Sex-based mutational landscape confers sensitivity to checkpoint blockade immunotherapy in cutaneous melanoma

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Running title: Sex-based TMB predicts response to immunotherapies.

Keywords: melanoma; sex differences; biomarkers; immunotherapies; tumor mutation burden; BRCA2.

Abstract

Anti-programmed cell death protein 1 (PD-1) therapy provided superior benefits in skin cutaneous melanoma (SKCM), but only a minority of patients responded. As SKCM had sex-based immunological differences, we sought to explore the potential interactions between sex and clinical responses to anti-PD-1 therapy in SKCM. We found that sex had significant effects on anti-PD-1 therapy. Tumor mutation burden (TMB) and neoantigen burden significantly correlated with the clinical responses to anti-PD-1 therapy only in males. Meanwhile, we recruited The Cancer Genome Atlas (TCGA) database to explore sex-based differences of the tumor microenvironment (TME) in SKCM. We observed that males with high TMB, especially in conjunction with interferon-gamma (IFN- γ) signaling, significantly correlated with increased PD-L1 expression, major histocompatibility complex (MHC) class I gene expression, and the infiltration of cytotoxic T lymphocytes (CTLs). In addition, TMB and anti-PD-1 efficacy in SKCM each correlated with homologous recombination repair (HRR) gene BRCA2 mutations, but only in males. Taken together, these data revealed that in SKCM, high TMB correlated with prominent immunotherapeutic TME only in males, and that gender should be taken into account when predicting the efficacy of anti-PD-1 therapy for SKCM.

1 Introduction

2 Sex-based immunological differences have been reported across multiple cancer types,
3 with males getting higher risks of mortality from malignant cancers than females (1, 2).
4 Notably, melanoma displays one of the greatest sex-specific outcomes among many
5 cancers (2, 3). These sex-based differences of tumorigenesis in melanoma probably
6 reflect genetic, hormonal, environmental, and microbial effects on the immune system
7 throughout life in humans (1, 4-6).

8 Recent clinical trials have demonstrated promising performance on PD-1 immune
9 checkpoint blockade (ICB) therapies in melanoma, but only a fraction of patients
10 responded (7-11). Previous reports indicated that tumor mutation burden (TMB) and
11 neoantigen burden (12, 13), PD-L1 expression (12, 14), interferon-gamma (IFN- γ)-
12 related signaling (7, 15, 16), major histocompatibility complex (MHC) gene expression
13 (10, 17), the abundance of cytotoxic T lymphocytes (CTLs) infiltration (18, 19), and
14 mismatch repair (MMR) deficiency (14) could be predictive biomarkers for ICB
15 therapies across multiple cancer types (14, 20). Among these biomarkers, TMB
16 performs much better and is being developed as an immunotherapeutic biomarker for
17 the oncology clinic (14, 21, 22). High TMB could potentially produce more
18 immunogenic neoantigens on the surface of tumor cells, and T cells were then activated
19 to proliferate and destroy these tumor cells presenting the recognized neoantigens (14,
20 21, 22). However, the predictive power of TMB for ICB response is still imperfect and
21 needs further improvement (14, 21, 22).

22 Previous studies have reported that increased TMB was associated with clinical

1 efficacy to cytotoxic T lymphocyte antigen 4 (CTLA-4) blockade but not PD-1
2 blockade in melanoma (8, 13, 18), and male patients had higher TMB than females in
3 melanoma (23, 24). Consequently, we hypothesized that male factor integrating with
4 known biomarkers, especially TMB, could provide more accurate predictive power of
5 ICB response in skin cutaneous melanoma (SKCM), one of the commonest subtypes of
6 melanoma (25). Although a recent meta-analysis concluded that males could derive
7 greater benefits from ICB therapies than females in many cancer types (23), the
8 conclusion was controversial (26-28). In addition, their work also did not consider and
9 analyze the sex-based association of TMB with the efficacy of immunotherapies.

10 To test our hypothesis, we captured two well-studied melanoma
11 immunotherapeutic cohorts (8, 9) and one non-immunotherapeutic The Cancer Genome
12 Atlas (TCGA) dataset. It has already been indicated that many public tumor databases
13 without immunotherapies were still informative regarding tumor immune evasion (11,
14 19, 29). Therefore, we fully integrated these three cohorts to comprehensively analyze
15 interactions between sex-based biomarkers and the efficacy of immunotherapies.
16 Significantly, we uncovered that males with high TMB, in conjunction with other
17 biomarkers, could be a superiority group to anti-PD-1 therapy in SKCM.

