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Genomic features of exceptional response in vemurafenib ± cobimetinib–treated patients with *BRAF*^{V600}-mutated metastatic melanoma

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Disclosure of Potential Conflicts of Interest

Y.Y., M.J.W., Z.S., H.K., J.J.H., I. Caro, I. Chang, and IR are employees at Genentech, Inc. C.R. reports consultancy and advisory board fees from Amgen, Bristol-Myers Squibb, GlaxoSmithKline, Merck, Novartis, and Roche. J.L. reports consultancy and advisory board fees from Bristol-Myers Squibb, Eisai, EUSA Pharma, GlaxoSmithKline, Kymab, Merck Sharp & Dohme, Novartis, Pfizer, Pierre Fabre, Roche/Genentech, and Secarna. P.A.A. reports consultancy and advisory board fees from Bristol-Myers Squibb, Roche-Genentech, Array, Amgen, Merck Sharp & Dohme, Merck Serono, Novartis, and Pierre-Fabre, and research grants from Bristol-Myers Squibb, Roche-Genentech, and Array. B.D. reports research grants from Roche and GlaxoSmithKline, and personal fees from Roche and Bristol-Myers Squibb. M.M. reports advisory board fees from Roche, Bristol-Myers Squibb, Astra Zeneca, and Incyte. C.G. reports consultancy and advisory board fees and honoraria from Bristol-Myers Squibb, GlaxoSmithKline, Roche, Amgen, Merck, Novartis, and Philogen, research grants from Bristol-Myers Squibb, GlaxoSmithKline, and Roche, and travel expense reimbursement from Bristol-Myers Squibb, GlaxoSmithKline, Roche, Amgen, Merck, and Novartis. P.B.C. reports advisory board fees from Roche/Genentech, consultancy fees from, LICR Research, Merck, Rgenix, and Scancell, and honoraria from GlaxoSmithKline. J.A.S. reports advisory board fees from Array Pharma, Bristol-Myers Squibb, and Curis. GM reports research grants (payable to his institution) from Roche-Genentech. A.R. reports consultancy fees from Amgen, Merck, and Pfizer, and stock ownership in Compugen, CytomX, Five Prime, and Kite Pharma.

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Abstract

Purpose: Previous investigations identified transcriptional signatures associated with innate resistance to anti-programmed cell death protein 1 therapy in melanoma. This analysis aimed to increase understanding of the role of baseline genetic features in the variability of response to BRAF and MEK inhibitor therapy for *BRAF*^{V600}-mutated metastatic melanoma.

Experimental design: This exploratory analysis compared genomic features, using whole exome and RNA sequencing, of baseline tumors from patients who had complete response versus rapid progression (disease progression at first postbaseline assessment) on treatment with cobimetinib combined with vemurafenib or vemurafenib alone. Associations of gene expression with progression-free survival or overall survival were assessed by Cox proportional hazards modeling.

Results: Whole exome sequencing showed that *MITF* and *TP53* alterations were more frequent in tumors from patients with rapid progression, while *NFI* alterations were more frequent in tumors from patients with complete response. However, the low frequency of alterations in any one gene precluded their characterization as drivers of response/resistance. Analysis of RNA profiles showed that expression of immune response-related genes was enriched in tumors from patients with complete response, while expression of keratinization-related genes was enriched in tumors from patients who experienced rapid progression.

Conclusion: These findings suggest that enriched immune infiltration might be a shared feature favoring response to both targeted and immune therapies, while features of innate resistance to targeted and immune therapies were distinct.

Translational relevance

Our exploratory analysis of baseline *BRAF*^{V600}-mutated melanoma samples from the coBRIM study aimed to identify genetic characteristics of patients with a complete response or no response to combined BRAF and MEK inhibition with vemurafenib and cobimetinib. No individual mutation was identified as a population-wide driver of exceptional response, but certain gene expression signatures were found to distinguish the two extremes of response. Melanomas from patients with complete response possessed higher preexisting tumor immunity features, while those from patients with rapid progression had a keratin signature similar to one previously associated with poor prognosis. These findings suggest that enriched immune infiltration of melanomas may be a shared feature of favorable response to both targeted and immune therapies, and distinct from features of innate resistance to immune and targeted therapies. We anticipate these findings could assist in optimization of treatment selection for patients with *BRAF*^{V600}-mutated metastatic melanoma.

Keywords

melanoma/skin cancers; cancer genome anatomy; comparative expression patterns; phase I-III trials; melanoma/skin cancers; molecular markers of metastasis and progression; pharmacogenetics/pharmacogenomics

Introduction

The introduction of small-molecule inhibitors of BRAF and MEK and monoclonal antibodies targeting programmed cell death protein 1 (PD-1) and cytotoxic T lymphocyte-associated antigen 4 have remarkably improved treatment outcomes for patients with metastatic melanoma (1–15). With the emergence of distinct classes of therapies, there is a need to better understand baseline features associated with response to either targeted therapies or immunotherapies to allow consideration of treatment that will optimize benefits for the individual patient. An investigation of genomic features in responders compared with nonresponders identified transcriptional signatures associated with innate resistance to anti-PD-1 therapy (16,17), although no common feature was identified to predict the response. While previous investigation of the mechanisms of acquired resistance to inhibitors of the MAPK pathway identified MAPK reactivation by various genetic and epigenetic alterations developed during treatment (18,19), baseline features predicting response to MAPK-targeted therapies have not been examined.

