The Immune-Related PRF1 May Interact with Component 3 to Facilitate a Favorable Prognosis of Skin Cutaneous Melanoma

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Abstract

Background Previous studies have revealed the immune cytolytic activity (CYT) mediated by Perforin 1 (*PRF1*) is associated with a favorable prognosis of skin cutaneous melanoma (SKCM) and displays significant differences between primary and metastatic SKCM. However, the role of *PRF1* in SKCM is poorly understood.

Methods By comparing the *PRF1* co-expression network between metastatic and primary SKCM, we identified the potential hub genes interacting with *PRF1* and then verified their correlation in lung metastatic melanoma cell lines. Survival analysis was performed for the four groups of SKCM defined by the median expression values of *PRF1* and *C3*. Meanwhile, the possible relationship of *PRF1* with immunotherapy response is investigated in the melanoma expression profile of an adoptive T cell therapy dataset.

Results Almost all primary co-expression genes (n= 502, 96.91%) and more than half of metastasis-specific co-expression genes (n= 830) were identified in metastatic SKCMs. Functional enrichment analysis revealed that extensive CYT-related biological processes were disturbed in the metastatic SKCM, including immune response activation and inflammation response. The hub gene *C3* showed a positive correlation with *PRF1* in multiple datasets. Prognosis analysis indicated that the group with high expression of both *PRF1* and *C3* showed the best prognosis. Moreover, high expression of *PRF1* and *C3* is associated with positive immunotherapy response. Besides, we identified five human leucocyte antigen (HLA) genes as the central nodes of the PPI network.

Conclusions Remarkable alterations in the CYT-related biological processes were observed between primary and metastatic SKCMs. Preliminary results demonstrate that *PRF1* may act synergistically with *C3* to facilitate the favorable prognosis of SKCM.

Background

Melanoma is a malignant form of skin cancer that develops from melanocytes, of which skin cutaneous melanoma (SKCM) is the most common type [1]. Recently, the incidence of SKCM presents a steady increase and has become one of the top 20 most common cancers, causing approximately 1.6% of all new cancers and 0.6% of all deaths each year even though the approved application of new treatment techniques has reduced its mortality [2]. Numerous complex factors contribute to the development of SKCM, whereas UV radiation exposure is generally considered to be the key risk factor [3]. Age is the other important factor related to the prognosis of SKCM, with the disease tending to occur more aggressively in older patients (> 70 years), having lower survival rates [4]. Juvenile or pediatric patients are usually diagnosed at an advanced stage, while the prognosis is better in comparison with adult patients. In addition, sex is also associated with SKCM incidence and men are more likely to develop melanoma than women [5].

Perforin 1 (*PRF1*), known as pore-forming protein, encodes a protein that forms pores in the cytoplasmic membrane, thus allowing cytotoxic lymphocytes or neutral killer (NK) cells to release granzyme (GZMA) in order to mediate the lysis of target cells [6]. *PRF1* acts with *GZMA* synergistically to perform the immune cytolytic activity (CYT), an essential component of adaptive immunity, thus allowing cytotoxic T cells and NK cells to induce cytolysis through programmed cell death, aiming to remove the infected or pre-cancerous transformed abnormal cells [7]. Mutated or expression defected *PRF1* can cause dysfunction of the immune system, leading to serious immune disorders such as familial hemophagocytic lymphohistiocytosis and macrophage activation syndrome [8]. Additionally, *PRF1* also exerts an important role in provoking skin inflammation, as evidenced by its increased expression in chronic inflammatory skin diseases such as atopic dermatitis (AD) and psoriasis [9]. The relationship of altered *PRF1* with primary cancers is not well-known. In a genotyping study, it was found that the genotype of *PRF1* was not associated with increased susceptibility to malignant tumors such as melanoma, lymphoma, colorectal cancer and ovarian cancer [10]. In head and neck squamous cell carcinoma (HNSCC), *PRF1* is recognized as a good prognostic predictor with an elevated expression, especially in HPV positive HNSCC [11].

Accumulated evidence has demonstrated that *PRF1* plays an essential role in the control of viral infections and tumor surveillance. However, the role of *PRF1* in SKCM is poorly studied. Extensive studies have focused on the CYT, which is denominated by the expression of the toxins *PRF1* and *GZMA* [12]. It has been reported that CYT is associated with a favorable prognosis of SKCM, and the CYT score, defined by average expression of *PRF1* and *GZMA*, is significantly higher in metastatic SKCMs than that in primary SKCMs [13, 14]. In the TCGA SKCM dataset, metastatic patients show a better prognosis than primary patients. This prompted us to propose a hypothesis that the different CYT between primary and metastatic SKCMs may have an important impact on the patients' prognosis, which can be mediated by shaping the adaptive immunity of tumor cells. By comparing the *PRF1* co-expression network between metastatic and primary SKCM, we identified the potential key genes that interact with *PRF1* in the network, and then verified their correlations in lung metastatic melanoma cell lines. Meanwhile, the possible relationship of *PRF1* with immunotherapy response is also investigated in the melanoma expression profile of an adoptive T cell therapy dataset.

