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Resistance patterns to anti-PD-1 therapy in metastatic melanoma

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Anti-PD-1 antibodies block the immune checkpoint programmed death 1 receptor to increase T cell specific anti-tumor responses (1,2). Anti-PD-1 therapy represents a major advance in the treatment of metastatic melanoma. However, the response rate (defined as shrinkage of tumor by greater than 30%) in these patients is still only approximately 30%. A key concept in immune-based therapy is that patients without responses may have long term survival due to the immune system attacking the tumors without a radiographic response seen on imaging studies. Therefore response and survival are not synonymous concepts for patients undergoing immune-based therapy. Patients who do respond to anti-PD-1 therapy tend to have very long responses to therapy compared to traditional chemotherapy in melanoma (3–5). Therefore, it is crucial to understand the mechanisms of resistance of anti-PD-1 therapy to both spare patients from unnecessary therapy and to facilitate the development of combinatorial approaches to overcome anti-PD-1 resistance.

A key resource that has provided the technical skill and knowledge base needed to understand the mechanism of response to anti-cancer therapies is the Cancer Genome Atlas (TCGA). TCGA is a database that describes the genetic patterns of malignant tumors. In particular, the genomic classification of cutaneous melanoma was described in 2015 (6). For the initial TCGA database, the investigators collected tumor samples from 333 cutaneous primary or metastatic melanomas. They performed six types of global molecular analysis on the tumor samples: (I) Solution-based hybrid-capture whole-exome sequencing; (II) DNA copy-number profiling; (III) mRNA sequencing; (IV) microRNA sequencing; (V) DNA methylation profiling; and (VI) reverse-phase protein array expression profiling. For melanoma, the authors established a genomic classification of malignant melanoma consisting of four subtypes based on the pattern of the most prevalent mutated genes. These subtypes are (I) mutant *BRAF*, (II) mutant *NRAS*, (III) mutant *NF1*, and (IV) triple wild-type (6).

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About 50% of cutaneous melanomas (but only a very small percentage of acral-lentiginous, desmoplastic, uveal and mucosal melanomas) fall into the first subtype as they harbor *BRAF* mutations (7). *BRAF* mutations lead to activation of the mitogen-activated protein kinases (MAPK) pathway (8). Many patients with *BRAF* mutant metastatic melanoma first receive treatment with BRAF inhibitors alone or with MEK inhibitors and some are then treated with anti-PD-1 therapy after they develop resistance to MAPK-targeted therapy. Interestingly, failure of MAPK targeted therapy predicted resistance to subsequent immune checkpoint blockade therapy in melanoma. Furthermore, resistance to MAPK-targeted therapy was found to be associated with depletion of intratumoral T cells, exhaustion of $CD8^+$ T cells, and loss of antigen presentation (9). Antigen presenting cells present foreign antigens (such as cancer cell antigens) in the context of the major histocompatibility complex type II (MHC-II) to T cells. Indeed, increased MHC-II expression in melanoma cells is associated with both PD-1 signaling and response to anti-PD-1 therapy (10). Therefore, it is not surprising that absence of the intratumoral T cell infiltration predicts resistance to anti-PD-1 therapy in melanoma (11).

In the TCGA database it was found that patients with a combination of T cell infiltration of tumors and increased levels of mRNA transcripts of immune-associated genes within the tumors tended to survive longer (6). With the TCGA dataset available and hints of the mechanism of resistance available from the literature, Hugo *et al.* investigated the factors that might correlate with response to anti-PD-1 therapy in metastatic melanoma and they discovered new features that appeared to be predictive of response to this therapy (12). In particular, they investigated the transcriptomic (analysis of mRNA) and genomic (analysis of DNA) features of the patient's melanoma samples and how they may predict resistance to anti-PD-1 therapy. The conclusions from the paper can be summarized in four main statements: (I) high mutational loads may not predict response to anti-PD-1 therapy by traditional response criteria, but may serve as an indicator as to which melanoma patients may have improved overall survival; (II) *BRCA2* mutations are frequently observed within the tumor specimens of melanoma patients responding to anti-PD-1 therapy; (III) in patients not responding to anti-PD-1 therapy, a transcriptomic signature referred to as the innate anti-PD-1 resistance (IPRES) signature is composed of RNA transcripts relating to mesenchymal transition, angiogenesis, hypoxia and would healing; (IV) the IPRES signature is also detectable in melanoma patients treated with BRAF targeted therapy and in other cancer types.

