diehn: Sure. I think what you're asking about the front-line data for [Osamercnib 00:26:41] showing that it's very effective, is that what you're getting-

Speaker 2:   Yes. Mm-hmm (affirmative)-

Max Diehn: ... at, yes. Yeah, okay-

Speaker 2:   ... it's clearly not, T-790 is clearly not the target.

Max Diehn: I think that's a complicated question because [Osamercnib 00:26:51] resists T-790 inhibitors, extremely potent inhibitor of the activating mutation as well.

[00:27:00]   Likely the result in the front line is in large part due to the strong activity of that drug on the activating mutation. Of course, not allowing the T790m mutation to arise. I think you're right, that does change the landscape. There will be more and more patients that will get [Osamercnib 00:27:11] front line and therefore likely won't get T790m, but they will get other resistance mechanisms such as C-797s, which is another point mutation you can find in the blood and likely we will be testing for that once we have inhibitors to that mutation.

[00:27:30]
Speaker 2:   What I'm saying is, the test is erroneous. It was meant to be marketed and accepted because it identified the target when it's really just the EGF-receptor and it's a better EGF drug-

Max Diehn: Yeah, I think-

Speaker 2:   [crosstalk 00:27:38] second [alumibid 00:27:39].

Max Diehn: Your question's a little more on the clinical side, and I'm happy to discuss that with you later. I think I can explain that to you, but let's do that in the one on one so that other people can ask questions.

Julia Beaver: Let's go to, last question, from Dr. Arteaga.

Carlos Arteaga:
[00:28:00]

Thank you for your very comprehensive talk. A point of clarification about your prenatal testing. I think the number of tests was 3% that were positive-

Max Diehn:

That was overall.

Carlos Arteaga:

Overall, but the number of patients with cancer was .0000 something, that raises question about the specificity of the test, can you comment on that?

Max Diehn:

Oh, I see. Yes. So that positivity was positive for Down's syndrome or other ... that included all those patients who had ... that the test was designed to detect abnormalities in the fetus. The positive test result was for that, the vast majority of patients who were positive had those abnormalities in the fetus, but a small sub-set of those patients actually had cancer. Does that make sense? Yeah.

[00:28:30]
Julia Beaver:

Great. Thank you so much, so we'll move to our next speaker.

Raghu Kalluri:
[00:00:30]

Thank you very much. I'm honored to be here, and I thank the organizers for thinking of exosomes as also a topic of discussion. The field really, as far as diagnostics, really came about in 2014, so it's a very young field. Lots of new work is just coming out. The whole area of exosome itself, I compare it to how immunology field was in the 1950s. The only difference is that we have millions of journals now, so we publish a lot, but that doesn't mean that the knowledge is increasing at any level that you would expect.

[00:01:00]

I think that this is a young field, so it would be great to see what your thoughts are. I have some disclosures to let you know. I'm involved with a company called Kodiak, which is developing some of these ideas into potential diagnostics.

[00:01:30]

Exosomes are these nanovesicles that are released by all cells. They have a lipid bilayer, and they're generated by all cells in the human body. There hasn't been a cell type identified that do no generate exosomes, including lower organism cells that can do this. The physiological role of these exosomes is unknown, so why do cells generate it? In fact, we can have about quadrillion of these vesicles floating in our blood, so that's a large number of these vesicles floating, and the question is, if we didn't have them, would we be normal human beings, etc. These are things that are really unknown.

[00:02:00]

There's some speculations that exosomes serve as a way to get rid of cellular constituents that are in excess. There is some evidence for that, but this is being challenged now. There seems to be some mechanism to specifically put out some constituents by the cells.

If you look at the size of exosomes, generally they're about 100 nanometers in size on average, so they're a size of viruses. This is what's floating in our blood as in relation to size of a cell here.

[00:02:30] If you were to think about exosomes, as I mentioned, and think of the biogenesis, just like thinking about lymphocyte in the 1950s. We are now collecting all of these exosomes through technology that is available in the range of 40 nanometers to about 180 nanometers, but there could be heterogeneity as shown in the schematics. Several different sizes of exosomes can be released. Now there's functionality to it. Would they have different content in them? All of these things have to be unraveled when new technology comes in using nanofacs, etc., because right now the technology is such that you cannot really differentiate some of these exosomes. I think when you can do that, then you'll identify new surface markers on them, etc.

[00:03:00]
The biogenesis, in general for exosome, is considered to involve endosomal pathway, somewhat similar to lysosomal pathway, and there are some proteins that have been identified that are involved in it, especially there's some evidence for RAP27, but generally, as you know in biology, when we don't know much, we draw schematics, and they become dogmas. In exosome, that is pretty much what happened. Everybody believes that this is the truth, but again, there's
[00:03:30] hardly any evidence that this is the only way that exosomes can be generated, let alone if this is the pathway. I think more and more work needs to be done to unravel various ways exosomes can be generated, and based on that, what their function heterogeneity might be.

If you were to look at all the proteomic data that people have collected, exosomes coming from different cell source, from dendritic cells to fibroblasts, epithelial cells, and put it all together, pretty much every constituent protein constituting that cell has been found in the exosome now, at one time or the
[00:04:00] other. It depends on who did the analysis and what cell type was used. It looks like everything in a cell can get out, including now, as I'll show you, all types of nucleic acid, lipids, metabolites, etc. Pretty much everything can get out into the exosomes.

[00:04:30]
Our laboratory began this work about in 2010, and we showed that there's functional mRNA present in the exosomes, and that this functional mRNA can be transferred to the recipient cells through entry into the cells and make a functional protein. This is also led to the identification. You could do a simple expression profiling between exosome from healthy individual versus a cancer individual and see a tremendous difference in those profiles. I think expression profiling is one advantage of exosomes, along with other things I'll show you.

