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IMMUNO-ONCOLOGY INSIGHTS

SPOTLIGHT ON:

Hunting for better biomarkers of response

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Volume 2, Issue 3

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INTERVIEW

Reflecting on Merck's I-O biomarker development journey

ERIC H RUBIN has focused on cancer drug development for over 25 years, initially as a faculty member at the DanaFarber Cancer Institute, then as a senior leader of the Cancer Institute of New Jersey, where he served as the Director of the Investigational Therapeutics Division of that institution. His research efforts focused on mechanisms of resistance to DNA topoisomerase-targeting drugs and his laboratory cloned TOPORS, a novel topoisomerase I- and p53-interacting tumor suppressor gene. In 2008 he was recruited to Merck to lead the clinical oncology development team. Under his leadership, the clinical oncology group underwent a transformational change in an effort to realize the potential of cancer immunotherapy. He led the initial development of the anti-PD-1 antibody pembrolizumab, which was the first anti-PD-1 therapy approved in the USA, and in the identification of the significant activity of this breakthrough therapeutic across several cancer types. In 2014 Dr. Rubin was asked to head up Oncology Early Development for Merck, and in this role he oversees development of a promising and expansive early pipeline, as well as translational oncology research activities. Dr. Rubin has authored over 100 original, peer-reviewed publications and book chapters related to oncology translational research, clinical trials, and drug development. He has served frequently as a member of National Cancer Institute and American Cancer Society study sections, as well as on program committees for the American Association of Cancer Research (AACR) and the American Society of Clinical Oncology. He is a co-chair of the Cancer Steering Committee of the Biomarkers Consortium, Foundation of the National Institute of Health, a member of the Science Policy and Governmental Affairs Committee for AACR, and was a member of the National Cancer Moonshot Initiative/Blue Ribbon Panel Working Group on Expanding Clinical Trials.

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Q What are you working on right now?

EHR: I oversee early oncology clinical development for Merck, as well as translational oncology.

I am working on new compounds that we are developing, many of them in combination with Keytruda® (pembrolizumab). And in the translational oncology space, we're working on continuing to develop companion diagnostics for Keytruda®, as well as developing new biomarkers for the earlier-stage pipeline. We are also focused on biomarkers in the combination setting.

Q You have been intimately involved in the clinical development story of Keytruda® – can you tell us about that journey?

EHR: It is an honor to have been involved in the first human trial with Keytruda®, which started in 2011.

It was quite remarkable in that very early on in that study, it became clear that Keytruda® was something special. We had received that feedback from investigators, some of whom were my friends (my background before coming to Merck was a long-standing one in academia).

It was also good timing in that the FDA, under Dr Richard Pazdur's guidance, was interested in accelerating the approval of highly active drugs – what would come to be known as breakthrough drugs. In fact, we were fortunate enough to receive the first breakthrough designation in oncology from the FDA. That allowed us to do some fairly unusual things in that first-in-human trial.

For example, we ended up enrolling 1,200 patients in that trial – quite unusual for a Phase 1 study. And ultimately, we were able to provide an evidence package to the FDA that led to accelerated approvals in melanoma and lung cancer, as well as the first companion diagnostic in the field: the PD-L1 immunohistochemistry (IHC) test, which we developed in conjunction with Dako (now Agilent).

Q Starting with the PD-1s, what can you tell us about Merck's ongoing efforts to discover and develop improved biomarkers of clinical response to immuno-oncology agents?

"very early on … it became clear that Keytruda® was something special."

EHR: That first biomarker – the PD-L1 IHC test – made sense from a biological perspective and what was understood about the pathway at the time. It was logical to look at PD-L1 expression on a tumor in order to determine whether that would be predictive of an outcome with an

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anti-PD-1 agent – and indeed, that proved to be the case. We also looked at PD-L2, the other ligand, but felt that developing a test looking at PD-L2 expression didn't seem to add much beyond what we were already getting with PD-L1. While there are some who would perhaps malign PD-L1 IHC today, I think it remains a pretty good test. It is able to identify patients who are most likely to respond to the drug as a monotherapy across multiple tumor types. And today, the test is approved across multiple tumor types as a companion diagnostic. But of course, over the years we have looked for additional biomarkers, and we continue to do so.

The other type of biomarker that we and others have found useful measures tumor mutational burden (TMB). This follows a concept that the immune system recognizes cancer because of mutations present in the cancer that create abnormal proteins, often referred to as neoantigens. The idea is that the more mutations you have in a tumor, the greater the number and variety of neoantigens and thus, the greater the likelihood that tumor will be recognized by the immune system. The tumor will block the immune system through the PD-1 pathway – however, if you can come in with a drug like Keytruda, the immune system would be released and be active.

This hypothesis was first tested in a collaboration with investigators from Johns Hopkins, and it turned out to be true. The first few patients were in colorectal cancer, a cancer that in general was not known to be responsive to I-O therapies. But the Hopkins researchers observed a very high response rate to Keytruda® – close to 50% – when they looked at patients who specifically had defects in DNA repair that led to very high mutational burden (known as mismatch repair deficient patients). That finding essentially validated the hypothesis. They then began to explore the idea that this might not be limited solely to colon cancers: all cancers have mutations, so other cancers that also had these defects in the mismatch repair pathway, would likely also be responsive to Keytruda®. And again, this was borne out.

We began to interact with the FDA around the idea that since we were now developing drugs that were potentially based on a biomarker rather than tumor type, perhaps we could consider what is now called a tumor agnostic approval. We pursued this and ultimately got that approval in microsatellite instability-high (MSI-H) or mismatch repair deficient (dMMR) cancer. This was the first pan-tumor biomarker approval.

Now around that time it also became clear that there were other ways to get to a high mutation rate, beyond just these defects in the mismatch repair proteins. This led to the notion that we were actually leaving patients behind, if we were only identifying patients through diagnostic tests that looked at the mismatch repair proteins or the microsatellite instability effect. The simplest approach to this would be just to count mutations and that number would called the TMB. However, this approach required quite a bit more work, because there was no standard definition of what could be considered a high TMB. Happily, though, again in conjunction with the FDA and also the Friends of Cancer Research, there was an effort to gain a consensus across the field and following a series of meetings, alignment was reached across multiple pharma companies and the FDA. A cutoff point of ten mutations per megabase, using the Foundation Medicine assay, was agreed upon.

This breakthrough enabled us to study those patients again across multiple tumor types and unsurprisingly, we found that Keytruda® was once again highly active in that scenario. This

ultimately led to a submission and approval in 2020 for the second tissue agnostic indication for Keytruda® within the high TMB disease state.

Having now done a lot of work around both the PD-L1 assay and the TMB assay, we know that these are predominantly independent of each other. For example, you can find patients who do not have a high level of expression of PD-L1, but whose TMB is high. That has further helped us to identify more patients than either test alone. However, it also allows us to classify patients by dividing them into four quadrants; some patients are positive for both, some are negative for both, and then you have two further variants where one is positive and the other is negative. A few years ago, we looked across our database trying to understand the genetics and biology of those four different quadrants, which resulted in a paper published in *Science*. We found that if you begin looking into those four different quadrants the biology is different, and you can find signatures that suggest it might be good to use a particular drug to target that particular biology. We have subsequently used that information to guide us in terms of target selection for new drugs that we want to combine with Keytruda®, as well as to study whether certain combinations that are now approved or under development might be preferable for a patient who is in a given quadrant.

That particular study, Keynote 495, is still underway. We classify patients using the TMB biomarker and a biomarker that is related to PD-L1, which is known as the gene expression profile. (It is an RNA-based test, but it correlates very strongly with the PD-L1 IHC test). We are looking at different combinations to see if there is a good match depending on how the patients are classified – we are variously studying Keytruda® in combination with Lenvima® (lenvatinib), with our own CTLA-4 inhibitor, and with our LAG-3 inhibitor. So this is a prospective study that is evaluating what you could term a precision medicine approach; trying to identify a particular combination that would match a specific patient's tumor biomarker state.

We are doing a lot of other things besides, but that is a summary of our most active efforts.

Q Can you expand on any recent advances in enabling technology innovation that you see as having the potential to create breakthroughs in this area?

EHR: There are efforts to combine biomarkers in multiplex assays. I think the technology is improving there, allowing one to look at multiple proteins at once instead of having to sample tissue multiple times for each individual test. That sort of advance will particularly enable the immunohistochemistry space, allowing a more sophisticated classification of patients than we are able to do currently as we typically look at one biomarker at a time.

The other technology area that I find interesting, and it's one where we have some collaborative work ongoing, is imaging. For example, there are some tests that will measure CD8 T cell activation that can be done through an imaging approach. The advantage there is you get a whole body perspective rather than having to rely upon a single biopsy, with the inherent issues of heterogeneity both within a given tumor location and across different metastatic sites.

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Q As Merck becomes more and more active and experienced with other established and emerging therapeutic modalities, how are your biomarker R&D activities evolving – and are there any specific examples you can tell us about?

EHR: We are certainly looking at combinations with our PARP inhibitor, Lynparza® (olaparib), which we co-own with AstraZeneca, and that includes a whole host of ongoing predictive biomarker work – of course, it is never clear at the outset whether a biomarker that is predictive with a monotherapy will also be predictive in the combination setting. So we have quite a lot of work going on across multiple tumor types with that particular combination, where we are looking at both the established biomarkers for both drugs as well as potential new ones.

I think you can extrapolate that approach to other combination settings. We have about 20 product candidates in our early-stage R&D pipeline and in every case, even before the drug enters the clinic, we are looking for potential predictive biomarkers based on our understanding of the biology of the target.

And of course, we are also bearing in mind the two established biomarkers for Keytruda® that we discussed earlier. We are always looking at whether those tests remain predictive in a combination setting or not.

Q Looking to the future, what is your expectation/vision for the convergence of immuno-oncology and precision medicine? What would be the key next steps forward, and what do you regard as attainable/realistic goals for the field as a whole in the foreseeable future?

EHR: To some extent, I think it is already there: as I mentioned earlier, for many tumor types, and for Keytruda as well as other companies' anti-PD-1s or

anti-PD-L1s, immunohistochemistry is now a fairly routine test that is now done for many patients with cancer – particularly where it is a companion diagnostic. And this is perhaps a good point to briefly mention complementary diagnostics, where the test is not required for use of the drug, but it is known that the biomarker is predictive. In those cases, it may be useful for a physician to do the test even if it is not actually required per the drug label. Again, this contributes to the increasingly widespread use of PD-L1 testing around the world.

It's a somewhat different story with MSI-H testing and TMB testing, although that is changing. MSI-H testing has also been around for a while now - in part because of prior work that established that for some patients (particularly in colon cancer, where it can be up to 10–15% of patients) it has prognostic information for the use of chemotherapy. There are actually two types of tests that are used there. One is an IHC test based upon looking at loss of mismatch repair proteins, which I mentioned previously. The other is actually a DNA-based test, which uses a polymerase chain reaction analysis to look for alterations in what are called microsatellites – small, repetitive sequences of DNA that are present in all of us. In cases where you have defects in these DNA repair proteins, they expand and they are unstable, which can be detected by that test.

So these tests are also in common use to identify patients. For us, that usage led to a product approval and one of the things that came with that approval was a commitment to develop FDA-approved versions of both DNA-based and protein-based tests. We are in the midst of collaborating with diagnostics companies to meet that commitment.

These tests are now fairly widespread then, and there are no real issues in terms of patients having access to them. However, an issue does arise (and I don't think it is unique to immuno-oncology) in cases such as pancreatic cancer. In these examples, in the absence of a high TMB or a microsatellite instability defect, the patients are generally not going to respond to I-O, and the likelihood of a patient with pancreatic cancer having a high TMB or MSI-H state is pretty low – probably in the range of 1–2%. So most of the time that test is going to be negative, and in light of that fact, you can see from both the physician's and the patient's perspective how they might conclude it is not worthwhile to conduct it.

I think part of our future effort as a field could be to try to enable such low prevalence biomarker testing. We need to mitigate frustration at the fact that 99% of the time it is going to be negative. Part of this will be to address the fact that the majority of TMB tests available today are relatively expensive - they tend to use next-generation sequencing approaches, which are more expensive than IHC tests, for example. However, along these lines, there is some interesting work that is now been published. Researchers are using artificial intelligence to look for patterns that would be present in a standard tumor biopsy slide specimen, which might engender confidence in predicting the likelihood of a positive TMB test or MSI-H test.

Q Finally, what will be your key priorities and goals in your work over the next 12–24 months?

EHR: I will continue to look for the next Keytruda® – I guess I would say is a large part of my work. I hope I find it! As we discussed, we do have a lot of product candidates that are either in late preclinical or early clinical evaluation.

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And the other part of my work moving forward will be assessing whether there are unique biomarkers that can be developed to allow us to identify those patients most likely to respond to a specific combination.

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EXPERT ROUNDTABLE

Hunting for better biomarkers of response: where is real progress being made in understanding why patients do and don't respond to I-O therapeutics?

ADIL DAUD
Professor of Medicine and Dermatology, University of California; and Director of the Melanoma program, Helen Diller Family Cancer Center, UCSF

Dr Adil Daud is an expert in immunotherapy and has pioneered the development of novel immunotherapeutics and targeted therapies. His fellowship in medical oncology was at the Memorial Sloan Kettering Cancer Center and was a faculty member at the Moffitt Cancer Center in Tampa, Fl. He is currently Professor of Medicine and Dermatology at the University of California, San Francisco. He is the director of the Melanoma Program and melanoma clinical research at the Helen Diller Family Cancer Center at UCSF. He led the development of IL-12 in melanoma and has developed novel technology to deliver it *in vivo*. He has played a major role in developing PD-1 antibodies in cancer therapy. Recently he has developed novel assays to determine immune responsiveness *in vivo*. These assays can determine the likelihood of response to immune therapy and provide novel insights into immune drug development. He has collaborated on numerous clinical and translational clinical trials that have yielded insights into the use of immunotherapy.

STEVEN FLING
Senior Staff Scientist, Fred Hutchinson Cancer Research Center; and Lab Director, Central Immune Monitoring Lab

Steven Fling, PhD serves as Lab Director for Central Immune Monitoring Lab (CIML), overseeing correlative biomarker studies for the NCI funded CITN (Cancer Immunotherapy Trials Network) and the industry funded ION (Immune Oncology Network). CITN/ION conducts early-stage, multicenter IO clinical trials, with a focus on agents/combinations to potentiate effective immune responses. Dr. Fling has >24 years research experience in T cell immunobiology, cancer vaccine and immunotherapeutic discovery, including ~13 years managing immunology research labs and directorship of collaborative research networks and consortia. Previously Dr. Fling served as Project Director for the IAVI's Neutralizing Antibody Consortium, an international consortium working to develop a vaccine for HIV and in 2009 established lab operations at IAVI's Neutralizing Antibody Center at the Scripps Research Institute (La Jolla, CA). In biotech R&D, Dr. Fling worked on vaccines for cancer and infectious diseases including adjuvant development at Corixa Corp (Seattle, WA) and as an Investigator in the Adjuvant Group for GSK Biologicals.

JOHN ROSSI
Senior Director, Department of Translational Medicine, Kite

I joined Kite in 2015 after 13 years at Amgen. I am currently a Senior Director in the Department of Translational Medicine at Kite and lead all pharmacology activities related to the clinical development of Kite's cell therapy pipeline. At Kite, I have built an effective translational team to support the clinical development of axicabtagene ciloleucel (Yescarta®) and KTE-X19 (Tecartus®). My team has contributed directly to the regulatory approval of these products through pharmacokinetic and pharmacodynamic evaluation. Among many achievements at Kite, I have represented the organization through external scientific presentations and collaborative manuscripts with leading academic researchers such as Steven Rosenberg and James Kochenderfer at the NCI. Accomplishments include the discovery of metrics to characterize CAR T based on functionality, novel biomarker knowledge of how CARs work in the clinic, mechanistic information on toxicities and insights into the biology of the TME, including immune checkpoints, and the role of IL-15 in the context of CAR T-cell function.

MAJID GHODDUSI
Senior Director, Clinical Biomarkers, Poseida Therapeutics

Dr Ghoddusi has over 15 years of experience in some of the most challenging areas of oncology with focus on drug discovery and clinical development. Dr Ghoddusi has broad and overarching insights into unmet therapeutic areas with expertise in translational sciences and clinical biomarker development which allows him to provide unique perspective on how to propel therapeutic projects from discovery to approval. Trained as a translational pathologist he has held numerous positions at large pharmaceutical and small biotech companies including Novartis, Celgene and Juno Therapeutics. His current focus at Poseida Therapeutic is Gene and Cellular Therapy.

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Q How would you each describe in broad terms the current 'state of the art' and the major challenges in the hunt for reliable biomarkers of patient response to I-O therapeutics?

AD: PD-1 has just been an enormous revolution in our field, but it still remains a mystery as to exactly how it's working.

We know it is expressed transiently on T cells and NK cells when they get activated, but exactly how it's working in human beings has been the focus of a lot of active research. And one of the issues is that in different study populations and different solid tumor malignancies, there are different biomarkers that seem to predict with variable accuracy what the likely response rate and long-term benefits of PD-1 will be. People have looked at tumor mutation burden (TMB), PD ligand expression, B-cell infiltration, the microbiome… My own group is very interested in the presence of exhausted T cells, or different types of T cell populations. Taking just one biomarker as an example, TMB seems to have a very different effect on PD-1 in different tumor types in lung cancer, but where the data from tumor mutation burden is strongest, there does seem to be a relationship. However, in some of the critical Phase 3 trials, it has actually not proven to be useful as a patient selection tool for treatment. There is an approval for pembrolizumab (Keytruda®) for

TMB-high patients (≥10 mutations/megabase), but it is based on something like a 29% response rate in that TMB-high population, agnostic of site.