Methods

Data collection and preparation

The gene expression profiles of SKCM were obtained from the Cancer Genome Atlas (TCGA) database (<https://cancergenome.nih.gov/>), which contains 456 primary (n=98) and metastatic (n=358) samples (**Supplementary table 1**). The overall survival time was shorter in primary patients than that of metastatic patients, and there was no significant difference between male and female proportions ($P = 0.55$). The other four expression profiles, GSE22155 (n=29) [15], GSE54467 (n=70) [16], GSE65904 (n=206) [17] and GSE100797 (n=25) [18] were retrieved from the Gene Expression Omnibus (GEO) database (<https://www.ncbi.nlm.nih.gov/geo/>). We merged GSE22155, GSE54467, and GSE65904 as an

independent validation set, as they had small sample sizes and survival time, and were generated by Illumina HumanHT-12 platform. The Z-score method was used to normalize the expression profiles before merging to remove batch effects. GSE100797 contains 25 samples that derived from melanoma patients after adoptive T cell therapy, including 5 complete responders (CR), 5 partial responders (PR), 10 stable disease (SD) and 5 progressive disease (PD).

Co-expression analysis

Pearson correlation coefficients between *PRF1* and all other genes were calculated in primary and metastatic SKCMs, respectively, meanwhile significant P values were estimated. Low-expression genes, defined as the FPKM values below 1 in more than 50% samples, were removed before the correlation analysis to avoid impacting the assessment of correlation coefficient. Multiplex test was conducted to evaluate the false positive discovery rate using the BH method, and genes with FDR < 0.05 were screened as co-expression genes. Gene ontology (GO) enrichment and gene set enrichment analysis (GSEA) were performed for the co-expression genes.

Identification of hub genes

The co-expression genes were used to construct a protein-protein interaction (PPI) network by integrating the interaction information from STRING (Search Tool for the Retrieval of Interacting Genes) database [19] to identify the potential hub genes. The connections with score ≥ 0.8 were selected as reliable protein-protein interactions. The Cytoscape software [20] was used to show the PPI network and the topological parameters of this network were estimated by the NetworkAnalyzer plugin [21]. The potential hub genes were determined based on the number of directed edges and betweenness centrality of nodes. Besides, the other four parameters, closeness centrality, clustering coefficient, average shortest path length and stress were also estimated.

Construction of recombinant vector

Since the *PRF1* gene is barely expressed on the MALME-3M melanoma cell line, the gene sequence could not be cloned by a cDNA library. The vitro recombination technique were used to clone the full length of *PRF1* cDNA. Briefly, two pairs of primers were designed to amplify two exons of *PRF1* respectively (**Supplementary table 2**), and then the two amplified fragments were ligated to a complete *PRF1* sequence. Then the complete *PRF1* fragment was inserted into the pcDNA3.0-3xFlag vector to construct the recombinant vector.

Cell culture and transfection

Human Malme-3M melanoma cells was purchased from ATCC (<https://www.atcc.org/products/htb-64>) in May 2019 and authenticated by STR profiling. Mycoplasma contamination was first tested for the cells and then they were revived on Dulbecco's modified Eagle's complete medium (DMEM), and incubated in a cell incubator at 37°C and 5% CO₂ for 4-6 hours. The revived cells were then digested by trypsin for 3-5 min at 37°C. 10ul of the cell suspension were added to the blood cell counting plate for cell counting (2.5×10^6 / ml). Then, 80ul of cell suspension was added to a six-well plate, incubating in a cell incubator at 37°C and 5% CO₂ for 24 hours. Solution 1 was prepared by mixing 100ul of Opti-MEM medium (without antibiotics and serum) with 3ul of transfection reagent (Lipo3000). Solution 2 and solution 3 were prepared by mixing 100ul of Opti-MEM medium with 0.1ug and 1ug recombinant vector. Solution 1 was mixed with solution 2 and solution 3 respectively, and stand for 15 min. Then, the mixed solutions and 800 ul of preheated DMEM medium (serum free and double antibody free) were transferred to the six-well plate that contained revived cells. The cells were firstly incubated in a 37°C cell culture incubator for 4-6h. The medium was subsequently replaced by complete medium (containing 10% fetal bovine serum and 1% double antibodies) and after 48h, the cells were collected for RNA extraction.