Combined MEK and BRAF inhibition with cobimetinib and vemurafenib has been shown to improve response rates, progression-free survival (PFS) (4,5), and overall survival (OS) (6) compared with BRAF inhibitor monotherapy. Responses to BRAF and MEK inhibitors vary between patients; while some patients achieve complete response (CR), a proportion of patients demonstrate rapid disease progression suggestive of some degree of innate resistance. A better understanding of the mechanisms underlying the variability of patient responses to these BRAF and MEK inhibitors is required in order to target therapy more effectively.

The objective of this retrospective, exploratory analysis was to compare genomic features of pretreatment tumors from complete responders with those from rapid progressors on treatment with cobimetinib combined with vemurafenib or vemurafenib alone in patients with *BRAF*^{V600}-mutated metastatic melanoma.

Methods

Analysis population.

Data from patients with *BRAF*^{V600}-mutated metastatic melanoma treated with cobimetinib combined with vemurafenib or vemurafenib in the BRIM-2, BRIM-3, BRIM-7, and coBRIM studies were included in the analysis. Detailed methods have previously been reported for each study. Briefly, BRIM-2 ([ClinicalTrials.gov ID, NCT00949702](https://clinicaltrials.gov/ct2/show/study/NCT00949702)) was a multicenter, single-arm phase II study in which patients were treated with vemurafenib 960 mg twice daily(1). BRIM-3 ([ClinicalTrials.gov ID, NCT01006980](https://clinicaltrials.gov/ct2/show/study/NCT01006980)) was a multicenter, randomized, open-label phase III study in which patients were randomly assigned to receive

vemurafenib 960 mg twice daily or dacarbazine 1,000 mg/m² every 3 weeks(2,3). BRIM-7 (ClinicalTrials.gov ID, NCT01271803) was a multicenter, single-arm phase Ib dose-escalation study in which patients received cobimetinib 60, 80, or 100 mg once daily, given on a schedule of 14 days on/14 days off, 21 days on/7 days off, or continuously, in combination with vemurafenib 720 or 960 mg twice daily(4). coBRIM (ClinicalTrials.gov ID, NCT01689519) was a multicenter, randomized, double-blind phase III study in which patients were randomly assigned to receive cobimetinib 60 mg once daily for 21 days followed by 7 days off or placebo in combination with vemurafenib 960 mg twice daily (5,6).

Key eligibility criteria for the four trials were similar, including age ≥ 18 years, unresectable stage IIIC or IV melanoma harboring a *BRAF*^{V600} mutation, an Eastern Cooperative Oncology Group performance status of 0–1, and adequate organ function. The BRIM-3 and coBRIM studies enrolled previously untreated patients only, whereas BRIM-2 enrolled patients who had received at least one prior systemic treatment for advanced melanoma and BRIM-7 enrolled both previously treated and untreated patients.

Baseline tumor samples were obtained from consenting patients before initiation of study treatment. Genomic features at baseline were compared between patients who had CR or rapid progression (defined as disease progression at the first tumor assessment) according to Response Evaluation Criteria in Solid Tumors, version 1.1, on treatment with cobimetinib combined with vemurafenib or vemurafenib alone.

Each of the trials was conducted in accordance with the Declaration of Helsinki and the principles of Good Clinical and Laboratory Practice and with the approval of appropriate ethics committees. All participants in each study provided written informed consent.

Whole exome sequencing

Baseline formalin-fixed, paraffin-embedded (FFPE) tumor samples from patients with complete response and patients with rapid progression were analyzed by whole exome sequencing (WES). Patient samples where *BRAF* mutations were not detected were excluded from the analysis.

DNA exomes were captured using the Agilent SureSelect 51 Mb Kit (SQ756; Agilent Technologies, Inc., Santa Clara, CA, USA) and sequenced to a target depth of 100×. Reads were aligned to human reference genome GRCh38 using genomic short-read nucleotide alignment program (20). Variants were called using LoFreq (21). For variant filtering, sequencing data pooled from normal tissue samples from 106 patients in the BRIM-2, BRIM-3, BRIM-7, and coBRIM studies and data from the Exome Aggregation Consortium (ExAC) database (22) were used as reference data in the variant calling algorithm. Copy-number alterations were identified using CODEX (23). Variants and copy-number alterations were annotated using the Ensembl Variant Effect Predictor tool (24).

RNA sequencing

A subset of baseline tumor samples from patients with CR and patients with rapid progression were evaluated by RNA-Seq to identify transcriptional signatures. mRNA

libraries were prepared using TruSeq RNA Access (Illumina, San Diego, CA, USA). Paired-end 2×100 base reads were generated on a HiSeq system (Illumina). Reads were aligned to human reference genome GRCh38 using a genomic short-read nucleotide alignment program, and reads that overlapped gene exonic regions were counted (20). Differentially expressed pathways were identified by gene set enrichment analysis using the Molecular Signatures Database v5.1 (25,26). For transcriptional signature analyses, counts were normalized to library size as counts per million using the voom function of the R limma package (27,28) and computed by taking the mean z-score of all component genes. Using RNA-Seq data, in-silico cell type enrichment analysis was performed using xCell to calculate the percentage of tumor cells, as well as stromal and infiltrating immune cells (29).

