The IO Quiz Show

The Complexity and Conundrums of the Tumor Microenvironment

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Thank you Naiyer and thank you for the invitation and to allow me to present here some of the work and some of the ideas around what's happening in the tumor microenvironment.

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Educational Objective

The hope of this next few minutes is that we will be able to learn about the clinical relevance of characterizing the tumor microenvironment in patients with cancer.

Cancer Moonshot NCI U24 CIMAC CA224319: A Multidisciplinary High-Dimensional Approach to Defining Biomarkers of Response to Tumor Immunotherapy

Recently the NCI has really been very sensitized to this question of why so many patients still fail to respond to immunotherapy despite these amazing successes and how can we define better markers of patients who respond but also find mechanisms and potential new therapies in those who don't.

They really emphasized, like we thought in this community of scientists, that it's not going to be enough to just find a single biomarker and that we will have to develop some signatures to define a really comprehensive idea of the immune response going on at the tumor site in particular.

It required this multidisciplinary approach, that is based originally on understanding the immune cells there because that's the mechanism of most of these new drugs, but also get immunopathology involved to really go in depth at the tumor site, in the genomics and microbiome analysis, and later we'll talk more about that, but also a lot of data analysis because we will generate more and more data as we go along, and we are at the point already where it's too much to handle.

Because the NCI recognized this, they funded the first Cancer Moonshot, this Joe Biden initiative to fund a clinical trial, and we were lucky to be one of the recipients of this

grant which is the U24 mechanism that will now allow many of these NCI-supported immunotherapy trials to be monitored in depth with all these categories.

Topics Addressed, Highlighting the Importance of Tissue-Based Biomarkers

The topics that I would like to address today are really trying to identify which type of biomarkers we can find in tissues and it all starts, as I said, with immune responses that are present there and the main hypothesis is the reason why they are at a tumor site is that the tumor is somehow recognized by T-cells and by other immune cells and so they carry tumor rejection antigens, and later we will talk much more about that.

There is also the hypothesis that maybe those tumors that respond better are the ones that have more infiltration to begin with, that are more so-called immune-competent already.

Of course, the reason why these tumors don't go away on their own is that there are also mechanisms that prevent those cells from acting, even if they are there and suppressing mechanisms.

We would like to explore these now with single cell methodologies at the tissue site. I have mentioned mostly multiplex immunohistochemistry and a little bit of CyTOF as well and these are basically sample-sparing methods that are very high-dimensional and give us a much greater idea of what's happening at a tumor site, and that requires this data analysis.

Many Different T-cell Types Within the Tissue Microenvironment, and Their Distribution Is Not Random [*Four Slides*]

If you look at the tumor, most of it is really not just tumor cells, and if you zoom in this non-small-cell lung cancer, you can see that there are really a lot of different tissues that are present there besides just the tumor cells, including lymphocytes that are organized and these areas as well as macrophages. Not even shown here are stromal cells and the vasculature as well as other structural components of the lung. These are, as I said, not randomly assigned and so what are those T-cells doing there?

What Is the "Immunoscore"?

It's time for the first question, so I'll read it and it is: What is the 'immunoscore'? A score for how well your immune system can fight infections; a technique to quantify dendritic cells in blood; a prognostic measurement of tumor infiltration by T-cell subsets or a new machine to measure immunoglobulin counts?

If you get this wrong, you will be eliminated!

Okay, fantastic, so most of you can stay – it was indeed a prognostic!

T-cell Tumor Infiltration as a Prognostic Marker in Various Tumors and a Predictive Biomarker of PD-1 Response in Melanoma

I will show you some examples where there is a lot of evidence. as now. Most of you are clearly aware that the more a tumor is infiltrated by T-cells, the better the prognosis of these tumors. That has been shown in a meta-analysis of the literature by the group of Hervé Fridman in France who looked at some 120 papers and found that those T-cells are doing better and so, the immunoscore itself is really the reclassification of colorectal cancer by the amount of infiltration present in these particular tumors.