[00:05:00]
Many others had already shown this. We also contributed to the idea that microRNAs are present in large amounts in the exosomes, and there's a difference between cancer exosomes versus normal exosomes, or what type of microRNAs it contains. We identified that double-stranded DNA's present, which was validated by many other groups immediately, that the double-stranded genomic DNA can be identified, and I'll show you some data here. And that there could be some markers on the surface of exosomes coming from cancer cells

that are not as enriched by the normal cell derived exosomes. That could be used as a way for pulling of ... using facts to identify them.

[00:05:30]   Now with respect to microRNAs, we identified that they actually have a capacity to perform microRNA biogenesis, but what was important is that we identified [isomer 00:05:35] 10B and 21 as potential microRNAs that are quite specific to cancer cell dervied exosome versus normal cell. And this is again being validated later, and I'll show you in a second.

[00:06:00]

[00:06:30]   While we were doing this work, it became clear that we were also getting some genomic DNA, which was a surprise to most people because one would not imagine that genomic DNA could get into the exosome. But then if you think about all the genomic DNA that is present inside of plasma, then it's not that surprising that some of them get into the exosome. We began this work looking at the DNA in the exosome using pancreatic cancer cell lines, and we noticed that in fact, large fragments of DNA can be detected inside the exosome after digesting with DNAs and others. This was, again, surprising that such large fragments of DNA can be present, and then a few months after we published our paper, another group in New York identified that in fact, that this is true, that there's tremendous amount of cell lines where you can identify large fragments of genomic DNA. Again, they also identified 10 kb fragments.

Then again, the question was what's the utility of it? So we quickly decided to do some proof of [inaudible 00:06:44] experiments, and this is just a [inaudible 00:06:46] sequencing of the DNA to identify the Ras mutation and P53 mutation that was identified in this patient from the primary tumor, suggesting that some of this DNA, it has some oncogenic value in identifying mutations.

[00:07:00]   Then we began whole genome sequencing work, and in fact, that was the biggest surprise, that exosomes contain DNA from all the chromosomes encompassing all of the genome, just like the cell DNA that you isolate from the nuclei. This was quite surprising to us as a collection of billions and billions of these exosomes that you isolate in the culture system, you actually can pretty much cover the entire genome.

[00:07:30]   So we started doing as many of the cell types. This is another pancreatic cell type. This is an ovarian cancer cell type. This is a mouse cancer cell type. Pretty much now, having looked at about 50 different cell lines, this is true for every cell line we looked at. They dump out the DNA into the exosomes and it encompasses most of the ... all of the spectrum of all the chromosomes.

[00:08:00]   Then we collaborated with the Broad Institute and did some studies also with the primary tumor and the [inaudible 00:07:52] isolation from pancreatic cancer patients, and again, you can see ... And this has been done now for hundred of patients, and we've never not seen the entire coverage of all of the DNA present in the exosomes. This is of some value, now, because you can actually combine this with the cfDNA analysis and see what the advantage and disadvantages are. I think that's where the field is at the moment.

[00:08:30] Not only that, but we found that, in fact, the DNA can be also used to identify methylation sites and methylome analysis, and we found, in fact, between the exosomes coming from normal ... Collected from healthy individuals versus cancer individuals, or metastatic cancer cells or non-metastatic cancer cells, you can see difference in there of some value.

[00:09:00] Our colleagues at MD Anderson, St. Luke's, and others also independently verified that, in fact, plasma exosomes from pancreatic cancer can be used to write a whole genome sequence and identify mutations. They went on to actually do a more elegant experiment, where they used pleural effusion from pancreatic cancer patients and did RNA whole exome sequencing, whole genome sequencing, and identified mutations, and, in fact, identified certain mutations that were not present in the primary tumor, giving to this idea that using exosome DNA from circulation, you can tackle this heterogeneity issue that exists and identify sometimes mutation that is not present in the piece of the tumor that is most often sequenced. This again parallels some of the work that people are doing with the cfDNA.

[00:09:30] To really rapidly use this in a way that sequencing will not allow, we [inaudible 00:09:28] digital PCR method to detect mutations from the exosome, and I'm just summarizing this, that we actually ... Without the knowledge of primary tumor now, the whole idea is to now do away with the idea that there'll be this thing you can hang onto for validation of primary tumor and just take serum and analyze and see if you find mutations. Use G12D as proof of cancer. You can see about 39% of the patients have this, which means it really parallels the number that one would expect in pancreatic cancer.

[00:10:00] What is interesting is that healthy individual blood from the blood bank that we received also had mutations, and three out of 114 of them had in the study, and these three were at the age of 18, 26, and 29. These are healthy people who come to give blood, so again, this brings a point that you can identify mutations, but the question is how do you counsel the person from which you identify. I think that's something that we'll have to tackle with any of this, and this sort of preventive screening detections that we would do with this sort of technology.

[00:10:30] Again, another [inaudible 00:10:29] group had done the same studies, and they found, in fact, a higher percentage. They found a 7.4% of people with mutations in the KRAS using exo-DNA, and then, of course, if you do that in the cfDNA, they found it's 14%. Here, I'm showing you this data because they suggest that in fact exosome DNA is better in identifying mutations in pancreatic cancer than cfDNA.

[00:11:00] All of this is not really yet been validated by others, and in fact, I'll show you some of the results that we've achieved doing these studies, and we don't see that sort of amazing difference. The question about exosomes has been always that are cancer cells generating more of it, and there's some evidence for that. If you were to look at the number of exosomes, like if you look at the amount of ctDNA or cfDNA, would that be of value? There is some increase in pancreatic

[00:11:30] cancer patients compared to healthy donors, but you don't really see that major difference that you can really use this as a diagnostic. The question is because the circulation contains exosomes from all cell types, including cancer cells, is there a possible way to enrich them for something that is present on exosomes from cancer cells not likely to be that high in the exosomes coming from normal cells?