Statistical analysis

Frequencies of mutations and copy-number alterations identified by WES were compared between biopsies from patients with CR and patients with rapid progression by Fisher's exact test (two-sided). Differential expression analysis was performed using the R limma package (27). Differential gene expression was defined as a raw *P* value ≤ 0.05 and at least a twofold change (increase or decrease) in expression between biopsies from patients with CR and patients with rapid progression. Transcriptional signatures and mutational load were compared by analysis of variance (two-sided analysis of variance). Associations of gene expression with PFS or OS were assessed by Cox proportional hazards modeling.

Results

Differences in baseline genetic alterations

A total of 130 baseline tumor samples underwent WES, with a median of 46.8 million reads (interquartile range, 40.7–50.7 million). Exome sequencing data were excluded because a *BRAF* mutation could not be identified ($n = 17$) or because of a high fraction of unmapped reads ($n = 4$). Thus, baseline tumors from 48 patients with CR and 61 patients with rapid progression were characterized by WES. A list of tumors, clinical features, treatment, response, and sequencing characteristics is provided in Supplementary File 1. Raw sequencing data are provided in Supplementary File 2, and transcript data of differentially expressed genes are provided in Supplementary File 3.

Large heterogeneity in mutation profiles was observed across the two subgroups (Fig. 1). The specific genes altered in tumor biopsies from patients with a complete response to therapy were different from those that were altered in tumor biopsies from patients with rapid progression; however, the frequency of alterations in any one gene was too low to warrant characterization as a common driver in these responses.

A preliminary analysis of genetic features distinguishing between biopsies from patients with CR and patients with rapid progression showed higher rates of *MITF* amplification (15% vs. 4%) and *TP53* mutations (15% vs. 4%) in biopsies from patients with CR than in those from patients with rapid progression. However, there were no significant differences in expression levels of *MITF/AXL* between biopsies from patients with CR and patients with rapid progression (Supplementary Fig. S1). Conversely, *NFI* alterations were more common

in biopsies from patients with CR than in those from patients with rapid progression (13% vs. 3%, $P=0.13$). No significant difference was observed between biopsies from patients with CR and patients with rapid progression in the frequency of *BRAF* amplifications (10% vs. 10%) or *CDKN2A* alterations (44% vs. 52%). Tumor mutational load, as assessed by exome-wide nonsynonymous single nucleotide polymorphisms, was similar between patients with CR and those with rapid progression (Fig. 2).

Association of response with signatures of preexisting tumor immunity

Since genomic differences could not fully account for the complete response vs. rapid progression, we investigated transcriptomic variation in these pretreatment tumor biopsies. Tumors from 32 patients with CR and 40 patients with rapid progression were evaluated by RNA sequencing (RNA-Seq).

The initial analysis identified 669 genes that were differentially expressed between tumors from patients with CR and patients with rapid progression (Fig. 3A). Among these differentially expressed genes, 370 were also associated with PFS, 59 were also associated with OS, and 44 were associated with both PFS and OS (Fig. 3B). Gene ontology analysis indicated that enriched expression of genes related to immune response processes was associated with CR, while enriched expression of genes related to keratinization was associated with rapid progression (Fig. 3C). The immune-related expression profile included gene signatures of CD8⁺ effector T cells, cytolytic T cells, antigen presentation cells, and natural killer cells, all of which were significantly enriched in tumors from patients with CR (Fig. 3D). Differential gene expression between patients with CR and rapid progression was similar when examined according to treatment with vemurafenib alone or cobimetinib combined with vemurafenib (Supplementary Fig. S2).

Consistent with the observation that immune-related genes are associated with CR, xCell analyses revealed higher levels of immune cell types such as CD8⁺ T cells in tumors from patients with CR compared with those with rapid progression (Supplementary File 3). Immunohistochemistry (IHC) data from a subset of patients ($n=37$) also showed a trend towards higher levels of CD8⁺ T cells in the tumor center in patients with CR than in those with rapid progression ($P=0.051$; Supplementary Fig. S3). There was good overall concordance between CD8⁺ T cells in the tumor center by IHC and CD8⁺ T cell levels inferred from RNA-Seq using xCell (Pearson's $r=0.88$, $P=8.1 \times 10^{-6}$; Supplementary Fig. S4).

Association of resistance with “keratin” subtype

There was no significant difference in innate anti-PD-1-resistance signatures (IPRES) between tumors from patients with CR and patients with rapid progression (Fig. 4A). However, keratin and kallikrein gene expression was higher in tumors from patients with rapid progression than in those with CR (Fig. 4B). These results suggest that features for innate resistance for targeted and immune therapies were distinct.

Discussion

Previous studies of melanoma disease progression have implicated tumor genomic heterogeneity in the development of resistance, mainly involving MAPK pathway reactivation³. Our exploratory analysis of pretreatment melanoma biopsies showed a wide heterogeneity of genomic alterations in the tumor biopsies of patients who had CR and those who progressed rapidly when treated with cobimetinib combined with vemurafenib or vemurafenib alone.

