Fundamentals and Future of Next Generation Sequencing for Biomarkers in Immuno-Oncology

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I am going to talk about sequencing, because I don't understand tumor micro-environment very well. I think you have to be much smarter to understand that, and I don't like looking in microscopes.

Disclosures

These are my disclosures.

Educational Objective

Educational objective is really to understand how we use next generation sequencing for assessing immune biomarkers. I think when we use sequencing it is really not to develop a biomarker, but tumors are genetically driven; ultimately, at the end of the day, they are all genetically driven. So if you can understand the genetics of a tumor, you can understand everything you need to know, because it is all the immune effects occur downstream of the tumor genetics, and I think we are doing a much better job learning the landscape of tumor genetics and how it influences the immune milieu.

Which of the Following is NOT True?

More "not true" questions: Which of the following is not true? Next generation sequencing has the capacity to detect multiple genetic biomarkers in one assay. Whole-exome sequencing is a read-out for gene expression levels. NGS can replace standard PCR tests to determine MSI-high status. TMB can be analyzed with comprehensive gene panels. RNAseq is used to quantify expression levels of mutated genes, and whole-exome or whole-genome sequencing can generate a map of tumor-specific mutations.

Okay, right on; let's keep going and let people know this stuff.

Which of the Following Is True?

Which of the following is true? High tumor mutation burden decreases the number of neoantigens on HLA molecules. TMB is higher in melanoma than in lung cancer. TMB correlates with PD-L1 expression in small-cell lung cancer. TMB is determined by counting silent mutations and non-synonymous mutations lead to the highest number of neoantigens per mutation.

TMB Varies Across Cancer Types

Okay; people got it. TMB is higher in melanoma than in lung cancer; not by that much, but it is definitely higher in melanoma.

Response to IO Treatment Correlates with TMB

Everyone has seen this TMB landscape curve a million times. I think it still is an important predictor of response based on tumor mutation burden. We now have a nice review that was in the *New England Journal* a few weeks ago, showing all the different tumor types, and they show in the different colours of the number of mutations. So if you have purple you will have 100, if you have orange it is 1,000, blue is in the 10,000 range. I am sure there are a number of tumors analyzed, but they show the response rate in the circles. They chose the median number of coding mutations, and you can see that, as you can expect, uveal melanoma, which typically has very few mutations, has a very low response rate, whereas MSI-high colorectal cancer, which is very mutated, along with melanoma has a very high response rate, and so does cutaneous squamous cell carcinoma as well. A clear correlation between IO therapies and response.

MCH-Antigen-TCR Interaction

Why does TMB equals immunotherapy response? I think it is important to really think about tumor mutation burden as a surrogate for neoantigens. If you recall from Mario's discussion what happens is tumors are shed, tumor proteins are shed in the bloodstream and the proteins are processed by antigen-presenting cells, broken down into small peptides and for MHC class 1 they are usually 9 to 10 amino acids. It is these 9 to 10 amino acids which are presented by MHC 1 to the T-cell receptor and leads to tumor rejection, but the body's immune system is all about tolerance; that is why we don't develop autoimmune disease. As soon as an antigen is typically presented, and T-cells respond, the T-cells shut off.

You have these hundreds of thousands of mutations which are very clearly not self, but your immune system is not doing anything because the homeostasis has kicked in and what these immune checkpoint inhibitors do is really restore T-cell rejection to these foreign peptides, but most of these mutations are not immunogenically relevant. Obviously, if they don't change the amino acid, they only change the sequence but not the amino acid, they are not going to be seen as different by the immune system, or you may have a mutation that could occur. For example, it could occur in the anchor residue of the peptide, so if you have a mutation that occurs in the anchor residue you are not going to bind to MHC, so you need a specific mutation that binds to MHC really well and binds to the T-cell receptor really well. When you have that occur then those are typically very clear neoantigens that can

incite a T-cell response and then a shut-off. When you get immune checkpoint inhibitors, you are actually turning T-cell responses to these very specific neoantigens.

There are probably not a lot of them. So if you have 1,000 mutations within your tumor, you probably have a handful of true neoantigens which are turning on, whereas if you have 100 mutations, you probably don't have any neoantigens, or you may not have any neoantigens. It is really a stochastic effect, which is, as Thomas Schumacher would say, really kind of a lottery ticket whether you have relevant neoantigens that can be reacted with immunotherapy.

