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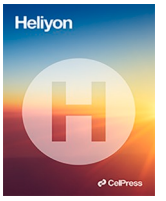

Research article

Integrative analysis of the immunological significances of guanylate binding protein family genes in microsatellite stability colorectal cancer

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A R T I C L E I N F O

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A B S T R A C T

Background: Microsatellite stability (MSS) colorectal cancer (CRC) has poor sensitivity to immunotherapy and its underlying mechanisms are still unclear. Guanylate binding proteins (GBPs) are a family of GTPase involving innate immune responses by providing defense against invading microbes and pathogens. However, the immunological significances of GBPs in MSS CRC remain unknown.

Methods: We utilized bioinformatic tools to comprehensively analysis the expression pattern, clinical relevance, prognostic value, biological function, and immunoregulation effect of distinct GBP members in MSS CRC.

Results: The expression of all seven GBPs in MSS samples are remarkably decreased compared to microsatellite instability-high (MSI-H) samples. Among them, GBP1/2/4/5 are obviously correlated with distant metastasis status. High expression of GBP1/4/5/6 was remarkably related to favorable overall survival (OS) and progression-free survival (PFS) in CRC patients with MSS tumor. Subsequent enrichment analysis revealed that Interferon-gamma (IFN-γ) and NOD-like receptor signaling are the most relevant functions. Besides, the expression patterns of GBPs are remarkably associated with several tumor infiltrated immune cells (e.g. regulatory T cells, $CD4^+$ T cells, and macrophages) and diverse immunoregulatory molecules (e.g. immune checkpoint biomarkers (ICBs) and major histocompatibility complex (MHC) molecules). Moreover, high GBP1/2/4/5 expression predicted better immunotherapy responsiveness in immunotherapy cohorts.

Conclusion: These findings might provide novel insights for the identification of therapeutic targets and potential prognostic biomarkers of GBP family in CRC with MSS samples.

1. Introduction

Colorectal cancer (CRC) is the most commonly diagnosed malignant tumor of the digestive system, which is responsible for more

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than 900,000 tumor-related deaths around the world in 2020 [1]. CRC is a highly heterogeneous tumor that can be classified into different subtypes according to diverse molecular characteristics, such as microsatellite instability/mismatch repair (MSI/MMR) status and consensus molecular subtypes (CMS) [2,3]. Among them, MSI/MMR is the most significant immune-related biomarker for CRC, patients with MSI-high/deficient MMR (MSI-H/dMMR) tumor possess increased new antigens production and greater immunogenicity compared to microsatellite stability/proficient MMR (MSS/pMMR) tumor, and the new antigens trigger T cell immune responses at the same time [3]. Tumor treatment has entered the era of immunotherapy, which focused on killing the tumor cells by activating host immunity and has achieved unprecedent efficiencies in various types of tumors, including CRC. In 2015, the KEYNOTE-016 clinical trial first reported that MSI-H/dMMR CRC patients could benefit from immune checkpoint inhibitors (ICIs), and can be used as an indicative biomarker for the efficacy of immunotherapy [4]. The Food and Drug Administration (FDA) then approved anti-PD-1 agents pembrolizumab and nivolumab as standard treatment strategy for CRC patients with MSI-H/dMMR tumor that was unresectable or metastatic [5,6]. Thus, the latest updated National Comprehensive Cancer Network (NCCN) and Chinese Society of Clinical Oncology (CSCO) guidelines propose MMR/MSI status as a global indicator for CRC classification. However, only a small proportion (15 %) of CRC patients are MSI/dMMR, and the remaining 85 % are MSS/pMMR, and questions remain concerning the role of immunotherapy in MSS/pMMR CRC [7]. Although the prevailing viewpoint is that MSS/pMMR CRC patients do not benefit from existing immunotherapy strategies, several studies also suggest that a small number of MSS/pMMR CRC patients could benefit from ICIs, and the combination of ICIs with other anti-tumor drugs is currently being evaluated in MSS/pMMR CRC [8,9]. These findings indicate that it may be possible to detect MSS/pMMR patients who are potentially effective in ICI therapy through the detection of predictive biomarkers, thereby expanding the population benefiting from immunotherapy. Thus, identifying effective biomarkers to improve the efficacy of immunotherapy in MSS/pMMR CRC is of great importance.

Guanylate binding proteins (GBPs) are a family of GTPases involving innate immune responses by providing defense against invading microbes and pathogens [10]. To date, a total of seven family members of GBPs have been identified in mammalian cells, and several of them play critical roles in inflammation processes. Besides, a few studies also reported that GBP family genes are involved in cancer progression, but the underlying mechanisms have yet to be clearly clarified. Considering the immunological roles of GBP genes in human disease, we obtained the transcriptome profile and clinical information of CRC from public platform and comprehensively analyzed the immunological significances of seven GBP family genes in MSS/pMMR CRC. Our findings provide valuable clues for further exploration of prognostic biomarkers and therapeutic targets in MSS/pMMR CRC in the future.

2. Materials and methods

2.1. Expression pattern

The transcriptome data of CRC were retrieved from TCGA GDC (https://portal.gdc.cancer.gov/) project, and the expression matrices of GBP family members was retrieved. Then, the clinicopathological parameters of CRC patients were obtained from the UCSC Xena (https://xenabrowser.net/) platform, and patients were classified into MSS and MSI-H clusters according to their microsatellite instability (MSI) status [11]. Subsequently, the expression patterns of distinct GBPs in patients with different MSI statuses were analyzed. Besides, we further determined the associations of GBP family members with clinicopathological features in MSS CRC.

2.2. Kaplan-Meier survival analysis

The follow-up data of overall survival (OS) and progression-free survival (PFS) of CRC patients were also retrieved from the UCSC Xena platform. Then, Kaplan-Meier survival analysis was conducted for investigating the capability of GBPs in forecasting the OS and PFS of MSS and MSI-H CRC samples.

2.3. Biological function analysis

To determine the underlying biological function of GBPs, we performed Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) and Reactome enrichment analysis by exploring the Database for Annotation, Visualization and Integrated Discovery (DAVID, https://david.ncifcrf.gov/home.jsp), a web-based functional annotation tool that allows researchers to investigate the biological meaning behind large numbers of genes [12,13]. Besides, the pathway diagram was retrieved from KEGG (https://www.kegg.jp/).

