Identifying Novel Targets for Enhancing Anti-PD1 Immunotherapy in Melanoma

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Abstract. Checkpoint inhibitors targeting PD-1/PD-L1 have become prominent treatments for advanced melanoma. However, these inhibitors are effective in less than half of patients, highlighting the need for new pathways and strategies to improve response rates. Analysis of RNA sequencing data from melanoma patients receiving anti-PD1/PD-L1 therapy revealed an enrichment of FGFR signaling in nonresponders, which was associated with a poorer prognosis. Additionally, it was found that FGFR signaling is negatively correlated with B cell signaling in melanoma tumors. Conversely, B cell receptor signaling is enriched in responders' tumors and associated with a better prognosis. This suggests that inhibiting FGFR or increasing the activation of B cells may be novel therapeutic targets for melanoma patients and aid in checkpoint inhibitor therapy. Further validation is necessary to assess the impacts on anti-PD1/PD-L1 therapy response. Overall, characterizing distinguishable pathways between responders and nonresponders may lead to more personalized immunotherapy selections and improved clinical outcomes for advanced melanoma.

Keywords: Melanoma, Anti-PD1/PD-L1 treatment, FGFR signals, B cell infiltration.

1. Introduction

Melanoma, the third most common skin cancer in the United States, has an average of 92,000 new cases each year [1]. Among our skin cells, melanocytes are responsible for the production of melanin, which typically happens during the wound-healing process. Melanoma can appear in many different ways, and it is often characterized by new irregular spots on the skin. Melanoma was considered the most dangerous skin cancer as the cancerous cells undergo rapid metastasis. Common causes include excessive ultraviolet light exposure and family gene inheritance such as the gene BRAF V600e. Although early identified localized melanoma is easy to treat through surgery and a survival rate over 99% is observed, only 32% of the patients are able to survive after five years with metastasis melanoma.

Currently, surgical removal of primary melanoma lesions is effective for localized melanoma. However, such treatment is ineffective for metastasis melanoma as it can spread to regional lymph nodes and migrate to distant sites across the body. The traditional method of palliative chemotherapy leads to a poor 5-year survival rate and is now replaced by the checkpoint inhibitors such as anti-PD-1/PD-L1 treatment. PD-1 is a member of the immunoglobulin family, and it delivers inhibition signals when it is engaged with the ligand PD-L1. PD-1 is commonly expressed in T cells, B cells, and natural killer cells, whereas its ligand can be found in T cells, B cells, APCs, and tumor microenvironments.

Checkpoint inhibitors promote the immune-mediated elimination of tumor cells through the inhibition of their respective ligand binding to checkpoint molecules. Treatment of nivolumab(anti-PD1) in stage III and IV melanoma patients has shown prominent results, as it reaches a median of 36.9 overall months of survival in nivolumab alone treatment. However, treatment of anti-PD1/PD-L1 was only effective in less than half of the patients even among patients that are strongly positive for PD-1 [2]. As a limited number of biomarkers are identified, further discoveries and experiments are needed to predict the response of the anti-PD1 treatment in patients. In this experiment, I tried to identify potential signaling pathways using silico analysis and predict their effects on the prognosis of patients who received anti-PD1 treatment.

2. Materials and Methods

2.1. Patient Information and Data Collection

Data of melanoma patients receiving anti-PD1/PD-L1 therapy was obtained from five publicly published studies [3-7]. The single-cell analysis data was obtained from the study. A total of 35 samples were used for analysis. The expression of FGFR 1 and B cell markers in the tumor tissue is manually extracted from the Bulk RNA sequencing profile. The correlation of FGFR 1 expression and tumor infiltrating B cells in melanoma is analyzed through timer, which provides comprehensive analysis of tumor infiltrating immune cells [8].