And if you look at PD-L1, which is probably the most commonly used biomarker, it seems to work in some tumor types but not others. (I think that partly has to do with meeting a threshold for PD-L1 expression but then beyond that threshold, you don't get a lot more in terms of response). Taking melanoma as an example, which is a highly mutated tumor, PD-L1 is perhaps not as useful. We actually had a paper showing more PD-L1 indicates a higher response rate, but there doesn't seem to be a floor to that – even if you have low PD-L1, you can still have responses. Equally, if you have high PD-L1, that is still not associated with response on occasion.

So, I think the criticism of PD-L1 and of IFN-γ profile, and also of T cell infiltration, is that if you are selecting patients for PD-1 therapy, which can be extremely effective in a tumor type like melanoma, if you have a negative result, does that mean you should give that patient the benefit of doubt and let them try it, or not?

Right now, we don't have a red light/green light type of biomarker available. What we have to work with are patterns of response that are associated with positive, higher TMBs, or PD-L1s, or IFN-γ signatures, or T cells, or the presence of exhausted T cells. I think one of the key challenges is just in conveying all of that information to physicians who are seeing cancer patients in clinics, and trying to make a decision as to whether or not they should be given immunotherapy. I personally struggle with this issue every day and I don't think we have a greater answer that provides the requisite degree of specificity.

SF: At a high level, I think one of the major challenges in terms of identifying biomarkers is simply the fact that there are so many clinical trials and only so many patients. Deriving statistically significant, meaningful data can be difficult when you have limited numbers to deal with. And that's complicated further by the fact there is such heterogeneity amongst all of these different tumors and cancers. Even an individual cancer has multiple phenotypes - and genotypes, for that matter.

On top of that, everybody's immune system is different as well. So there are just multiple levels of complexity, which means that, ultimately, much of our success in identifying biomarkers is going to depend on precision medicine and an individualized approach to patients. That is another major challenge that I see.

AD: Just to expand on that comment, I think part of the issue with PD-1 mechanism of action is that there is definitely a systemic contribution as well. We know that those exhausted T cells in the tumor microenvironment (TME) were systemically primed T cells from throughout the body – in other words, a lot of what you see in that TME is a product of what is happening in the rest of the body. We have published data on this previously, about the presence of liver metastases, the presence of obesity, the male gender, ECOG performance status, lactate dehydrogenase (LDH), tumor size, tumor extent, sites of metastases… All of these seem to matter just as much as the presence of T cells in the TME, or PD-L1 positivity, or IFN-γ profile, or TMB.

I think that part of that complexity of immunotherapy has to do with the possible need to meet a threshold and yet sometimes, once we've exceeded that threshold, we still have a non-productive immune response. In those instances, perhaps we are just not setting off the fuse. We don't fully understand why we can have all the ingredients – a high TMB, T cells in the TME, etc. – but we are just not getting a productive immune response.

MG: The world of oncology biomarkers was probably easier when there were just targeted therapies, and the focus was mostly on tumor cells and their

characteristics. But as things have shifted towards immuno-oncology, with the individual variation between individual patient's immune systems, the complexity has multiplied quite considerably, as has been discussed. That has made statistical analysis and finding meaningful correlations a major challenge. And as has been pointed out already, threshold of presence or expression of particular biomarkers and its correlation with the immune response has also been a challenge for the field.

These are really difficult obstacles to overcome. But then adding to the complexity is the fact that, more or less as soon as the first I-O agents were approved, we as a field started gravitating towards combinations to try to generate better responses. That really enormously complicates things in terms of finding and validating a biomarker of response.

SF: A major challenge in research as we seek to identify biomarkers is the reality that our sampling of tissue is static, for the most part. For example, if we want to look at the TME, we are just taking a single snapshot (or two snapshots, if we are lucky) over a period of time. But the biology is very dynamic – things are in transition, they are moving. So it becomes difficult to assess what is really going on, biologically speaking, and therefore, what might be really meaningful from a biomarker standpoint.

Furthermore, just obtaining post-treatment biopsies can be difficult in many instances, depending on the indication, so you don't know really what is happening, or not happening, in the tumor at all during treatment. I think sampling is a major obstacle in immune biomarker research.

JR: I agree. We've covered a lot of ground here on a very difficult concept, but I'll add just a couple more points.

One of them is that immune function in the context of tumor biology and tumor clearance is also very poorly understood. I'll give you an example from the CAR T cell therapy world. We've published on the fact that we see activated, non-CAR transduced T cells in the TME, around the time that we are seeing peak activity by cytokines and serum, and measuring levels of systemic CAR T cells in blood. That was a surprise to us. And we have also seen the role of Fas ligand and other pathways that we didn't think would be important for CAR T cell-mediated killing. This is just to illustrate that, in this particular case, the mechanism of action is very poorly understood for what is a commercial product. (And now there are four CAR T cell therapies that have been approved).

When you think about it in this context – that the general immune function in the context of cancer is not as well understood as we might think – then discovering biomarkers that are predictive becomes that much more challenging. And that is on top of heterogeneity of disease, on top of heterogeneity of the immune system, sampling issues, points in time… All of these pieces that have been brought forward by the other panelists.

Lastly, when I think about biomarkers in general, my first question is, this is a biomarker of what, for what? Because there are going to be different diseases, different treatment modalities – and of course, we are not always looking at response. We're trying to predict toxicities, for example, or understand relapse and the changes that occur in the tumor at that time.

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- **John Rossi**

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So it's very challenging, a tough question for the whole field. And the further point was made earlier that as we move away from a single pathway-targeted therapy – a HER-2-directed antibody, an EGFR small molecule, or what have you – then the pathways become more complex. And that continues to raise the bar.

Q Diving deeper into a couple of specific areas, what would be your assessment of current progress in, and remaining challenges or directions for, the harnessing of peripheral blood biomarkers in the I-O space?

SF: I think that in terms of the periphery, what's happening and needed in the field is multi-parametric and longitudinal analysis of highly multiplex datasets of cell subtypes by flow cytometry, which when combined with machine learning and, for example, CyTOF (cytometry by time of flight) analysis with tetramers, can provide us with the ability to pick out antigen-specific cells in the periphery that might be associated with a response to tumor. I think including cross-analyses of those types of data with plasma protein profiles and metabolites in the periphery will allow the field to more clearly dissect dynamic biomarkers that would be indicative of clinical response. That's the way I see the field going.

AD: Adding to that, I think there is definitely a strong contribution being made by peripheral priming. T cells from the periphery seem to come and replace exhausted T cells, once they've been stimulated by PD-1, and once you get rid of that initial batch of T cells.

I think the challenging thing now with T cells in the periphery is that you probably have that kind of priming happening in most patients with melanoma, which is highly immunogenic, and the question is, how do you turn that into a biomarker? I think Steve's answer illustrates some of the complexity in answering that question. Yes, you have those cells. But are they going to come in and kill tumor on cue, or are they not?

I follow the dendritic cell field, peripherally (my primary interest is in T cells) and in that field, it is clear that you need to have these type 1 or type 2 dendritic cells, and they kind of license activity in the tumor microenvironment. One of the interesting things I see in the CAR T cell literature is that one of the reasons they don't seem to work in solid tumors is they don't appear to attack the tumor when you have an ice-cold TME, despite being in position to do so. For instance, some of the work from Elizabeth Jaffee at Johns Hopkins shows that when you have CAR T cells directed to mesothelin, which pancreatic cancer cells do express, those CAR T cells don't seem to get engaged and kill cells in the TME. And presumably, that is because they are lacking some sort of signal or license from dendritic cells that it is OK to go and attack.

SF: The dendritic cell field is obviously highly complicated. We know there are so many different types of dendritic cells, and their characterization is currently insufficient to tell us what the various types are responsible for priming, either in the TME or the periphery.

That highlights to me the importance of and need for computational analysis of these minor subsets – to be able to analyze which cells are involved with priming the T cells, for example.

"For immuno-oncology and cellular therapies ... we are still far away from being able to identify one or two truly breakthrough biomarkers to tell us what is going on, or to predict what is going to happen in future."

- **Majid Ghoddusi**

We recently published a vaccine study with Nina Bhardwaj on expanding dendritic cells with Flt3 ligand, then vaccinating with an antigen NY-ESO-1 vaccine so that it was targeted to dendritic cells [1]. Within that study, we looked at our flow cytometry and carried out some detailed analysis. We were able to pick out in the periphery a minor subset of dendritic cells that appear to be associated with optimal T cell responses. The only point I'm trying to make here is that I think it is useful to sample the periphery, because it is a dynamic system. It's easy to sample and if we apply some high computational approaches to it, we can hopefully dissect what's going on in the tumor.

We also have a collaboration with Garry Nolan and Darci Phillips at Stanford, which is another pembrolizumab study submitted for publication. It is a spatial analysis of the TME, which we have subsequently paired with sampling of plasma from the periphery. To make a long story short, what we saw in the TME was the release (due to the spatial organization and the relationship of the cells within the tumor) of certain cytokines and chemokines that correlate with response. And we also found those same chemokines and cytokines in the periphery correlating with response.

So, if I were to make an assessment of where the field is moving, and what the big picture is for the periphery, I would highlight these very complex (and admittedly, expensive) technologies that allow us to investigate these spatial relationship – tools like multiplexed ion beam imaging (MIBI) and CODEX. These types of analysis allow us to tease out what's going on interactively between cells in the TME. We can then pair that with data we can assess from the periphery and perhaps ultimately find biomarkers in the periphery that we know would be associated with those types of interactions in the TME. I think that's where and how the field will grow, because I don't think we can afford to assess every patient by spatial topography assays. We need simpler, more affordable assays.

MG: In my view, the biggest progress in circulating biomarkers is still ctDNA (circulating tumor DNA), although that really still relates mostly to targeted therapies with single genetic aberrations or mutations – cell and gene therapies have been a little more challenging than the rest in this regard. For immuno-oncology and cellular therapies in particular, and the immunophenotyping of immune cells, ctDNA definitely gives you a clue, but I would say we are still far away from being able to identify one or two truly breakthrough biomarkers to tell us what is going on, or to predict what is going to happen in future.

My biggest hope in this area is towards circulating tumor cells (CTCs). Unfortunately, the yield is still a challenge there – it varies from individual to individual, from tumor to tumor, and across different indications. However, there are improvements in terms of technology – how we better extract those CTCs and what we do with them – especially in the shape of single-cell analysis. We really have seen a lot of improvement in that field – I can now obtain a wealth of data from just a relative handful of CTCs that we manage to get from the periphery.

SF: On that note, what do you know about the exosome field as reflective of tumor burden and so on, Majid? Do you

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follow that? I know that is a field worth investigating.

MG: Yes, it's been getting hotter and hotter - I see more people focusing on it and papers coming out. I think it's a promising field, but it's really too early-stage to see something concrete coming out of it just yet. It definitely looks to be a promising avenue, though, and something that we hadn't put very much research effort into previously.

AD: I think that if you are looking for a dynamic picture, as Steve was discussing earlier – gaining multiple snapshots rather than a single snapshot in time – then I think CTCs and the kind of analysis Steve talked about makes a lot of sense. Because you might be able to biopsy a tumor once, but are you really going to be able to do sequences? Probably not, due to the difficulty in getting hold of tumor samples.

Q The challenge obviously becomes still greater in the combination therapy setting – what should be the next steps in biomarker R&D in that particular sphere?

JR: I think about addressing this in terms of what treatment are we using as the backbone, and what are we going to combine it with. I will use CAR T cell therapy as an example again, and the comparatively easy, low-hanging fruit of combining it with a checkpoint inhibitor such as an anti-PD-1. We ran a study with Genentech using atezolizumab (Tecentriq®) and even with biomarkers available – we looked at PD-L1 expression in lymphoma tissue, specifically – it was not predictive of response.

With CAR T, your overall response rates tend to be high, in the 80–90% range, and the complete response rates are relatively high as well. So what we were hoping to achieve through this combination was to extend the durability of response – to bring the complete response rate up through enhancement of our PK profile, by blocking cell exhaustion through the PD-1 pathway. It didn't work at all, but the results were equivocal. So that led to different questions on our end about what biomarkers should we have been looking at. Well, it turns out that we were looking at the wrong constellation of checkpoint markers. And there is more to the story both in terms of the final CAR T product itself pre-infusion and how activated an individual patient's T cells were at the other end of the manufacturing process, and what the tumor was expressing in terms of ligands to activate those checkpoint pathways. In other words, it becomes a much more complex question.

Of course, the other key aspect with combination studies is going to be toxicity. Sometimes toxicity, particular with an I-O agent, can tell you a lot about the mechanistic function of the immune system in the face of whatever therapy you put forth – a spike in IFN-γ with a checkpoint, for example. With CAR T, looking very broadly at serum biomarkers, cytokines, chemokines, evidence of effector molecules, granzyme release, etc. is important. The pharmacokinetic profile of the living drug plays a role: what does the expansion look like? What does our E:T ratio actually look like? And then, what is happening within the tumor?

This last question is very important because highly potent I-O agents such as CAR T cells bring a very, very strong IFN-γ signature upon activation in the TME, and that very quickly drives up a whole constellation of compensatory pathways as checkpoints. We see changes within 4–5 days when we serially biopsy patients in that setting. And then

there are changes that continue until the tumor is either cleared or relapses.

It turns out that a good way to start to track that, and to think about what is happening in the tumor, is a systems biology approach. We look at product features, pharmacodynamic features. We are starting to explore metabolomics assays using mass spectrometry as a means of looking more broadly, but we are also using things like CAP-Seq, which is a plasma-based ctDNA assay that allows you to track many mutations at once. And we can actually see the disappearance and emergence of dominant mutations over time, which tell us that the tumor is changing, And that's just done with plasma, for example.

To go back to the very challenging question of what biomarkers do you use for a combination, I think it should be fit-for-purpose based on the treatments that you are working with. The patient population and indication have to be considered too, of course. However, I am not sure there is a clear answer as yet, other than to take your best hypotheses and test them in the clinic. That is where I sit, personally: within the translational team on the development side. But again, it's challenging. You become limited by the number of samples you can take over time: you can only draw so much blood from patients; you can't biopsy everyone. Getting tumor samples is incredibly difficult. For one thing, obtaining samples from the treatment centers is becoming harder and harder, because nobody wants to give them to the drug developer who is running the study - they want to keep them for their own research.

I think we can look to harness these other approaches we have discussed – using plasma, more accessible sample types – to try to find changes in biomarkers we think are important in the combination setting. But in terms of making reliable up-front predictions, while we have good ideas there (for example, if I was going to combine CAR T with PD-1, I would look at PD-L1 in the tumor, as I described earlier) I don't think we can say with certainty that we know what to look at for any of our drugs.

That said, I think we are learning more in the I-O space every day, and I do think we will have predictive biomarkers of response – and very strong ones – in the relatively near-term. I believe we will start to move away from a lot of what we have been discussing, which are really more prognostic markers of outcomes than actual predictive biomarkers.

MG: The message for me there is that the complexity goes up quite enormously when you combine things together. And agents that may work as monotherapies may not work in the same way when combined together. It's really a huge challenge for the field.

Q Do you see the regulatory environment shifting in relation to the acceptance of I-O-related biomarkers, currently? And what would you like to see as the focus for future regulatory evolution?

MG: Looking at what is in play in the regulatory environment, both in terms of guidelines and requirements, I don't really see that anything unreasonable is being asked of I-O drug developers. But of course, that doesn't make it easy, either!

From my point of view, the main challenge I have faced is to ascertain what exactly is the level of validation I need to put in

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place for a particular assay to make the data presentable and acceptable to an agency. I don't think there is a really clear indication as to how far you need to go in that regard, especially for a developing field such as CAR T cell therapy where we are all still learning. So, we put something forward, and we get knocked back, and we go back to the drawing board and do it again but differently. It would be nice to have a better mechanism or better, more clear and precise guidelines up front, which clearly set out standards for assays, so that when we collect data, we don't need to repeat everything – we don't need to go back and design another prospective study to gain validation, we can just go ahead with the data we have already collected. I think that piece will be put in place as the field continues to grow, and we have more conversations with the involved agencies and other organizations.

Additionally, there has been some talk around CAP/CLIA assays and laboratories, which to a large extent are bread-and-butter for rapidly evolving fields like immuno-oncology. There was some indication that the FDA might step in and more directly regulate those assays and those labs in order to bring the standards even higher. Of course, everybody wants to make sure an assay is good, that a biomarker actually works for the intended purpose. But raising that bar would also bring with it a lot of challenges, particularly in the form of logistical issues and costs related to getting everything to the new higher standard and making it acceptable to the agency. I am not sure if anything further has happened with regard to this issue, or if anything concrete has been put forward, but at least the rumors have not gone away either.

> "...the field needs to be holistic, but ... in order for this to happen there has to be a greater emphasis on the myeloid component. I don't think we have appreciated just how important that is yet."
>
> - **Steven Fling**

Q Broadly speaking, do biomarkers in I-O need to be more adaptive or innate immune system-centric, for you?