18

19 **Results**

20 **Clinical and genomic characteristics of the study cohorts**

21 After extensive survey of cancer databases and literature, we were able to collect only
22 two melanoma PD-1 blockade cohorts with both clinical and genomic characteristics:

34 SKCM patients in Hugo et al study (8) and 44 SKCM samples in Riaz et al study (9). Pre-therapy biopsies from SKCM patients were determined by whole-exome sequencing (WES). Given the small number of samples in these cohorts, we integrated the data into one Cell-SKCM dataset according to their common features, such as patients' TMB, neoantigens, gender, clinical response to anti-PD-1 therapy, histology, and age (n=78; Fig. 1A and B; Table S1). In addition, we also recruited TCGA-SKCM datasets containing multidimensional non-immunotherapeutic characteristics to further explore the tumor immune escape in Cell-SKCM cohort (n=472; Fig. 1C; Table S1). Importantly, in Cell-SKCM, the quantity and range of TMB (i.e., the total number of somatic non-synonymous single nucleotide variations) were similar to that in the TCGA-SKCM dataset ($P=0.71$; Fig. S1A), suggesting the homogeneity of these two cohorts as previously described (12, 30).

Sex differences correlated with TMB and neoantigen burden in patients with differential response to anti-PD-1 therapy

Compared with females, TMB was markedly increased in males in the TCGA-SKCM cohort; a similar trend was observed for the Cell-SKCM cohort although the difference was not statistically significant possibly because of smaller sample size (Fig. S1B). Consistent with these, males but not females displaying good therapeutic responses (CR/PR: complete/partial response to anti-PD-1 therapy) showed significantly higher TMB compared with those who had no response (SD/PD: stable/progressive disease for anti-PD-1 therapy) in Cell-SKCM cohort (Fig. 2A). Receiver operator characteristic

(ROC) curves also validated high associations between TMB and clinical efficacy to PD-1 blockade in males (AUC=0.74, $P=0.005$; Fig. 2B). The discovery of the sex-based immunotherapeutic differences of TMB was consistent with neoantigen burden (AUC=0.73, $P=0.007$; Fig. 2D and E); neoantigens are generated as a consequence of TMB and can elicit effective immune responses (12, 31).

To further reveal the potential clinical usefulness of TMB and neoantigen burden for predicting PD-1 blockade response, we used the Youden index (7, 11, 12) to determine the optimum cutoff point. The cutoff points of both TMB and neoantigens were significantly lower in males than in females, suggesting that these two biomarkers were more sensitive to males for predicting PD-1 blockade efficacy (Fig. 2B and E). Additionally, univariate logistic regression analysis also demonstrated that TMB and neoantigens were significantly associated with clinical efficacy to anti-PD-1 therapy in male patients (Odds Ratio=7.96, $P=0.009$ and Odds Ratio=8.01, $P=0.01$, respectively) (Fig. 2C and F; Table S2). Using multivariate logistic regression analysis with adjustment for age, we also observed a significant association between male-based TMB and clinical efficacy to anti-PD-1 therapy (Odds Ratio=7.77, $P=0.01$; Table S2). Of note, given its significant correlation with TMB, neoantigens were not included in the multivariate regression analysis (Fig. S2A and B).

To assess the clinical utility of TMB in Cell-SKCM cohort, patients were classified into four TMB-defined groups based on Youden index-associated cutoff points (11): females with high TMB (F_High), ≥ 402.5 ; females with low TMB (F_Low), < 402.5 ; males with high TMB (M_High), ≥ 265.5 ; males with low TMB (M_Low), < 265.5 .

1 The same methodology applied to neoantigen-defined groups. This stratification
2 scheme revealed that the percentage of overall survival (OS) was significantly better in
3 males with high TMB and neoantigen burden ($P=0.03$ and $P=0.05$, respectively; Fig.
4 S3A-D).