[00:12:00] That became our quest for us, and we decided to do masspic analysis of exosomes coming from normal cells versus cancer cells, and found several proteins that are usually enriched on cancer cell derived exosomes, including a protein called Glypican-1, which is known to be highly expressed by pancreatic cancer cell and also is present in higher levels on exosomes coming from cancer cells. We went and did a FACS analysis to use an antibody to pull out after we isolate exosomes from the serum. [inaudible 00:12:16] positive exosome versus negative. This EM shows you that there's a lot of positivity in the positive fraction, as you'd expect, and if you look at ... With respect to healthy donors,

[00:12:30] you can see that most of the pancreatic cancer patients are positive for it, suggesting that it is a highly expressed, at least at more higher levels than the healthy individual exosomes.

Then in a validation cohort, another 56 patients, we found that from a different center, and then a second validation cohort coming from another center, we found this to be true. So looks like most of the exosomes from these at least pancreatic cancer setting have an elevated level of Glypican-1, and that allows us to then pull them out and enrich the material within them to do analysis.

[00:13:00] Of course, one thing that we wanted to do is to ask the question if the levels would drop post-surgery. So after seven days after surgery, you can see that the levels drop, and if you relayed that to survival, the ones that drop the most, that do better than the other, so this is some ... There's some parallel to be able to track this survival, and so looks like this could be an interesting marker and this should be further studied.

[00:13:30] Now the question for us was can we use a simple qPCR, because remember that exosomes also have RNA, so just qPCR and see the negative fraction verus a positive fraction, where would the mutations be? We were able to enrich in the positive fraction the mutations with G12D that we know from the primary tumor and the G12V, suggesting that this one-step enrichment allows us to use simple techniques to identify the mutation.

[00:14:00] The question again was whether this level of increasing levels of these Glypocan-positive exosome would correlate with MRI. For that we used a genetically engineered mouse model of pancreatic cancer and looked to see when the MRI becomes positive here what would be the status of the Glypocan-postivie exosome in circulation, and they are positive, but before even MRI picks up, we're able to pick up in the circulation. There's a large study going on now with many different mouse models and some patient studies that we're doing now which we'll report soon.

[00:14:30] The question with this particular technology was that ... Whether all antibodies will work the same that are commercially available, and here is a study that we did, which we published that the original antibody we used and the other antibodies, including this monoclonal antibody here, all work well to differentiate very well. There are many different reagents available to do these [acids 00:14:41].

[00:15:00] At this point, we received a call from [inaudible 00:14:46] and Mike [Gogen 00:14:47] saying that they would like to send us blinded sets of samples, completely coded from Hopkins and whether we can do the analysis and they'll un-blind it. We did that study, and they sent us the samples, and we performed analysis, sent it back, and this is the analysis that they provided to us. You can see that in fact we were able to detect the [inaudible 00:15:05] patients with the Glypocan positivity versus disease controls and healthy inviduals, and also [SMIPMNs 00:15:12] which showed little elevation of it. These validation, blinded studies also convinced us that this is a marker that could be potentially pursued, and this is the ROC curve for that.

[00:15:30] Meanwhile, some other studies have come out where Glypocan-1 has been shown to be a sensitive marker for breast cancer with very highly sensitive acid, and here is another study for colon cancer where people have shown that it's elevated in patients with colon cancer and after therapy, it goes down. This is another study where a chip technology was used to identify Glypocan-positive exosome in pancreatic cancer.

[00:16:00] Some other studies as a disclosure so that we're discussing the utility of this, are suggesting that Glypocan may not be as good as some microRNAs are detecting, and here's a study where this is normal individuals compared to three patients and you can see an elevation, but they don't think that this is that impressive, just three patients. But in the microRNA analysis they did 10B and 21, which we also detected earlier. If you remember, I mentioned that, and you can see that here, they use 29 patients to derive this difference. I think if they were to do more patients here, they probably will see that.

[00:16:30] There's another study that Ralph [inaudible 00:16:23] published from their group using a proprietary of [microfielding 00:16:28] technology that they have through a company that they have founded. There they identify five markers that they think are present on the cancer ... pancreatic cell derived exosome, and Glypocan-1 was one of them, tbough they think that this was a weaker of the others, but it did definitely improve sensitivity and specificity when they used it. So this is another study that utilized it, and you can see here Glypocan-1 in the cohort here versus the healthy subjects.

[00:17:00] Another study was published for MD Anderson by [inaudible 00:16:56], and they show in fact that when they used ... They came up with their own set of six markers on the exosome and they used a capture with the six markers, and if they use total circulatory exosomes, they see 51% of metastatic disease and ...

[00:17:30] Locally invasive 50%, whereas they use a six marker to enrich it, 71% of positive versus 76%, so they think that this enrichment will give them a better detection here as an example to KRAS mutation. They also in their proteum analysis found that the Glypocan-1 is present, but they felt that the other six markers were better because they're better reagents to study that.

What's interesting is that it's becoming clear that every group that is doing this is identifying their own set, and so nobody is able to really validate the other group. I think what's important here is that reagents have to be shared, because you can ... The previous study I just mentioned, only one of the markers shares with them. I think that one of these cross validations must happen between groups.

[00:18:00] We decided to do a study where we took tumor tissue cfDNA and serum exosomes, and this study is ongoing, and I just want to summarize it. We didn't see the stunning advantage of using serum exosomes compared to cfDNA. When we fractionated, we saw some advantage, but really I think the cfDNA continues to still be a viable one at least, a mode of analysis at least for DNA at the moment.

[00:18:30] With that, I want to summarize what I think needs to be done in this field. As I said, the identification of DNA and the exosome only happen in 2014, so this is a young field that's growing, but I think more validation studies are required with larger patient sets. We need to have a standard procedure for exosome isolation that can create heterogeneity. Everybody uses their own favorite technique to isolate exosomes. And we have to have a standard method of identifying the surface proteins. All of the studies I've shown you use different techniques. Nobody has used the same ones, so that also gives you a lot of heterogeneity.