2.4. Tumor immune cell infiltration

CIBERSORT is a bioinformatic algorithm that enables deconvolute tumor-infiltrating immune cell (TIIC) type proportions from bulk RNA sequencing profiles [14]. In the present research, we utilized the CIBERSORT algorithm to deconvolute the proportions of 22 TIICs in each CRC sample and compared the differences of these TIICs between MSS and MSI-H subgroups. Then, the associations of GBP family members with TIICs' abundance in MSS CRC were analyzed.

2.5. Immunomodulatory molecules expression

Based on the transcriptome data retrieved from TCGA project, we extracted the expression matrices of diverse immunomodulatory

molecules, including immune checkpoint biomarker (ICB) and major histocompatibility complex (MHC) molecules. We first determined the expression patterns of these immunomodulatory molecules in MSS and MSI-H CRC samples. After that, the relationships between the GBP members and immunomodulatory molecules were assessed.

2.6. Differential expression analysis in immunotherapy cohort

IMvigor210 cohort contains transcriptome profiles and detailed clinical information of urothelial carcinoma patients treated with anti-PD-L1 agents [15]. PRJEB25780 is an immunotherapy cohort where patients with gastric cancer received anti-PD-1 therapy [16]. We downloaded the transcriptome data and clinical information of these two immunotherapy cohorts and classified patients into responder (including partial response (PR) and complete response (CR)) and non-responder (including progressive disease (PD) and stable disease (SD)) subgroups. Then, differential expression analysis of GBP members in IMvigor210 and PRJEB25780 cohorts was performed to determine whether the expression of GBP genes correlated with immunotherapy responsiveness.

2.7. Statistical analysis

Statistical analysis was carried out by using R (version 4.2.1, https://www.r-project.org/) and the corresponding feature packages. Among them, Differential analysis between subgroups was conducted by the "ggpubr" package and evaluated by the Wilcoxon test, survival analysis was conducted by the "survival" and "survminer" packages and estimated by the Log-rank test, correlation analysis was conducted by the "corrplot" package and assessed by the Spearman test, and pathway enrichment analysis was estimated by Fisher's test. A p-value <0.05 was considered to indicate a statistically significant.

3. Results

3.1. Expression patterns of GBPs in CRC with different microsatellite status

We first analyzed the expression patterns of GBP members in CRC to determine its correlation with the MSI status, the raw data of GBP genes in CRC was presented in Supplementary Table 1. As shown in Fig. 1A–G, all seven GBP members showed significantly decreased expression levels in MSS CRC samples compared to MSI-H samples. Besides, except for GBP3, the expression levels of GBPs in MSI-H samples were remarkably elevated compared to normal controls. These findings suggest that GBP family genes have totally different expression patterns between MSS and MSI-H status in CRC.

3.2. Correlation with distant metastasis

Considering that gene expression level may regulate cancer progression, we determined whether the expression levels of the GBP family genes were connected with distant metastasis of MSS CRC. As displayed in Fig. 2A–G, the expression of GBP1/2/4/5 was remarkably downregulated in patients with distant metastasis, whereas the differences in GBP3/6/7 expression did not reach a statistical significance. We then investigated the expression relationships among GBP family genes and found that these seven GBP genes

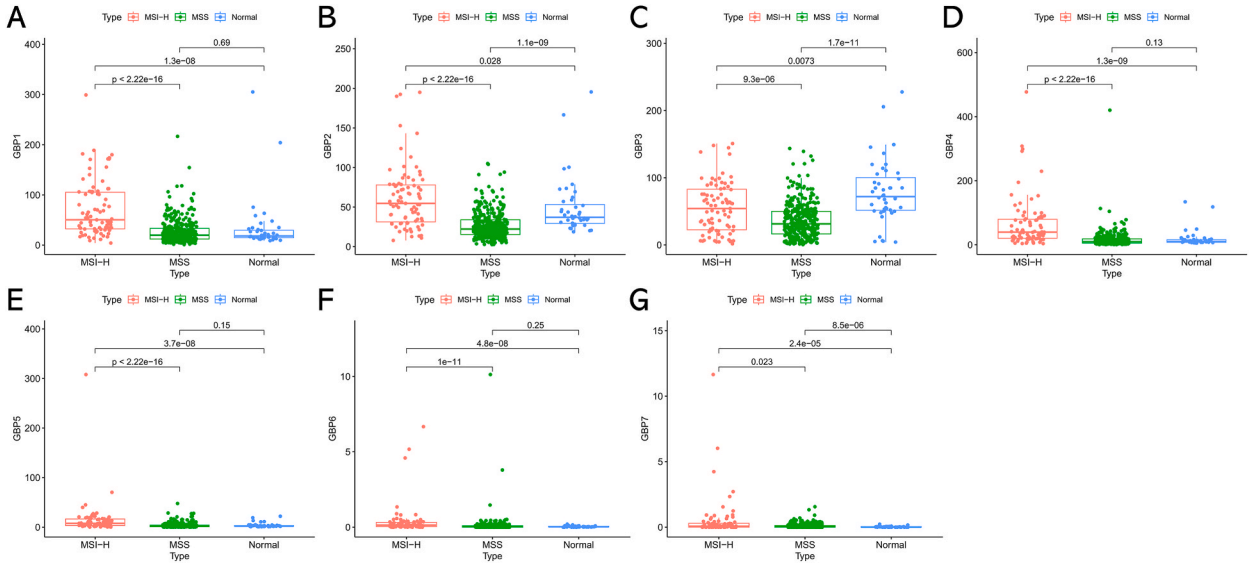


Fig. 1. The expression patterns of (A) GBP1, (B) GBP2, (C) GBP3, (D) GBP4, (E) GBP5, (F) GBP6, and (G) GBP7 in human CRC.

were significantly positively associated with each other (Fig. 2H). These results imply that GBP1/2/4/5 may participate in the metastasis of MSS CRC.