JR: Neither. I think it needs to be holistic, and you need to look at both arms of the immune system – the adaptive and the innate.

You need to look at myeloid-related activity. The full gamut of immune cells that are present in the periphery and in tissues and tumors needs to be understood, because each of these immune cell subsets plays a role, one way or another, in the outcome for the patient.

We know from the work of Jérôme Galon and others with their Immunoscore and Immunosign that they can look just at the presence of CD8 cells, and higher levels are prognostic for a good outcome, for example. We know that the balance of myeloid-derived suppressor cells and M1 and M2 macrophages in the tumor is important. We have touched on dendritic cells and their role, class 2 mediated immune responses in the tumor… all of these play in together. So, I don't think you can focus on one or the other, they are all important. And I think that is the case for cancer treatment in general, not just immuno-oncology. The immune system is playing a role whether you are using radiation, chemotherapy, or a checkpoint inhibitor or a CAR T cell.

SF: I agree completely. I come from a background of antigen processing and presentation and I really do believe that the intersection of the innate and adaptive immune systems is where we control everything, so it has to be holistic.

I think, though, that the field of cancer immunotherapy has initially been biased towards a focus on adaptive responses. For the most part, if you are a cancer patient and you are looking for treatment, most of those available are associated with adaptive-type modalities. To my knowledge, there aren't that many myeloid-affecting therapies out there yet. So, on the whole, the field needs to be holistic, but I would add that in order for this to happen there has to be a greater emphasis on the myeloid component. I don't think we have appreciated just how important that is yet.

JR: Absolutely. Gilead just acquired Forty Seven, Inc., whose lead drug candidate is a monoclonal antibody that essentially cloaks the CD47 on the surface of tumor cells – the "don't eat me" signal – and the data I've seen and that I think has been published looks quite compelling. It does clearly show the role of myeloid-related inflammation, and what happens in terms of the response to a therapeutic: it builds, and then we have JAK/STAT inhibitors that not only reduce T cell-related inflammation, but also myeloid-related inflammation. And we can measure down-regulation of myeloid-related cytokines and chemokines with these agents, just as much as we can see the impact on IFN-γ, IL-2 and other type 1 cytokines.

So again, while you may focus on one area more than the other based on the treatment modality, I think you are going to find the interplay between innate and adaptive immune systems sooner or later. And this goes t our earlier discussions around the complexity of the human immune system, layered on top of the complexity of tumor biologies, creating a Mount Everest of a challenge for biomarker R&D. It means we do need to continue to look as broadly as we can, but in a smart, directed way – not just generating huge datasets that we then try to mine. We are always going to find interesting things in an RNASeq experiment, for example, but when we are searching so broadly, I think it is important to really try to define our hypotheses up front and then test them.

AD: I think that myeloid and dendritic cell biology is very important. For me, one of the holes in our current biomarker approach is just trying to understand exactly where myeloid biology is the limiting factor, and not adaptive response.

Steve mentioned the fact that we haven't had a successful therapeutic in that area yet – it's certainly not for lack of trying: there have been the CSF1 (colony-stimulating factor 1) drugs, and multiple attempts at getting the myeloid biology or DC biology right. But we just don't know where it becomes the limiting factor. I think it goes back to our biomarker discussion and just trying to figure out from human tumor types where exactly do we have an adaptive response. For example, if we look at the TLR agonists, we are seeing effects in tumors that are already pretty hot, but not in tumors that are presumably in need of that innate system signal or license we spoke about earlier. And why is that? We don't really have a good answer at the moment, but I think that maybe in the next few years, we will get a better idea of where a certain innate pathway is a limiting factor.

> "...one of the things that we are maybe failing in right now is not providing clinicians with a simple tool to assess how hot or cold a tumor is."
>
> - Adil Daud

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Q What will be the important tools to harness in the ongoing search for novel biomarkers in the I-O space – for example, single cell sequencing – and what will be the keys to their successful deployment?

MG: I mentioned earlier that I think single-cell analysis is what the field will most likely move towards – that, and using the peripheral blood as matrix, because it is so easy and non-invasive for the patient.

In addition, I would highlight the importance of artificial intelligence and machine learning in helping us interpret the data and find relationships. I think harnessing those tools will be key for success because as Steve mentioned, we get so much data at the moment that we simply don't have the capacity or capability to mind effectively.

Q In our research quest, how do we best balance the need for information/data and protect patient and clinical trial patient rights and confidentiality in such a rapidly moving field?

SF: Scientists are inquisitive. It's our job to ask questions, to want to analyze a sample for a particular scientific question or hypothesis. And that drives the field forward, that drives knowledge. But of course, you are constrained by what was agreed to within the protocol, and what the patient agreed to in terms of access to information.

That's a difficult challenge because the field is moving so rapidly. Bureaucracy can be a four-letter word, I suppose, but you do need that bureaucracy to make sure inquisitive scientists adhere to IRBs (Institutional Review Boards) and patients' rights.

Q Finally, what do you each see as the future of 'precision I-O' in clinical routine? And what steps should therapeutic developers be taking today to ensure the biomarkers and diagnostics of the future are fit for purpose in that environment?

AD: The one organizing concept I see is 'hot versus cold'. I recognize that is overly simplistic: there are lots of varieties of 'hot' – it's not at all clear to me that PD-L1-positive lung cancer is the same thing as a PD-L1-positive melanoma, or a PD-L1-positive gastric cancer, for example, and we are seeing that there are very different response rates there, as there are with TMB as the marker. But I do feel that one of the things that we are maybe failing in right now is not providing clinicians with a simple tool to assess how hot or cold a tumor is. I hope that in the next few years, the community could come up with a simpler

assay that just integrates things like TMB, PD-L1, and IFN-γ. It wouldn't answer the peripheral question and you would still need to do a lot of work on integration and understanding the context, but it would give you usable tumor-specific insights. I feel that this could be achieved with the help of machine learning and the like.

JR: Yes, I think we are heading towards having those types of assays. I mentioned the Immunoscore, which is essentially CD8 immunohistochemistry – looking at the density of the CD8 cells in the context of checkpoints, location, border, infiltrated, etc. is fairly useful, although it's not the whole picture.

More broadly, what we really need to be asking is why is a tumor cold and why is a tumor hot? And how could you change a cold tumor to a hot tumor, if we all believe that a hot tumor is going to be more sensitive to any given I-O agent – X, Y, or Z? That is the real question, for me. We know that TMB, microsatellite instability, etc. all plays a role, but that's not the whole story. Steve described his work earlier with the Stanford team, and we looked at the same thing – cytokine production in the TME. We know that patients who locally produce IL-15 in the TME do better with CAR T cell therapy. Now, we could have measured IL-15 systemically all day long, but it wasn't until we looked in the tumor and found that there are a number of chemokines, which are homing signals for immune cells, that are either up or down. In other words, they play a role. Why are they there? Why are they not there? We don't know.

For me, those are the important basic science questions at this point. I think that before we can start talking about what is a good biomarker of a hot tumor, we need to discover just why it is hot. What does that mean versus a cold tumor? I don't think we have all of the answers right now. There is a good grant in there for someone to think about funding!

SF: I believe that most of what we do in this I-O world is going to need to be individualized. And I think the only way to understand and apply it to the individual is to have highly complex algorithms that take this enormous amount of data we generate and integrate it to come up with answers as to why a particular patient doesn't have a hot tumor, for example.

In my experience, we have more data than we can handle. There is information out there, there are answers waiting within those datasets – we just haven't figured out exactly how to dovetail all these different types of peripheral data, tumor data, and other information together. I think the future of precision medicine in the I-O space will be having algorithms that can analyze that data rapidly and come out with an interpretation.

MG: In my view, the future will see us move towards having an algorithm of biomarkers – an array of different biomarkers for individual patients, rather than a just one or two, which can tell us how an individual patient is going to benefit from an I-O therapy.

This is because we are moving towards tools that generate more and more data, as the other panelists have mentioned. For example, single-cell analysis is moving towards gaining us genomic, transcriptomic, and proteomic data from one cell. And I fully agree that it's really beyond us to sit down and analyze all of that in a meaningful way. We will need to create data lakes and harness machine learning tools that connect all these elements and data from various sources together, so that when we ask the system to analyze for a certain patient, it will come back with a panel of maybe 20 biomarkers, perhaps with different thresholds based upon their stage of disease, what line of therapies they are receiving, etc.

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IMMUNO-ONCOLOGY INSIGHTS

HUNTING FOR BETTER BIOMARKERS OF RESPONSE

SPOTLIGHT

REVIEW

CTLA-4 expression by human tumor cells and its impact on immunotherapeutic strategies: a systematic review

Farah Abdulkhaleq, Niss Larossi, Okanda Ogbonda, Rasha Abu-Eid & Frank James Ward

Background: Cancer is a leading cause of death worldwide and its development is closely related to immune dysfunction. Immune checkpoint (IC) receptors maintain immune homeostasis to protect normal tissues, but cancers use several immune escape mechanisms including altered IC expression to evade destruction by the immune system. Cytotoxic T-lymphocyte associated antigen-4 (CTLA-4) is one such IC, which downregulates T-cell activation. There are at least two isoforms of CTLA-4 in humans; the full-length receptor isoform and an alternatively spliced soluble CTLA-4 (sCTLA-4) isoform. The aim of this systematic review is to investigate whether or not human tumor cells express CTLA-4, and to examine if there are any consistent retrospective correlates of increased CTLA-4 expression with disease outcome.

Methods: We searched Medline, Scopus, Embase and Web of science for original research articles that investigated CTLA-4 expression by human primary tumor cells or tumor cell lines, from 1987 to April 2020. Forty-five records were deemed eligible and data describing tumor site and stage, CTLA-4 isoform studied, test sample and control groups involved, methods and level (mRNA or protein) of detection, location and any retrospective association with disease outcome were extracted.

Results: Of the forty-five eligible manuscripts, thirty-eight studies focused on the full-length isoform, one study focused on the soluble isoform and six studies investigated both. Forty-two studies reported an increase in CTLA-4 detection by cancer cells. Twenty-one manuscripts performed a retrospective comparison of patient outcomes in CTLA-4 high and low groups in terms of overall survival; eleven studies found that high tumor CTLA-4 expression correlated with poor outcome while seven studies found an opposite correlation. Three studies, however, reported no association.

Conclusions: This review provides strong evidence that a variety of cancer cells express both CTLA-4 transcripts and functional CTLA-4, detectable in the cytoplasm or on the cell surface. Overall, the data suggest that CTLA-4 expression levels in cancer cells are an important but variable feature of the disease phenotype, which will be both increasingly important to evaluate in the context of immune CI therapeutics, and may also be a useful response biomarker.

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INTRODUCTION

Cancer is one of the leading causes of death worldwide, causing an estimated 9.96 million deaths in 2020 [1]. The most common types of cancer include lung, breast, colorectal, prostate, leukemia, lymphoma and skin cancers (carcinomas and melanomas). Limitations of both cancer diagnosis and effective treatment place a colossal strain on those affected, as well as healthcare budgets for middle- and low-income countries [2].

Our current understanding of how cancers develop points to an initial failure of immune surveillance and elimination of transforming cells, followed by an equilibrium period in which nascent cancer cells are kept in check by the immune system, and finally the evolution of molecular mechanisms that allow the cancer to evade the immune system to proliferate and metastasize uncontrollably [3,4]. Cancer cells can escape detection by the immune system through a number of potential mechanisms that can model the tumor microenvironment to tolerate growth of the tumor. They can secrete immunosuppressive factors, such as TGF-β and IL-10 [5,6] or promote recruitment of immunosuppressive cells, such as regulatory CD4 T cells (Treg) [7] and myeloid-derived suppressor cells (MDSC) [8] to the tumor microenvironment. Intrinsically low or loss of MHC class I molecules also allow escape from detection [9]. Moreover, cancer cells can take advantage of immune checkpoints by usurping either directly or indirectly their function, including CTLA-4 on regulatory T cells and programmed cell death-ligand 1 (PD-L1) on tumor cells, leading to dampening of the anti-tumor immune response [10,11]. Maintained high exposure to antigens in the tumor microenvironment, induces a state of dysfunction in anti-tumor effector T cells, called T cell exhaustion [12]. Exhausted T cells are terminally differentiated T Cells that lose their functionality and consequently fail to effectively eliminate cancer cells. They increasingly and sustainably express multiple inhibitory receptors, including CTLA-4 and programmed cell death-1 (PD-1) [13], which suppress their effector function.

The emergence of effective immunotherapy by antibody-mediated checkpoint blockade now offers new opportunities for improving patient outcomes in a range of cancers [14]. Immune checkpoints are typically surface receptors on T cells that aid in maintaining homeostasis, particularly during resolution of an immune response [15]. Unlike traditional cancer therapies that exhibit direct cytotoxic effects, e.g., chemotherapy and radiotherapy, blockade of immune checkpoints functions indirectly by boosting anti-tumor immunity [16].

Cytotoxic T-lymphocyte associated antigen-4 (CTLA-4) or CD152 is a well-known immune cell checkpoint receptor. This full-length receptor isoform, also called transmembrane CTLA-4 (tmCTLA-4), is constitutively expressed in homodimeric form on the surface of regulatory T cells and activated effector T cells [17]. A second less well-known

isoform, soluble CTLA-4 (sCTLA-4), is secretable and produced by alternative mRNA splicing of the *CTLA-4* gene [18,19].

Ipilimumab, a monoclonal anti-CTLA-4 antibody and the first approved checkpoint inhibitor (CI), was approved for the treatment of malignant melanoma in 2011 by the FDA [20]. Immunotherapy with anti-CTLA-4 CI antibodies has been somewhat overshadowed by the emergence of anti-PD-1 and anti-PD-L1 antibodies that have seen much greater clinical and commercial success [21,22]. These antibodies, first introduced in 2014, target PD-1 on anti-tumor effector T cells or PD-L1 on tumor cells. Patient response frequency and stratification are aided by PD-L1 staining levels on tumor biopsies [23]. Since their inception, the use of antibodies to inhibit the PD-1: PD-L1 axis has been approved for the treatment of over 20 cancers including non-small cell lung cancer [24]. Anti-CTLA-4 antibodies, in comparison to the anti-PD-1/PD-L1 antibodies have received fewer FDA approvals despite their potential to completely eradicate disease and provide an enduring remission from disease. Ipilimumab is currently approved as a monotherapy solely for melanoma but has also been partnered with nivolumab (anti-PD-1) for several cancers including advanced renal cell carcinoma [25], metastatic colorectal cancer, non-small cell lung cancer [26] and malignant pleural mesothelioma [27]. This has resulted in a significant increase in the number of patients receiving long term survival benefits [28,29] compared with monotherapy. Therefore, it is now imperative to understand the role of anti-CTLA-4 therapy as well as CI therapy more broadly, particularly its effects on the tumor microenvironment including effector immune cell activation or regulatory T cell depletion in order to optimize treatment. Indeed, a combination of tumor intrinsic, immune cell specific and even tissue contextual biomarkers may need to be combined in future bioassays to both stratify responsive patients and refine dosing strategies for an optimum outcome [30].

Although CTLA-4 is generally associated with immune cells, particularly T cells, it is also expressed by a number of non-immune cells including pituitary gland cells [31] and cancer cells [32]. The aim here was to survey and review systematically which tumors have been reported to express increased tumor cell levels of tmCTLA-4 or sCTLA-4 and further to determine whether patient outcome was influenced by the level of CTLA-4 expression by tumor cells.

METHODS

We conducted and reported this systematic review following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) recommendations [33].

Search strategy

A systematic search of Medline, Scopus, Embase, and Web of science biomedical and pharmacological databases of published literature from 1987 (discovery of CTLA-4 [17]) to April 2020 (date when search performed) was conducted. The search was restricted to studies published in the English language and studies conducted on humans and for studies related to the expression of CTLA-4 and/or sCTLA-4 by cancer cells. The following keywords were used in our search strategy: (CTLA-4 OR sCTLA-4 OR "soluble CTLA-4" OR CD152 OR tm?CTLA-4 OR "transmembrane CTLA-4" OR CTLA-4delTM OR "cytotoxic T lymphocyte associated protein?4" OR "soluble cytotoxic T lymphocyte associated protein?4" OR "cytotoxic T lymphocyte associated antigen?4" OR "soluble cytotoxic T lymphocyte associated antigen?4") AND (cancer* OR malignan* OR tumor* OR tumor* OR neoplasm* OR "cell line"). The final search was performed on 11 April 2020.

Our inclusion criteria were:

1. Original research articles

2. Articles published in English
3. Studies assessing the expression of human full-length and/or soluble CTLA-4 by cancer cells
4. Studies conducted on human samples or human cell lines

Our exclusion criteria were:

1. Case reports, case studies, letters to the editor, conference abstracts, comments, review and systemic review articles
2. Studies conducted on animals
3. Studies assessing the expression of transmembrane and/or soluble CTLA-4 in the tumor microenvironment including infiltrating lymphocytes

Duplicates were removed (based on authors, title, journal, volume, issue and page numbers), using the referencing software Mendeley. Titles and abstracts were screened for potential relevance. 101 records were passed to the second stage (full-text screening) for further screening and data extraction.