[00:19:00] I think there's multicenter collaborative assessment is required, like the one that we did with Hopkins, they sent us things, we did analyze it, send it back. I think we should do it multicenters, and I think that will give us an idea of where this sits with respect to cfDNA. That's the biggest question, but I think if you ask me right now today, I think that the case for cfDNA is much better to analyze and standardize compared to exosome DNA, but again, more and more work will be done, I'm sure, to see what the utility is.

[00:19:30]
[00:20:00] Having said that, as I mentioned to you, exosomes come from cancer cells and they contain DNA. You can do [P 00:19:29] genetic analysis on it. They have intact mRNA that's functional, so it's a very great amount of mRNA that can be analyzed. All types of non-coating RNAs and small RNAs are present. Proteins are present. Lipids are present in them that have been identified. Metabolites are present. I think of exosome as a platform analysis. You just don't one thing. If you identify something with DNA, you can use the same material to confirm that the RNA are present, and then you can also continue to use some other things to create an algorithm. I think that would be the utility of exosomes that ... Not just the DNA, where cfDNA might be doing a pretty good job of that at the moment.

[00:20:30]

I want to end by showing you that, by looking at metabolites in exosomes released by hypoxic breast cancer cells versus [nomoxic 00:20:18], you can see that there's a nice correlation in the exosome metabolites. You can detect all of the amino acids and their differences there. I think this gives another way you can track how your anti-metabolite therapy or anything is working, looking at exosomes in circulation.

With that, I want to end with the people who did the work, many of the people who contributed to this. Kate [inaudible 00:20:40] she was the one who started some of this work and other collaborative that participated in this. This is our group at MD Anderson, and I'm happy to answer any questions you have. Thank you very much.

[00:21:00]
Speaker 3:

Hi. I have a question. Have you thought, or is it in the works of doing a kind of longitudinal study to determine how early you can see these? I know you did it in the mice, but it was only a few weeks before you could see something on an MRI. I'm talking about for a true, early detection. Is there a longitudinal study in the works?

Raghu Kalluri:
[00:21:30]

Yes, the answer is yes. We're trying to do it in pancreatic cancer with IPMNs and early lesions and see if we can track them in high-risk individuals, people over the age of 60 with diabetes, etc. We're doing this, and I think the study's ongoing. You saw that in pieces I've shown you, 10 samples here, 5 samples there, 10 samples there with IPMN, and there is a trend to detect it with this Glypocan positivity, including the blinded set of samples that was sent from Hopkins. But I think what you're asking is appropriate and correct, and we have to do a larger study and track and see whether this is of any value.

[00:22:00]

Speaker 3:

In relation to that, I think you mentioned using similar reagents, but I think very importantly, you need to use similar samples.

Raghu Kalluri:

I agree.

Speaker 3:

Is it necessary for you to have fresh blood to do this, or can this be something that can be gotten, stored, transported ...

[00:22:30]
Raghu Kalluri:

Yes, that's one of the great advantages of exosomes. They're very stable, and we can do it from ... We did it from serum stored 10-15 years ago in minus 80 that have probably gone through a couple of freeze-thaws. That's one great advantage is that it's quite stable and you can do it from frozen samples.

Julia Beaver:

Great, we'll go to microphone four.

Speaker 4:

Hi. Is this genomic DNA that you see, is that primarily from cells that are dying or dead, or do you know?

[00:23:00]
Raghu Kalluri: Not necessarily, because at least in the culture system, we've very extensively done the studies to prove that they are released by life cells. In fact, the dead cells released apoptotic bodies that are very large, and they don't contain as much DNA as you would think they would have, but these are from live cells. There's a tremendous amount of cellular DNA that people are identifying now in the cytoplasm, and I think this is one way that is probably getting out. People are correlating this with a sting response and things like that, so maybe this is a way
[00:23:30]   to get rid of it, to avoid the sting response. I think this mechanism, it probably existed for that, and it's shutting down the cellular DNA, and we're doing some work to tag the DNA and track it, and it's quite impressive how much cellular is left behind after cells duplicate as aborted DNA because the [inaudible 00:23:48] going to abort it sometimes. I think that all of this is a collection is what we're seeing.

Speaker 4:   Do you have any sense of the kind of ratio? Like if you had evenly mixed dying
[00:24:00]   cells and live cells, would more of the DNA come from the ...

Raghu Kalluri: That's exactly ... I didn't specify, that's exactly the type of experiments we have done. It truly comes from live cells. The number of live cells, the more DNA you get in the exosome. This actually work is getting reviewed at the moment, so hopefully you'll see it soon.

Julia Beaver: Microphone 2.

Speaker 5:   Question on early detection again. You suggested that the cell-free DNA may be
[00:24:30]   a better tool at this time over the exosomes for detection. Would that also fit in the early fields, or is that more in the later stages of cancer?

Raghu Kalluri: I say that not because I have great evidence that that's the real issue. I say that because first, cfDNA can [inaudible 00:24:48] compared to exosomes. You have to isolate exosomes and then isolate DNA. All of that is process, so what I meant
[00:25:00]   is that if you look at the technologies today, cfDNA has been around for a long time. There's some standardize technology developed. I think that there's more utility there to identify mutations, etc. With respect to that, I would say that as of today, the evidence is suggesting that it is not that much superior to cfDNA for DNA analysis, right? But if the technology improves, we're able to detect more smaller number of exosomes with heterogeneity aspect within it that's going to
[00:25:30]   be [inaudible 00:25:28] solved, that means that we can pick up exosomes that have more DNA versus not, maybe with some other discoveries, then it may ... We may change this idea.