3.3. Correlations with the prognosis in CRC patients

The capability of GBP genes in forecasting the clinical outcomes of CRC patients were assessed by Kaplan-Meier method. Results indicate that increased expression levels of GBP 1/4/5/6 were strongly correlated with favorable OS in MSS CRC patients (Fig. 3). In terms of PFS, GBP1/2/4/5/6/7 were found to be obviously associated with favorable prognosis (Fig. 4). Besides, we also evaluated the prognostic value of GBP genes in CRC patients with MSI-H tumor. Except for GBP3, six GBP genes were found to be significantly correlated with unfavorable OS and PFS in MSI-H patients (Supplementary Fig. 1 and Supplementary Fig. 2). Thus, the GBP1/4/5/6 might serve as novel reliable prognostic biomarkers for CRC patients with different MSI status.

3.4. Underlying biological function enrichment analysis

Reactome, GO and KEGG enrichment analyses of GBP genes were conducted by using the DAVID platform. As displayed in Fig. 5A, Reactome enrichment analysis suggested that GBP family genes were all enriched in R-HSA-168256 (Immune System), R-HSA-1280215 (Cytokine Signaling in Immune System), R-HSA-913531 (Interferon Signaling), and R-HSA-877300 (Interferon-gamma Signaling). In addition, significantly enriched GO terms including GO:0071346 (cellular response to interferon-gamma) and GO:0042742 (defense response to bacterium). In terms of KEGG analysis, GBP family genes were mainly enriched in hsa04621 (NOD-like receptor signaling pathway), and the roles of GBPs in NOD-like receptor signaling pathway was displayed in Fig. 5B. These enrichment results suggest that GBP family genes are mainly involved in the process of immune regulation.

3.5. Correlation with tumor infiltrating immune cell

Considering the significant roles of TIIC in tumor initiation and progression, we first compared the differences in 22 types of TIIC between MSS and MSI-H subgroups. As shown in Fig. 6A and B, the infiltrating abundances of CD8 T cells, activated CD4 memory T cells, resting NK cells and M1 Macrophages were remarkably elevated in MSI-H samples, while the infiltrating of Plasma cells and regulatory T cells (Tregs) were decreased. Among these differentially infiltrated TIICs, higher infiltrating densities of activated CD4 memory T cells and M1 Macrophages were correlated with favorable clinical outcomes of MSS CRC patients, while higher infiltrating densities of resting NK cells and Tregs were unfavorable (Fig. 6C). Then, we assessed the relationships between GBP genes and TIICs in

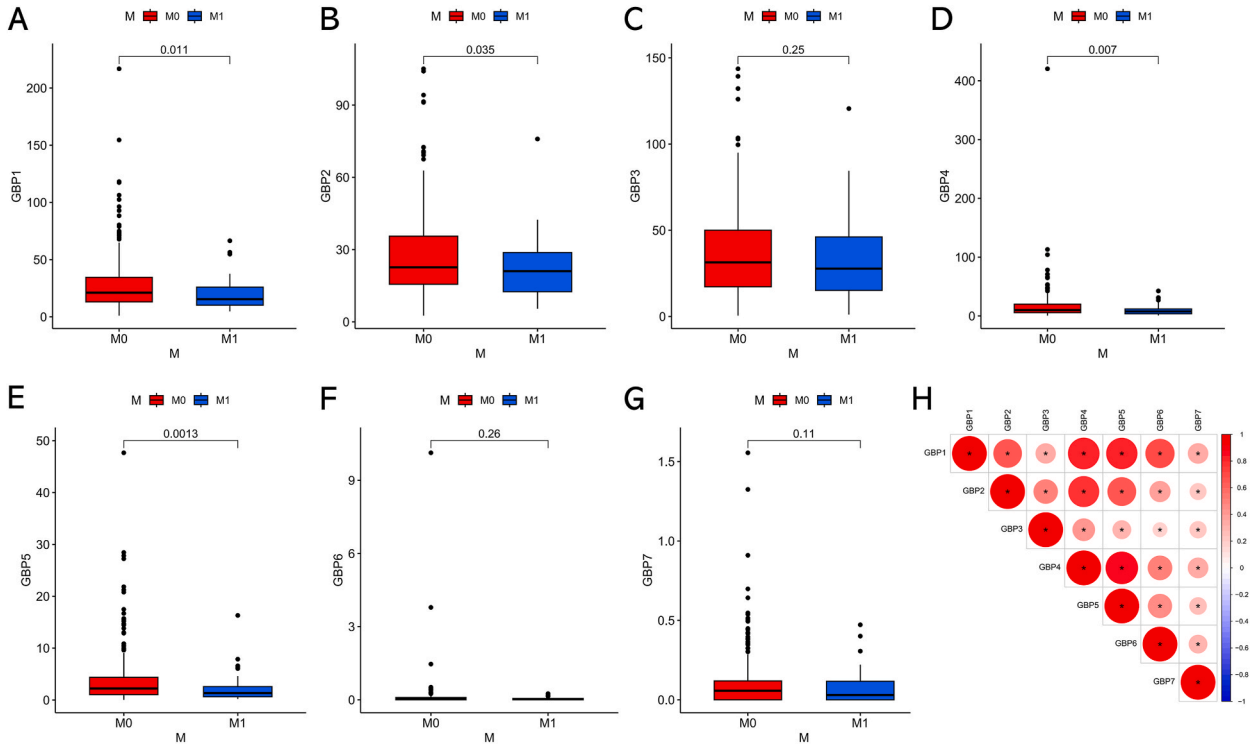


Fig. 2. The associations of (A) GBP1, (B) GBP2, (C) GBP3, (D) GBP4, (E) GBP5, (F) GBP6, and (G) GBP7 with distant metastasis status in MSS CRC. (H) The Spearman correlations of GBP family genes with each other in MSS CRC. M0: without distant metastasis; M1: distant metastasis.