For these 101 entries, the full text of the articles was obtained. In case of articles without full text, we searched for the relevant full-text articles using the authors' names and/or combinations of the title words or requested a full-text from the authors. Full texts were subjected to the inclusion and exclusion criteria listed above. All entries and full texts were evaluated independently by members of the study team; the senior author (FW) checked for accuracy and settled any cases of disagreement.

Data extraction

The studies which met the inclusion criteria were summarized and data extraction was performed independently by three investigators, using a pre-defined form and accuracy checks were performed by FA, RAE and FW. Data extracted included: First author, year of publication, sample size, control group, tumor site, clinical stage, study design, method of sample analysis, CTLA-4 isoform analyzed and association of CTLA-4 expression with tumor progression.

RESULTS

Manuscripts included in the systematic review

Of 4911 identified citations from the search results, we identified 101 articles which met the inclusion criteria by title and abstract screening. Most of the identified studies did not discriminate whether CTLA-4 was expressed/produced by the tumor cells or the microenvironment (immune cells), or if the studies only focused on CTLA-4 in immune cells. These studies therefore, had to be excluded as they did not meet the inclusion criteria. It was not possible to refine the search strategy to address this as this can be only identified upon screening the manuscripts. Following full text screening, 45 articles were deemed to be eligible for inclusion in this study. Figure 1 shows the flow diagram of the studies retrieved for this systematic review. The characteristics of these studies are listed in Table 1 [32,34–77].

Manuscripts excluded from the systematic review

As illustrated in Figure 1, a total of 12,174 results were obtained from the search from different databases. Following removing the duplicate, of the 4911 identified citations, we excluded 4810 articles that did not meet our inclusion criteria by title and abstract screening. Following full-text screening, 56 articles were excluded due to the reasons listed in Figure 1.

Data summary

The full characteristics of the study populations in the included manuscripts are displayed in Table 2.

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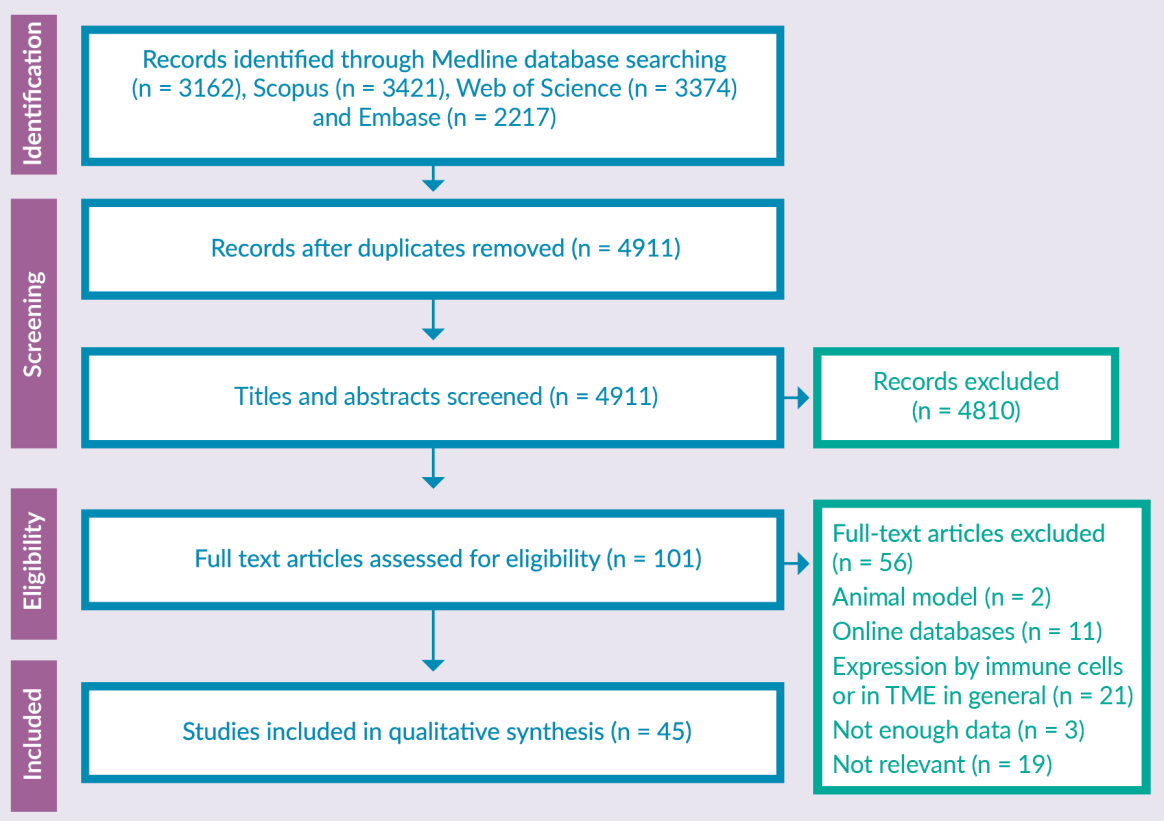
FIGURE 1

Flow diagram of the studies retrieved for the review.



Samples & controls

All studies were conducted on human samples, either by extracting tumor cells and tissues by surgery from patients (n = 34), by using commercially available cancer cell lines (n = 5) or by both (n=6). 23 out of 45 studies included control groups, either tissues or cells from healthy volunteers or normal tissues adjacent to tumors from the same patients. However, the remaining 22 studies did not mention any information about including controls. Table 2 summarizes the study population and the control group.

Tumors expressing CTLA-4

The studies assessed CTLA-4 expression mainly in leukemia/lymphoma (n = 12) (two of the studies assessed the same cohort of CLL patients [36,37]), breast cancer (n = 7), lung cancer (n = 7) and melanoma (n = 6) while the remaining articles were about gastric cancer (n = 3), esophageal (n = 2), uterine (n = 1), cervical (n = 2), ovarian (n = 1) and nasopharyngeal cancers (n = 2), thymoma (n = 1), mesothelioma (n = 1), testicular cancer (n = 1), salivary cystic carcinoma (n = 1), osteosarcoma (n = 1), rhabdomyosarcoma (n = 1), neuroblastoma (n = 1), renal (n = 1), colorectal (n = 1), bladder (n = 2) and bile duct cancers (n = 1). Figure 2 summarizes the different types of cancers that express CTLA-4 which were reported in the manuscripts included in our study.

Twelve included articles discussed the expression of CTLA-4 in leukemia/lymphoma. The subtypes of leukemia/lymphoma studied were: CML (n=1) [34], ALL (n=2) [34,42], AML (n=2) [34, 36], CLL (n=5) [34,45,47,48,67] with two studies assessing the same cohort ([47,48]), ATL (n=3) [35,39,77], CTCL (n=1) [37] and mantle cell lymphoma (n=1) [69]. All these studies showed that malignant cells express CTLA-4, apart from

TABLE 1

Main characteristics of eligible studies.

Author	Year	Cancer subtype	CTLA-4 isoform	Studies conducted on mRNA or protein	Method for CTLA-4 detection	CTLA-4 expression
Pistillo *et al.* [34]	2003	AML CML B-ALL T-ALL B-CLL T-CLL	Tm & s	mRNA and protein	IHC, Flow cytometry, RT-PCR, Western blot	Expressed in 25–85% of AMLs and CMLs; positive expression in B-ALL, T-ALL and B-CLL; few negative cases in T-CLL
Contardi *et al.* [32]	2005	Colorectal adenocarcinoma Breast carcinoma Lung carcinoma Ovarian carcinoma Uterine carcinoma Renal carcinoma Bladder carcinoma Neuroblastoma Rhabdomyosarcoma Melanoma Osteosarcoma	Tm & s	mRNA and protein	Flow cytometry, RT-PCR IHC, flow cytometry, RT-PCR Flow cytometry, RT-PCR Flow cytometry, RT-PCR Flow cytometry, RT-PCR Flow cytometry, RT-PCR Flow cytometry, RT-PCR Flow cytometry, RT-PCR Flow cytometry, RT-PCR Flow cytometry, RT-PCR IHC, Flow cytometry, RT-PCR, Western blot	Expressed in high levels in all the tested cell lines
Matsubara *et al.* [35]	2006	ATL	Tm	Protein	Flow cytometry	ATL cells from Foxp3-high cases expressed considerable levels, while those of Foxp3-low cases expressed no or very little CTLA-4
Laurent *et al.* [36]	2007	AML (M0-M7 subtypes)	Tm & s	mRNA and protein	Flow cytometry, nested RT-PCR (semi quantitative)	Consistently expressed by leukemic blasts (M0, M1, M2 and M5 subtypes), although at different levels by flow cytometry, Extracellular domain detected while no full-length CTLA-4 detected by nested RT-PCR
Capriotti *et al.* [37]	2008	CTCL	Tm	mRNA	qPCR	Expressed in 21% of the samples
Shah *et al.* [38]	2008	Melanoma	Tm & s	mRNA & protein	RT-PCR, RT- qPCR, Western blot, Flow cytometry	Positive expression
Shimauchi *et al.* [39]	2008	ATL	Tm	Protein	IHC, Flow cytometry	Elevated expression on 13.33% of the patients
Mao *et al.* [40]	2010	Breast cancer	Tm	mRNA & protein	IHC and RT-PCR	Strong expression in 100% of all the samples at both the protein and mRNA levels
Salvi *et al.* [41]	2012	NSCLC	Tm	Protein	IHC	Expression increased in 52.8% (non-squamous) and 35.7% squamous NSCLC
Simone *et al.* [42]	2012	ALL	s	mRNA and protein	Flow cytometry, ELISA, Western blot, RT-PCR	Positive expression in 70% of B-ALL patients
Antczak *et al.* [43]	2013	NSCLC	Tm	mRNA	q PCR	Expression increased in 74.65% of the patients
Laurent *et al.* [44]	2013	Melanoma	Tm & s	mRNA & protein	IHC, flow cytometry, ELISA, RT-PCR, qPCR	Positively expressed in all the tested cell lines; sCTLA-4 transcript was expressed at lower levels than the full-length, in all cell lines except MECO
Mittal *et al.* [45]	2013	CLL	Tm	mRNA and protein	Flow cytometry, RT-PCR (semi-quantitative), qPCR, Western blot	Positively expressed; with CLL cells having different levels of expression (high CTLA-4 and low CTLA-4 expression)
Yu *et al.* [46]	2015	Breast cancer	Tm & s	Protein	IHC	Positively expressed
Ciszak *et al.* [47]	2016	CLL	Tm	Protein	Flow cytometry	Significantly higher levels expressed in patients compared to the controls
Ciszak *et al.* [48]	2016	CLL	Tm	Protein	Flow cytometry	Patients expressed significantly higher levels in comparison to the controls
Huang *et al.* [49]	2016	Nasopharyngeal carcinoma	Tm	Protein	IHC	Expressed with different intensities in 97.4% of the patients
Kim *et al.* [50]	2016	Gastric cancer	Tm	Protein	IHC	Positive expression in 65.8% of the patients
Roncella *et al.* [51]	2016	Mesothelioma	Tm	Protein	IHC	Expressed in 56% of the samples with variable intensity
Schloβer *et al.* [52]	2016	Gastric adenocarcinoma	Tm	Protein	IHC, fluorescence microscopy, targeted sequence	Positive expression in 86% of the sample
Zhang *et al.* [53]	2016	Esophageal carcinoma	Tm	Protein	IHC	Expressed in 87% of the patients. Elevated CTLA-4 expression (“+” and “++”) was detected in 52.6% of the samples expressing CLTA-4
Chakravarti *et al.* [54]	2017	Melanoma	Tm	Protein	IHC	Highly expressed

Tm, Transmembrane (Full length) CTLA-4; s: Soluble CTLA-4; AML, Acute myeloid leukemia; ATL, Adult T cell leukemia; B-ALL, Acute lymphoblastic leukemia of B cell lineage; T-ALL, Acute lymphoblastic leukemia of T cell lineage; CLL, Chronic lymphocytic leukemia; CML, Chronic myeloid leukemia; EHBD, Extrahepatic bile duct cancer; ESCC, Esophageal squamous cell carcinoma; TGCTs, Testicular germ cell tumors; MCL, Mantle cell lymphoma; NSCLC, Non-small cell lung cancer; SCLC, Small cell lung cancer; MIBC, muscle-invasive bladder cancer; NMIBC non–muscle-invasive bladder cancer; N/A, Not available; IHC, Immunohistochemistry; PCR, Polymerase chain reaction; RT-PCR: reverse transcription PCR; qPCR: quantitative real-time PCR; RT-qPCR: reverse transcription quantitative real-time PCR; ELISA, Enzyme-linked immunosorbent assay.

TABLE 1 (CONT.)

Main characteristics of eligible studies.

Author	Year	Cancer subtype	CTLA-4 isoform	Studies conducted on mRNA or protein	Method for CTLA-4 detection	CTLA-4 expression
Chen *et al.* [55]	2017	Breast cancer	Tm	Protein	Flow cytometry	Expressed by breast cancer cell lines, especially MDA-MB-231 and MCF-7
Le Goux *et al.* [56]	2017	Bladder urothelial carcinoma	Tm	mRNA	Real-time RT-qPCR	CTLA-4 over-expressed in 84.5% in MIBC and in 35.2% in NMIBC samples
Karpathiou *et al.* [57]	2017	Laryngeal and pharyngeal squamous cell carcinoma	Tm	Protein	IHC	Positive expression
Kim *et al.* [58]	2017	Breast cancer	Tm	mRNA	Whole exome sequence, RNA-Seq, gene enrichment analysis	Positive expression
Lafuente-Sanchis *et al.* [59]	2017	NSCLC	Tm	mRNA	IHC, RT-qPCR	Expression is detected in all the samples (100%)
Lim *et al.* [60]	2017	EHBD	Tm	Protein	IHC	Positive expression in 95% of the patients
Paulsen *et al.* [61]	2017	NSCLC	Tm	Protein	IHC	Over-expression in 50% stromal-CTLA-4 and 43% epithelial-CTLA-4
Yang *et al.* [62]	2017	Gastric cancer	Tm	Protein	IHC, Western blot	Positive expression in 43.7% of the sample by IHC
Kassardjian *et al.* [63]	2018	Breast cancer (ductal carcinoma in situ, invasive ductal carcinoma, invasive lobular carcinoma and invasive tubular carcinoma)	Tm	Protein	IHC	Over expressed in 52.7% of the all the samples with variation depending on tumor type and grade
Lan *et al.* [64]	2018	Breast cancer	Tm	Protein	IHC	Expressed in 41.2% of the samples
Mo *et al.* [65]	2018	Melanoma	Tm	mRNA and protein	Confocal microscopy, flow cytometry, RT-qPCR, Western blot	Highly expressed by most human melanoma cell lines
Santoni *et al.* [66]	2018	Thymoma	Tm	mRNA and protein	IHC, RT-qPCR, confocal microscopy	CTLA-4 expression was statistically found to progressively increase in A, B1, B2, AB and it was maximal in B3 thymomas
Do *et al.* [67]	2019	CLL	Tm	mRNA and protein	qPCR, flow cytometry, confocal microscopy	CTLA-4 expression in CLL B-cells was one of the most differentially expressed genes, average 19-fold change over normal B-cells (microarray); constitutive expression in CLL B cells compared to control (qPCR and confocal microscopy); constitutive intracellular expression in 61% patients (flow cytometry)
Gutiérrez-Hoya *et al.* [68]	2019	Cervical cancer	Tm	Protein	Flow cytometry	Positive expression
Harrington *et al.* [69]	2019	MCL	Tm	mRNA and protein	qPCR, flow cytometry	Very low mRNA expression No Surface protein expression
Inozume *et al.* [70]	2019	Melanoma	Tm	mRNA and protein	IHC, flow cytometry, confocal microscopy, RT-PCR	Expressed in 50% of tested the cell lines
Lobo *et al.* [71]	2019	TGCTs	Tm	Protein	IHC	Positive expression
Mosconi *et al.* [72]	2019	ACC of salivary gland	Tm	Protein	IHC	No expression
Regzedmaa *et al.* [73]	2019	SCLC	Tm	Protein	IHC	Expressed in 89.5% of the samples
Zhang *et al.* [74]	2019	ESCC	Tm	Protein	IHC	Elevated expression in 48.8% of the patients
Zhang *et al.* [75]	2019	NSCLC	Tm	Protein	IHC, Western blot	Expressed in high levels in A549, H460, HCC827 and H1975; very low levels in H661 and no detectable expression in H1650
Karpathiou *et al.* [76]	2020	uterine cervix cancer	Tm	Protein	IHC	Expression was found in 61.5 % of the invasive cases; CTLA-4 tumor cell expression was more often found in squamous cell carcinomas than in adenocarcinomas
Takeuchi *et al.* [77]	2020	ATL	Tm	Protein	IHC	No IHC stains with greater than 50% staining detected

Tm, Transmembrane (Full length) CTLA-4; s: Soluble CTLA-4; AML, Acute myeloid leukemia; ATL, Adult T cell leukemia; B-ALL, Acute lymphoblastic leukemia of B cell lineage; T-ALL, Acute lymphoblastic leukemia of T cell lineage; CLL, Chronic lymphocytic leukemia; CML, Chronic myeloid leukemia; EHBD, Extrahepatic bile duct cancer; ESCC, Esophageal squamous cell carcinoma; TGCTs, Testicular germ cell tumors; MCL, Mantle cell lymphoma; NSCLC, Non-small cell lung cancer; SCLC, Small cell lung cancer; MIBC, muscle-invasive bladder cancer; NMIBC non–muscle-invasive bladder cancer; N/A, Not available; IHC, Immunohistochemistry; PCR, Polymerase chain reaction; RT-PCR: reverse transcription PCR; qPCR: quantitative real-time PCR; RT-qPCR: reverse transcription quantitative real-time PCR; ELISA, Enzyme-linked immunosorbent assay.