2.2. Gene Sets Enrichment Analysis

GSEA is an effective analytical tool that interprets the gene expression data we obtained (https://www.genepattern.org/modules/docs/GSEA/14#gsc.tab=0). GSEA derives an enrichment result by comparing groups of genes that share common biological functions or pathways. By comparing the level of gene expression in responder patients and nonresponder patients, GSEA is able to identify genes that demonstrate great similarity and differences in both the responder group and the nonresponder group [9]. In our experiment, the expression datasets are the five publicly published studies that used anti-pd1 as the treatment in melanoma patients, and the phenotype labels are in accordance with the phenotype of each patient in each dataset. Reactome was used as the gene sets database of the research, as it provided numerous cells signaling pathways and served as a solid foundation for our pathway-based data analysis. Bulk RNA analysis provides one unique advantage over single-cell RNA-seq analysis as the process of collecting single-cell data, such as fluorescence-activated cell sorting, is often limited by the requirement of advanced equipment, skilled operators, and a higher research cost [10].

2.3. Survival Analysis Based on FGFR 1 Expression and B Cell Marker

Gene concentration analysis of FGFR 1 and B cell markers (CD19, CD22) in nonresponding patients and responding patients was conducted using two methods. One, the bottom 25% and top 25% concentration of FGFR 1 and B cell markers in patients were separated into low concentration group and high concentration group. Within each concentration group, the number of patients who were identified as nonresponder or responder were recorded using a segmented bar chart. FPKM (Fragments per kilobase of transcript per million mapped reads) analysis was used in evaluating different expressions of FGFR and B cell markers in nonresponding patients and responding patients [11].

3. Results

3.1. FGFR signals are enriched in nonresponders' tumors and are associated with poor prognosis

To identify factors that contributed to immune surveillance of anti-PD1 therapy in melanoma patients, we compared the transcriptome data of the nonresponding patients and the responding patients using GSEA analysis. I then collected top altered pathway between the nonresponder and responder that falls under the p-value <0.1 and has a high enrichment score. During the analysis stage, GSEA would first rank the expression level of each gene in respective of the two phenotypes. The enrichment score was then obtained based on the most represented gene in the entire gene list, which was often accompanied by a large set of genes that overexpression in one phenotype but not the other. A permutation test was then used to obtain the p-value of the finding and its statistical significance.

According to the enrichment results we obtained and organized, 23 genes were enriched in 3 of the datasets, 18 genes were enriched in responder, and 5 genes were enriched in nonresponder. One gene found to enrich in four datasets was FGFRs signals. As shown in **Figure 1**, multiple genes of

FGFRs signal were found to overrepresent in nonresponders compare to responders. Evidenced by FGFRs signals' prominent enrichment result across nonresponding patients, we then performed a survival analysis to further verify our GSEA analysis results. Using the 25% grouping method, the FGFR1 low-concentration group was found to have 15 nonresponders and 17 responders, whereas the high-concentration group was found to have 21 nonresponders and 11 responders. A significant difference is identified between the FGFRs high concentration group and low concentration group in responding to the anti-pd1 treatment among melanoma patients. According to **Figure 1**, FPKM analysis demonstrated a significantly higher concentration of FGFRs signals across all nonresponding patients compare to all responding patients.