                 But right now, my feeling is that for detecting a cancer derived DNA in the blood, I think cfDNA is far ahead in showing its utility than exosome DNA, but if you were to want to do mRNA analysis, microRNA, others, I think that exosomes are much more valuable, obviously because they have this protective cargo.

[00:26:00]

Speaker 5:   So another way to ask the question is, are early cancers producing a significant amount of exosomes as they are detectable?

Raghu Kalluri: Yes, they do produce, it's detectable, but I think as Max mentioned in his talk, if they find very little rDNA, we find much less, so this is about sensitivity issue.

Julia Beaver: Thank you. I think actually we have to move on to the next, but thank you so much.

Raghu Kalluri: Thank you.

[00:26:30]
Julia Beaver: Dr. Haber?

Daniel Haber: Okay. So, thank you very much for the opportunity to present. I think we're gonna go from molecules to cell fragments to whole cells, so if your eyesight isn't so good, this'll be a little easier on all of us. In terms of disclosures, the important one to mention is that there is a diagnostics company, [ANewCo 00:00:37], which is being launched now by both Mass General and Johnson & Johnson to disseminate the technology that our group has developed, which hopefully will be helpful to the field in general.

[00:00:30]

[00:01:00]   So, this is what cells look like. Circulating tumor cells are shed from the primary metastatic tumors into the blood. They travel into the blood, where most of them die, and a small subset will go on to give rise to metastases. So they're three big questions in the field. Obviously, understanding bloodborne metastases, looking for targets, trying to understand how blood ... how cancer cells can survive in the blood and give rise to metastases, and obviously the questions we're interested in today, noninvasive monitoring of cancer, as well as early detection.

[00:01:30]   I'm gonna divide this talk in three. First, I'll give you an update on the technology, primarily negative depletion, which is a technology to get rid of all the normal blood cells in a blood sample so that you leave behind the untagged and intact tumor cells, and that's a technology that's particularly important, we feel, for the field, but also for the other two points, which I'm gonna describe. One of them is the ability to study cultured viable CTCs and the biology we can learn from them. And thirdly, the possibility of using RNA based diagnostics based on these intact cells.

[00:02:00]   So, as you can know, this is a tube of blood, has about 10 mils of blood, 50 billion red blood cells, 50 million white blood cells, then anywhere from zero to 100 CTCs. So that's the kind of engineering challenge, if you will. This is a device which was developed by my close collaborator Mehmet Toner at MGH, and basically, it involves using physics principles to purify cells so well that you can get rid of normal blood cells, leaving behind the tumor cells.

[00:02:30] So, the first step is that you add antibodies against white blood cells that are conjugated with magnetic beads directly into a tube of cold blood. Now, my tumor may be different from your tumor, but our blood cells are exactly the same. So, from that act alone, you're able to use well-characterized antibodies that are constant from one individual to the other.

[00:03:00] The first step of this chip is a size base fractionation. All the red cells, the platelets, the unconjugated beads are all discarded by size in the first few seconds, and you're left with nucleated cells, which go through these channels. This is the beauty of the device. As the cells go through these channels that are curved and have a particular diameter, inertial forces from the walls will force them from a broad laminar flow into a single row. And when the cells are traveling in a single file down a [microfluid 00:03:13] channel, it's very easy, then, to apply a magnetic force and to divert them with either CTCs or the antibody-tagged white blood cells. So, this is the key that enables us to get that kind of level of purification without touching the tumor cells.

[00:03:30] The chip is now made by Sony Blu-ray. They don't make quite as many DVDs as they used to, so they're very open to making plastic devices for us. The company is now called [Stratech 00:03:38]. Blood comes in here, the product comes in there, it's divided in two, and you can see this is the chamber for size base fractionation. This is the inertial focusing to get the cells in a single row, and then the magnet is applied here. And then we have a device, so the disc, if you will,

[00:04:00] fits within this cartridge, and the cartridge fits right here within this processor. The blood is up here, buffer is here, and white blood cells come out here, antibody-tagged white blood cells here, and the CTCs come out in the middle. At this point, it's automated in that we can process about 10 mils of blood in one hour. It's an on/off switch, and you can process up to 30 mils of blood with this device.

[00:04:30] So, to recap the technology, this is the famous kind of needle in the haystack. I've seen that picture earlier today. Depending on how big you make the needle, it's easier to find, but the idea, really, is to get rid of the hay. So, by unbiased enrichment, if you get rid of all the white blood cells, you don't have to guess or know what's expressed on the tumor cells. The second point is that the cells, the procedure is automated at this point, so it's relatively quick and [inaudible 00:04:47] in terms of purifying the cells. And finally, the cells are viable or untouched, which is particularly important if you're trying to isolate RNA or make

[00:05:00] viable cells. And you can see in this picture here, this is the CTC, this is the [inaudible 00:05:00] biopsy from the same patient, and because the cells are in solution and nothing's ever touched them, they do quite well.

So let me move now, quickly, to some part of discussion about cultured CTCs, because that's, again, another important aspect of the field. Can we actually interrogate these cells, not just genetically, but functionally?

[00:05:30] So, this is work that was done by a former postop in the lab, [Minyu 00:05:24] who was very, very patient, and was able to culture cells, primarily from ER-

positive breast cancer. And, at this point, I would say we are successful in about 10-15% of patients, and it takes about two to three months to generate a cell line. And the conditions that we've developed are primarily hypoxic, and anchorage independent conditions, and once you get a cell line established, you can generate tumors orthotopically in NSG mice.