The observation of greater *MITF* amplification in tumors from patients who experienced rapid progression is consistent with previous evidence that high *MITF* expression is associated with a proliferative (rapidly progressive) phenotype in melanoma (30,31) and can contribute to resistance to MAPK pathway inhibition (32). However, in this analysis, there were no significant differences in baseline expression levels of *MITF/AXL* between tumors from patients with CR and patients with rapid progressions. *TP53* alterations were more common in tumors from patients with rapid progression than in those with CR in the current analysis, in contrast with previous findings suggesting that *TP53* mutational status has no impact on overall response rate, PFS, and OS among patients with *BRAF*^{V600}-mutated metastatic melanoma treated with a first-line BRAF inhibitor (33). *NFI* alterations were more common in tumors from patients with CR, consistent with evidence suggesting that melanomas lacking *NFI* expression are dependent on MAPK signaling and respond to MAPK inhibitors (17,34,35). Although *CDKN2A* mutations have been associated with worse PFS and OS outcomes in patients treated with trametinib combined with dabrafenib (36), we observed no significant difference in *CDKN2A* alterations between response groups in this analysis. However, the low frequency of occurrence of any one mutation within this study suggests that, while individual mutations may drive progression in individual patients, there are no mutations that are population-wide drivers of response. Mutational load was similar between patients with CR or rapid progression, although there was a trend towards improved survival observed in patients treated with cobimetinib combined with vemurafenib who had higher mutational load in the coBRIM trial. This is in line with previous findings that high mutational load is not associated with tumor response to treatment in melanoma but does correlate with improved patient survival (16,36), implying that factors beyond mutational load influence shorter-term tumor responses and longer-term patient survival.

Melanomas from patients with CR possessed higher preexisting tumor immunity features than those from patients who experienced rapid progression. Multiple lines of evidence point to the involvement of the BRAF/MEK pathway in the regulation of the host antitumor response in melanoma (37–41). Oncogenic BRAF signaling contributes to immune escape in melanoma (37,38), while BRAF inhibition has been shown to improve the host immune response to melanoma (39–41), and this immune response appears to be downregulated prior to the emergence of resistance (41). Taken together, the evidence suggests that presence of a preexisting immune response may be an important component of the clinical activity of BRAF/MEK inhibition. Given that pretreatment melanomas from patients with CR have greater tumor immunity features, the addition of anti-PD-1 therapy to cobimetinib combined with vemurafenib is currently under investigation.

The innate PD-1 resistance (IPRES) transcriptional signature describes pretreatment genomic features associated with response to anti-PD-1 therapy in metastatic melanoma; tumors nonresponsive to anti-PD-1 therapy were enriched for upregulation of expression of genes associated with mesenchymal transition, cell adhesion, extracellular matrix remodeling, angiogenesis, and wound healing (16). In the current analysis, expression of groups of genes identified as IPRES were not significantly different between tumors from patients with CR and from those with rapid progression. This suggests that there is not a complete overlap in tumor resistance to anti-PD-1 therapy and tumor response to BRAF/MEK inhibition.

Melanomas from patients with rapid progression may be overrepresented by the “keratin” molecular subtype. Melanomas expressing high levels of keratins and genes associated with epithelium have been associated with worse outcomes (17). The higher expression of keratin and kallikrein genes in tumors from patients who experienced rapid progression in this analysis is reminiscent of this “keratin” signature associated with poor prognosis.