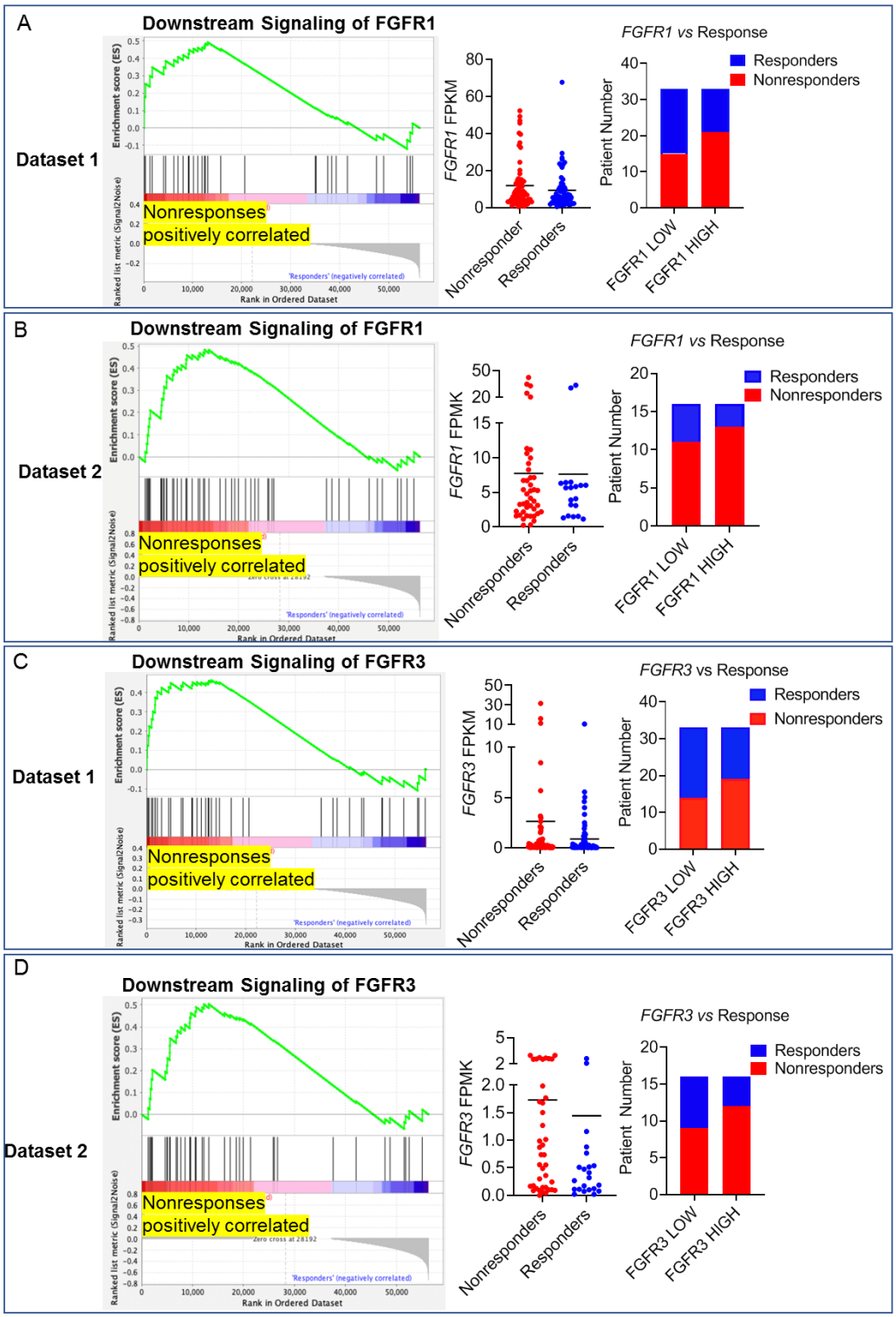

Figure 1. FGFRs signals are enriched in nonresponses.

3.2. B Cell signals are enriched in responder's tumors and associated with better prognosis

Adopting the same filtering condition under p<0.1, we were able to identify the B cell receptor signal pathway to enrich in responding patients (**Figure 2**). According to the enrichment score result, multiple genes were found to express at a high level in responding patients compare to nonresponding patients. To assess the role of the B cell signaling pathway in patients' response to the anti-PD-1/PD-L1 treatment, several distinguishable markers present in the B cell signaling pathway were identified (CD19, CD20, CD22). CD19, CD20, and CD22 all encode transmembrane proteins that are essential for the function of B cells, and their unique presence in B cells enables us to draw a correlation between the markers' concentration level and the role of the B cell signaling pathway (**Figure 2**). From the result we derived from the FPKM analysis, there is a significant difference between the concentration of B cell markers (CD19, CD20, CD22) in responding patients and the non-responding patients. In addition, 19 patients responded to the treatment at a high expression level of CD19, while only 15 patients responded to the treatment at a low expression level.