[00:06:00]

[00:06:30]

The interesting thing, of course, is that when you have a lot of tumor, you can ask about mutations, and you can got through various panels, which are harder to do during the course of breast cancer treatment. And what you're seeing here are a whole bunch of mutations that are present in these cancer cell lines that were not present at the time of biopsy. And we know that they come from the patient, because we can repeatedly sample the same patient, and if we find the same mutation on multiple independent tries, then we know that they came from the patient and not from in vitro. The types of mutations that are important in breast cancer include acquired mutations in the estrogen receptor gene, as well as activating mutations that we found, for example, in FGF receptor [NPI3 kinase 00:06:34]. And that then raises the question as patients are treated for breast cancer and other cancers, and they acquire these mutations, are they drivers? Are they passengers? What do we do about them? And having the viable cells gives you the opportunity to ask that question.

[00:07:00]

So in this particular example, this was a patient where half the alleles had an FGF receptor mutation, half had a NPI3 kinase mutation, and we could tell that these were heterozygous mutations in the same cell. We could test them against drugs, and you can see FGF receptor inhibitors, NPI3 kinase inhibitors, and the two together being cooperative. And you can show this in vitro, as well as you can show this in vivo by reconstituting the tumors.

[00:07:30]

Now, mutations are not the only things that we've seen in these CTC cultured lines. We also see changes that are more likely to be epigenetic in nature. This is an old conundrum in the field, which is that you have patients with ER-positive breast cancer whose cells acquire her to expression. They don't have amplification of her too. They don't seem to be driven by her too, but what are they actually doing? And you can confirm that with biopsies.

[00:08:00]

But, somehow, the significance of acquired HER2 expression in ER-positive breast cancer isn't clear. You can see here a cell, so this is a CTC, which is EpCAM positive. This one's HER2 positive, this one is not. And if you pick these cells, you can actually sequence the RNA reads, and you'll find is that some of those cells have absolutely zero HER2 reads, and some of them have quite significant numbers of HER2 reads as if they're two distinct, discrete subpopulations of CTCs within these women with breast cancer. And if you look at the tumor cell lines from these patients, you see exactly that. You see a peak that's HER2 negative, a peak that's HER2 positive. Again, in multiple cell lines, you see these two discrete populations.

[00:08:30]

So, what happens now, if you separate them, you can purify the HER2 negative, or the HER2 positive lines. The HER2 negatives grow more slowly. The HER2

[00:09:00] positives grow more quickly, but they both give rise to tumors at 200 cell inoculum. So it's not a cancer stem cell type of proliferative effect. You could see tumorigenic cells, but if you take HER2 negative cells, you give rise to a tumor slowly. If you take HER2 positive cells, you give rise to a tumor more quickly. And then something very bizarre happens. If you look at the mature tumors, the HER2 positive cells show acquisition of HER2 negative cells, and the HER2 negative cells show acquisition of HER2 positive cells.

[00:09:30] So we've taken this, actually, further in vitro in terms of mass spec characterization and single cell cloning, and to summarize the story, what we find is that there really are two subpopulations that exist, or coexist in these advanced breast cancers. There is a proliferative population, which is characterized by HER2 plus staining, but it's not addicted to HER2, it doesn't amplify HER2, it has multiple RTKs that are activated. They proliferate very quickly, but they're very sensitive to chemotherapy or to multiple kinase inhibitors.

[00:09:57] The other population, HER2 minus, grows more slowly, is more resistant to chemotherapy, but shows expression of notch and notch-driven pathways, and is much more sensitive to gamma secretase inhibitors, which inhibit . And the balance between these two depends on what's happening to the tumor. The more you let the tumor grow, the more you have HER2 positive cells. The more you add chemotherapy, the more there's reactive oxygen damage or other stresses, the more you have the balance towards the notch positive cells. And these cells coexist in a tumor. In fact, if you make a tumor in a mouse that's both

[00:10:30] HER2 positive and negative, so parental cell, you can see that it grows very quickly. If you add a gamma secretase inhibitor in green, nothing happens. If you give four weeks of chemotherapy, you suppress the tumor, and then it grows up again. If you give four weeks of chemotherapy plus a gamma secretase inhibitor, you suppress the tumor.

[00:11:00] So, the point here is that cancer is much more complicated than we ever thought, and particularly in breast cancer, where you've treated women for multiple years on multiple different regimens, the type of cancer that you have after years of treatment is very different than what you may have had initially, and you may be looking at multiple populations with multiple mutations as well as epigenetic differences. There's no way of telling that by plasma DNA, by exosomes, you really need the whole cell, and you need to be able to look at the culture from these patients.

[00:11:30] So, let me switch now to work which is, some of it published, most of it unpublished. But, looking at RNA based digital scoring of CTCs, because the second thing you can do with CTCs is study the RNA that's intact within whole tumor cells. So, to do these kinds of studies, we take cells through the chip, then do live staining of cell surface with epithelial, mesenchymal, or whatever marker we want, and we pick a single cell and then we can do single cell sequencing.

[00:12:00]

[00:12:30]

Overall, to give you the background, a chip purifies cells about 10 to the four, 10 to the five. There's about 500 white blood cells left contaminating [inaudible 00:11:53] of blood that went in. So, the purity that we're talking to at the end is from 0.1 to 10 percent, depending on how many CTCs were present. If we do single cell sequencing of single CTCs, in this case in prostate cancer, you get very, very high quality reads. In fact, if you don't touch the cells, you get about 10 times more reads than if you add an antibody and capture the cells and start degrading the cells. Out of all these studies, the most interesting things that we found was in prostate cancer. For example, a single patient may have multiple CTCs with different variations in the androgen receptor. Some have the AR7 deleterious variant, others don't. You see everything within the same CTCs from a given patient.

[00:13:00]

So, this is the big challenge that we have in the field. We feel really good about this chip, and there are a number of other technologies out there that are now being commercialized for automated [inaudible 00:12:50] enrichment of CTCs. But then comes this part. So this is a really pretty picture, but getting this picture of a CTC cluster will take you three hours in front of a microscope. So, the microscopic scoring and analysis of CTCs is antibody dependent. You have to threshold the signal, a fluorescent signals, it's manual, and it's been one of the major limitations to clinical deployment of CTC based technologies.