6 **Correlations of sex differences with interferon- γ (IFN- γ) signaling and related** 7 **gene signatures in SKCM**

8 As Cell-SKCM cohort lacks transcriptomic profile, we turned to multidimensional
9 TCGA-SKCM datasets, which carry both genomic and matched transcriptomic profiles,
10 to further explore the sex-based tumor immune evasion. We stratified the TCGA-
11 SKCM samples into four TMB-defined groups as described above for the Cell-SKCM
12 cohort (Fig. S1C; Table S3), observing significant sex-based differences in neoantigens
13 in different groups, consistent with a tight correlation between TMB and neoantigens
14 (Fig. S2C and D).

15 Furthermore, males (but not females) with high TMB were significantly enriched
16 in genes associated with immune system and IFN- γ signaling (such as PD-L1, IFN- γ ,
17 GBP1, HLA-B, HLA-C, and CTLA4; Fig. 3A-C), a finding reinforced by unsupervised
18 hierarchical clustering of differential expression genes revealing that only males with
19 high TMB significantly increased expression of genes related to IFN- γ signatures (such
20 as IFN- γ , STAT1, CXCL9, CXCL10, CXCL11, and IDO1; Fig. 4A-C). Our finding
21 supports the notion that IFN- γ signaling and related gene signatures play important
22 roles in mediating tumor rejection for ICB therapies and can predict clinical response

1 to PD-1 blockade in melanoma (7, 10, 15, 16). Finally, a T cell-inflamed gene
2 expression profile (GEP) has been described for predicting PD-1 blockade response
3 across multiple cancers (7, 11). Indeed, T cell-inflamed GEP score tended to increase
4 in males with high TMB ($P=0.07$; Fig. S4A). The low statistical significance might be
5 attributed to the fact that T cell-inflamed GEP (as a pan-tumor biomarker) is not as
6 specific and effective as IFN- γ signatures in predicting PD-1 blockade efficacy in
7 SKCM (7).

8

9 **IFN- γ -induced immune microenvironment facilitates MHC-I gene expression in** 10 **males with high TMB**

11 IFN- γ -mediated signaling can upregulate or induce the expression of MHC-I molecules
12 on non-immune cells in tumor tissues, which can accelerate immune recognition and
13 removal of malignant cells (16). Additionally, the reduction in the MHC-I gene copy
14 number can facilitate immune evasion via defects in neoantigen presentation (32), and
15 patient MHC-I molecules can influence cancer response to ICB therapies (10, 17).
16 Consequently, we next examined MHC-I gene expression in the TCGA-SKCM dataset.

17 Unsupervised clustering of differentially expressed genes in TCGA-SKCM
18 samples showed that MHC-I genes (such as HLA-A, HLA-B, and HLA-C) were
19 significantly enriched in males but not females with high TMB (Fig. 4A), concordant
20 with the pathway analysis (Fig. 3A). MHC-I gene copy numbers were similar among
21 the four groups (Fig. 5A, C, and E), but MHC-I gene expression was highly upregulated
22 in males with high TMB (Fig. 5B, D, and F), presumably as a result of IFN- γ stimulation

1 which should facilitate antigen presentation and T cell recognition in the tumor
2 microenvironment.

3

4 **CTLs infiltration in IFN- γ -induced immune microenvironment**

5 The presence of cytotoxic T lymphocytes (CTLs) is an important biomarker for
6 predicting response to ICB therapies (18, 19). We therefore examined the crucial
7 markers of CTLs (GZMA, GZMB, CD8A, CD8B, and PRF1) as described (19), finding
8 that CTLs infiltration tended to increase in males with high TMB compared with low
9 TMB ($P=0.21$; Fig. S4B).

10 CTLs infiltration can be affected by its dysfunctional state and
11 immunosuppressive factors (19). Using Tumor Immune Dysfunction and Exclusion
12 (TIDE) algorithm (19), we found that males with high TMB displayed significantly
13 decreased exclusions of T cells ($P=0.0067$) but no T cell dysfunction, suggesting that
14 the infiltrating CTLs were functional and thus beneficial to these patients (Fig. 6A and
15 B). Consistent with this, these patients also showed decreased TIDE scores and
16 increased IFN- γ scores ($P=0.0069$ and $P=0.0059$, respectively; Fig. 6C and D), which
17 are known to correlate with the efficacy of anti-PD-1 therapy.