In summary, we identified specific transcriptomic signatures distinguishing patients who have complete responses and patients who progress rapidly on treatment with the BRAF/MEK inhibitors cobimetinib and/or vemurafenib for *BRAF*^{V600}-mutated metastatic melanoma. Melanomas from patients with CR possessed higher preexisting tumor immunity features, while those from patients with PD may have the “keratin” signature associated with poor prognosis. These findings suggest that enriched immune infiltration might be a shared feature favoring response to both targeted and immune therapies, while features of innate resistance for targeted and immune therapies were distinct. These results provide a rationale for the combination of BRAF and MEK inhibition with immune checkpoint inhibitors in clinical studies, and may assist in optimization of treatment selection for patients with *BRAF*^{V600}-mutated metastatic melanoma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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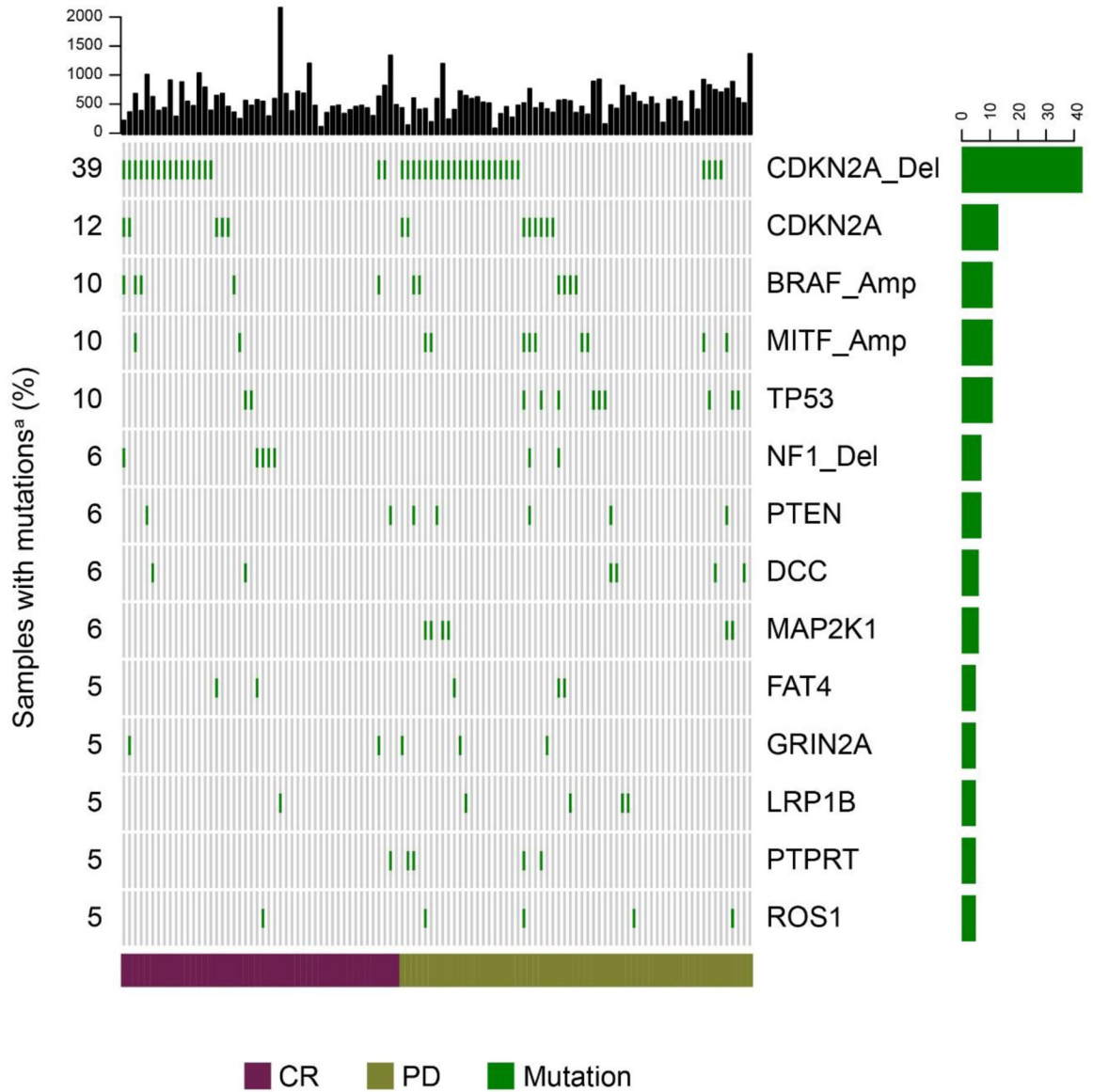


Figure 1. Most frequently mutated genes in tumor samples from patients with complete response ($n = 37$) or rapid progression ($n = 70$).

Whole exome sequencing results for the most frequently mutated genes are shown. CR, biopsies from patients with complete response; PD, biopsies from patients with rapid progression.

^a Mutations include single nucleotide variations, insertions, and deletions.