TABLE 2

Study population and control groups used in the included studies.

Author	Cancer subtype	Stage or grade	Sample		Control	
			Type	Size (n)	Type	Size (n)
Pistillo *et al.* (2003) [34]	AML CML B-ALL T-ALL B-CLL T-CLL	N/A	Donor patients (Primary samples) and cell lines (CEM, Jurkat, Molt-4, Daudi, Raji, HOM-2, HL60, KG1a, K562)	100 patients and 9 cell lines	Healthy donors	10
Contardi *et al.* (2005) [32]	Colorectal adenocarcinoma, breast carcinoma, lung carcinoma, ovarian carcinoma, uterine carcinoma, renal carcinoma, bladder carcinoma, neuroblastoma, rhabdomyosarcoma, melanoma, osteosarcoma	Grade 1 and grade 2 (breast carcinoma), grade 4 (osteosarcoma), N/A (colorectal adenocarcinoma, lung, ovarian, uterine, renal and bladder carcinoma, neuroblastoma, rabdomyosarcoma and melanoma)	Donor patients (primary samples from osteosarcoma and breast cancer) and cell lines (4 colorectal adenocarcinoma cell lines: HCT-8, HT-29, COLO 205 and CACO-2; 4 breast carcinoma cell lines: MCF-7, MDA-MB-231, T-47D, BT-20; 3 lung carcinoma cell lines: CALU-1, CALU-6, A549; 2 ovarian carcinoma cell lines: SKOV-3 and A2780; 1 uterine carcinoma cell line; 5 neuroblastoma cell lines: NB100, SJNKP, CHP212, SY5Y, SKNBE-2C; 3 renal carcinoma cell lines: SKRC-10, SKRC-52, SKRC-59; 2 uterine carcinoma cell lines: TG, HELA; 1 bladder carcinoma cell line: T24; 2 rabdomyosarcoma cell lines: RD/18, TE671; 4 osteosarcoma cell lines, HOS, MG-63, U2-OS, SaOS-2; 3 melanoma cell lines, MEL-1, ALO-39, F0-1; 2 nontumorigenic human breast epithelial cell lines: MCF10A, HC11)	6 Osteosarcoma samples 5 breast cancer samples and 34 cell lines	PBMCs from healthy donors; for osteosarcoma cell lines, HSSCs from healthy donors stimulated to differentiate toward the osteogenic lineage; for breast tissue, non-malignant tissue adjacent to tumor	10 HSSC; 5 non-malignant breast cancer tissue adjacent to tumor
Matsubara *et al.* (2006) [35]	ATL	I-IV	Donor patients (primary samples) and cell lines (ATL-T, ATL-2, ATL-43T, ATL-48T^{+}, ATL-55T^{+}, ED-40515^{+}, MT-1)	20 patients (9 patients of the acute type, 10 of the chronic type, and 1 of the lymphoma type) and 7 ATL derived cells lines	CD4^{+} and CDD4^{+}CD25^{+} T cells purified from PBMCs from healthy donors	N/A
Laurent *et al.* (2007) [36]	AML (M0-M7 subtypes)	N/A	Donor patients (primary samples)	25 (15 untreated and 10 chemoresistant patients)	PBMCs from healthy donors	N/A
Capriotti *et al.* (2008) [37]	CTCL	I-III	Donor patients (primary samples)	28	PBMCs from healthy donors	6
Shah *et al.* (2008) [38]	Melanoma	N/A	Donor patients (primary samples) and cell lines (UACC 1273, A2058)	N/A (patients) and 2 cell lines	N/A	N/A
Shimauchi *et al.* (2008) [39]	ATL	N/A	Donor patients (primary samples)	21	PBMCs from healthy donors	8
Mao *et al.* (2010) [40]	Breast cancer	N/A	Donor patients (primary samples)	60	Normal breast tissue from patients with benign breast disease or external breast injury	30
Salvi *et al.* (2012) [41]	NSCLC	I–III	Donor patients (primary samples)	81	Tumor-adjacent normal tissues	N/A
Simone *et al.* (2012) [42]	ALL	N/A	Donor pediatric patients (primary samples)	80	Age-matched normal serum samples from healthy donors	45
Antczak *et al.* (2013) [43]	NSCLC	N/A	Donor patients (primary samples)	71 (23 adenocarcinoma, 41 squamous cell carcinoma and 7 large cell carcinoma)	N/A	N/A
Laurent *et al.* (2013) [44]	Melanoma	N/A	Donor patients (primary cell lines from metastatic lesions of cutaneous melanoma and melanoma tissue sections) and long term cell lines (C23, MeWo, FO-1)	14 primary cell lines, 3 long-term cell lines and 33 tissue sections	N/A	N/A
Mittal *et al.* (2013) [45]	CLL	N/A	Donor patients (primary samples including peripheral blood, bone marrow and lymph node samples)	105	N/A	N/A
Yu *et al.* (2015) [46]	Breast cancer	I-III	Donor patients (Primary samples)	130	N/A	N/A
Ciszak *et al.* (2016) [47]	CLL	N/A	Donor patients (primary samples)	38	B cells purified from healthy donor PBMCs	15
Ciszak *et al.* (2016) [48]	CLL	I–IV	Donor patients (primary samples)	38	B cells purified from healthy donor PBMCs	6
Huang *et al.* (2016) [49]	Nasopharyngeal carcinoma	UICC I–IVc; WHO II & III	Donor patients (primary samples)	191	N/A	N/A
Kim *et al.* (2016) [50]	Gastric cancer	I–III	Tissue microarrays from donor patients (primary samples)	243	Non-neoplastic gastric mucosa specimens	N/A

n, number; N/A, not available; CLL, Chronic lymphocytic leukemia; ATL, Adult T-cell leukemia/lymphoma; PBMCs, Peripheral blood mononuclear cells; HSSCs, human stromal stem cells; MIBC, muscle-invasive bladder cancer; NMIBC non–muscle-invasive bladder cancer .

TABLE 2 (CONT.)

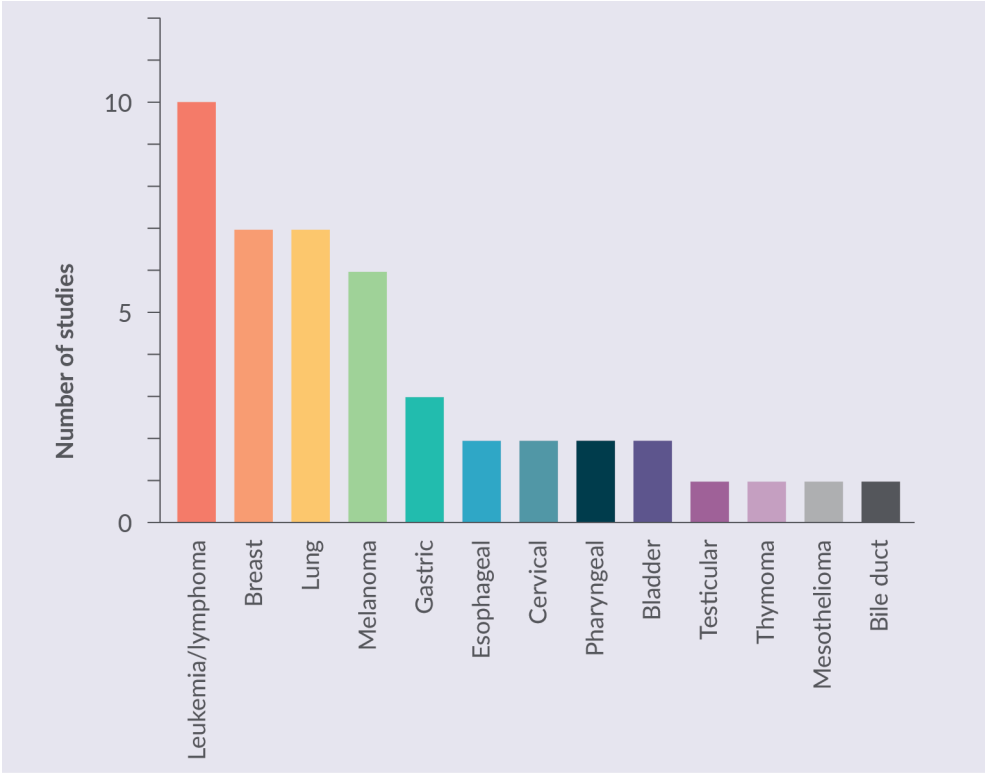
Study population and control groups used in the included studies.

Author	Cancer subtype	Stage or grade	Sample		Control	
			Type	Size (n)	Type	Size (n)
Schloβer *et al.* (2016) [52]	Gastric adenocarcinoma	I-IV	Donor patients (primary samples)	127	N/A	N/A
Zhang *et al.* (2016) [53]	Esophageal carcinoma	I-IV	Donor patients (primary samples)	158	N/A	N/A
Chakravarti *et al.* (2017) [54]	Melanoma	N/A	Donor patients (primary samples)	81	N/A	N/A
Chen *et al.* (2017) [55]	Breast cancer	N/A	Cell lines (MDA-MB-231, SKBR3, MCF-7, T47D)	4 cell lines	N/A	N/A
Le Goux *et al.* (2017) [56]	Bladder urothelial carcinoma	Ta-T3, low grade and high grade	Donor patients (primary samples)	155 (84 with MIBC and 71 with NMIBC)	Normal bladder tissues from surgery unrelated to bladder tumors	15
Karpathiou *et al.* (2017) [57]	Laryngeal and pharyngeal squamous cell carcinoma	I–IV	Donor patients (primary samples)	152	N/A	N/A
Kim *et al.*(2017) [58]	Breast cancer	Stage IV or recurrent after curative treatment	Donor patients (primary samples)	37	N/A	N/A
Lafuente-Sanchis *et al.* (2017) [59]	NSCLC	I–III	Donor patients (primary samples)	78	Tumor-adjacent lung tissues	78
Lim *et al.* (2017) [60]	EHBD	T1–T4	Donor patients (primary samples)	77	N/A	N/A
Paulsen *et al.* (2017) [61]	NSCLC	I–IIIA	Donor patients (primary samples)	536	N/A	N/A
Yang *et al.* (2017) [62]	Gastric cancer	N/A	Donor patients (primary samples)	48	Tumor-adjacent normal tissues	48
Kassardjian *et al.* (2018) [63]	Breast cancer (ductal carcinoma *in situ*, invasive ductal carcinoma, invasive lobular carcinoma and invasive tubular carcinoma)	I–IV	Commercially obtained breast tissue microarray sections	93 (73 invasive ductal, 10 invasive lobular, 2 invasive tubular, 8 ductal carcinoma *in situ*)	Normal breast tissues from the same tissue microarrays	6 (2 normal and 4 with fibrocystic changes)
Lan *et al.* (2018) [64]	Breast cancer	I–III	Donor patients (primary samples)	102	N/A	N/A
Mo *et al.* (2018) [65]	Melanoma	N/A	Cell lines (Hs 936.T, A2058, COLO679, WM983(B), 451 Lu, WM3918 and WM3912)	7 cell lines (in addition to 61 melanoma cell lines from the cancer cell encyclopedia database)	Human primary neonatal foreskin melanocytes	N/A
Santoni *et al.* (2018) [66]	Thymoma	N/A	Donor patients (primary samples)	68	PBMCs from healthy donors	N/A
Do *et al.* (2019) [67]	CLL	0–IV	Donor patients (primary samples) and cell lines (Mec1, OSU-CLL)	28 N/A	B cells and T cells purified from blood from healthy donors	N/A
Gutiérrez-Hoya *et al.* (2019) [68]	Cervical cancer	N/A	Cell lines (HeLa (HPV 18), CaSki (HPV 16), C33A (HPV-), INBL)	4 cell lines	N/A	N/A
Harrington *et al.* (2019) [69]	MCL	N/A	Donor patients (primary samples)	16	PBMCs from healthy donors	N/A
Inozume *et al.* (2019) [70]	Melanoma	N/A	Donor patients (primary samples) Melanoma cell lines	13 melanoma tissue sections (5 shown in manuscript) 10 cell lines	N/A	N/A
Lobo *et al.* (2019) [71]	TGCTs	I–III	Donor patients (primary cells)	271 tumour samples from 162 patients	N/A	N/A
Mosconi *et al.* (2019) [72]	ACC of salivary glands	I–III	Donor patients (primary samples)	36	N/A	N/A
Regzedmaa *et al.* (2019) [73]	SCLC	I-IV	Donor patients (primary samples)	38	N/A	N/A
Zhang *et al.* (2019) [74]	ESCC	I–IV	Donor patients (primary samples)	84	N/A	N/A
Zhang *et al.* (2019) [75]	NSCLC	N/A	Cell lines (A549, H460, HCC827, H1975, H1650, H661)	N/A	N/A	N/A
Karpathiou *et al.* (2020) [76]	Uterine cervix cancer	0–IV	Donor patients (primary samples)	63 lesions from 52 patients	N/A	N/A
Takeuchi *et al.* (2020) [77]	ATL	I–IV	Donor patients (primary samples)	69	N/A	N/A

n, number; N/A, not available; CLL, Chronic lymphocytic leukemia; ATL, Adult T-cell leukemia/lymphoma; PBMCs, Peripheral blood mononuclear cells; HSSCs, human stromal stem cells; MIBC, muscle-invasive bladder cancer; NMIBC non–muscle-invasive bladder cancer .

FIGURE 2

Studies that reported detectable CTLA-4 in tumor cells.



2 studies, which stated that mantle cell lymphoma [69] and ATL [77] do not express CTLA-4, and this might be due to the small sample size [69] or the method used (only IHC was used) [77], in addition to the lack of control group [77].

A study that investigated the expression levels of CTLA-4 in adenoid cystic carcinoma of salivary gland founds that CTLA-4 expression in tumor cells is negative [72]. It is worth noting that only one method was used to assess protein expression (IHC).

On the other hand, we included seven studies about lung cancer which have clearly demonstrated positive expression of CTLA-4 by cancer cells; the majority were focused on NSCLC (n=6) [32,41,43,59,61,75] with a single study on SCLC (n=1) [73].

Breast (n=7) [32,40,46,55,58,63,64], gastric (n=3) [50,52,62] and melanoma (n=6) [32,38,44,54,65,70] cancer cells were confirmed for positive CTLA-4 expression by all the included manuscripts.

All the remaining types of cancers included in this systematic review were positive for CTLA-4 expression. Expression patterns are summarized in Table 1.

In terms of cytoplasmic vs surface expression, twenty-two out of the forty-five studies looked at the intracellular localization of CTLA-4. One study examined only the cytoplasmic CTLA-4 [63] while the other twenty-one studies investigated both cytoplasmic and surface CTLA-4 levels, sixteen of them observed higher CTLA-4 levels in the cytoplasm than on the cell membrane [32,34,44,46–48,51,55,60–62,65,67,70,71,76], which is consistent with what we know about the endosomal/lysosomal vesicular localization within cytoplasm previously reported in T cells, where CTLA-4 is rarely expressed on the membrane and is rapidly internalized into the cytoplasm by means of endocytosis [65]. The other five studies, however, did not specify where the highest levels of CTLA-4 are localized [40,41,53,64,66].

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CTLA-4 isoform studied

With the exception of seven studies that investigated the soluble isoform of CTLA-4, either alone [42] or together with the full-length isoform [32,34,36,38,44,46], the majority of the studies focused on the full-length isoform (n = 44).

Methodologies used to detect CTLA-4 expression by cancer cells

As summarized in Table 1, CTLA-4 was detected either at the mRNA level (by real-time PCR and/or RT-PCR) and/or at the protein level (by Western blotting, immunohistochemistry, flow cytometry, ELISA and/or fluorescence microscopy). Most studies measured CTLA-4 at the protein level (n = 27) [35,39,41,46–55,57,60–62,68,71–77] with two examining the same cohort [47,48] while four studies measured CTLA-4 only at the mRNA level [37,43,58,59]. Fourteen studies, however, measured CTLA-4 at both levels [32,34,36,38,40,42,44,45,56,65–67,69,70].

Correlation of CTLA-4 expression by tumor cells with clinical outcome

The outcomes of the studies analyzing the potentially prognostic role of CTLA-4 in cancers are varied, especially with regards to whether increased expression signifies a better or poorer outcome for the patient cohort (Figure 3). Out of the forty-five papers included in this study, twenty-one papers looked retrospectively at cancer progression including overall survival. In general, eleven studies found that high tumor CTLA-4 expression correlated with poorer outcome compared with lower CTLA-4 expression [42,46,49,52–54,57,64,66,71,74]. Conversely, seven studies found an opposite correlation [42,45,47,51,60,61,73]. In mesothelioma, only the sCTLA-4 in the pleural effusion, rather than serum, was found to be a statistically significant positive predictive factor [51]. Three studies, however, reported no association between tumor expression levels of CTLA-4 and tumor progression [56,59,76]. Table 3 illustrates the correlation between CTLA-4 expression levels in cancer cells and disease outcome, in the twenty-one articles which reported that.