Analysis of the Neoantigen-Specific T-cell Repertoire in Human Cancer

When we think about identifying neoantigens there are ways for us to determine whether a mutation is, in fact, a neoantigen or not. The way that we do that is we obtain genetic information; we do whole exome sequencing, determine which mutated genes are expressed, and then we can use prediction algorithms and look at the location of the mutation in 9 to 10 amino acid stretches and match that with the patient HLA, so the mutation can be anywhere within that 9-amino acid peptide. If you use a 17-amino acid stretch and then you virtually move the mutation up and down, you can identify the location of that mutation matched to that patient's HLA, to predict what could be a neoadjuvant or not. That doesn't actually tell you whether they are neoantigens or not, because you are only defining the binding to MHC class 1, you are not defining the binding to the T-cell receptor.

The way you need to do that is really with in vitro assays where you make the peptide of choice, and you do multimer assays, specific for HLA, with the patient's autologous T-cells and look for T-cell response. It is a laborios process, but that is the only way that we have right now to identify a real neoadjuvant. When you see papers calling everything "neoantigens", they are talking about predicted neoantigens – so predicted based on software algorithms. They are not true neoantigens; you really need to validate them to make sure they are neoantigens.

Identification of Neoantigens in Pembrolizumab-Treated NSCLC

This is one of my patients who had a very good response to immunotherapy. You can see they had a beautiful IHC; even Sacha will agree that is a beautiful IHC, and you have very good PD-L1 expression within the tumor microenvironment, you have CD3 T-cells within the tumor and on the invasive margin. You can almost guarantee this patient will respond to immunotherapy with that perfect T-cell and PD-L1 stain. The patient had a great response to PD-L1, and we ended up sequencing his tumor. He had 494 mutations; 324 were non-synonymous, 226 were candidate class 1 peptides, and then we ended up screening Tom Schumacher's group with us – 80 HLA peptides.

Identification of Neoantigens in Pembrolizumab-Treated NSCLC

From screening 80 HLA peptides we only found one true neoantigen; it was a mutation in the HERC1 gene, and you can see that we saw a very good tumor response with a reduction in the tumor showing in purple. Paralleled with that, we saw a very nice specific spike in the neoantigen specific T-cell response in the peripheral blood multimer assays, which paralleled tumor regression.

Neoantigen Clonality and Pembrolizumab Benefit

That tumor, that HERC1 mutation, was also work that we did with Charlie Swanton; it was also a highly clonal mutation, so I think that my theme for the rest of this discussion is yes, sure TMB, but there is so much more that we are understanding about TMB to define the biology of those responders.

I think that in the near future we are going to be much more exact in saying what parameters of the mutation landscape for each patient will dictate response or not. This was the same dataset of our 34 patients that we treated with pembrolizumab, and the ones on the left-half are the responders and the right-hand they are the non-responders. When we published our paper, we are a little perturbed that those handful with the arrows beside were tumors that didn't respond, but they are highly mutated. They had 600/400, a couple of hundred mutations. What Charlie showed us is that those tumors actually had very heterogenous tumors; they had a significant number of subclonal neoantigens, so it is not so straightforward to do clonality measurements. But you need more than just exome data; you need some copy number variability data. It has to be off exome; you can't do targeted panels, but they were able to show that those tumors that have large subclonal fractions did not respond as well to immunotherapy, which is clinically important, because one of the things chemotherapy does, especially drugs like temozolomide, is it induces mutations, but you know what those mutations are. They are actually subclonal mutations, so it all becomes relevant in terms of our clinical practice, when we use chemotherapy, what line of therapy to give these therapies.

Total Exome Mutations Versus Genes in FoundationOne Panel CheckMate 026 TMB Analysis

Obviously, in clinical practice we can't do whole exome sequencing on everyone – well, we may in the future, we may next year, but certainly, for today, we are not doing whole exome sequencing on everyone, and there are targeted panels that we can use. The FoundationOne panel is, instead of 35 megabases, which is the typical number for whole exome sequencing, you can sample the FoundationOne panel – it ends up being a little over 1 megabase rather than 35 megabases of sequencing. You can see here that, with a more

limited analysis, you can get pretty good correlation, or very good correlation between whole exome sequencing data and response.