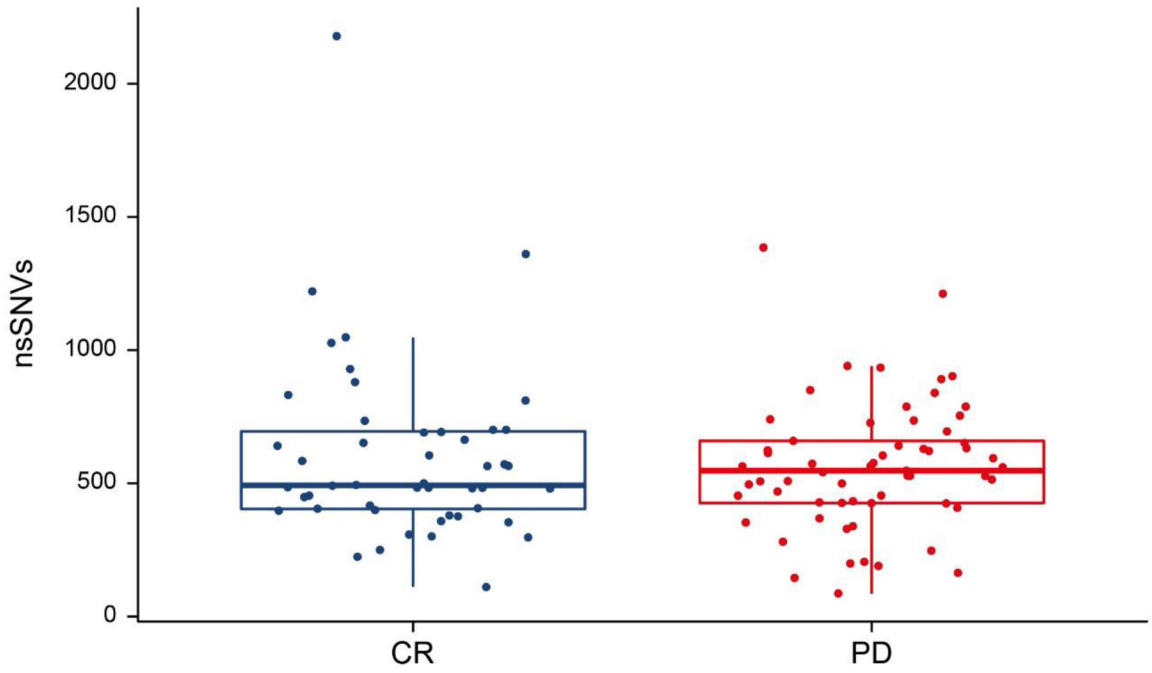


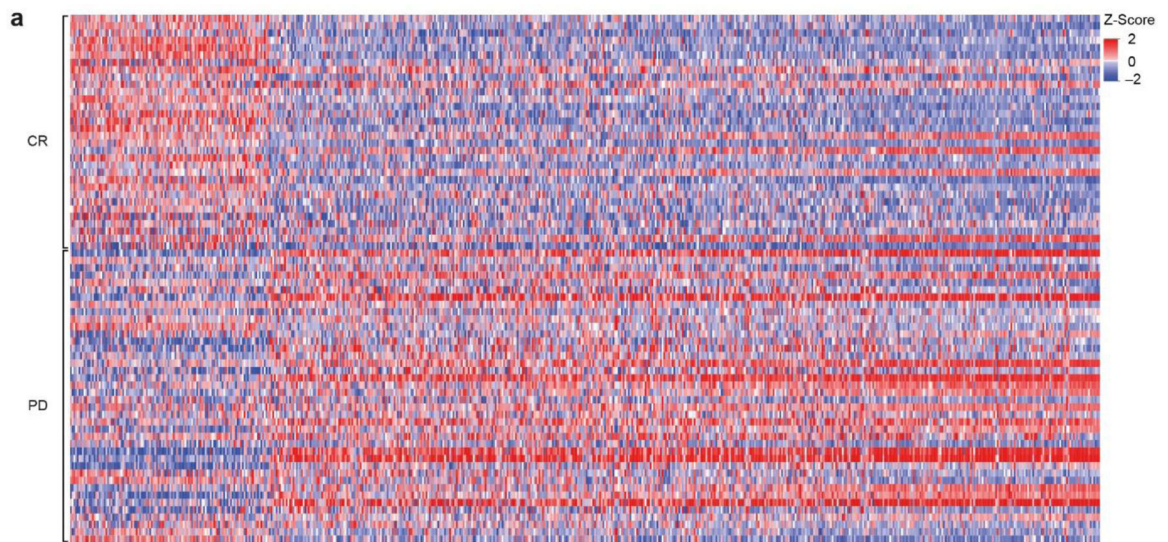
Figure 2. Mutational load in patients with complete response ($n = 37$) or rapid progression ($n = 70$). CR, biopsies from patients with