If you look at the regular TNM staging and now if you include, instead, the presence of T-cells inside the tumor margin and within the centre of the tumor and the invasive margin having a high level of memory T-cells or CD8 T-cells can re-categorise patients, regardless of stage almost with one, two, three there doing as well as the Stage One patients, they are above this 80% line while if they are low in infiltration, they are doing as poorly as metastatic patients.

This has been shown in a variety of tumors and even validated in a multi-center fashion, but also importantly it has been shown to be relevant in the context of immunotherapies. This paper from Tony Ribas's group has shown that the melanoma patients treated with PD-1 blockade, pembrolizumab, the ones who responded already started out with a higher infiltration of T-cells in their tumors as well as in the invasive margin that only increased with treatment while the patients who were progressors mostly weren't.

Heterogeneity of Lymphocyte Density in Patient Tumors and Prognosis: Immune Exclusion

This is really not new when we think about even though for a long time when you looked at the tumors, pathologists used to ignore mostly those lymphocytes, because they weren't sure what to do with them, but some of the first signs that they are important were just looking in melanoma brisk infiltration versus no infiltration and already seeing overall survival benefit as well as colorectal cancer even before the immunoscore having shown that some tumors really do poorly, especially when they are not infiltrated. The same thing with ovarian cancer, so there is a great prognostic factor of having T-cells but conversely, not having them is actually a bad thing, and so why aren't some cells infiltrating those tumors?

One of the first papers of PD-1 blockade hypothesized that there will be three types of profiles of T-cells – either those tumors that do not get infiltrated at all and that are immunologically ignorant in some ways or maybe the T-cells are there but they are not functional or they are excluded somehow physically.**Tumor Immunogenicity and Intrinsic Signatures Promoting Immune Infiltration or Recognition**

What would contribute to these types of exclusions? One is that the T-cell may not be recognising the targets they are supposed to recognise, and there is now more and more evidence mounting that the machinery of antigen presentation, in particular the Class 1 pathway is really important to be intact in order for a good infiltration to occur and that alteration in this machinery really leads to potentially, a poor immunogenicity and, therefore, poor T-cell infiltration.

More recently, Tim Chan has also shown that if you have, for example, a very varied HLA profile, haplotype, you are probably more likely to have clinical benefit than if you don't, if you have a homozygous at any of the HLA Class I loci, and if you lose one of these loci with loss of heterozygosity, that's also a core prognostic factor.

There are also, besides the intrinsic factors directly causing loss of immunogenicity, potentially indirect factors as well that are also dictated by the tumor, and you know there are mechanisms such as beta-catenin and Wnt pathways that can be up-regulating the tumor or CDK5 that contribute to this exclusion of T-cells, and if you disrupt them you can get more infiltration of cells as well.

That has been shown in these two papers from Tom Kyewski's group, and basically through indirect mechanism of chemokine production and cytokine up-regulations, you can modulate the T-cell microenvironment of the tumor.

Which one of these statements is false?

Time for a second question; which of these statements is false – high CD3 T-cell infiltration shows improved survival in ovarian cancer, in lung cancer, in melanoma patients treated with PD-1 blockade, in renal cell carcinoma or in head and neck cancer? One of them is false.

T-cells Are Not Always a Good Prognostic Marker; It Is Important to Quantify Other Cell Types (B cells, DCs)

Alright, so actually in ovarian, I just showed you a bit earlier, it's actually a good prognostic factor to have T-cells, but what it is, is really the renal cell, although there is some controversy about this data. But this renal cell is one of the rare exceptions where having T-cells is actually associated with a higher grade of disease and overall worse survival which is interesting because that means that it's not really enough to just measure one type of cell in the microenvironment. In this particular case, NK cells are actually doing better in prognostic value for these patients.