High mutational loads as measured by non-synonymous nucleotide variations (nsSNVs) may not predict response to anti-PD-1 therapy by traditional response criteria, but may serve as an indicator as to which patients may have improved overall survival. Malignant tumors all have mutations in their somatic DNA that are not present in the patient's germline DNA. Some have relatively few mutations, others have many. Cutaneous melanoma in particular is a tumor type associated with very high numbers of somatic mutations due largely to ultraviolet exposure. These mutations, when they result in the production of mutated proteins expressing neoantigens not found in normal cells, could increase the likelihood of response to immunotherapy such as anti-PD-1 antibody treatment. In the first set of experiments, the authors analyze the nsSNVs (which are a class of mutations that alters the nucleotide on the DNA strand that ultimately results in translation of a different amino acid

at that position). Hugo *et al.* analyzed pre-treatment tumor tissues of 38 patients with metastatic melanoma who were treated with anti-PD-1 therapy. The number of nsSNV mutations in the tumors was investigated by whole-exome sequencing and RNA-Seq methods. A median of 489 non-synonymous somatic mutations (range, 73–3,985) were detected. Tumor specimens obtained from melanoma patients responding to anti-PD-1 therapy harbored more nsSNV mutations and more human leukocyte antigen (HLA) class I and class II neoepitopes compared to the non-responding patients, but this association did not reach statistical significance. The overall survival was significantly increased in those patients whose nsSNV mutational load fell in the top third of cases (P=0.005). Although this is a small study, the patients who had a low nsSNV mutational load but still had a response to anti-PD-1 therapy had an increased overall survival compared to those patients who had a high mutational load and were non-responsive to therapy. As might be expected, the greatest difference in survival was between responding patients with a high nsSNV count and those patients who did not respond to therapy with a low nsSNV count (8). These same investigators had previously attempted to find signatures that were associated with response to anti-CTLA-4 therapy but were unsuccessful (12,13). Therefore the biomarkers predictive of response or resistance are likely different across the different classes of checkpoint inhibitors.

BRCA2 mutations are frequently observed within the tumor specimens of melanoma patients responding to anti-PD-1 therapy. Hugo *et al.* then went on to analyze the genetic variations (nsSNVs, small insertions and deletions, copy number changes) in tumors prior to treatment, and then looked for differences between responding and non-responding patients on anti-PD-1 therapy. The background mutation rate of each gene was calculated from the whole exome sequencing data of 469 melanoma tumors from the TCGA database (4,10). *BRCA2* copy number changes, nsSNV, mutations resulting in the net change in the number of nucleotides resulting from the insertion and deletions of nucleotides (INDEL) or mutant allele copy number gain were significantly more frequently found in tumors from patients responding to treatment. The pattern of *BRCA2* mutations suggested that they were loss of function mutations. Loss of function mutations in BRCA2 are known to lead to defects in homologous recombination and double-strand break repair (14). Therefore, it is possible that the loss of *BRCA2* leads to an environment where the cancer cell may undergo apoptosis or has preferential selection of mutations that lead to anti-PD-1 responsiveness (12).

In patients not responding to anti-PD-1 therapy, a transcriptomic signature referred to as IPRES is composed of RNA transcripts relating to mesenchymal transition, angiogenesis, hypoxia and wound healing. Hugo *et al.* also investigated whether expression of RNA transcripts in tumor specimens prior to initiation of anti-PD-1 therapy would identify patients who would respond to anti-PD-1 therapy. They first identified differentially expressed genes in those samples from patients not responding to anti-PD-1 therapy. Mesenchymal transition genes (*AXL*, *ROR2*, *WNT5A*, *LOXL2*, *TWIST2*, *TAGLN*, *FAP*), immunosuppressive genes (*IL-10*, *VEGFA*, *VEGFC*), monocyte and macrophage chemotactic genes (*CCL2*, *CCL7*, *CCL8*, *CCL13*), wound healing genes, and angiogenesis genes were up-regulated in pre-treatment tumors from patients who proved to be non-responders (12). Indeed, it has been shown in previous studies that genes associated with wound healing, angiogenesis, and mesenchymal transition were also potentially T cell