complete response; PD, biopsies from patients with rapid progression.

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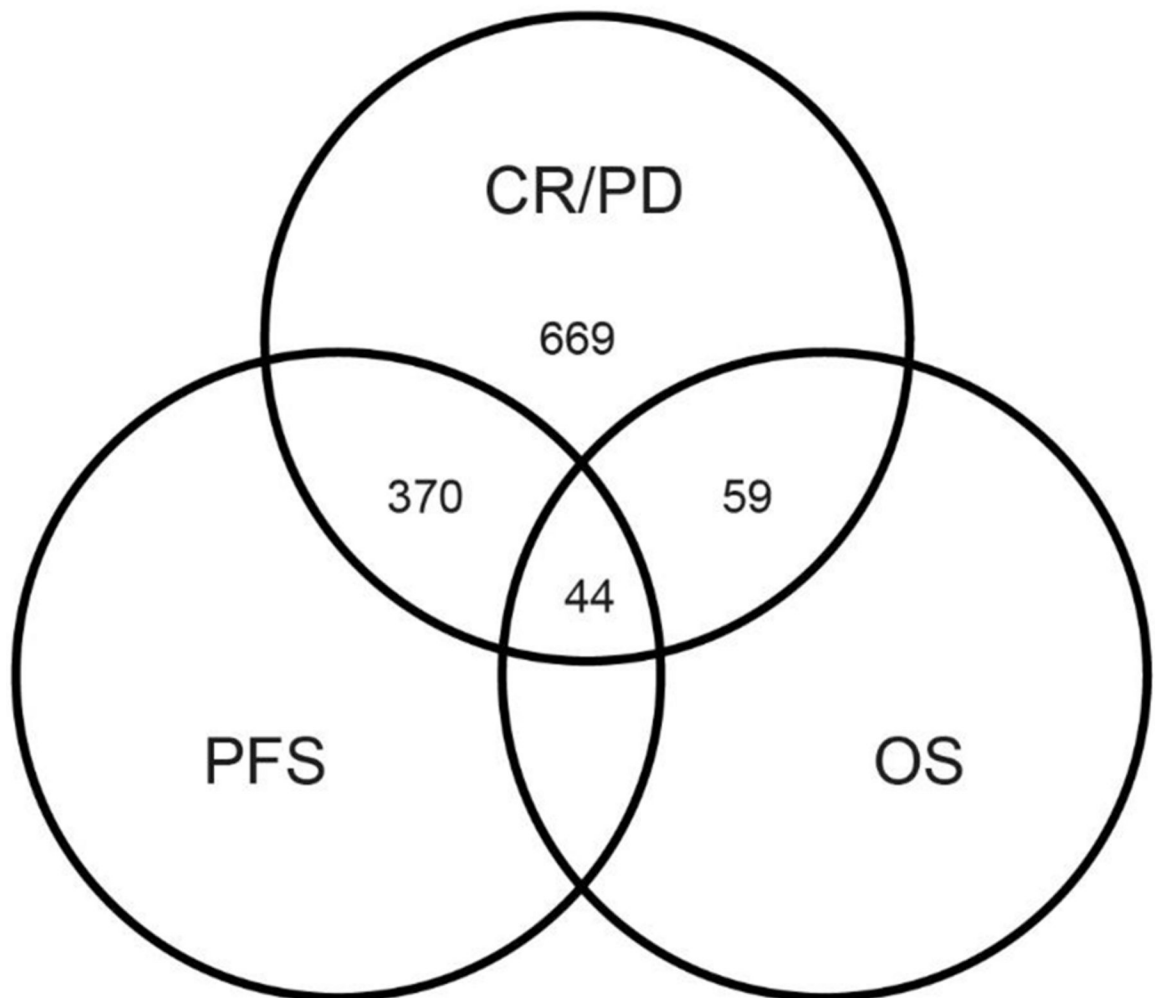
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b