[00:13:30]

So, given that the RNA is so good, why can we not develop an automated digital droplet PCR readout for CTCs? So this is the technology that we established now. We put the blood through the chip, we purified the cells, we lysed the cells, make RNA, do a whole transcriptome amplification to get a mini library of the cDNA, and then we pass it through a droplet digital PCR machine.

[00:14:00]

[00:14:30]

So, the first thing is, what would you look for? So, let me tell you the liver story. It turns out, as you know, that albumin is the most abundant protein in your blood, but if there's a cell in your blood and it has albumin RNA, it comes from your liver. So, by the very fact that you start with a whole cell and then you interrogate the RNA, you can not only identify the cancer, but see where it comes from. And this, to give you an example, are the liver is particularly good, because it not only makes albumin, it makes a bunch of enzymes that are not made by any other tissue in the body. Liver cells, for example, this is the blood cells that contaminate the chip. You can see how clean it is. You also get signal amplification, so for any given transcript from one cell you can get from 10 to 1,000 droplets. If you add 10 different genes and 10 different types of droplets that you're looking at, you get massive amplification of the signal compared to looking for one cell under the microscope.

[00:15:00]

And finally, if you do the whole transcriptome amplification right, and you calibrate this correctly, you can preserve the distribution among the various markers. So you can ask intracellular questions of relative abundance of different RNAs within the CTCs. So, this is liver. Liver is particularly interesting for us, because they aren't very good markers. They aren't very good mutations or other ways to follow this, and people who develop liver cancer primarily have

cirrhosis or liver disease. So, there's a very high risk population with a chance of getting liver cancer, which ranges from 0.5 to 8 percent per year, depending on how bad the liver disease is.

[00:15:30] If we apply CTC based RNA diagnostic with 10 different markers, you can see healthy patients, healthy donors. You can see patients with cirrhosis. These are patients who all followed in the MGH hepatology clinic, because they have cirrhosis and are being watched for the development of liver cancer. With alpha-fetoprotein, you can see that that signal is quite low. Patients with new diagnosis of liver cancer, patients being treated for liver cancer, and then patients whose liver cancer has either been resected or embolized.

[00:16:00] So, again, you can see it's a nice trend. If you look at patients who are being treated for liver cancer, you can compare the AFP, which is the standard way of monitoring it, together with the CTCs in red. In some cases, they're concordant. In about a quarter of patients, their liver cancer does not make alpha-fetoprotein, so you can see there's no signal for AFP, but you can see that you can monitor this with CTCs.

[00:16:30] And turning to early detection of liver cancer, these were 15 patients, newly diagnosed with liver cancer, and you can see that either AFP or CTCs can pick up five of them, if P alone can pick up one, and CTCs alone can pick up four. So by combining AFP protein at a high cutoff and combining that with CTC RNA, you can now start to develop these kinds of composite early detection modules with the idea that you can do blood screening and ultimately validate that with imaging.

[00:17:00] Now, I'll give you a couple other cancers. This has been published recently, these other studies are unpublished, and I'll first talk about breast cancer. And breast cancer is a different kind of challenge, because breast cancer when it's diagnosed is relatively straightforward. When it's first treated, it's relatively straightforward. And then when it recurs, it's not so clear what to do. So if you look at the second line treatment of ER-positive breast cancer, physicians will select from a number of endocrine therapies. Then they'll select chemotherapies or targeted therapies or combine them, and there really isn't a very good way of identifying what markers. What should you give to whom? And by the time you

[00:17:30] find out if it's worked, you've actually spent three to six months on a particular treatment that may or may not have worked.

So, this is our test for breast cancer. Again, we have about 20 markers in breast cancer, and what we're able to do is to show that the baseline digital CTC markers in breast cancer are highly predictive of overall survival with [>>>00

[00:17:53] 00:17:49]:17:50]second line therapy. This was actually shown by Dan Hayes and with cells and cell search a long time ago. We're now applying this to hormonal

[00:18:00] therapies as well, and you see a very significant difference in survival, depending on the baseline CTC numbers. If you look at response, you can see that those who have high CTCs but whose CTCs drop do as well as those who don't have CTCs.

[00:18:30]

[00:19:00]

And finally, the most interesting aspect is if you look at who are the people who don't respond well to hormonal therapies. These are the patients who still have this signature, and this signature turns out to be an estrogen response signature. So what we can look at within RNA signals, we can see did the hormonal drug turn off signaling within the CTCs. If it did, you do okay. If it didn't, you don't do so well. So, to our knowledge, this is the first time that you can look at intracellular estrogen receptor signaling on treatment to ask the question, "Did you new drug turn off ER signaling?". Of note, about a third of these patients with persistent ER signaling have estrogen receptor mutations, so there clearly are other ways of activating ER other than mutations in these patients.

[00:19:30]

Now I wanna touch quickly on prostate cancer. Again, the big challenge is in setting of castrate resistant disease, metastatic prostate cancer within multiple choices. This is our assay here, and what we've been able to show is that the CTC score, in particular HOXB13, which is a measure of [a barren 00:19:26] signaling, is highly predictive of poor response to first line abiraterone. So again, these are the patients who are HOXB13 low. These are the patients who are HOXB13 high. And they have a real adverse outcome on first line abiraterone.

[00:20:00]

[00:20:30]

There is another marker in the field, AR-V7, this is a variant of the androgen receptor in the group at Hopkins, as well as Sloan Kettering and Epic, have shown that this is a very reliable measure of response to abiraterone, particularly in advanced patients. And when we compare it in our field, in our own assay, you can see that if you have AR-V7, and we can quantify this with a number of droplets, patients do very poorly on abiraterone compared to those who don't. Then when we compare the two metrics, what we can say is having AR-V7 is a very bad sign for response to abiraterone, but many patients don't do well without having AR-V7. HOXB13 is more sensitive for those who do poorly. But again, some patients don't, so what you can see if you compare these two metrics is the positive predicted value for AR-V7 is particularly good. The negative predicted value for [B13 00:20:35] is particularly good, and combining those two is hopefully gonna be very helpful in prostate cancer.