These observations led to the obvious question of whether or not any associations between patient outcome and CTLA-4 expression were specific to particular tumor types. Out of the twelve leukemia/lymphoma articles included in our review, only three investigated the association of tumor CTLA-4 expression with patient clinical outcome. Two studies found that high tumor CTLA-4 expression in CLL is a good prognostic factor [45,47]. Another study suggests that increased tumor sCTLA-4 expression in ALL correlates with poor outcome [42]. Two studies by Ciszak *et al.* assessed the same cohort for CTLA-4 expression in CLL [47,48] and only one of them studied the correlation with disease progression [47].

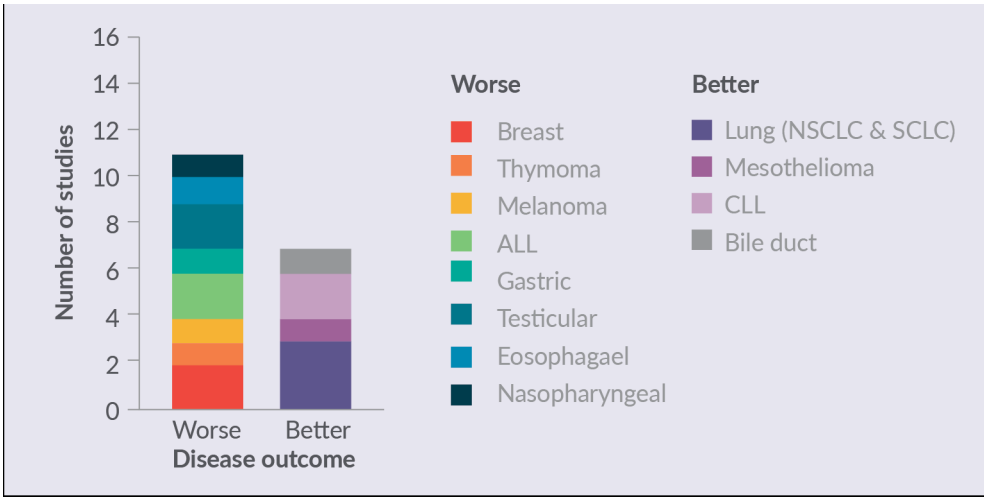
In lung cancer, increased tumor CTLA-4 expression was associated with better patient outcomes in three studies [61,73,78], including one small cell lung cancer study [73]. One study found a diverging prognostic impact of CTLA-4 expression in metastatic NSCLC lymph nodes versus primary tumor; while high stromal CTLA-4 was a positive prognostic factor in the squamous cell carcinoma (SCC) subgroup, no association with survival was found in the adenocarcinoma (ADC) and large cell carcinoma subgroups [61]. However, a study by Lafuente-Sanchis *et al.* demonstrated no association between tumor CTLA-4 expression levels and patient clinical outcomes [59].

Conversely, the two studies that examined the effect of increased CTLA-4 expression in breast cancer found a negative correlation with patient clinical outcome, suggesting that CTLA-4 might be a negative prognostic factor in breast cancer [46,64].

In esophageal carcinoma, increased tumor CTLA-4 expression is an independent predictor of shorter overall survival [53,74].

FIGURE 3

Cancers in which relatively high expression levels of CTLA-4 correlate with disease outcome.



Regarding gastric cancer, the prognostic effect of CTLA-4 was only studied in one article, which found a negative association between tumor CTLA-4 levels and overall survival [52].

In uterine cervix and bladder urothelial carcinoma, researchers could not find any correlation between CTLA-4 expression levels and clinical outcome [56,76]. On the other hand, increased CTLA-4 tumor expression predicted longer overall survival in patients with mesothelioma [51] and EHDC [60], and shorter overall survival in patients with melanoma [54], thymoma [66], nasopharyngeal carcinoma [49], testicular germ cell tumors [71] and laryngeal and pharyngeal squamous cell carcinoma [57]. However, we cannot build strong evidence collectively from these studies and more should be conducted to ascertain the relationship between CTLA-4 expression in tumor cells, disease progression and patient outcomes. Furthermore, different methodologies were applied in the analysis of CTLA-4 in these studies, and at different levels (gene and/or protein) further complicating a generalized conclusion.

DISCUSSION

One of the most important recent advances in cancer treatment has been the emergence of cancer immunotherapy, which is based on boosting the anti-tumor immune response rather than directly targeting tumor cells. Despite its impressive successes over the last decade, in some patients the response is limited or short-lived and indeed, protocols that consistently identify and stratify patients that will respond well to this type of therapy remain a high priority. These limited responses are mainly due to multiple tumor-mediated immune escape mechanisms which tumor cells use to suppress anti-tumor immunity. One of the major and most important immune escape mechanisms is by expressing co-inhibitory molecules, called immune checkpoints (IC). CTLA-4 in the context of the tumor microenvironment has typically been associated with infiltrating T cells, not least increased recruitment of regulatory T cells [79], but less attention has been paid to any role CTLA-4 may have when expressed by tumor cells directly. The clinical significance of the existence of this immunosuppressive molecule in both tumor and immune cells within the tumor microenvironment remains to be fully elucidated, and its potential as a prognostic marker or a therapeutic biomarker, in addition to any functional role it might have, needs to be further examined.

In this systematic review, we assessed the body of available peer-reviewed literature

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regarding CTLA-4 expression, both tmCTLA-4 and sCTLA-4, by a wide variety of cancer subtypes with the aim of understanding its expression by tumors and its correlation with disease progression and clinical outcome.

We found that the vast majority of studies demonstrated CTLA-4 expression was detectable, at the mRNA and/or protein levels, in tumor cells compared to its counterpart healthy cells. Three studies, however, observed no CTLA-4 expression, although this might be because they only investigated its expression at the protein level using only one methodology, IHC [72,77], because of the small sample size [69] or because the type of the tumor cells they investigated might not express CTLA-4. In contrast, sCTLA-4 was not studied as thoroughly as its counterpart receptor; only seven studies investigated sCTLA-4 expression by cancer cells, but these studies confirmed the possibility that cancer cells secrete this naturally immunosuppressive protein, perhaps as an immune evasion strategy [32,48–50].

Overall, this survey of CTLA-4 expression in tumor cells points to an area, which could yield a useful biomarker for CI therapy as part of the ongoing drive to generate predictable bioresponse profiles to treatment, but it also demands further comprehensive

TABLE 3

The correlation of CTLA-4 expression levels in tumor cells (mRNA and/or protein) with the disease outcome.

Author	Cancer subtype	Studies conducted on mRNA or protein?	Correlation of higher levels of tumor CTLA-4 with outcome
Salvi *et al.* (2012) [41]	NSCLC	Protein	Good outcome
Simone *et al.* (2012) [42]	ALL	mRNA and protein	Poor outcome
Mittal *et al.* (2013) [45]	CLL	mRNA and protein	Good outcome (Low-CTLA-4 CLL was associated with poor outcome, while high-CTLA-4 CLL was associated with good outcome)
Yu *et al.* (2015) [46]	Breast cancer	Protein	Poor outcome
Ciszak *et al.* (2016) [47]	CLL	Protein	Good outcome
Huang *et al.* (2016) [49]	Nasopharyngeal carcinoma	Protein	Poor outcome
Roncella *et al.* (2016) [51]	Mesothelioma	Protein	Good outcome
Schloβer *et al.* (2016) [52]	Gastric adenocarcinoma	Protein	Poor outcome
Zhang *et al.* (2016) [53]	Esophageal carcinoma	Protein	Poor outcome
Chakravarti *et al.* (2017) [54]	Melanoma	Protein	Poor outcome
Le Goux *et al.* (2017) [56]	Bladder urothelial carcinoma	mRNA and protein	No correlation
Karpathiou *et al.* (2017) [57]	Laryngeal and pharyngeal squamous cell carcinoma	Protein	Poor outcome
Lafuente-Sanchis *et al.* (2017) [59]	NSCLC	mRNA	No correlation
Lim *et al.* (2017) [60]	EHBD	Protein	Good outcome
Paulsen *et al.* (2017) [61]	NSCLC	Protein	Good outcome
Lan *et al.* (2018) [64]	Breast cancer	Protein	Poor outcome
Santoni *et al.* (2018) [66]	Thymoma	mRNA and protein	Poor outcome
Lobo *et al.* (2019) [71]	TGCTs	Protein	Poor outcome
Regzedmaa *et al.* (2019) [73]	SCLC	Protein	Good outcome
Zhang *et al.* (2019) [74]	ESCC	Protein	Poor outcome
Karpathiou *et al.* (2020) [76]	Uterine cervix cancer	Protein	No correlation

study. In particular, it will be useful to definitively resolve the impact of high CTLA-4 tumor cell levels both on patient outcome for each type of cancer and whether or not it affects CI therapy performance. Soluble CTLA-4 for instance, is bound by anti-CTLA-4 antibodies such that high serum levels of this immunosuppressive molecule could affect the amount of antibody engaging with tmCTLA-4. Moreover, our data suggest that antibodies specific for CTLA-4 expressed by T cells could also target cancer cells directly.

We looked for any correlation between CTLA-4 levels, disease progression and patient outcome in this study. Eleven studies found that high tumor CTLA-4 expression correlated with disease progression while lower CTLA-4 expression correlated with better outcomes [42,46,49,52–54,57,64,66,71,73]. Conversely, seven studies found an opposite correlation, where high CTLA-4 expression correlated with better clinical outcomes [45,47,51,60,61,73,78]. Three studies, however, reported no association between tumor expression levels of CTLA-4 and tumor progression [56,59,76]. The data from these studies are not robust enough to define clearly why these differences in outcome exist, but it is interesting to note that the cancers in which a worse outcome was observed do not overlap with those with a better outcome (Figure 3). This suggests that increased CTLA-4 expression has different, yet to be determined, effects in different types of cancer. Other reasons might be differences in methods used for CTLA-4 detection and whether it was at an mRNA or protein level. Additionally, there is a significant variation in the assessment of different CTLA-4 isoforms with sCTLA-4 being understudied.

Accordingly, we suggest a more robust streamlined protocol to assess CTLA-4 expression in tumors and its correlation with disease progression and clinical outcome.

Another possible biomarker could be the secretable sCTLA-4, which has not received the same level of examination in terms of immune regulation that its receptor counterpart has over the years and any role it might play particularly with regard to cancer progression is still unclear. Interestingly, it has been previously shown that selective blockade of sCTLA-4 exhibited a stronger and more consistent, significant enhancing effect on Ag-driven PBMC responses than pan-specific blockade of total CTLA-4 [80]. However, most of the studies included in this review which investigated sCTLA-4 expression used the ELISA assay method to measure serum levels [40,42,44,51] or pleural effusion [51], which does not discriminate whether it is produced and secreted by cancer cells or immune cells. This emphasizes the need to further study the expression of the soluble isoform by different tumor cell types with selective antibodies, as well as the need to use more than one method to detect its expression and to study its role in cancer and how cancer cells potentially use it to escape the immune system.

TRANSLATIONAL INSIGHT

Taken together, data from this systematic review provide evidence that CTLA-4 is expressed not only by immune cells but also by many types of cancer cells. Further, the data emphasize the importance of assessing the correlation between CTLA-4 levels and a patient's clinical outcome by using a more robust streamlined protocol to assess CTLA-4 levels in cancer cells, together with correlating both mRNA and protein levels with the disease progression. Moreover, there are only few studies which investigated the expression of the soluble CTLA-4 isoform by cancer cells, which means that the role of this key molecule might be underestimated, and further studies should be conducted to understand its role and function in cancer. Therefore, our findings suggest the need to define better and more robust methods to detect soluble CTLA-4 expression by tumor cells, in a wide variety of tumor types, and to deeply study its role in immune cells as well as in cancer cells.

Checkpoint inhibitor antibodies represent a novel type of cancer immunotherapy that

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has proven obvious success in the treatment of different cancers. As one of the major targets of checkpoint inhibitors, CTLA-4 needs to be studied more thoroughly in regards of its expression by cancer cells to assess its full potential, not only as a therapeutic target, but also as a biomarker for patient stratification, predicting prognosis and response to therapy within a broader set of biomarkers, which help to delineate the tumor microenvironment as a prelude to CI therapy. Despite the huge clinical benefits that CTLA-4 offers in both cancer and autoimmune disease immunotherapy, its role and function especially in non-immune cells remains largely unexplored.

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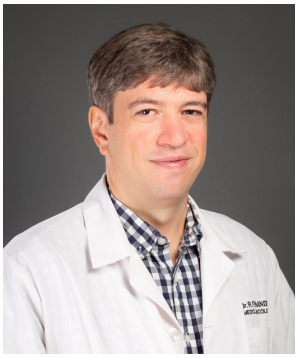
HUNTING FOR BETTER BIOMARKERS OF RESPONSE

SPOTLIGHT

INTERVIEW

Driving the development of microbiome-based biomarkers of patient response to immunotherapy



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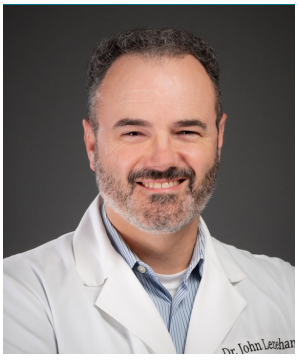

JOHN LENEHAN is a medical oncologist at the London Regional Cancer Program who specializes in treating melanoma and GI cancers such as pancreatic cancer. He is an assistant professor in the Department of Oncology at Western University and an adjunct scientist with the Lawson Health Research Institute.

SAMAN MALEKI is an Assistant Professor in the Departments of Oncology, Pathology and Laboratory Medicine, and Medical Biophysics at Western University. He is also a Scientist at Lawson Health Research Institute. Saman obtained his Bachelor of Science (BSc) degree in Microbiology from Arak Azad University in Iran in 2004. He then completed his Master's degree in Microbiology in 2008 at the University of Isfahan in Iran. For his Master's degree, Saman studied the effect of *Helicobacter pylori* (*H.pylori*) on suppressing human innate and adaptive immunity. As part of his MSc degree, he spent several months as an exchange student studying the effect of various *H.pylori* strains on human neutrophils at the University of Freiburg in Germany. In 2014, Saman obtained his Ph.D. degree from The Department of Microbiology and Immunology at Western University. During his post-doctoral training at Western and Lawson, Saman worked on several novel Immuno-Oncology drugs at the pre-clinical stage before those drugs entered the Phase 1 clinical trial in patients. His studies also helped inform the design of some of the arms in those clinical trials.

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Q Firstly, can you each tell us what you are currently working on?

SM: My lab's research is focused on finding novel approaches to sensitize treatment-refractory advanced or metastatic cancers to immunotherapy with checkpoint inhibitors. We do this by combining immunotherapy drugs with strategies that affect the tumor genome or its microenvironment. This includes inducing DNA repair defects in tumor cells that create immunogenic peptides, or modifying a patient's gut microbiome before treatment with immunotherapy drugs and studying the immune priming effect of the microbiome modification.

RF: I am leading an early-phase clinical trial project, namely the PERFORM trial, investigating an important and relevant question: is there a potential role for microbiome modification in preventing the onset of severe immune-related adverse events in patients with advanced renal cell carcinoma (RCC) patients treated with immunotherapy? The current study proposes a safe method, namely fecal microbiota transplantation (FMT) from a healthy donor to potentially prevent immune-related adverse events, allowing patients with advanced RCC to finish immunotherapy treatment.

JL: I am currently working with Saman on two main projects. The first is the MIMic trial, where we are combing Fecal Microbiome Transplant (FMT) with standard anti-PD-1 immunotherapy for patients with advanced melanoma. The main purpose of the trial is to ensure the safety of combining the two, although we will also be monitoring changes in the microbiome and immune profile.

Unlike other groups conducting a similar trial in the US and in Israel, we are treating people with FMT prior to starting treatment with immunotherapy. The other groups are focusing on

trying to rescue patients with primary or secondary resistance to anti-PD1 therapy. Another significant difference is that we are using stool from healthy donors instead of stool from patients who are themselves responders to anti-PD1 therapy. From the results of the Israeli and Pittsburgh groups, we know that the combination is safe when used as a rescue, and it seems to provide benefit for some patients – very exciting. We are hoping that FMT with healthy donor stool will re-set the immune system before we give the immunotherapy and lead to a better response.

The other project that Saman and I are working on together is an FMT trial in pancreatic cancers. These cancers are very aggressive and the overwhelming majority of patients do poorly. We participated in a pan-Canadian clinical trial that combined immunotherapy with standard chemotherapy to treat metastatic pancreatic cancer. The results showed that there was no clear benefit to adding immunotherapy, and this has also been the experience of others. Pancreatic cancer has a tumor microenvironment that does not support an immune response against the cancer. A few groups have analyzed the intestinal microbiome in patients with pancreatic cancer and have found that the microbiome is 'unhealthy', as we would expect in these patients.

The definition of a healthy microbiome is very broad and saying that a person with pancreatic cancer has an unhealthy microbiome doesn't tell us quite what is different about the microbiome of patients with pancreatic cancer versus any other cancer, or any other disease. Our study is designed to assess the microbiome of patients with incurable pancreatic cancer before standard chemotherapy, and then after 3 months of chemotherapy – we will see if there is a signal in the microbiome that predicts who will do better or worse with standard chemotherapy.