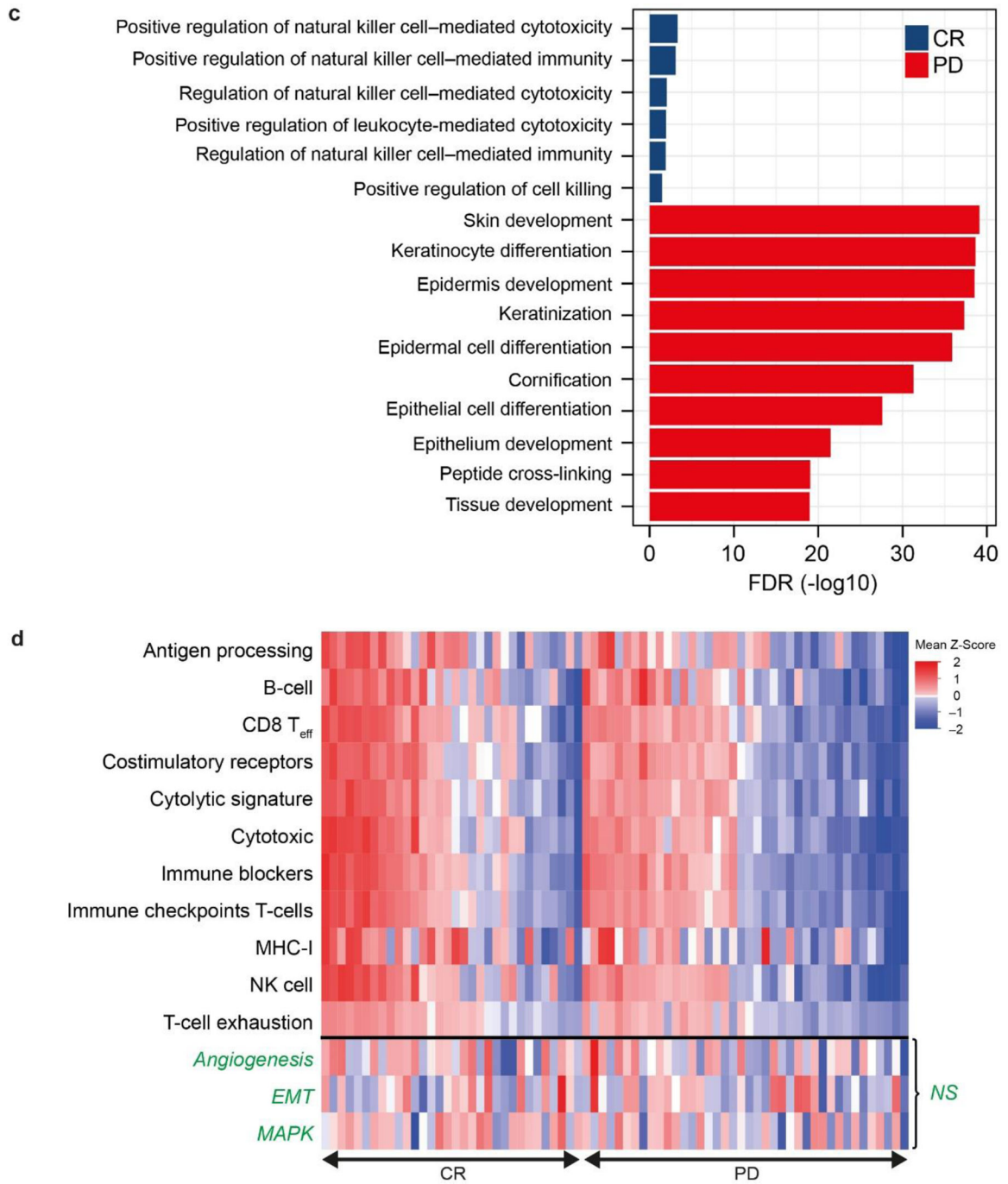


Figure 3. Differential gene expression by **A**, RNA sequencing in patients with complete response ($n = 32$) or rapid progression ($n = 40$), **B**, number of differentially expressed genes associated with response (complete response/progressive disease), progression-free survival, and/or overall survival, **C**, ontology analysis of differentially expressed genes, and **D**, immune-related gene signatures. Gene ontology analysis from <http://geneontology.org/>.³⁵ CR, biopsies from patients with complete response; FDR, false discovery rate; PD, biopsies from patients with rapid progression.

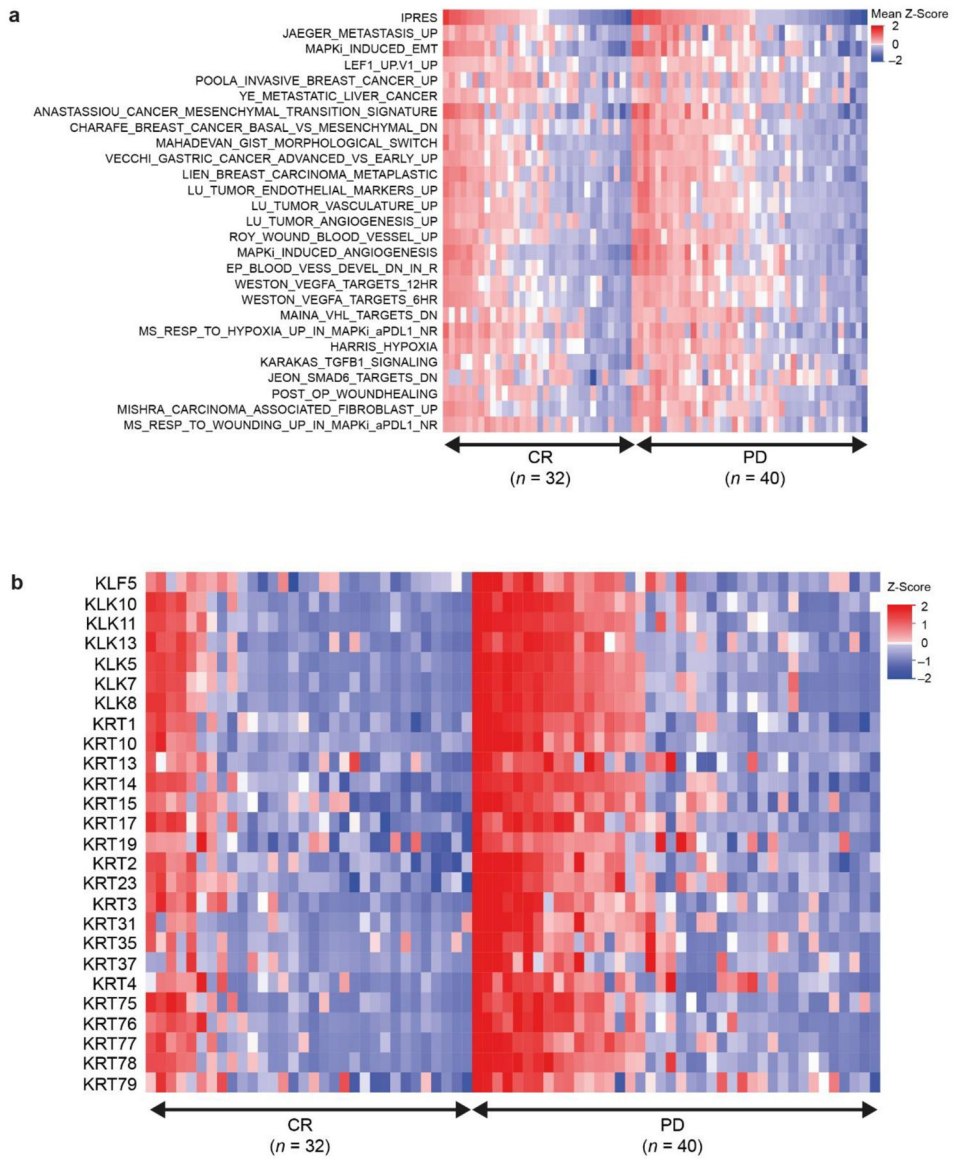


Figure 4. Expression of resistance gene signatures by RNA sequencing in tumors from patients with complete response ($n = 32$) or rapid progression ($n = 40$). Gene signatures constitute **A**, innate anti-PD-1-resistance signatures and **B**, keratin and kallikrein genes. CR, biopsies from patients with complete response; PD, biopsies from patients with rapid progression.