suppressive genes (15–17). Interesting, other T cell related genes such as *CD8A/B*, *IFNG*, *GZMA*, and *PRF1* were not differentially expressed in those patients responding to anti-PD-1 therapy. Furthermore, the transcripts for checkpoint receptors (PDL-1 and LAG3) were not up-regulated in patients responding to anti-PD-1 therapy. This is in contrast to the study by Van Allen *et al.*, where they found that T cell regulated genes (*GZMA*, *PRF1*, *PDL-2*, *CTLA4*) were up-regulated in the tumor specimens obtained prior to therapy from patients responding to anti-CTLA-4 treatment (14). Given the known differentially expressed genes in this study, the authors attempted to match the up-regulated genes to known biochemical processes. A traditional gene ontology search demonstrated that the tumors from patients who were non-responders to anti-PD-1 therapy had the processes of cell adhesion, ECM organization, wound healing and angiogenesis up-regulated before receiving treatment. Following this, the authors used the Molecular Signature Database to determine which biological processes were enriched in the tumor specimens of patients not responding to anti-PD-1 therapy. The Molecular Signature Database at the Broad Institute is a collection of manually and computational curated gene sets that can be utilized to find transcriptomic signatures of biological processes (18). They found 26 transcriptomic signatures associated with resistance to anti-PD-1 therapy. The authors named this signature the IPRES signature, and once again transcripts relating to mesenchymal transition, angiogenesis, hypoxia and wound healing were found to be up-regulated in the tumor specimens obtained from patients who were non-responsive to anti-PD-1 therapy. The authors clearly state that the results need to be validated in prospectively collected cohorts of melanoma patients.

The IPRES signature is detectable in melanoma patients treated with BRAF targeted therapy and in other cancer types. Hugo *et al.* analyzed four additional cohorts of RNA-Seq data derived from patients with (I) tumor specimens derived from patients prior to anti-PD-1 treatment; (II) tumor specimens derived from patients prior to anti-CTLA4 treatment; (III) tumor specimens derived from patients prior to MAPK inhibitor treatment; and (IV) four other cohorts of cancers present in the TCGA database (lung adenocarcinoma, colon adenocarcinoma, kidney clear cell carcinoma, and pancreatic adenocarcinoma) to evaluate whether the IPRES transcriptional signature was present in these other cohorts (6,9,12,19). The authors found that the IPRES signatures were found at increased frequency in metastatic (90/282) *vs.* primary (6/69) melanomas. In addition, it was determined that the IPRES signature was increased in the anti-PD-1 non-responding tumors (odds ratio =4.6) and was decreased in tumors responding to anti-PD-1 therapy (odds ratio =0.15). The IPRES signature was not associated with response to anti-CTLA-4 antibody. In addition, it was found that the IPRES signature can also be found in lung adenocarcinoma, colon adenocarcinoma, kidney clear cell carcinoma, and pancreatic adenocarcinoma.

Conclusions

In this study, Hugo *et al.* discovered a potentially new transcriptional signature, IPRES, associated with non-responsiveness to anti-PD-1 therapy. *BRCA2* loss of function mutations were found to be associated with anti-PD-1 responsiveness. The mutational load of melanoma may be associated with overall survival, but there is insufficient evidence to predict responsiveness to anti-PD-1 therapy based on this paper. These results may be potentially applicable to other tumor types, as the IPRES signature was also found in lung,

kidney, colon and pancreatic cancers. This study shows how careful analysis of changes at the gene and RNA transcription level in tumors prior to treatment can potentially be utilized to develop as biomarkers to predict who may have a higher or lower chance of responding to anti-PD-1 therapy. While we have a long way to go before these types of analyses become standard of care, it is an exciting field of research that could have broad applicability to multiple tumor types and multiple different kinds of anti-cancer therapy, not just anti-PD-1 in melanoma.

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