18

19 **BRCA2 mutation correlated with therapeutic response only in males**

20 Melanoma is one of the most typical mutation-caused malignant tumors and shows a
21 higher prevalence of C>T mutations through ultraviolet exposure and other lesions (33).

22 We found that both males and females with high TMB showed a higher proportion of

1 C>T mutations, suggesting no gender bias in ultraviolet-induced mutation signature
2 (Fig. S5A and B).

3 In contrast, Gene Set Enrichment Analysis (GSEA) showed prominent enrichment
4 of pathways associated with cell cycle and DNA replication only in males with high
5 TMB (Fig. 3A; Fig. 7A and B), which could potentially facilitate the accumulation of
6 DNA mutation and damage.

7 We further examined the DNA damage response (DDR) pathways essential for
8 genomic integrity (34), focusing on the genes whose mutations can induce a
9 hypermutational phenotype and durable clinical benefits from ICB therapies, such as
10 base excision repair (POLE), MMR (MSH2, MSH6, ARID1A, MLH1, and PMS2) and
11 HRR (BRCA2) (34, 35). We found that mutations in POLE and MMR-related genes
12 were increased in both females and males with high TMB (Fig. 7C and D). However,
13 BRCA2 mutations were specifically enriched in males with clinical benefits from anti-
14 PD-1 therapy ($P=0.009$) and with high TMB ($P=0.006$), indicating that males who had
15 high TMB and BRCA2 mutations were more sensitive to anti-PD-1 therapy in SKCM
16 (Fig. 7E and F). Furthermore, to assess the biologic and oncogenic effects of these
17 genetic alterations, we performed the analysis with MutationMapper (36), PolyPhen-2
18 (37), and SIFT (38). The result revealed that most of these genetic mutations (POLE,
19 MMR-related genes and BRCA2) were loss-of-function in nature (results of
20 MutationMapper, PolyPhen-2, and SIFT not shown).

21

22 **Correlations of estrogen and estrogen receptors (ERs) with tumor immune escape**

1 in SKCM

2 We have found that only in males was high TMB correlated with other known
3 biomarkers for predicting PD-1 blockade efficacy in SKCM (Fig. S6A and B). To begin
4 to explore the molecular basis of this gender-specific phenomenon, we focused on ER β ,
5 the predominant subtype of ERs in melanoma (39). ER β acts as a tumor suppressor (39)
6 partly via promoting IFN- γ secretion in the TME (1, 40), and its expression decreases
7 as melanoma progresses. We noted a marked decrease in ER β expression in females
8 with high TMB compared with males with high TMB (Fig. S7A and B), and there were
9 tighter associations between ER β and IFN- γ expression in females with high TMB than
10 males with high TMB ($r=0.24$, $r=-0.03$, respectively; Fig. S7C). Thus, in females with
11 high TMB, ER β and IFN- γ expression were compromised, which might help disrupt
12 the correlation of TMB and other biomarkers with the therapeutic efficacy, and this
13 disruption may be exacerbated after menopause, when the estrogen levels decline
14 rapidly (41). Of note, in the female SKCM patients, PD-L1 expression and IFN- γ score
15 also decreased with age, which could further disrupt the above-mentioned correlation
16 (Fig. S8A-D).

17

18 Discussion

19 Very little is known about sex-associated differences for ICB therapies. Although a
20 recent meta-analysis reported that males could derive greater value from ICB therapies
21 than females in multiple cancers (23), their conclusion is controversial (26-28). For
22 instance, Wallis and colleagues' meta-analysis collecting more clinical trials showed no

1 statistically significant differences between patient sex and immunotherapy efficacy in
2 many advanced cancers (26). Another recent study also reported no significant
3 association of sex with the efficacy of immunotherapies in metastatic renal cell
4 carcinoma (28). A caveat of these studies is that anti-CTLA-4 and anti-PD-1
5 immunotherapeutic cohorts were not separately analyzed in each cancer type, which
6 might have obscured the effects of sex (14, 42).

7 PD-1 blockade is effective to only a minority of melanoma patients, and the
8 mechanisms are unclear (8, 9, 43). Note that in the previous studies, the gender factor
9 has been ignored (8, 9, 22, 23, 26, 43). Importantly, for melanoma patients treated with
10 anti-PD-1 therapy, we showed that high TMB correlated with durable clinical benefits
11 only in males, providing a partial explanation for the heterogeneity in the patient
12 responses.