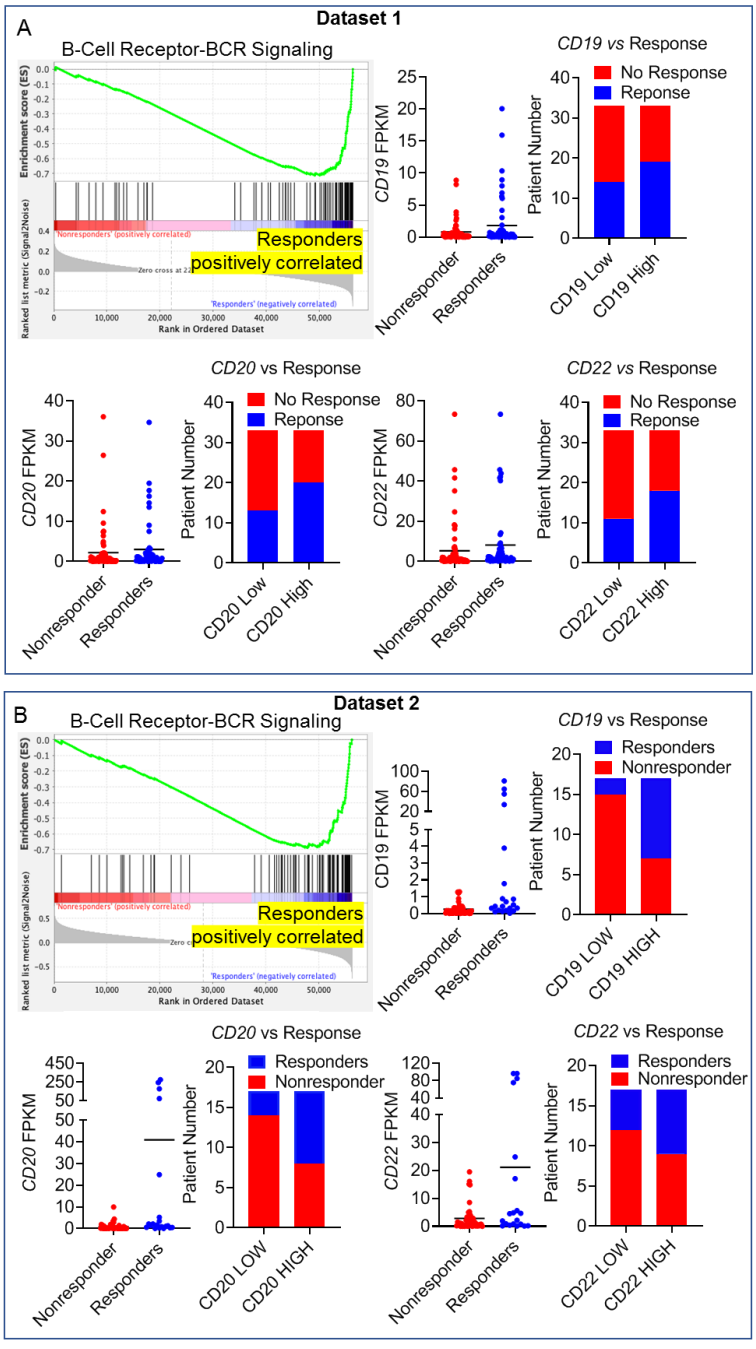

Figure 2. B Cell response signals are enriched in responses.

3.3. Tumor Infiltrated B Cell Proportion Correlated with Anti-PD-1 Response in Melanoma

In one paper, the researchers performed a single-cell RNA-seq analysis of the melanoma tissues that present the marker of CD45. CD45 is a marker that presents exclusively on the immune cells that comprise both the complement system and adaptive immune system. In the study that I collected data from, the researcher divided the concentration of immune cells into eleven groups and examined their concentration in a cohort of 35 melanoma patients. I analyzed the data of the 35 patients and divided them into groups of nonresponders and responders. Among 14 responding patients, a significant proportion of B cell concentration was identified. In three responding patients, half of their immune cells within melanoma tissue were constituted of B cells, and the average B cell concentration across all 14 patients reached over 22%. By contrast, the average B cell concentration found in nonresponding patients only constituted less than 2% of the entire immune cells **(Figure 3)**.