To act as a potentially more suitable control, we are also going to sample the microbiome of someone who is 'healthy' and lives in the same house as a cohabitant with the patient. There is evidence showing that the microbiomes of people who cohabitate are similar. The microbiome of the patient will have more similarities with the microbiome of the cohabitant than it would with that of a healthy control. We will compare the microbiome of the patient with that of the cohabitant and potentially identify differences that are the result of dysbiosis specifically related to the cancer. Adding this extra piece of information may show us something important about the microbiome of the patient.

A follow-on study will include modifying the intestinal microbiome of mice with pancreatic tumors, and then treating them with standard chemotherapy. The modification will be FMT with stool obtained from the first part of the study from either a patient who responded to chemotherapy, or from a patient who did not respond. We expect that responses in mice will mirror the response from the patient who is the stool donor, providing support to the concept of using FMT to influence the response to treatment.

There is pre-clinical evidence that the pancreatic tumor microbiome overlaps with the intestinal microbiome and using FMT to change the intestinal microbiome can change the pancreatic microbiome. If FMT helps in mice, then we will study the potential benefits of FMT in patients before systemic therapy, including standard chemotherapy and possibly immunotherapy. Interestingly, there is ample evidence that bacterial enzymes in the pancreatic tumor microbiome can metabolize the chemotherapy drug gemcitabine, commonly used to treat pancreatic cancers. Reducing the presence of bacterial species containing the enzyme may improve responses to this drug. FMT may prove to be an effective method of altering the tumor microbiome and promote the antineoplastic activity of gemcitabine.

These are going to be sequential studies to learn more about the pancreatic tumor microbiome and how to manipulate it. We have the funding for the first part, the observational part. I am not aware that anyone studying the microbiome in patients has considered the microbiome of a cohabitant, which I hope will give us more insight into how the microbiome is different in this cancer.

Q Can you give us some more background to your IO biomarker-related research to date, particularly in relation to the role of the microbiome as a biomarker for immunotherapy?

SM: I have always tried to learn from patients. We can develop better therapeutics by studying biomarkers of response or resistance to a particular group of drugs. For example, in 2015, a seminal publication by Le *et al.* in the *New England Journal of Medicine* showed that patients with mismatch repair (MMR)-deficient or microsatellite instability (MSI)-high tumors had a high response rate to anti-programmed cell death-1 (PD-1) agent pembrolizumab. This started a path for using MMR deficiency or MSI status of tumors as a biomarker of response to anti-PD-1 therapy and later on, the US FDA approved pembrolizumab for the treatment of MMR-deficient or MSI-high tumors. In 2015, as I was working on a project to identify biomarkers of response to anti-PD-1 therapy, I realized that we might be able to induce MMR deficiency in some tumors that are MMR proficient to sensitize them to immunotherapy – that became a major focus of research in my lab.

In 2016–17, I realized another biomarker area was promising, following reports that showed patients with certain gut microbiome profiles showed a better response to immunotherapy with checkpoint inhibitors. Mechanistic studies confirmed these observations in patients and established a causative role for the gut microbiome in conferring sensitivity or resistance to checkpoint inhibitors, allowing us to use the gut microbiome as a biomarker of response to these drugs. It also provides us with a unique opportunity, in my opinion, to modify the gut microbiome to induce a more robust anti-tumor immune response and sensitize tumors to immunotherapy. That is exactly what we have set out to achieve here at the London Regional Cancer Program (LRCP) in Ontario, Canada.

> "Mechanistic studies ... established a causative role for the gut microbiome in conferring sensitivity or resistance to checkpoint inhibitors..."
>
> - Saman Maleki

Q Studying the microbiome in the context of cancer therapy is a relatively novel and niche area of research – how and why is your location in London, Ontario and the local scientific community there so suited to it?

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RF: This project is a collaboration between the Departments of Oncology, Pathology and Laboratory Medicine, Microbiology and Immunology as well as Infectious Diseases at Western University. The goal is to improve the quality of healthcare delivery to patients with advanced renal cell carcinoma. I have been lucky to have Drs Silverman, Burton and Maleki in London, who have been highly involved in this project.

> "The goal is to improve the quality of healthcare delivery to patients with advanced renal cell carcinoma."
>
> - Ricardo Fernandes

I am a medical oncologist who treats genitourinary malignancies, with special interest in biomarkers and immunotherapy. I have been able to leverage these different Departments and use their expertise to mechanistically examine the changes in the immune and microbiome profile of these patients. That would help us determine potential clinical biomarkers that are related to healthy outcomes/less frequent toxicities in patients receiving combination immunotherapy.

JL: Our ability to pull this off when other, much larger centers struggled, is due to the fact that we already had the required expertise in the four main specialties. Other centers may have some if not all four of these components, but not necessarily the opportunity to bring them all together.

Dr. Silverman, The Chair/Chief of The Infectious Disease Department, has been doing FMT in *C. difficile* patients since 2003, and his team is excellent. FMT is the unique part – the greatest variable – but they have a robust system that works well. It includes the screening the donors, the safety measures, the preparation of the capsules, and actually getting them into people.

Dr. Burton is a microbiome expert who brings the microbiome analysis to the table. We can change a microbiome, but he tells us what we had, what we changed, and what that might mean.

I am oncologist who has experience using immunotherapy. I-O was first available to those who treat melanoma, and I was lucky enough to start practice right around the time we started to have access to these drugs. Saman and I crossed paths initially when I gave grand rounds on the latest in melanoma treatment in 2016. The key piece, though, is that this is something Saman wanted to do for years before I met him. It was through the force of his will that we were all brought together to do MIMic. I was the first oncologist he convinced to work on it, but not the first one he tried to get on board! He never gave up.

Saman is the fourth part of our trial team: the tumor immunologist. His skill is in measuring the immune response, teasing out the details of what is actually changing, and determining whether that means anything. He is good at what he does. So the reason it was possible here is because we have the four essential components, but in the end, it happened because Saman worked to get us together for a common goal.

Q Tell us about the translational R&D path which has led to John and Ricardo's current clinical studies – firstly, what are the distinctive

features of your biomarker studies as opposed to most others in the IO space?

SM: Most biomarker studies have shown that microbiome diversity is the best marker for the prediction of a good response to immunotherapy in patients. Most cancer patients have some sort of dysbiosis and their gut biome is less diverse compared to healthy individuals. We use healthy donors to re-establish a healthy gut microbiome in patients while other studies have focused on patients who showed a response as donors. Regardless of being responders, patients still have a limited gut microbiome diversity compared to healthy individuals. We are also doing these studies in treatment-naive patients upfront, while almost all the other studies are doing similar trials in patients who have failed I-O treatment.

Q Can you tell us more about your clinical strategies and methods as you explore the potential for manipulation of the microbiome to improve immunotherapy patients' responses? Firstly, can you share a few details of your respective studies and their current status?

JL: The MIMic trial has now accrued 12 patients – the goal is 20. Accrual has been slow because we do not allow patients being treated with combination immunotherapy into the trial given the high rate of serious toxicity. For those who are fit enough, combination immunotherapy is the standard, and single-agent anti-PD1 therapy is generally used in patients who are less fit. High rates of significant toxicity, like that of dual immunotherapy, would complicate assessing the safety of combing upfront FMT with healthy donor stool and standard immunotherapy.

We have two sites that have joined our study (both in Montreal) and combined, they have added 7 to our 5 patients. The intention of adding two larger centers was that they would increase the rate of accrual and add further evidence for this approach to the results that have been published to date. So far, we have not seen any side effects just after FMT, nor have we seen any side effects during the I-O treatment that are unusual, more intense, or more frequent than we would normally see. The first 6 patients we treated have all had radiographic responses. The third patient has had a complete response. Two patients had grade 3 toxicity. In both cases, these were symptoms they had before, but they seemed to be more intense on treatment - something I would have expected if they did not have FMT.

The pancreatic study has funding, but we are in the process of drafting the protocol for submission to the ethics review board. I anticipate that we will quickly accrue our goal of 50 patients and 50 cohabitants once the trial has started: I see a high proportion of the pancreatic cancer patients that come through our center, and there is nothing different that they have to do except give us some poop!

RF: The PERFORM study will involve fecal microbial transplantation (FMT), from a healthy donor, before the start of the immunotherapy combination and during the four cycles of ipilimumab and nivolumab treatment in patients with advanced renal

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> "So far, we have not seen any side effects just after Fecal Microbiome Transplant, nor have we seen any side effects during the I-O treatment that are unusual, more intense, or more frequent than we would normally see."
>
> - John Lenehan

cell carcinoma, as supportive therapy to prevent toxicity associated with immunotherapy combination.

The goal of this project is to prove the safety of such FMT combination treatment and reduce occurrence of immune-related toxicities in patients, allowing them to continue their cancer treatments in the hope of a better outcome. We will also be looking at changes in the immune populations, microbiome profile of patients, response to treatment, and patient survival as our secondary end points.

Q Can you tell us more about your choice of I-O agents for these studies?

RF: By way of background, cancer immunotherapy has become more common place and largely adopted in oncology patient management in the last decade. The responses to immunotherapy drugs have accelerated the approval of these drugs across multiple disease sites. A combination of two immunotherapy drugs (ipilimumab and nivolumab) has been approved for the treatment of patient with intermediate- and poor-risk advanced renal cell carcinoma. This combination provides not only survival benefit but also symptoms relief to these kidney cancer patients – therefore, it has been approved and funded for this patient population.

JL: For MIMic, we use either pembrolizumab or nivolumab, because these are the standard treatments and funded by the government. We have no industry sponsorship, so we are free to choose one or the other. As mentioned earlier, we are not allowing patients who are on combination I-O, unlike Ricardo's trial.

Q Moving forward, what are your respective plans for advancing your research beyond safety – e.g. to study the microbiome's relationship with the clinical effectiveness of I-O therapeutics?

RF: The current studies are feasibility trials with the aim of confirming the safety of FMT in advanced melanoma and renal cell carcinoma patients receiving

immunotherapy. The next step would be to conduct large Phase 2 trials to focus on the efficacy of altering the gut microbiota by assessing and comparing the response and survival rates across two groups of patients.

JL: Once the MIMic trial is completed, and we can say that there are no safety concerns with using FMT from a healthy donor before I-O therapy, we can then move to a Phase 2 trial to see if it this intervention benefits anyone. We will add it, or not, to standard therapy. The plan is to study patients with non-small cell lung cancer (NSCLC) receiving immunotherapy, as well as melanoma patients. This will allow us to have a large enough population of patients to obtain results in a reasonable amount of time. It also makes sense because for patients with NSCLC with a PD-L1, >50% are eligible to receive pembrolizumab as first line treatment. They are different cancers, but it is rational to include both in the trial.

SM: We have already secured funding for a Phase 2 trial of the MIMic study. We are also conducting more observational studies in different cancers that currently don't have immunotherapy approvals, such as prostate and pancreatic cancers. The main strategy for us is to pair our clinical data with our pre-clinical lab data moving forward to build more robust translational studies.

Q Finally, can you each share your long-term vision for microbiome-based cancer immunotherapy R&D? What will be the considerations for exploring this field across the broader range of I-O agents and/or tumor types?

RF: The ultimate goal is to design and execute studies that will help us find novel approaches to prevent immune-related adverse events in cancer patients resulting in less hospitalization and intensive care needs, thus reducing the significant burden on our healthcare system. This pilot project can provide the rationale for conducting large multi-arm studies that aim to change how we manage toxicity in patients who receive I-O therapeutics

JL: Long-term, if we show that FMT is helpful in a randomized trial, then we will likely branch out to other cancers to see if it is a general effect and not tumor specific. More importantly, the cancers that are resistant to immunotherapy such as some breast and colon cancers will be a focus, to see if FMT can stimulate the immune system and allow I-O therapy to be active in these cold tumors. It is not likely to be as simple as 'FMT, then off you go' – we may need to combine with other treatments such as chemotherapy and identify the optimal sequencing. Maybe combining with radiation first. These questions will keep us busy for years to come.

SM: I envision a time when, following a patient's visit to the clinic, we will run a comprehensive microbiome and immune analysis on them as part of their treatment planning. They will then receive a personalized I-O treatment, perhaps in combination with

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a microbiome modifying strategy to maximize the potential for a better clinical outcome and reduced toxicity.

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AUTHORSHIP & CONFLICT OF INTEREST

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IMMUNO-ONCOLOGY INSIGHTS

HUNTING FOR BETTER BIOMARKERS OF RESPONSE

SPOTLIGHT

EXPERT INSIGHT

Digital pathology for the identification of prognostic biomarkers in head and neck cancer

Rasha Abu-Eid & Frank James Ward

Head and neck cancers comprise a group of diseases with the most common being oral cavity and oropharyngeal cancers. The incidence of these cancers is on the rise but vary globally due to differences in risk factors such as alcohol, tobacco and betel quid consumption in addition to human papilloma virus infection. Despite advances in treatment, including cancer immunotherapy, the mortality rate remains high, which is mainly attributed to late diagnosis. Early detection of malignancies and prediction of malignant transformation in potentially malignant lesions are therefore vital to improve patient outcome. Digital pathology, which uses pre-defined algorithms to generate consistent and faster histopathological analysis, has made great strides in the quantification and identification of different markers capable of predicting disease progression, patient prognosis and response to therapy in head and neck cancer. The combination of digital pathology with different novel technologies including omics platforms, artificial intelligence and machine learning holds great translational potential for identifying prognostic biomarkers for head and neck cancer and beyond.

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HEAD & NECK CANCER.... A QUICK OVERVIEW

Head and neck cancers comprise multiple types that affect different sites with two major subtypes being oral cavity cancer and oropharyngeal cancer. The incidence of oral cavity cancer is increasing globally with the steepest increase observed in the developing world [1], while human papillomavirus (HPV) driven oropharyngeal cancer is increasing in the developed world [1]. Changes and geographical variation in risk factors account for this rise, in particular, changes in alcohol, tobacco and betel quid consumption in addition to a major rise in HPV infections. The latter is worrisome as it is presenting at an earlier age group than what is traditional for these cancers and its prevalence is increasing independent of sex, race or ethnicity [2]. The incidence of HPV positive oropharyngeal cancers is increasing at an alarming rate leading to the suggestion that it is an 'emerging cancer epidemic' [3].

Despite advances in diagnosis and treatment modalities, the mortality rate remains high. In 2020, 377,713 new cases and 177,757 deaths of lip and oral cavity cancer were reported, in addition to 98,412 new cases and 48, 143 deaths of oropharyngeal cancers [1]. The high mortality rate is mainly due to late diagnosis with many patients presenting at a stage where curative treatments are no longer an option. Therefore, screening tests and programs that can identify high risk patients at an early stage are essential.

Up until last year, it was thought that HPV driven oropharyngeal cancer could not be detected early and this was one of the reasons as to why there is no screening program for this cancer type. Tang *et al.* last year, for the first time, detected 2 mm occult HPV driven oropharyngeal cancer in an asymptomatic individual, which paves the way forward for initiating a screening program for HPV driven oropharyngeal cancer [4,5].

Some head and neck cancers are preceded by potentially malignant lesions that carry a higher risk of developing into malignancy. Screening for these lesions is also important to aid in the early detection of malignant transformation. Currently, there is a lack of biomarkers that can predict disease progression in these lesions and prognostic and predictive biomarkers to determine patient outcomes in an objective manner are also needed.

There are many attempts to introduce robust grading and classification systems for potentially malignant disorders to aid treatment planning and predicting disease progression. Assessed lesions were traditionally classified into mild, moderate and severe grades of epithelial dysplasia depending on the severity of cellular and tissue changes observed at a microscopic level. Although several iterations have been introduced to improve it, this classification system is not reproducible, has proven subjective, poorly transferable and unreliable in predicting malignant transformation [6–8]. There is, therefore, a move towards a simpler binary classification system that classifies epithelial dysplastic lesions into low- and high-grade dysplasia. Assessment of a binary system that classified dysplasia into low-risk and high risk lesions improved reproducibility, inter-pathologist agreement and was associated with 85% sensitivity and 80% specificity for predicting malignant transformation [9]. However, a reliable unified system that can accurately predict disease progression remains elusive.

CANCER IMMUNOTHERAPY IN HEAD & NECK CANCER

Cancer immunotherapy has proven successful in the management of many cancer types. There has been evidence of the potential for immunotherapy in head and neck cancer, however, there is a variable outcome in terms of response frequency and efficacy in patients. One of the main factors that lead to this variability is the type of tumor. While oral cancer has a significantly worse prognosis than oropharyngeal cancer, the latter's prognosis varies significantly depending on the HPV status, which is indirectly assessed based on

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the overexpression of p16, where p16 positive (assumed HPV positive) oropharyngeal cancers have a better prognosis and respond better to therapy [10–12].