If you go in lung cancer, you can see also that there may be other markers than T-cells that could be highly predictive of disease-free survival. For example, a high density of follicular B cells, meaning B cells organized as follicles at the tumor site as well as a high

density of dendritic cells, both in early- and late-stage cancer has been shown to be a good prognostic factor. That's the group from France, Marie-Caroline Dieu-Nosjean and her colleagues. Besides that, Mario will probably touch much more upon that.

PD-L1 on Immune Cells as a Predictive Biomarker of Response to PD-1 Blockade

There are also other mechanisms; just PD-L1 expression in particular on the immune cells that could be predictive of response as well, depending on the amount of PD-1 expressed on these cells.

All this points to really the need to define much better the variety of these cells at the tumor site, and the way to approach this in an agnostic fashion in some ways is to maybe go directly at the genomic sequencing at the single cell level and really get a good sense of the true heterogeneity of the tumor marker environment.

Single Cell Analyses Reveal Impaired Immune Profiles at the Tumor Site vs Adjacent Non-Involved Tissue in Early NSCLC

This is work from my colleague, Miriam Merad at Mount Sinai who had access to early non-small-cell lung tumors where we got the tumor tissue as well as the adjacent non-involved tissue, and we did a whole bunch of analyses that I will show you also a bit later, but one of them was looking at 10X single cell sequencing and trying to define what are the various myeloid cell populations present within this tumor microenvironment and how does it distinguish from normal tissue as well.

There were several categories of cells that could be grouped together by their transcriptional profile, but some of them were more highly found in the tumor cells and those genes and markers that are associated with those are now maybe important to include in some of our analyses, back to immunohistochemistry and other methods to characterize those cells.

There were some surprises, like PD-L1 for example, that was found highly in mast cells, and I don't think people study those cells that much, so maybe that's something of importance as well and particular macrophage populations that were shown to be up-regulated that may be either targets but also potentially something that we should monitor in order to find out whether they may be responsible for some of the prognostic factors or predictive factors to treatment.

MICSSS: Multiplex Immunohistochemical Consecutive Staining on Single Slide

Because of all these particular markers that we need to think of, we need to really at the tissue site, since most of what we have is paraffin embedded tissue, we need to find methods to multiplex immunohistochemistry so that we can look at more markers with fewer samples.

One method that we also developed together with Miriam Merad was to re-use these FFPE slides, these formalin-fixed paraffin embedded slides, and put them through a cycle of regular immunohistochemistry with chromogen development and then after taking a picture of the first marker we can de-stain those slides chemically and block the previous step and then go back into iterative cycles of staining.

That allows us, for example, to stain for tissue here with CD20, then this disappears after de-staining and then we re-stain, and I will skip all the controls that we did to make sure that there is no cross-reactivity between each step and then the sensitivity is preserved. We tried permutation of order of staining, etc, etc, but eventually what's nice about this is that you get potentially up to ten markers before the tissue starts maybe degrading on a single slide, to be able to co-localize these markers, but also look at them in much greater depth.

Lymphocytes, Macrophages in Small Bowel Neuroendocrine Tumors (With Michelle Kim and Paolo Boffetta)

This is an example of the side-by-side markers. This is to show a neuroendocrine tumor that was collected in Mount Sinai, and there are areas like these lymph nodes that are organized by T-cells around the B cell follicles as well as macrophages that are in there, but then when you go in other parts of the tumor you can see also where these T-cells accumulate again not randomly across the tissue

That's really the strength of these approaches – that it's full slide, and it's also very similar to what you would do as a single step in the histochemistry, so it allows you to much more easily compare those and quantify them.

Multiplex IHC on Tissue Microarrays to Identify New Prognostic Biomarkers

We applied this method to tissue microarrays which is a very good way to do this and have quickly potential clinical results, and so this particular microarray had 75 non-small-cell lung cancers assembled on it and we could do all these markers consecutively on the single microarray and look at density of CD3, CD20, FOXP3 Tregs, etc, etc and then see how these markers associate.