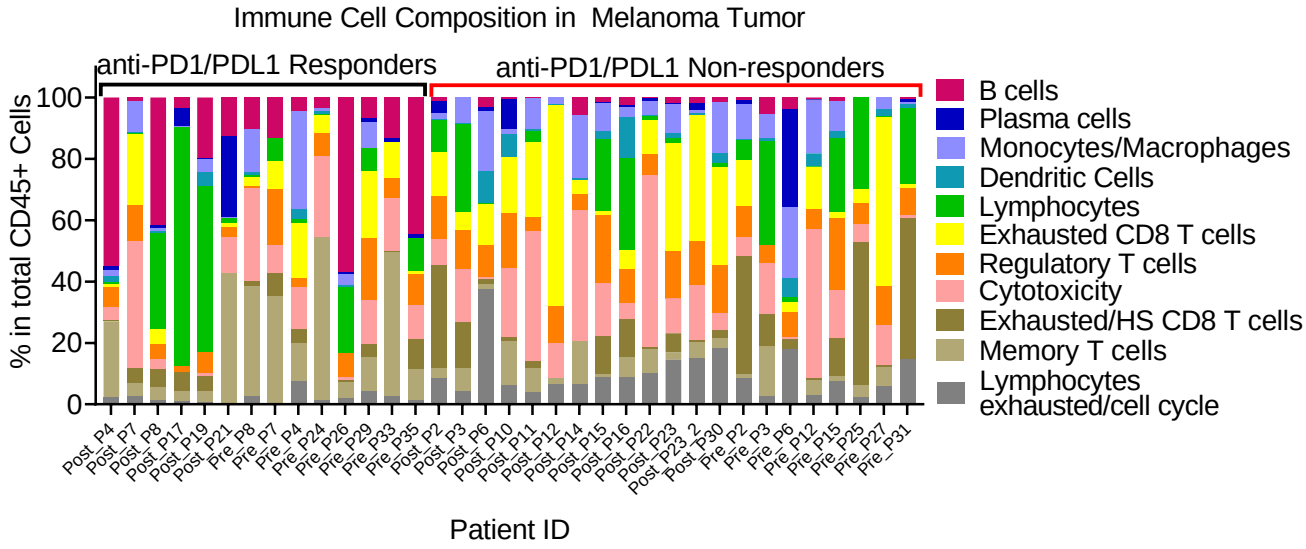

Figure 3. B cell proportion is high in immunotherapy responders

3.4. Tumor Cell FGFR Signals Are Negatively Correlated with B Cell Infiltration

At the single-cell level, the concentration of FGFR within tumor cells was examined. Across nine clusters of cells, the expression level of FGFR1 was significantly higher within the malignant melanoma cells. Using logarithm TPM analysis based on the X-cell dataset, the correlation between the FGFR expression level and B cells infiltration level was examined. At the significance of $p < 1.91\ e^{-2}$, the B cell infiltration level was found to be negatively correlated to the expression level of FGFR1 **(Figure 4)**.