Immune checkpoint inhibitors have encountered success in the treatment of head and neck cancer and newer therapies are showing great promise [13]. Anti-PD-1 antibodies in particular have shown therapeutic efficacy. Pembrolizumab, was approved in 2016 following the KEYNOTE-012 Phase 1b clinical trial in which head and neck cancer patients with progressive disease following platinum-based therapy received pembrolizumab and showed a promising response with 18% of patients achieving an overall response with a duration of 12·2 months and overall survival of 13 months. These results compared favorably with cetuximab, an anti-epidermal growth factor receptor antibody [14]. Interestingly, pembrolizumab, demonstrated antitumor activity in both HPV positive and HPV negative oropharyngeal cancers [15]. A larger Phase 3 KEYNOTE-040 clinical trial compared pembrolizumab as a monotherapy to methotrexate, docetaxel, or cetuximab [16] and reported 19% improvement in overall survival in patients treated with pembrolizumab, although not significantly different from standard treatment [17]. Pembrolizumab as a monotherapy or in combination with chemotherapy (cisplatin or carboplatin) and 5-fluorouracil was compared with cetuximab with the same chemotherapy combination in the Phase 3 KEYNOTE-048 trial in recurrent or advanced metastatic head and neck cancer patients [18]. Data from the trial suggest that pembrolizumab as a monotherapy or in combination, significantly improved overall survival especially in PD-L1 positive tumors [19]. The National Institute of Health and Care Excellence (NICE) in UK has recommended the use of Pembrolizumab as a monotherapy as a treatment option for untreated metastatic or unresectable recurrent head and neck cancers that express PD-L1 [20].

Nivolumab was approved in 2016 as a result of the Phase 2 clinical trial (CheckMate-141) in which patients with recurrent progressive post-platinum treatment head and neck cancer were treated with nivolumab as a monotherapy and once again, compared with methotrexate, docetaxel and cetuximab [21]. Nivolumab was associated with an improved overall survival and tumors that were positive for PD-L1 showed a better response to this anti-PD-1 antibody [22]. Interestingly, irrespective of the HPV status, patients with oropharyngeal squamous cell carcinoma responded better to nivolumab [22].

As oral potentially malignant disorders carry a higher risk of progression into malignancy, cancer immunotherapies are being assessed to treat these lesions before transforming into overt cancers. Targeting PD-1/PD-L1 in oral premalignant lesions was found to prevent their progression into malignancy in a murine oral squamous cell carcinoma model [23], suggesting the potential use of these immune checkpoint inhibitors in the treatment of oral potentially malignant disorders as well as established malignancies.

Despite being the first immune checkpoint inhibitors to be approved [24], antibodies targeting CTLA-4 did not meet the same success as those targeting PD-1 and PD-L1. As a consequence, there have been very few studies that looked into the potential of immune checkpoint inhibitors targeting CTLA-4 in head and neck cancer. A Phase 2 clinical trial that compared a combination of nivolumab and ipilimumab to nivolumab as a monotherapy in oral squamous cell carcinoma reported that patients in both arms of the trial showed evidence of response with the combination arm showing a marginally stronger response [25]. Furthermore, in a murine oral squamous cell carcinoma model, targeting PD-1 alone or in combination with CTLA-4 inhibition hindered the progression of oral premalignant lesions into malignancy [23].

However, the results of a more recent open label Phase 3 clinical trial that compared the anti-PD-L1 antibody (durvalumab) on its own or in combination with the anti-CTLA-4 antibody tremelimumab to the standard of care treatment (cetuximab, docetaxel, paclitaxel, methotrexate, 5-fluorouracil, TS-1, or

capecitabine) in recurrent or metastatic head and neck cancer patients did not find any improvement in overall survival [26].

Although anti-CTLA-4 antibodies received little attention in the treatment of head and neck cancer, there is abundant evidence that CTLA-4 plays an important role in the pathogenesis of these tumors. CTLA-4 genetic polymorphisms have been reported to be associated with susceptibility to head and neck cancer [27,28] and certain genotypes, such as CTLA-4 A/A at position +6230 A/G (CT60), were associated with poorer prognosis [29].

Head and neck cancers recruit regulatory CD4 T cells (Tregs) as an important immune escape mechanism. Despite the known suppressive role of Tregs in the tumor microenvironment, there are contradictions about their role in head and neck cancer [30]. Objective quantification and proteomic analysis of Tregs within the tumor microenvironment is essential to understand their role and identify important biomarkers on these suppressive cells. This is especially important as Tregs expressing high levels of CTLA-4 in head and neck cancer were reported to be associated with a high proliferative profile and were found to be highly suppressive [31] and the highest level of CTLA-4 expression in tumor infiltrating T cells in these tumors was observed in Tregs [32].

DIGITAL PATHOLOGY & BIOMARKER IDENTIFICATION & QUANTIFICATION

It has become apparent that identifying biomarkers that predict disease progression, patient prognosis or patient response to therapy as a stratification tool is essential for head and neck cancer patients.

Digital pathology has made great contributions in the quantification of various biomarkers including morphological, immunological and phenotypic markers that have enhanced our understanding of the pathogenesis of different cancers.

Markers for detecting disease progression

Oral potentially malignant disorders carry a higher risk of malignant transformation. Therefore, identification of markers that can predict disease progression is essential to aid in the early detection of cancer formation and ultimately improving patient outcome.

Cell & tissue morphology

Changes in tissue and cellular morphology are important parameters in diagnosing potentially malignant head and neck lesions. Understanding these changes is essential to help identify markers for malignant transformation. To help improve the classification systems used for these lesions, image analysis was used to quantify various descriptors of tissue and cell architecture in normal, premalignant and malignant oral tissues. Fractal geometry quantified changes in the complexity of the basement membrane and different morphological parameters of cellular shape and size were objectively assessed to reveal gradual increase in basement membrane irregularity and changes in cell morphology associated with disease progression [33]. Our group is currently investigating the use of newer image analysis software packages to assess morphological changes in oral and oropharyngeal tissues in an attempt to identify markers associated with disease progression.

Angiogenesis

Changes in blood vessels are associated with disease progression where increased vascularization is observed in premalignant [34] and malignant oral lesions [35], suggesting their use for predicting disease progression [36]. Our group used digital pathology for the quantification of collagen IV, a marker of blood vessel basement membranes, and reported significant changes in the spatial distribution and morphometry of collagen IV expression in normal, premalignant and malignant oral [37] and oropharyngeal tissues with differences observed between HPV positive and negative

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tumors [38]. Our findings highlighted the potential for using collagen IV as a marker for detecting disease progression and potentially a positive response to therapy given the role of blood vessels in drug delivery.

Immune markers

CTLA-4 has not received the same attention as PD-1/PD-L1. However, there is strong evidence of its involvement in head and neck cancer pathogenesis. Current anti-CTLA-4 antibodies can bind both the full-length receptor isoform and an alternatively spliced isoform, called soluble CTLA-4 (sCTLA-4) [39,40]. We have developed reagents that are selective for sCTLA-4 [41–43], allowing the study of the two forms of CTLA-4 in detail. We have recently shared our early data applying digital pathology in quantifying the expression of sCTLA-4 in normal, potentially malignant and malignant head and neck tissues. Our preliminary findings clearly show changes in the expression level and distribution of this immune checkpoint associated with disease progression suggesting sCTLA-4 as a promising marker for predicting disease progression in head and neck potentially malignant disorders [44].

Given the role that Tregs play in immune escape in head and neck cancers, digital pathology is a very useful tool in the quantification of these cells within the tumor microenvironment. Indeed, our group is currently using different image analysis techniques to assess Treg infiltration and distribution in normal, potentially malignant and malignant head and neck tissues to identify changes in these cells associated with disease progression.

Markers for predicting prognosis & response to therapy

Markers that can predict patient prognosis and response to therapy in head and neck cancer are needed to help stratify patients for different treatments, personalize therapies and improve patient outcome.

Clinical trials have shown that the response to anti-PD-1 therapy in head and neck cancer is stronger in PD-L1 positive tumors [19,22]. Furthermore, PD-L1 and PD-L2 expression was found to be positively correlated with Aldehyde dehydrogenase family 1 member A1 (ALDH1A1) expression with PD-L1 possibly involved in ALDH1A1 mediated poor prognosis [45]. Therefore, Quantifying PD-L1 expression is essential, and digital pathology and advanced image analysis techniques can provide robust and objective tools to assess the expression of this immune checkpoint.

Digital image analysis is also important for quantifying T cell infiltration in the tumor microenvironment including calculating the immunoscore as defined by CD3 and CD8 T cell infiltration in the tumor core and invasive margin in head and neck cancer [46]. Additionally, image segmentation algorithms were applied in multiplex immunohistochemistry in oropharyngeal cancer samples leading to better detection of different T cell subsets including, TH1-like and TH2-like TH17 T cells. TH2-like TH17 cells were more prominent in HPV negative cases and were spatially correlated with CD66b+ granulocytes suggesting a suppressive tumor environment [47].

Image analysis was also used to quantify tumor infiltrating lymphocytes within the epithelial compartment and in the stroma in head and neck cancer. This was coupled with the analysis of the immune cell infiltration based on RNAseq data and PD-L1 mRNA expression. Tumor infiltrating lymphocytes from hematoxylin and eosin-stained sections were found to be positive prognostic markers. Furthermore, sequencing data identified T cells as positive prognostic markers while PD-L1 was a negative prognostic marker [48]. Additionally, liquid biopsy-based techniques are coming to the forefront, as potential prognostic biomarkers. As an example, Kulasinghe *et al.* has shown that PD-L1 expression on circulating tumor cells can be used as a potential biomarker to determine response to immunotherapy in a head and neck cancer patient [49].

Digital pathology & omics platforms

Technological advancements have enabled the incorporation of image analysis with proteomic and genomic analyses. The Hyperion™ imaging system for high dimensional proteomics analyses, which combines the simultaneous detection and quantification of over 40 markers with localization on histological sections, was used to quantify the tumor microenvironment in oral squamous cell carcinoma and highlighted the potential of this technology in predicting patient prognosis [50].

The NanoString GeoMx™ Digital Spatial Profiling technology, which combines image analysis with spatial genomic analyses, was used to analyze head and neck cancer samples from patients who received immune checkpoint inhibitor therapy and identified immune cell types and markers associated with disease progression [51]. In addition to analyzing immune cell types, these systems have a great potential in quantifying tumor mutation burden thus helping in the identification of therapeutic targets and predicting response to various therapies.

Digital pathology & artificial intelligence

Developments in artificial intelligence and machine learning have further revolutionized the applications of digital pathology.

Machine learning was used to create a model for predicting treatment in oropharyngeal squamous cell carcinoma while taking into consideration variables related to the tumors, socioeconomic, regional, and institutional factors [52]. Artificial intelligence is also used for intensity-modulated radiation therapy (IMRT) treatment planning [53] and radiomics in head and neck cancer [54]. Furthermore, artificial intelligence was used to predict microsatellite instability and deficient DNA mismatch repair in hematoxylin and eosin stained colorectal cancer sections with high accuracy in uniform datasets [55]. Artificial intelligence and machine learning have also been used in developing biomarkers for early detection of head and neck cancer by assessing metatranscriptomic data from saliva samples from normal, potentially malignant and malignant oral tissues [56].

The applications of digital pathology go beyond microscopic analyses of histopathological markers. Moderate associations have been found between PD-L1 expression and parameters of dynamic contrast enhanced MRI in head and neck cancer [57]. Weak associations were also observed between PD-L1 expression and diffusion-weighted imaging as quantified by apparent diffusion coefficient parameters [58]. This suggests the potential of applying artificial intelligence and machine learning at various levels including microscopy and clinical imaging to detect prognostic biomarkers that can predict disease progression and response to therapy.

Further studies that apply the power of machine learning and artificial intelligence are needed with the ultimate goal of improving patient outcome.

TRANSLATION INSIGHT

Digital pathology coupled with various technologies has great potential for identifying biomarkers that can help in detecting disease progression, predicting patient outcome and stratification of patients for specific treatments in head and neck cancer. Furthermore, these novel technologies have the potential to identify new targets for developing novel therapies based on quantifiable and objective assessment of cancer tissues.

As can be observed from this quick journey into the applications of digital pathology in head and neck cancer studies, the translational potential of these technologies in identifying diagnostic, prognostic and predictive biomarkers has become apparent, not only for head and neck cancer but for different malignancies and beyond.

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IMMUNO-ONCOLOGY INSIGHTS

HUNTING FOR BETTER BIOMARKERS OF RESPONSE

SPOTLIGHT

VIEWPOINT

Leveraging the advantages of liquid biopsy for predictive and prognostic biomarkers in R&D

Associate Professor Chamindie Punyadeera

Immune checkpoint inhibitors (ICIs) therapy has steered in a new era of anti-tumor therapy, with significant survival outcomes observed for multiple tumors. Anti-programmed cell death-1/programmed cell death-ligand 1 (PD-1/PD-L1) antibody has been approved for second-line or first-line treatment in melanoma, lung cancer, renal cell carcinoma (RCC), head and neck squamous cell carcinoma (HNSCC) and gastroesophageal cancer. However, despite the breakthrough in clinical treatment with ICIs, most patients do not benefit. As an example, pembrolizumab or nivolumab showed 40–45% response as a first-line treatment in melanoma patients and 20% response as a second-line treatment in non-small cell lung cancer (NSCLC) patients. Therefore, attention has given to identifying and validating predictive biomarkers for the efficacy of ICIs. Liquid biopsy (the use of biomarkers in body fluids in place of traditional tumor tissues) approached has also been investigated as potential predictive biomarkers. In recent years, the tumor microenvironment, tumor genome through next generation sequencing, and neoantigens have been investigated to comprehensively understand tumour biology. The field is now moving towards multi marker predictive panels in place of single marker as previously done. The advent of single cell RNA sequencing and 3D spatial biology technology will fast track the progress of identifying predictive biomarkers to stratify cancer patients who are responders vs non-responders for ICT treatment.

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A key reason why immuno-oncology therapeutics continue to be given only as second- or even third-line treatments in indications with relatively high unmet need, such as head and

neck cancer, is the lack of a good biomarker to identify which patients would respond to these $150,000 treatments.

The current gold standard for identifying which patients would respond to immuno-oncology is to use tumor expression levels of PD-L1 as a surrogate biomarker. However, recent clinical studies, field trials, and literature all indicate that tumor PD-L1 expression level, as measured using the Immunoscore system, is of limited value.

Recent research, including that conducted by Associate Professor Punyadeera's research team at the Queensland University of Technology (QUT), is focused on demonstrating that PD-L1 expression on circulating tumor cells (CTCs) or CTC clusters (the cells disseminated from primary and metastatic sites, thus representing both the primary and metastatic cancer) can be a good prognostic marker.

While our pilot clinical studies conducted to date have been in small clinical cohorts, we believe that looking into PD-L1 expression in CTCs and adjacent white blood cells may be a key future avenue for identifying responders and non-responders to immunotherapies.

THE ADVANTAGES OF LIQUID BIOPSY

The translational research program that I lead at QUT is using human saliva and blood methods of sampling as an alternative to traditional tumor biopsy testing for cancer diagnosis, prognosis, and predicting outcomes.

Having started my independent research career in Australia, my own work in pioneering the use of saliva as a liquid biopsy began in 2010, having received a Queensland Government Smart State Senior Fellowship. My research focused initially on understanding how biomolecules entered saliva, and their physiological status and its effect on saliva secretion. I then investigated links between these salivary biomarkers and systemic disease. In the first instance, this specifically involved the development of non-invasive saliva-based biomarkers for the early detection of heart failure where we have created novel intellectual property. Our IP is now licensed to an Australian biotech company. Today, though, my efforts are focused on head and neck cancer, glioblastoma, liver, and lung cancer. We are studying the utility of CTCs, circulating tumor DNA, exosomes, and high-risk human papillomavirus (HPV) DNA as biomarkers to detect cancer early, predict outcomes, and also for disease surveillance.

There are several key advantages to using liquid biopsy instead of tumor tissue, including the fact that tumor tissue biopsies are generally very difficult to access. Furthermore, there is also the issue of tumor heterogeneity to consider: a tumor tissue sample only represents approximately one-fifth of the overall tumor, which means that one is not able to obtain a true representation of the whole tumor. It is like trying to view the world through a keyhole.

BUILDING AN OPTIMAL CTC DETECTION & SEPARATION PLATFORM

When we began investigating CTCs, we employed the FDA-approved CELLSEARCH® system from Veridex, LLC, a technology based on beads that pick out tumor cells in the circulation that overexpress EpCAM (epithelial cell adhesion molecule). However, we found that we couldn't obtain high yields of CTC with this system, which we later discovered could be explained by the fact that most CTCs from head and neck cancer don't overexpress EpCAM.

Subsequently, we sought to compare the state-of-the-art CTC detection technologies – CELLSEARCH® and ScreenCell®. The latter separates cells based on size – CTCs, which are larger cells, should remain on the filter, while all smaller cells (e.g. white blood cells, platelets, etc.) filter out. We also compared microfluidic devices, including the technology we currently use, which is a spiral

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microfluidic technology. Microfluidic technology uses inertial focusing/microfluidics which takes into account cell size and deformability properties to separate CTCs from smaller cells. This microfluidic technology is a highly promising approach for size-based cell separation due to its ease of operation and high separation resolution.

The result of these studies is an established workflow, which we have validated in head and neck cancer, lung cancer, glioblastoma. We are now adapting the platform for liver cancers.

FUTURE STEPS

Moving forward, single-cell RNA-Seq data carries the potential to grant us insights into the expression levels of different immunomarkers on tumor tissue and microenvironment, as well as CTCs and other immune cells in circulation.

This is critical because of the importance of a holistic approach in this area of research. We cannot just focus on tumor cells alone, or on white blood cells alone: I believe a combination of the two will be required. As much as cancer biologists (such as myself) would love to go with CTCs, and immunologists would love to go with white blood cells, I think the field will continue to move towards a marrying of immunology and tumor biology.

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