13 One limitation of our study lies in the small number of PD-1 blockade samples
14 from different clinical trials, but our datasets are much more homogeneous than
15 previous studies (8, 9, 22, 23, 26, 43) and thus more reliable for discovering the
16 determinants of therapeutic efficacy. In addition, we recruited multidimensional
17 TCGA-SKCM datasets to corroborate our analysis. Although a recent study based on a
18 melanoma immunotherapeutic cohort (22) also revealed sex-based TMB for predicting
19 survival (HR: hazard ratio; females: HR=0.6, $P=0.1$; males: HR=0.49, $P=0.005$), we
20 abandoned this cohort because it was based on targeted panel sequencing (MSK-
21 IMPACT) and multiple ICB therapies.

22 Taken together, our study was the first to explore sex-related differences of TME

1 in SKCM, revealing a significant positive link of males with high TMB to IFN- γ
2 signaling, MHC-I gene expression, CTLs infiltration, tumor immunogenicity, and
3 better anti-PD-1 response in SKCM.

4

5 **Abbreviations**

6 SKCM: Skin cutaneous melanoma; TMB: tumor mutation burden; TCGA: The Cancer
7 Genome Atlas; TME: tumor microenvironment; MHC: major histocompatibility
8 complex; CTLs: cytotoxic T lymphocytes; ICB: immune checkpoint blockade; WES:
9 whole-exome sequencing; CR/PR: complete or partial response to anti-PD-1 therapy;
10 SD/PD: stable disease or progressive disease to anti-PD-1 therapy.

11

12 **Conflict of interest**

13 The authors declare that they have no conflict of interest.

14

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Figure and table legends

Figure 1. Study design. (A and B) two PD-1 blockade immunotherapeutic cohorts

published on *Cell* were integrated into a single Cell-SKCM cohort. Pre-therapy biopsies from patients were assessed by WES before treatment with the PD-1 blockade therapy. We abandoned those samples that were on-therapy or non-SKCM. (C) One TCGA non-immunotherapeutic SKCM dataset with both WES and RNA-seq data available was defined as the TCGA-SKCM cohort. SKCM, skin cutaneous melanoma; WES, whole-exome sequencing; TCGA: the cancer genome atlas. ICB, immune checkpoint blockade.

Figure 2. Correlations of sex differences with TMB and neoantigens in patients with differential responses to anti-PD-1 therapy. (A and D) Sex-based differences of TMB and neoantigens in Cell-SKCM cohort with differential responses to anti-PD-1 therapy. (B and E) Sex-based differences of ROC curves for correlations of TMB and neoantigens with CR/PR to anti-PD-1 therapy. Youden Index–associated cutoffs for TMB and neoantigens in each group are shown. The area under the ROC curve (AUC); CI (confidence interval). (C and F) The logistic regression of TMB and neoantigens with clinical response to anti-PD-1 therapy. The S-shaped curves in males are clearly better fits for most of the data than in females. Probability, probability of getting clinical benefits from anti-PD-1 therapy. F_CR/PR (n=8), females with complete/partial response; F_SD/PD (n=21), females with stable/progressive disease; M_CR/PR (n=23), males with complete/partial response; M_SD/PD (n=24), males with stable/progressive disease; All_CR/PR (n=31), all samples with complete/partial response.

Figure 3. Correlations of sex differences with interferon- γ (IFN- γ) signaling in TCGA-SKCM dataset with differential TMB. (A) Pathway enrichment analysis of differential expression genes ($\log_2$ fold change ≤ 0.5 and P value ≤ 0.05) between males with high TMB and low TMB (pathways with q value ≤ 0.05 significant). (B and C) Quantitative analysis of mRNA expression of PD-L1 and IFN- γ . F_High (n=39); F_Low (n=141); M_High (n=169); M_Low (n=123).

Figure 4. Correlations of sex differences with IFN- γ -related gene signatures in TCGA-SKCM with differential TMB. (A) Hierarchical clustering of differentially expressed genes between males with high TMB and low TMB (differentially expressed genes with P value ≤ 0.05). (B and C) Quantitative analysis of two IFN- γ signatures associated with clinical response to anti-PD-1 therapy among four groups. F_High (n=39); F_Low (n=141); M_High (n=169); M_Low (n=123).