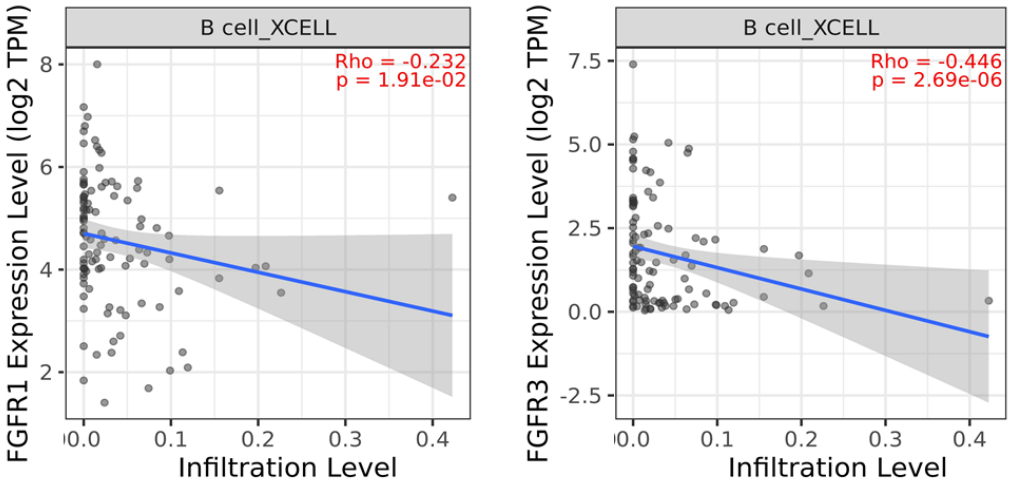

Figure 4. FGFR signals are negatively correlated with B cell infiltration.

4. Discussion

The fibroblast growth factor receptor (FGFR), a member of the RTK family, is a common transmembrane receptor present on the surface of human cells. The dimerization of the receptor induces various pathways that are implicated in the normal function of cellular proliferation, survival, and migration [12]. The FGFR signaling pathway is found to influence the activity of RAS, BRAF, and MEK-ERK pathways as well as cellular invasiveness through the interaction with the N-cadherin. N-cadherin, in the presence of FGF-2, increase migration, invasion, and metastasis of solid tumors [13], which constantly activates the RAF-MEK-ERK signaling cascade. The cascade constantly activates the cell cycle and promotes cell proliferation while inhibiting the apoptosis of the cells through a high density of regulatory T cells. While anti-PD1/PD-L1 treatment relies on the T cell immune function, abundant Treg cells within the tumor microenvironment might contribute significantly to the unresponsive rate of checkpoint inhibitor treatment.

The importance of FGFR1 in melanoma growth and proliferation was identified in several studies. In one study, inhibition of basic fibroblast growth factor (BFGF) and FGFR1 were performed and cloned in nude mice in vitro through antisense cDNA. The study concluded that melanoma tumors with these constructs were unable to continue intratumoral angiogenesis and were also arrested in the current stage [14] FGF2/FGFR1 might also contribute to melanoma metastasis through angiogenesis. FGF2 was identified to promote the expression of vascular endothelial growth factor (VEGF), and a high density of microvessels was discovered in an experiment in 2002 [15]. The increased expression level of FGFR1 may contribute to immune surveillance as well as melanoma cells' ability to react to the targeted therapy. FGFR1 helps melanoma cells to survive under treatment and enter the senescence stage. In one study, the secretion of FGFR was identified as resilient toward long-term BRAF inhibition and limited the apoptotic effects of the BRAF inhibitor [16]. As suggested by the paper, cancer-associated fibroblasts are stimulated to secrete hepatocytes growth factor (HGF), which further contribute to the resistance of melanoma cells to the drug. The release of HGF couples with c-MET signaling is crucial for the tissue regeneration and progress of melanoma. The presence of HGF ensures the survival of the melanocytes while promoting the expression of the melanocytes. While normal melanocytes that secrete melanin don't release HGF, cancerous melanoma cells are able to secrete both c-MET and HGF and thus are able to proliferate and metastasize in an autocrine manner [17]. FGFR's contribution to the resistance of the treatment such as anti-PD1 can be examined through the combination treatment of both the FGFR inhibitor and the checkpoint inhibitor. As evidenced in the experiment, resilience to the treatment of BRAF inhibition was broken after the treatment was combined with the FGFR inhibitor, suggesting the lateral application of combined therapy [16].