We confirmed what was already known in the literature that a higher T-cell count in lung tumors is actually a good prognostic factor but there are potentially better associations

we should think about such as high dendritic cell content with low neutrophil. CD66, is actually a good prognostic factor while high neutrophils, low dendritic cells is very poor.

Again, these are now potential hypotheses that can be tested thanks to these higher dimensional approaches.

We can also apply this for treatment before and after in comparing what may have happened, and so one question you may have is why don't we do all these things with genomic profiling and just transcriptional profiling, and I will show you now that the problem is that you really need to know the architecture of the tissue and where those cells are.

Applying Multiplex IHC to Query Effect of Checkpoint Blockade

This is an example that we've done with Jedd Wolchok at Memorial Sloan Kettering from a patient with melanoma who actually responded to ipilimumab, but one lesion was left over and kept growing, and it was biopsied, and this lesion actually had a lot of PD-L1 expression on it.

What I am showing here is the same method except we are showing pseudo fluorescence just to visualize it more easily.

And we looked then at macrophages with CD68 that formed this ring around that tumor nest that was PD-L1 positive, then the dendritic cells that were kept outside of the tumor, B cells in large numbers, but again completely outside of the center of the tumor mostly forming these germinal centers and around it, a huge number of T-cells as well. So if you were just to look at these by genomic transcription of CD3 counts, you would say it is a highly infiltrated tumor, but again none of it goes within the tumor itself; it's all around it. The same thing for Tregs, and so now you really get the full picture of why this particular tumor kept growing this large number of T-cells and response overall in the patient.

Artificial Intelligence and Deep-Learning Algorithms Are NOT Well Suited For:

The last question today is about the analysis part, so I will spend the few next slides showing you some algorithms to make sense of all this data.

Artificial Intelligence and deep-learning algorithms that are used to now analyze this data are not well suited for segmenting nuclei and cells to count them, identifying and annotating tissues by areas, clustering cells based on co-expression of markers, reducing dimensionality and analyzing cell-cell interactions or automated staining of tissues by IHC.

That's good, so yes, that's right; most of the other methods I will show you but really automated staining is done with just simple machines and don't require deep-learning algorithms, or it can be done by hand as well.

Automated Quantification of Staining by Deep Neural Network Learning

One of the ways that we approached this is – this is an example for our tissue microarray I showed earlier and we had two people independently count one-by-one these cells painstakingly and then have an algorithm trying to identify which cells are actually stained and whether they are co-expressing markers like CD3 and Ki67 and this is the automated count which looked very similar to the count from the people who did it manually. So this already is like a much more facilitated step to be able to quantify those without too many problems.

Automated Quantification After Tissue Microarray Grid Detection and Single-Cell Segmentation

So then on new software like Qpath, for example, that we are now trying to optimize for these purposes and they can not only find directly the grid of the microarray and within it quantify and segment each particular nuclei and then distinguish those nuclei that are stained by your marker, in this case CD3 or not, and then quantify them.

This is a recent work that we have just done in a large series of head and neck tumors, 500 cases or more, collected with Simon Laban in Germany, in Ulm, and Sven Perner in Lübeck, and we could confirm what was known here, that CD3 T-cells are actually of good prognostic value, but with annotation now you can look at things like correlation with HPV status where there are more T-cells in the HPV positive.

Interestingly, there are actually fewer cells in the patients who smoked versus the non-smokers which is maybe an interesting thing. I am not sure if Naiyer will talk about that or Mario about the association between more mutations caused by smoking and yet maybe fewer T-cells as well. These are things that now we are increasing the number of markers that we will do on this particular one, and it's a work in progress.

Ultimate Goal: Generating Analytic Pipeline That Will Help Automate Analyses of Multiplex Images

But as we increase those markers, how do we really deal with this information? One of the things we had turned to is what has been already done with other instrumentation such as CyTOF or mass cytometry.