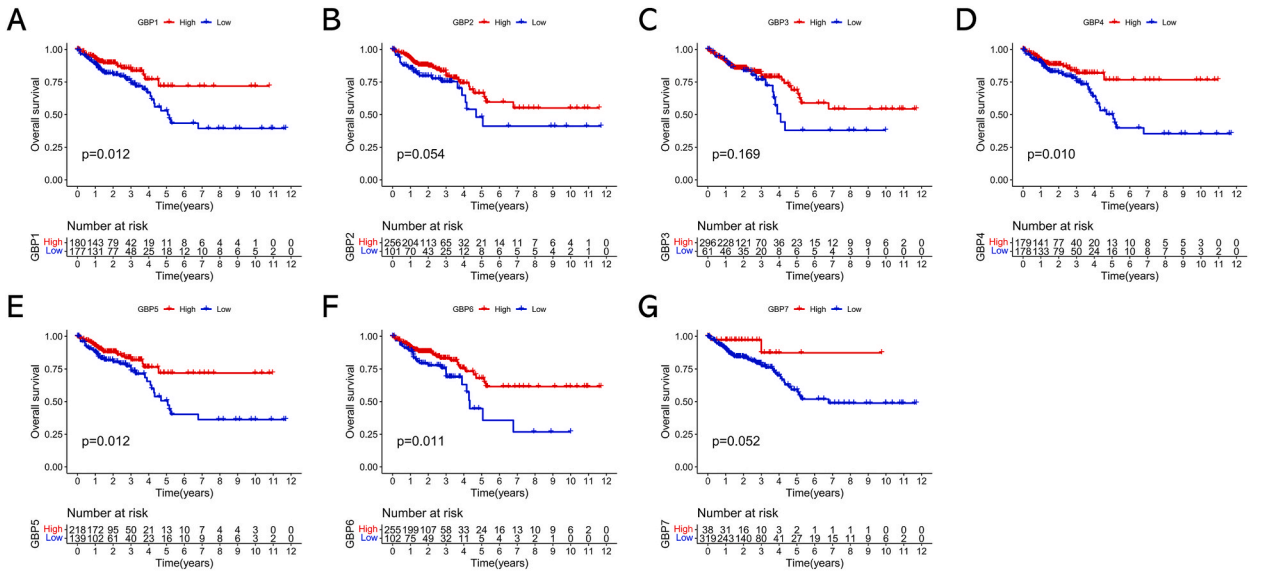


Fig. 3. The prognostic values of seven GBP family genes for OS of MSS CRC.

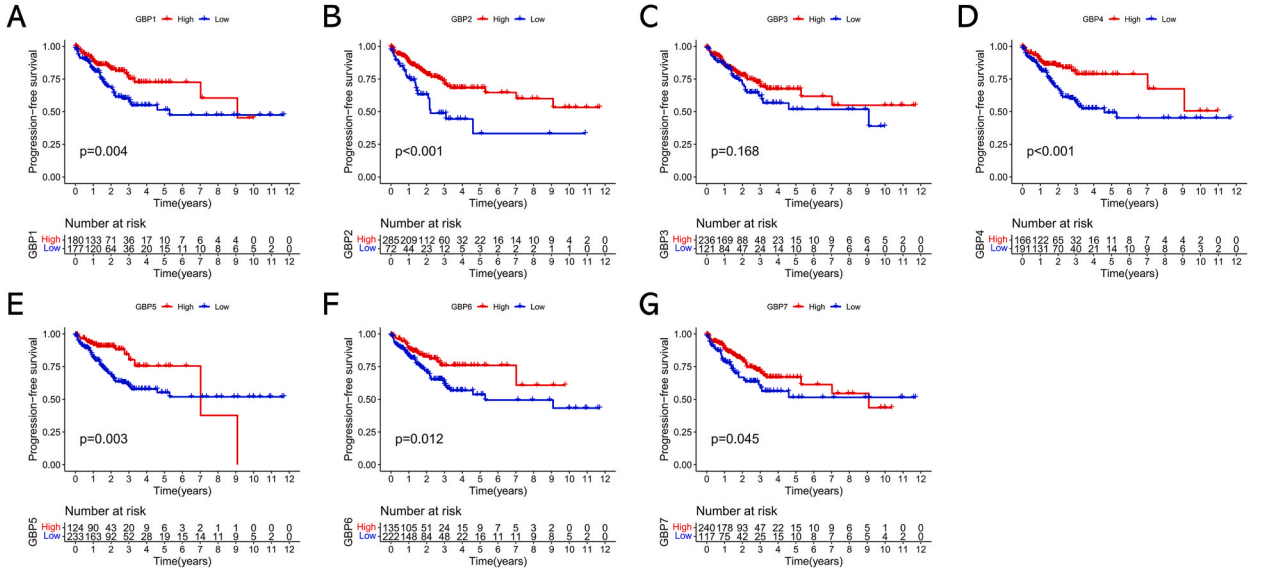
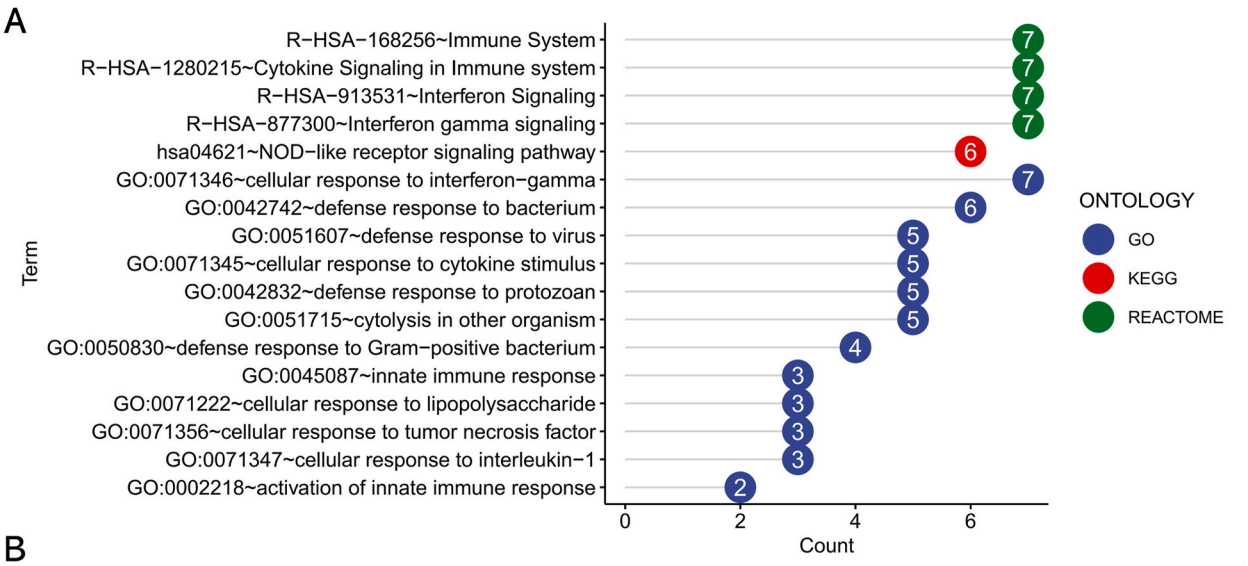


Fig. 4. The prognostic values of seven GBP family genes for PFS of MSS CRC.