I think it is useful to do; I think that the Foundation assay's trip is totally reasonable. I think when you dial down the number of mutations more than that, it does break down. So you can't use a Guardant panel of 70 genes, or a TruSeq panel of 50 genes, or a snapshot. You need to have one of the larger panels, otherwise it will be too unreliable.

Defining TMB Threshold

We get a lot of these questions around – "What does this mean?" There is a total number and mutations per megabase – what is the threshold? You can see that there is a fair range, but it is not that big a deal. It is a gradient, so if you pick a number – let's say you pick 5/8 or 10 mutations per megabase, I think that is probably a reasonable threshold for you to think about. For our paper, our median number of mutations was a little over 200, and that was the threshold that we used, which ended up being about 5 mutations per megabase. Foundation assay uses a higher threshold when they report out mutations per megabase. Basically, it is the 5 to 10 mutations per megabase.

Which of the following is true?

Which of the following is true? Next question. PD-L1 expression is a good predictive biomarker for response to nivolumab in small-cell lung cancer. The HLA genotype of a patient does not affect outcome with anti-PD-1 therapy. Blood-based TMB can be used to predict benefit in lung cancer patients treated with atezo. High intra-tumor heterogeneity is a good predictor of increased PFS with pembrolizumab treatment.

The heterogeneity is actually a poor – tumor heterogeneity is important, but if you have a fairly heterogenous tumor, you have a number of subclones. They don't respond as well. I think that probably what happens is if you have a large subclone fraction, you may get one or two neoantigens; you may be able to wipe out a couple of the clones, but it is hard to wipe out the whole tumor, which is why tumors with a large subclone fraction don't do as well. Blood-based TMB, I think can be used to predict in lung cancer response. I don't think a year ago I would have thought that you would be able to use a blood-based assay to estimate TMB, but where there is a will there is a way, I guess.

PFS by TMB With First-Line Nivolumab Treatment for NSCLC

Just to go through TMB a little bit – Mario talked about this already, so I won't belabor it, but certainly high TMB is important. This data really suggests that it is not linear. If you basically divide it by tertiles, and high is anywhere around 250 mutations; so as a threshold for the upper tertile, it is not linear, so if you have high mutations you do really well, but if you

have low or medium, it doesn't seem to do any different. There does seem to be a threshold number of tumor mutations or neoantigens that we need.

Analysis of the Association Between TMB and PD-L1 Expression

Then also from 026 and other reports have already shown this, there is no association between TMB and PD-L1 expression; there is no co-association, so there are, I think, independent biomarkers which really give different information.

PFS by TMB Subgroup and PD-L1 Expression

But I think that they can be complementary. I like this slide, because I think simplistically one can use this slide to say, "Okay; if you have high TMB and high PD-L1, maybe you should just get single agent PD-L1 therapy." If you are in the middle, you have high TMB, and you have low PD-L1, or zero PD-L1, maybe you should get combination immunotherapy with PD-L1 and CTLA-4, or PD-1 plus chemotherapy, some sort of combination approach, but I think that if you have low or medium TMB, it doesn't really matter what your PD-L1 expression is, you are not going to respond very well. This is certainly for lung cancer. I think that it is different paradigms for different tumors, but for those patients who have low or medium TMB, irrespective of PD-L1, I think we need to find better therapies.

SCLC: PD-L1 Expression

Small-cell I think is a really fascinating tumor, and Sacha was discussing this earlier, where certain tumors like squamous cell don't have a good PD-L1 expression. Small-cell has even worse PD-L1 expression – 80% of small-cell tumors are PD-L1 zero expression, and there is no correlation between response with immunotherapy and PD-L1 expression if you look at the monotherapy with nivolumab or combination with ipilimumab, no correlation at all.

ORR by Tumor Mutation Burden Subgroup

What is cool is that if you do whole exome sequencing and look at tertiles, TMB was pretty robust. So of all the patients you can see in the left – TMB-evaluable – the response rate was 11%with nivolumab, 28% with ipi/nivo, but if you look at the high TMB group, you can see a very good response in the high TMB upper-third of patients. What was even better was the survival.

OS by TMB Subgroup: CM 032 SCLC

For all of the nivolumab – single agent nivolumab for low, or medium, or high TMB – the OS was 3 to 5 months. With ipi/nivo, low TMB-3 months, medium TMB-3months, high TMB-22 months; so small-cell I think you really need ipilimumab to drive response. They are

PD-L1 negative, they are more immunosuppressed, even though they are highly mutated. It really made a difference. This was one of the most important datasets showing why we need ipilimumab.