You may know that mass cytometry is this method that uses these metal-conjugated antibodies and allows you to put up to 50 antibodies on the same cell and yet to analyze those 50 markers you need to be able to do it semi-automatically otherwise you will spend days and hours trying to do 2D graphs.

The way it's done is it takes all these markers and then algorithms that are unbiased without user input, will cluster them by what looks to be more commonly co-expressed than

not, and each little dot on these plots represents a cell that was clustered based on all of these other markers, and so you can quickly characterize cells by co-expression of these but then also use a further pipeline algorithm of statistical comparisons between cohorts, for example either pre- and post-treatment or in this case between a normal lung and a tumor and blood, and see what are the populations that are most different across those.

Again, this is a nice way to look at maybe a subset of a particular myeloid cell that you may not have thought earlier on and let the computer find for you which are the markers of interest.

Immune Composition of Early Non-Small Cell Lung Carcinoma (NSCLC) by CyTOF Mass Cytometry

This is applied again to lung cancer by this work that Miriam Merad did in early tumors, and we can see here in three examples of patients that there are populations that are different from the tumor to the normal lung and then you can quantify those subsets here as canonical ones where B cells and T-cells are higher in the tumor versus normal while NK and NKT-cells are lower.

Goal: Generating Analytic Pipeline That Will Help Automate Analyses of Multiplex Images

Again we went then further into a much more granular understanding, but these types of algorithms are now also applied for the tissue analysis as well where you can look at each cell with its x-y coordinate a bit like on a flow plot by the SSC and FSC markers and then look at overlay and co-expression and then use this algorithm to try to cluster them. Here it doesn't look very nice because it's only a few markers, but as we add more, we'll have better clustering.

Annotating Tumor Areas With the Help of Artificial Intelligence and Deep Learning Tools

These deep-learning algorithms also help you to annotate tissues by area and this is an example again in lung where you can train the system to recognize areas of necrosis, tumor, normal tissue, airways, and even immune cells. That's important because then you can quantify them, as I mentioned earlier, not just overall in the tumor but by where they are in the tumor by tumor versus normal tissue and invasive margin.

Next Frontier in Tissue Imaging: Higher Multiplexing, 3D-4D Analyses, and Neural Network Image Learning

And finally we are moving now to also the next generation which is, for example the use of these metal-conjugated antibodies to actually do tissue analysis as well, so it's a type of mass cytometry based on tissues. This is the MIBI machine that produced these results,

so this is an ion beam instrument that can basically scan the surface of these antibody-labelled cells and get now up to 50 markers within one tissue.

We can also think about 3D characterisations and basically with this method, go all the way from the MRI scan or CT scan to the tissue where they were; annotate them; and then go into the genomic, proteomic, and tissue analysis; and try to correlate all this in a type of 3D, even 4D atlas by time.

Integrating Multiplex IHC, MIBI, CyTOF, and Single-Cell RNA-Seq analyses

The challenge that is remaining is to integrate the various datasets that we are generating from the tissue, so we are starting to do this with multiplex immunohistochemistry, then zooming in by this MIBI machine to get 50 markers, looking at the same tissues by CyTOF and the single cell suspension and even at a single cell level sequencing by transcriptomics, trying to characterize that this particular population it looks like fibroblasts were present within these tumors.

Take-Home Messages

To finish I would like to take some take-home messages. I hope I convinced you that there is a need for comprehensive high-dimensional immune monitoring strategies and that we will, hopefully with these new pipelines, be able to find better biomarkers of response and that this multiplexing and sample-sparing techniques are really important.

Again I don't think that one marker will be sufficient. We probably have to find a variety of markers, and we are hoping the next frontier is really to increase further these dimensions and generate data across samples and time, so that's where also the clinical trial design becomes important to make sure that we can do that with biopsies along the way.

Acknowledgments

I want to acknowledge people from our Immune Monitoring Center as well as my colleagues who contributed some of these slides and if you have any questions, I'll take them. Thank you.