MSS CRC. Results indicate that all GBP family genes showed a significant negative correlation with Tregs. Besides, significant positive correlations with activated CD4 memory T cells, M1 Macrophages, and naïve B cells in most GBP genes were observed (Fig. 6D). These findings indicate that GBP genes may participate in cancer progression via modulating immune cell infiltration in tumor microenvironment (TME).

3.6. Association with immunomodulatory molecules

We analyzed the relationships between MSI status and expression of immunomodulatory molecules, including ICBs and MHC molecules. As shown in Fig. 7A, most of the ICBs such as CD274, PDCD1, LAG3, HAVCR2, and CTLA4 were obviously overexpressed in MSI-H subgroup. With regard to MHCs, HLA-A/B/C were remarkably overexpressed in MSS samples, whereas the rest of the molecules were mainly overexpressed in MSI-H samples (Fig. 7B). Then, the relationships between GBP family genes and immunomodulatory molecules in MSS CRC were investigated. As presented in Fig. 7C and D, all seven GBP genes showed significant positive associations with the expression of ICBs and MHC molecules. These results imply that GBP family genes may be involved in the regulation of tumor



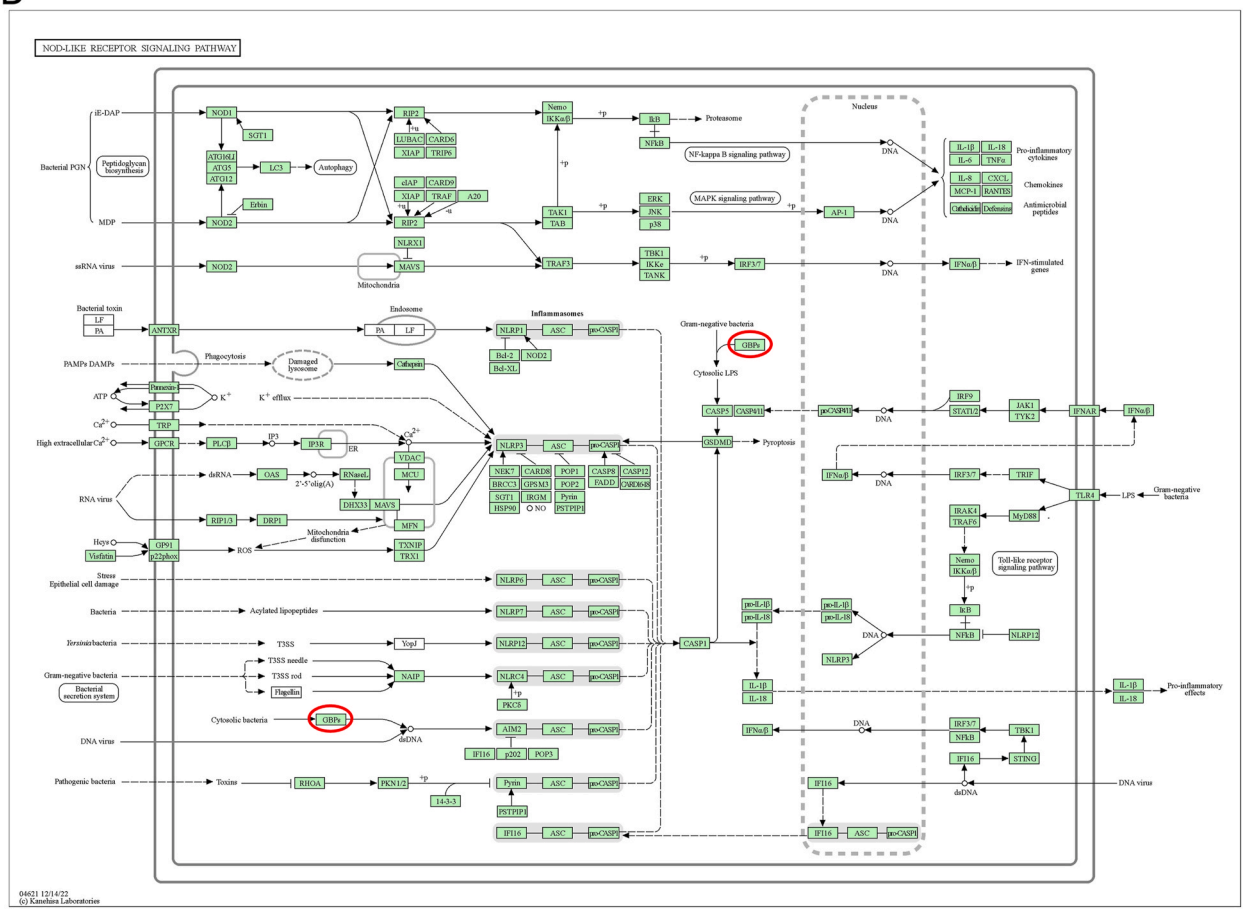


Fig. 5. Functional enrichment analysis of GBP family genes. (A) Reactome, KEGG and GO enrichment analysis of GBP family genes. (B) The roles of GBP family genes in NOD-like receptor signaling pathway.