[00:21:00]

[00:21:30]

And my last example is on melanoma, where, again, the major treatment in melanoma is checkpoint immunotherapy, and the big challenges are that there are multiple treatment options and the biomarkers are not well established. In this case, we may, because melanoma is so diverse, we have a 30 gene panel. And what we can show is that the baseline level of melanoma cells in the blood is not predictive of anything before immunotherapy. If you look seven weeks after immunotherapy, what you see is a tremendous difference in those who have a decline in CTC score, have much better disease survival, as well as overall survival compared to those who don't. It's particularly helpful, because immunotherapy is very delayed in giving you responses that you can measure by x-ray, so this is an early blood base marker of response within six or seven weeks of treatment.

So, I'm running out of time. Let me just say that what you're looking at here are these particular, this is an old picture that Victor and I put together in a review

article. We've talked primarily about molecules at the DNA level in plasma. You can do that to some extent in cells, but the key applications for cells is looking at RNA, looking at protein markers, or looking at cultured cells.

[00:22:00]   And finally, my acknowledgements. This is work from the MGH Cancer Center in partnership with Shyamala Maheswaran, and I've mentioned the people who did the work as I talked, and on the bioengineering side, with Mehmet Toner at MGH, and our collaborators here. Thank you very much.

Julia Beaver: If you come to microphone two?

Speaker 6:   So, for showing some promising data on the early detection of the cancer by
[00:22:30]   using the CTCs, can you draw any comparison how that compares to the circulating tumor DNA? 'Cause we are now in the era of circulating tumor DNA, and everybody's saying is the best approach to detect early cancer, of course CTCs have advantage, because you can do RNA. But in general, for detecting cancers early, do you see any advantage of using the CTCs?

Daniel Haber: Well, I think the places we've looked at, we've looked in liver cancer, because obviously, that's a place where very few markers, and that's where we're
[00:23:00]   applying, potentially, a very large study to look at patients with cirrhosis. We've looked in prostate cancer, and in prostate cancer, we don't see a lot of signal. We have to change the assay. We don't see a lot of signal in patients with indolent prostate cancers. We do see a signal in patients who have more invasive
[00:23:17]   types of localized prostate cancer, but we need a larger number to be able to go with that. And then, and our group just got a Stand Up To Cancer grant to look at patients with nodules in smokers with the idea of combining plasma DNA, as well
[00:23:30]   as cells. And I think it's gonna be the combination of these algorithms of multiple markers that are gonna be helpful for early detection.

Speaker 6:   [inaudible 00:23:34] detection that you can see different mutations in circulating tumor DNA [inaudible 00:23:40]

Julia Beaver: Can you come to the microphone, 'cause there's a webcast?

Daniel Haber: So, the question was in terms of mutations versus [crosstalk 00:23:48] in cells. So, we've done two things. We did one study of T790M EGFR mutations, and
[00:24:00]   there we found 70% concordance between the tumor and the blood, and 70% concordance between the blood and the CTCs. But that's an acquired mutation that's quite heterogeneous. We've just completed a separate study of estrogen receptor mutations in breast cancer, where we compared blood and CTCs, and there the concordance was above 95%. So, I think the big difference is partly technology, but it's also biology. The driving mutations are there. If you're talking subclonal acquired mutations, you're gonna start seeing variation.

Speaker 6:   Thank you.

Julia Beaver: Okay, yeah.

[00:24:30]

Speaker 7: It's good to hear all the detection, because these, they don't hear much about all the detection. So I'm very happy, and I have two questions, but let's start with the liver cancer. You have CTC, EFP, as you know, there are many other biomarkers. AFP, Alpha2, and also DCP. The question is the following. So ... it is also very important to know the vital etiology of liver cancer, so you test CTC plus

[00:25:00] EFP. Did you know about the etiology after samples?

That's question number one.

Daniel Haber: Yes we did, and in fact, none of the patients that we showed here had Hepatitis C, so that was an underrepresented population in our samples. We're now planning a very, very large study, potentially in Asia, that would try to identify patients with Hepatitis B versus C versus the more [crosstalk 00:25:22]

Speaker 7: [inaudible 00:25:23] on liver cancer on [inaudible 00:25:25] on [inaudible
[00:25:30] 00:25:26]. I would love to talk to you about this. The second question is that when you spoke about the [inaudible 00:25:33] HER2 positive, HER2 negative of CTC and very fascinating the [inaudible 00:25:39] described, so my question to you is how soon in natural progression [inaudible 00:25:44] you can see that sort of divergence that you can see how to [inaudible 00:25:50] 45%? Because I'm
[00:26:00] thinking, it may not be correct, that that might be one of the driving force for DCIs to become invasive, because as you know, so many parts [inaudible 00:26:02] of DCIs goes nowhere or only a few fraction of those become invasive. So, do you have any comment on ...

Daniel Haber: It's an interesting question. I think the biology of breast cancer evolves very quickly, and the more treatments you have, the more it evolves. The traditional field has been primarily from biopsies, which as you know, are not done that often in breast cancer and CTCs have been monitored over time. If you look back
[00:26:30] to the cell search and the HER2 staining days, I think you start to see acquisition of HER2 after two or three cycles of treatment, so it's rare at the onset of metastatic disease, but it's more common after multiple courses of treatment.

Julia Beaver: Okay, I think [crosstalk 00:26:44] we have to stay on time.

Speaker 7: Okay. How [crosstalk 00:26:47]

Julia Beaver: You can ask after. Come up to the podium after. So, we're gonna take a break now, and we'll reconvene at 10:15. Thanks so much.