Mutational Burden, MSI, and Response to Pembrolizumab

MSI we talked about – for time reasons we won't go into it,– but basically MSI is just a surrogate for hyper mutation. So if you are MSI positive, the median number of mutations was 1,700, if you are MSI low, it is 73 mutations, so it is simply a surrogate for TMB.

Blood-based TMB

Blood-based TMBs, – this data was presented at ESMO last year. There are plasma samples that were collected prospectively on a phase 2 POPLAR study and phase 3 OAK study; these were both atezolizumab versus docetaxel chemotherapy. They found that there was usable plasma samples in about two-thirds of patients that they looked at. Basically all they did was – they did more than this – but they modified the methods, but they basically used their foundation assay on plasma samples. It was pretty impressive.

Atezolizumab PFS Benefit in bTMB Subgroups Validated in OAK Study

If you look at the progression free survival in TMB with more than 16 – these numbers are confusing, because that is just total number of mutations; it is not per megabase, or it is not per exome, it is just what they found in the blood – if you had more than 16 you actually did really well with atezolizumab versus docetaxel. If you had less than 16 you didn't do well, and the hazard ratio using this blood TMB of more than 16 was 0.65, so I think certainly one can envision blood TMB assays going forward.

Which Mutations to "Count" in TMB

Just a couple of words on mutations types to "count". It is a sophisticated group, so I don't need to go into this, but in general, when you look at all of these TMB reports, they are mostly reporting missense mutations or non-synonymous mutations, which are missense mutations mostly. There are very few nonsense mutations. If you have a synonymous mutation you are changing the genetics, but you are not changing the protein, so it is not really detected immunologically as different, whereas the missense mutations change the amino acid.

Frameshift mutations are getting more interest; there is some speculation that renal cells may be more dependent, more frameshift mutation-dependent, and these frameshift mutations are probably the most immunogenic mutations, and much more so than non-synonymous mutations, because if you get an indel, –an insertion or deletion mutation – you can change the entire reading frame and if you change the reading frame then all your

amino acids downstream are going to be different until it stops. They can be very immunogenic mutations.

MHC Polymorphisms Effect on Immune System

This seems completely out of left field showing an HIV survival curve, but I think that we are starting to see that the host genetics are actually really important. It is very clear that MHC polymorphisms do affect the immune system. HLA-class 1 presents antigens to CD8 cells – you get it from your parents – so there are three main HLA-classes, A, B, and C. For class 1 you will have six alleles, so three from your dad, three from your mum; they are very polymorphic. We see that from HIV and other hepatitis and other literature, if you have all six of your HLA alleles are different, if you have maximal heterozygosity, if you have HIV you actually fight the HIV better than if you have maximal homozygosity, and you have less number of alleles – you have loss of one allele, two alleles or more.

Patient HLA Class I Genotype and Impact on IO benefit

Our group, along with Tim Chan and Diego Chowell, looked at this in cancer patients, in melanoma, in lung cancer, as well as in other tumor types, so this is one part of the paper that was published late last year. If you look at a group such as – this is discovery and validation sets – if you have heterozygosity of all loci in high mutation load in purple, your survival is really good. If you are homozygous in at least one loci and low mutational load, you do really poorly.

We also looked at things like LOH, where you may just lose half of your alleles, which is relatively common in many tumor types; you do dramatically worse. It is not just tumor genetics, it is going to be host genetics as well, which I think is just different pieces of the puzzle coming together as we learn more and more. If your HLA genotype is heterozygous, your TMB threshold may be half of what it is if you are homozygous or if you have LOH. So I think we are going to have to do a combined genetic analysis to really use TMB effectively.

Conclusions

Brief conclusions: I think TMB burden is really important. Similar to MSI-high, I think you are probably going to see in the near future some sort of pan-cancer TMB approval, where we screen everyone for TMB and treat based of that. It is clearly a surrogate for neoantigens. I think it is clinically important to learn how to identify neoantigens and it is a hot topic around personalized vaccine development, where we figure out what are the important neoantigens and boost T-cells responses to those specific neoantigens with different vaccination approaches.

TMB and PD-L1 I think have different information; they don't co-associate, but together they can be useful.

We have talked a little bit about host genetics, but I think there will be more to come.

I think blood-based TMB is an exciting, clinically useful tool, and I will stop there. Thank you.