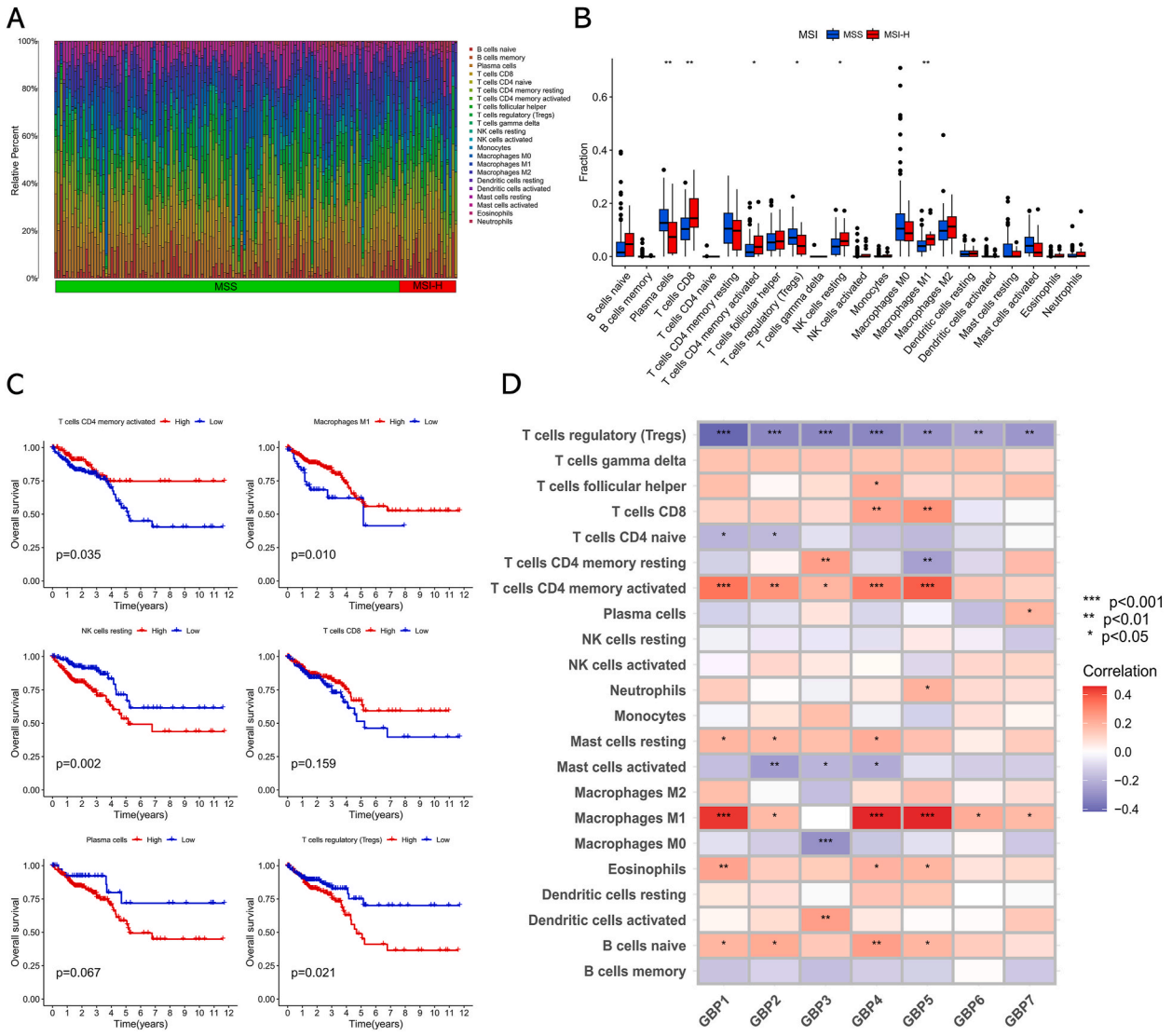


Fig. 6. Immune infiltration analysis. (A) Relative proportion of TIIC infiltration in MSS and MSI-H subgroups. (B) The differences of 22 types TIIC between MSS and MSI-H CRC. (C) The prognostic values of differentially infiltrated TIICs in MSS CRC. (D) The associations between GBP family genes and 22 types TIIC.

immune evasion in cancer progression, and they are potential biomarkers for immunotherapy in MSS CRC.

3.7. Validation in immunotherapeutic cohorts

We further analyzed the expression patterns of GBP genes in immunotherapy cohorts IMvigor210 and PRJEB25780. In IMvigor210 cohort, the expression of GBP1/2/4/5 were remarkably elevated in CR/PR subgroup of patients receiving anti-PD-L1 therapy (Fig. 8A). In PRJEB25780 cohort, the expression of GBP1/2/4/5/6 were remarkably elevated in CR/PR subgroup of patients receiving anti-PD1 therapy (Fig. 8B). These findings suggest that the GBP1/2/4/5 may be novel biomarkers for immunotherapy, which guiding the individualized treatment of CRC with MSS tumors.

4. Discussion

GBP family genes are supposed to have complex and distinct significances in human cancers, including CRC. For example, high GBP1 gene expression was found to be remarkably correlated with favorable OS in epithelial ovarian cancer, with the transfection of GBP1 partially suppressing the viability of ovarian cancer cells *in vitro* [17]. Unterer et al., reported that GBP1 could inhibit the proliferation of human cells through suppressing the Hippo signaling pathway [18]. In triple-negative breast cancer, GBP1 expression