My research finding has confirmed the result of previous researchers and identified FGFR1 as a crucial target that can be inhibited to promote the efficacy of current checkpoint inhibitor therapy. In addition, current clinical trials demonstrated prominent progress in the development of FGFR inhibitors, including small molecules drugs like Brivanib Alaniate and Dovitnib. Both of the drugs demonstrated excellent inhibition effects on the amplification of FGFR1 and phosphorylation effects of the downstream signaling molecules. In one study that uses Brivanib Alaniate in a breast cancer cell line, Brivanib Alaniate was found to have significant impacts on the bFGF growth stimulation as well as decreased receptor autophosphorylation and reduced phosphorylation of the ERK pathway [18]. In another clinical trial, Dovitnib showed strong inhibition effects in FGFR-amplified breast cancer patients, where quantitative PCR demonstrated a 21.1% reduction in target lesions [19]. Those established FGFR inhibitors can be readily assessed in melanoma patients, and perhaps improve the patients' long-term survival rate and response to checkpoint inhibitors while coupling with the target therapy and BRAF inhibitor.

While B cells serve an important role in the adaptive immune system, however, B cell targeting have been long neglected as most studies focused on the activation of CD8 T cells to kill the cancerous cells. Treatments such as checkpoint inhibitors target PDL1/PD1 or binding between CTLA-4 and CD80 ligands to activate the killing mechanism of T cells. My study identified the B cell signaling

pathway as an essential target in the response and prognosis to the checkpoint inhibitor therapy, and a significantly higher expression level of B cell markers is identified by the FPKM analysis. This result is validated by earlier research that the majority of melanoma lesions are comprised of huge amounts of B cells and about 86% of primary melanomas contain at least 10 stromal B cells per mm2 tumor [20].

Multiple B cell markers such as CD20 and CD22 also demonstrated higher mRNA expression in melanoma lesion compared to normal skin and their expression continue to arise as metastasis continues [21]. Continuously increasing the level of B cells in metastasis melanomas implies the presence of humoral immune response in melanoma while suggesting B cell signaling pathway can be an effective target to improve the prognosis of the patients. One previous research that focused on the role of B cells and tertiary lymphoid structures in promoting immunotherapy response has identified pathways upregulated in responders [22]. Many of the pathways such as CXCR4 signaling, and chemokine signaling pathway increase immune activity while regulating hematopoietic stem and progenitor cell function in bone marrow. The Bulk DNA analysis also identified a higher frequency of memory B cells in the responders compared to nonresponders. And this finding was corroborated by a group of melanoma patients who were treated with adjuvant checkpoint inhibitors. In addition, tertiary lymphoid structures, developed in non-lymphoid tissues upon chronic exposure to inflammatory signals, may also explain the effect of humoral immune response in responders. Among the cohorts of melanoma patients they examined, the Helmink study found that a higher density of CD20+ B cells was present in TLS of responders compared to nonresponders and that the B cells were also co-localized with higher expression level of CD4, and CD8 T cells. That evidence supports the role of B cells in responding to patients and how it can be utilized to predict the prognosis of patients and give treatment accordingly. To promote anti-tumor B cell subsets in tumor rejection, one method is to inhibit immunosuppressive TME cytokines such as IL-10, VEGF, or TGF-ß to promote the production of Th2 T cells and the proliferation of B cells. Another way is to reduce the affinity of CPI antibodies to inhibitor FC receptors and increase their binding to activatory FC receptors [23].

5. Conclusion

In conclusion, we can observe that FGFR signaling is enriched in nonresponders' tumors and associated with a poor prognosis. On the other hand, B cell receptor signaling is enriched in responders' tumors and associated with a better prognosis. FGFR signaling is negatively correlated with B cell signaling in melanoma tumors. Therefore, inhibiting FGFR or increasing the activation of B cells may serve as novel therapeutic targets for melanoma patients and aid in checkpoint inhibitor therapy.

One limitation of my study is that the results are primarily based on silico analysis and need to be further confirmed by experimental studies. Some future directions we can explore include determining the effects of FGFR expression in cancer cells on anti-PD1/PDL1 therapy response and investigating the effects of B cell population/activation on anti-PD1/PDL1 therapy response.

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