Figure 5. Correlations of sex differences with MHC-I gene expression in TCGA-SKCM with differential TMB. (A, C, and E) Quantitative analysis of MHC class I gene copy number variations (CNVs) among four groups. (B, D, and F) Quantitative analysis of MHC class I gene mRNA expression among four groups. F_High (n=39); F_Low (n=141); M_High (n=169); M_Low (n=123).

Figure 6. Correlations of sex differences with the infiltration of CTLs in TCGA-SKCM with differential TMB. (A and B) Relative quantification of T cell dysfunction

1 and exclusion among four groups. (C and D) Relative quantification of TIDE and IFN-
2 γ score among four groups. F_High (n=39); F_Low (n=141); M_High (n=169); M_Low
3 (n=123).

4

5 **Figure 7. Correlations of sex differences with DDR pathways in TCGA-SKCM**

6 **with differential TMB.** (A and B) Males with high TMB upregulated genes involved
7 in cell cycle and DNA replication pathways. NES, normalized enrichment score. (C and
8 D) Mutation percentage of POLE and MMR-related genes among the four groups in
9 TCGA-SKCM. (E) Males who responded to anti-PD-1 therapy showed significantly
10 increased mutations of BRCA2 in Cell-SKCM cohort. (F) Males with high TMB
11 showed significantly increased mutations of BRCA2 in TCGA-SKCM cohort. F_High
12 (n=39); F_Low (n=141); M_High (n=169); M_Low (n=123).

13

14

Figure 1

A

Hugo et al, *Cell*,
SKCM with WES
(n=38, anti-PD-1)

On-therapy
(n=4)

Pre-therapy,
SKCM with WES
(n=34, anti-PD-1)

Cell-SKCM,
Pre-therapy, SKCM with WES
(n=78, anti-PD-1)

B

Riaz et al, *Cell*,
melanoma with WES
(n=68, anti-PD-1)

Non-SKCM
(n=24)

Pre-therapy,
SKCM with WES
(n=44, anti-PD-1)

C

TCGA, SKCM
with WES and RNA-seq
(n=472, without ICB)

TCGA-SKCM,
SKCM with WES and RNA-seq
(n=472, without ICB)

Figure 2

Cell-SKCM

Figure 3

Figure 4

B **IFN-γ Signature (6-gene)**

C **IFN-γ Signature (10-gene)**

Figure 5

Figure 6

Figure 7

Table S1. Summary of clinical and genomic characteristics in Cell-SKCM and TCGA-SKCM cohorts.

	No. (%)	
Clinical cohorts	Cell-SKCM	TCGA-SKCM
No. of patients	78	472
Immunotherapy	Yes	No
Median age, years(range)	58(27-84)	58(15-90)
Gender		
Male	48(62)	292(62)
Female	30(38)	180(38)
Histology		
Cutaneous Melanoma	78(100)	472(100)
Treatment	anti-PD-1	No
Best overall response(BOR)		
Complete/partial response(CR/PR)	31(40)	
Stable/progressive disease(SD/PD)	45(58)	
Not evaluable	2(2)	
Mutation profiles	WES	WES
RNA-seq profiles	No	Yes

Table S2. Table for univariate and multivariate logistic regression of TMB, neoantigen burden, and age on BOR in Cell-SKCM cohort.

BOR (as a binary variable) **Female**

Model	Covariates	Odds Ratio	P value
Univariate	TMB	1.21	0.8
	Neoantigen burden	1.03	1
	Age	1.01	0.8
Multivariate	TMB	1.23	0.8
	Age	1.01	0.8

BOR (as a binary variable) **Male**

Model	Covariates	Odds Ratio	P value
Univariate	TMB	7.96	0.009**
	Neoantigen burden	8.01	0.01*
	Age	1.02	0.4
Multivariate	TMB	7.77	0.01*
	Age	1.01	0.85

Figure S1

Figure S2

Figure S3

Figure S4

Figure S5

Figure S6

Figure S7

Figure S8

TCGA-SKCM (Female)

TCGA-SKCM (Female)