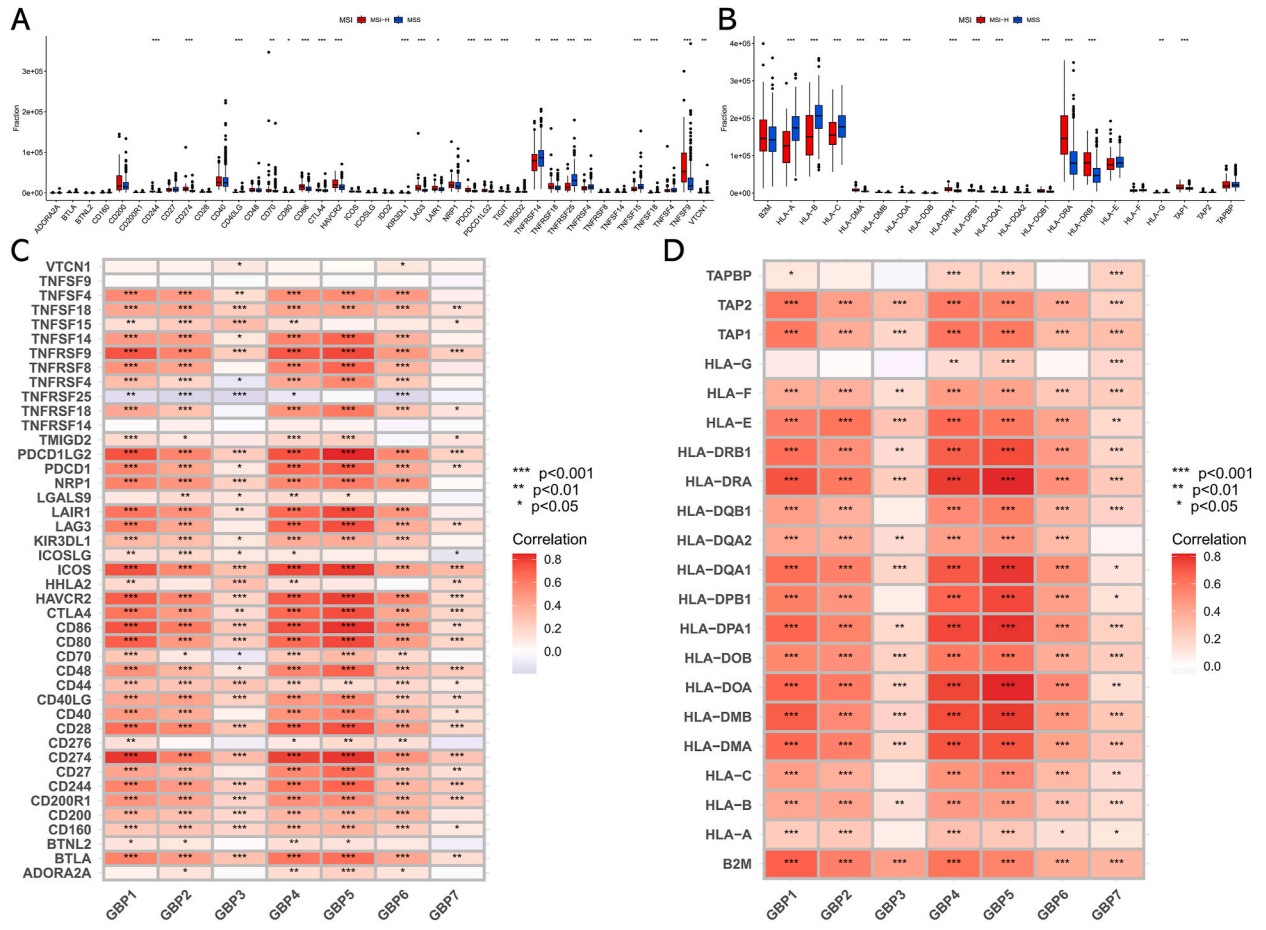


Fig. 7. Correlations between GBP family genes and immunoregulatory molecules. (A) The differences of ICBs between MSS and MSI-H CRC. (B) The differences of MHC molecules between MSS and MSI-H CRC. (C) The associations between GBP family genes and ICBs in MSS CRC. (D) The associations between GBP family genes and MHC molecules in MSS CRC.

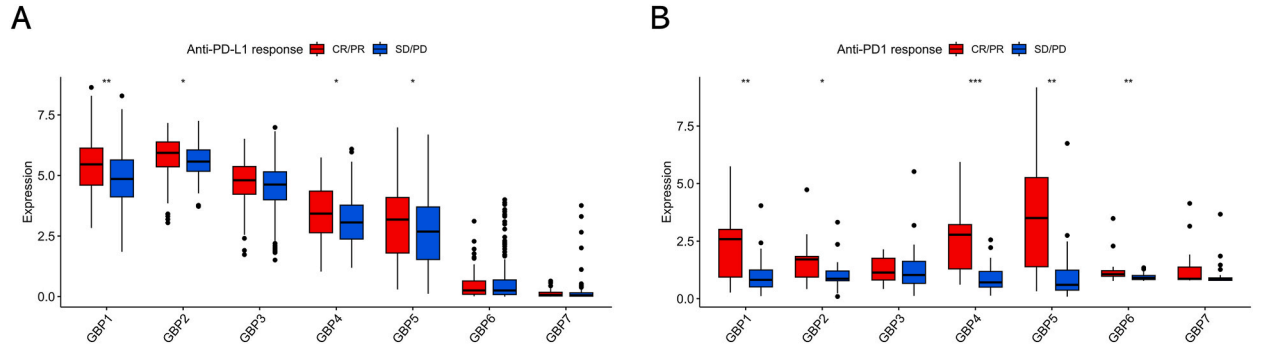


Fig. 8. Predictive capability of GBP genes for immunotherapy responsiveness. (A) The differences of GBP genes between responder and non-responder subgroups in IMvigor210 cohort. (B) The differences of GBP genes between responder and non-responder subgroups in PRJEB25780 cohort.

was regulated by EGFR and strongly correlated with the proliferation of triple-negative breast cancer cell lines [19]. Besides, Cheng et al., discovered that GBP1 promoted erlotinib resistance and invasiveness of non-small cell lung cancer by activating PGK1-mediated EMT signaling pathway [20]. GBP2 expression was highly expressed in glioblastoma and obviously correlated with unfavorable prognosis, with the silencing of GBP2 remarkably suppressing glioblastoma migration and invasion via STAT3/fibronectin pathway [21]. In clear-cell renal cell carcinoma, GBP2 expression is revealed to correlated to poor survival outcome and aggressive

characteristics [22]. Nevertheless, Zhang et al., reported that GBP2 overexpression inhibited cell metastasis via suppressing mitochondrial fission in breast cancer both *in vitro* and *in vivo* [23]. GBP3 has been reported to be expressed at high levels in skin cutaneous melanoma and is obviously correlated with favorable OS [24]. Xu et al., revealed the abnormally overexpressed GBP3 in glioblastoma predicts poor clinical outcomes, GBP3 knockdown enhanced the sensitivity of glioblastoma to anti-cancer treatment by modulating DNA damage repair [25]. Previous study indicated that GBP5 is highly expressed in oral squamous cell carcinoma, GBP5-knockdowned cells showed significantly reduced cell proliferation and invasion [26]. In gastric cancer, GBP5 expression was upregulated in tumor tissues, and GBP5 overexpression could promote cell migration by JAK1-STAT1 signaling [27]. Liu et al., indicated that low expression of GBP6 was correlated with lymph node metastasis and poor survival outcomes in oral squamous cell carcinoma patients [28]. Both GBP4 and GBP7 have the ability to repress the innate immunity to virus infection, but their functions in tumors have not been reported to date [29,30]. In terms of CRC, GBP1 has been identified as a tumor suppressor that contributes to inhibit tumor growth *in vivo*, and the loss of GBP1 expression mediate tumor escape from antitumorigenic immunity [31]. Wang et al., performed *in vitro* and *in vivo* experiments and pointed out that high GBP2 expression correlate with enhanced $CD8^+$ T cell migration and favorable response to immunotherapy in MSS CRC [32]. Besides, a recent study reported that GBP2 could inhibits cell growth and enhances the sensitivity to paclitaxel in CRC cell lines by blocking Wnt signaling [33]. In the present study, we found that the expression of all seven GBP genes are downregulated in MSS samples compared to MSI-H in CRC, and low expression of GBP1/2/4/5 obviously associated with distant metastasis as well as unfavorable clinical outcomes in MSS CRC patients. Taken together, these findings indicate that GBP1/2/4/5 are underlying diagnostic and prognostic biomarkers in MSS CRC.

We conducted GO, KEGG and Reactome enrichment analyses to investigate the biological significances of GBP genes further. We revealed that IFN-γ and NOD-like receptor signaling are the most relevant functions. IFN-γ is a cytokine that is mainly produced by T lymphocytes and natural killer cells and can eventually contribute to both protective immune responses and immunopathologic processes [34,35]. IFN-γ signaling induces anti-tumor response of host immunity in cancer progression, and mutations in the IFN-γ signaling generally lead to immunological dysfunction and resistance to ICIs in cancer [36]. In CRC, lack of IFN-γ signaling predicts poor survival outcomes [37]. In addition, Cui et al., indicated that IFN-γ produced by natural killer cells could inhibit cell growth and induce apoptosis of CRC by targeting IL-15 [38]. The activation of NOD-like receptor signaling has been reported to play a significant role in both innate and adaptive immune responses [39]. Previous studies have indicated that NOD-like receptor signaling involved in pathological process of diverse human diseases, including autoimmune, inflammatory disorders, and malignant tumor. Collectively, these results reveal that GBP family genes involved in modulating immune responses in cancer progression may be partially through IFN-γ and NOD-like receptor signaling. However, the underlying mechanisms about how GBP genes regulate the host immunity in MSS CRC have not been reported to date.

Tumor-infiltrating immune cells are the most critical components in TME, and the composition of TIICs within CRC tumors that are infiltrated is highly heterogeneous. T cells exhibit critical anti-cancer immunity and act as an essential role in tumor control, previous studies have proved that highly infiltrated CD4 and CD8 T cells were correlated with favorable prognosis in patients with CRC [40,41]. Macrophages in the TME can be generally classified into two functionally contrasting clusters, classical activated M1 macrophages and alternatively activated M2 macrophages. The former exhibits important anti-tumor immunity by producing inflammation-promoting cytokines and cytotoxic substances, such as TNF-α and IL-12, whereas the latter acts as an immunosuppressor to promote cancer progression by secreting cytokines such as TGF-β and IL-10 [42]. In CRC, the highly infiltrated M1 macrophages in tumor matrix predicted favorable clinical outcomes and prolonged survival time [43]. Besides, *in vitro* experiments revealed that inducting M1-polarization of macrophages enhances the oxaliplatin sensitivity of CRC [44]. In contrast, M2 polarization of macrophages was reported to accelerate the cell migration and invasion, and was significantly correlated with chemotherapy resistance to 5-fluorouracil in CRC [45,46]. Tregs are important immunosuppressor that inhibit anti-tumor immune responses by depressing antigen-presenting cells, exhausting the essential cytokines for cytotoxicity T lymphocyte activation, and secreting immunosuppressive molecules, thereby constructing an immunosuppressive TME to promote tumor cell growth [47]. Tumor-infiltrating Tregs was reported to generate an immunosuppressive TME in CRC by suppressing TNF-α and IFN-γ expression and was significantly correlated to tumor recurrence [48]. In our study, expression of GBP family genes in MSS CRC showed significant positive associations with CD4 T cells and M1 macrophages, and negative correlation with Tregs. These findings suggest that GBP genes might induce the TIICs to generate a tumor-suppressing TME, thereby inhibiting tumor growth and metastasis in MSS CRC, the immunoregulatory roles of GBP genes in MSS CRC deserve more exploration in the future.

Considering that ICIs have made great progress in anti-tumor therapy and are widely applicated in clinical as first/second-line treatment strategy, we investigated the associations between GBP genes and immunoregulatory molecules such as ICBs and MHC molecules. As expected, significant positive correlations between GBP genes and immunoregulatory molecules were revealed. Besides, we further validated the predictive capability of GBP genes for immunotherapy responsiveness by analyzing immunotherapy cohorts. As a result, we found that high GBP1/2/4/5 expression predicted better immunotherapy responsiveness. In line with our findings, Zhao et al., reported that GBP1 expression is a predictive biomarker for immunotherapy pan-cancer by a comprehensive bioinformatic analysis [49]. GBP2 was reported to be a promising therapeutic target for ICI treatment in MSS/pMMR CRC [32]. In addition, a recent study indicated that GBP5 is a novel immunotherapeutic target that could modulate PD-L1 expression in triple-negative breast cancer [50]. In sum, these findings suggest that GBP1/2/4/5 are potential biomarkers for enhancing the efficacy of patients' response to ICI therapy.

5. Conclusion

In conclusion, our study comprehensively investigated the significant roles of GBP family genes in MSS/pMMR CRC from the

perspectives of expression pattern, prognostic value, biological function, and immune infiltration. We proposed that GBP1/4/5/6 are potential prognostic biomarkers for MSS/pMMR CRC and GBP1/2/4/5 are promising targets for combinatorial therapy with ICI. These results provide valuable clues to guide individualized treatment of CRC and deserves further research in the future.

Funding

No funding was received.

Ethics statement

Not applicable.

Data availability statement

The datasets generated and analyzed during the present study are available in TCGA (https://www.cancer.gov/tcga), UCSC Xena (https://xenabrowser.net/), GEO (https://www.ncbi.nlm.nih.gov/geo/), and TIDE (http://tide.dfci.harvard.edu/) projects.

The code used in this article is deposited in GitHub (https://github.com/Bass-Lee1226/HELIYON-D-24-34758).

CRediT authorship contribution statement

Xin Wang: Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Ting Han:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Yinchun Wang:** Visualization, Methodology, Investigation, Data curation. **Rui Yang:** Visualization, Methodology, Investigation, Data curation. **Qingqiang Yang:** Visualization, Supervision, Methodology, Investigation. **Jianxin Li:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Not applicable.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e37741.

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