Quantitative PCR

Real-time PCR was used to quantify the expression of *PRF1* and *C3* in transfected cells. Total RNA was extracted from the collected cells using Trizol reagent according to the instructions. The HiScript Q Select RT SuperMix for qPCR kit (vazyme, Nanjing, China) and SYBR Green Master Mix kit (vazyme, Nanjing, China) were utilized for cDNA synthesis and quantitative PCR, respectively. Real-time qPCR was performed in the BioRad CFX96 PCR system. The primers used in the study were listed in **supplementary table 3**. *GAPDH* was selected as an internal reference, and $2^{-\Delta\Delta Ct}$ was calculated to quantify the relative expressions of *PRF1* and *C3*.

Survival analysis

Firstly, samples with survival time less than 30 days were removed. For categorical variables, the log rank test (R package 'survival') was used to evaluate the prognostic survival difference among groups. For continuous variables, the samples were divided into high-expression and low-expression groups according to the median expression value, and then the prognostic survival difference between the two groups were assessed. The survival curve and the corresponding 95% confidence interval was plotted in GraphPadPrism 8 (v8.0.0) software. Each point in the survival curve represents a censored value.

Statistical analysis

The data preparation, processing and statistical analysis were performed on R software (v3.6.2). Survival curves were plotted on GraphPadPrism software (v9.0.0). For continuous variables, wilcox rank sum test and krusk Wilma test was conducted for the comparisons between unpaired two groups and multiple

groups, respectively. The chi-square test was used for the comparisons of category variables between groups. Spearman's *rho* statistic is used to estimate the correlation of two continuous variables.

Results

The association of *PRF1* with SKCM prognosis

The primary SKCM patients had shorter overall survival time than the metastatic SKCM patients ($P < 0.001$, **Supplementary table 1**) (**Figure 1A**) with *PRF1* expression upregulated in metastatic samples (**Figure 1B**). Patients in the *PRF1* high expression group showed a better prognosis than that in the low expression group (**Figure 1C**). As one of the key marker genes of immune cytolytic activity (CYT), the *PRF1* expression exhibited a strong positive correlation with lymphocyte infiltration score (**Figure 1D**). These results reflected a tight association between *PRF1* and the metastasis and prognosis of SKCM, implying the remarkable varied CYT between primary and metastatic SKCMs.

The CYT-related biological processes in primary and metastatic SKCM

We identified 518 and 1332 co-expression genes in primary and metastatic SKCMs, of which 502 genes were shared by both (**Supplementary figure 1**), accounted 96.91% and 37.69% of the total co-expression genes respectively. Functional enrichment analysis revealed that both the primary and metastatic co-expression genes were enriched in the processes of T cell activation, lymphocyte activation, etc. (**Figure 2A**). For primary specific co-expression genes, they were mainly related to tumor necrosis, while immune response activation and inflammation response were the major enriched processes for metastatic specific co-expression genes (**Figure 2B**). The results of GSVA enrichment indicated that higher scores of LIPASE ACTIVATOR ACTIVITY, POSITIVE REGULATION OF PROTEIN LOCALIZATION TO CELL SURFACE and CELL CYCLE G2 M PHASE TRANSITION were enriched in primary SKCMs, whereas TOLERANCE INDUCTION, REGULATION OF B CELL PROLIFERATION and REGULATION OF ANTIGEN RECEPTOR MEDIATED SIGNALING PATHWAY obtained higher scores in metastatic SKCMs (**Figure 2C**).

Identification of *C3* as the hub gene in PPI network

Since extremely few primary specific co-expression genes ($n=16$) were identified, the metastatic specific co-expression genes ($n=830$) were selected to construct a protein-protein interaction network based on the StringDB database. The network consists of 948 edges and 257 nodes (**Figure 3A**). The topological parameters of PPI network estimated by the NetworkAnalyzer plugin in Cytoscape indicated that *C3* gene had the largest degree, the number of node connections (**Figure 3B**). The betweenness centrality of *C3*, which reflects the amount of control the node exerts over the interactions of other nodes in the network, ranked second (**Figure 3C**). In addition, the other four parameters including closeness centrality, clustering

efficiency, average shortest path length and stress, also suggested the key role of *C3* in the network (**Supplementary figure 2**).

The association of *PRF1* with *C3*

The expression of the potential hub gene, *C3*, was found upregulated in the metastatic SKCMs (**Figure 4A**), and its high expression group exhibited a better prognosis than the low expression group (**Figure 4B**), suggesting that *C3* might be a favorable prognostic predictor. Besides, the expression of *PRF1* and *C3* presented a moderate correlation (**Figure 4C**). Then, we divided SKCM samples into four groups, PRF1-high/*C3*-high, PRF1-high/*C3*-low, PRF1-low/*C3*-high, and PRF1-low/*C3*-low according to the median values of *PRF1* and *C3* expressions. Survival analysis indicated that PRF1-high/*C3*-high group showed the best prognosis and PRF1-L/*C3*-L group had the worst prognosis (**Figure 4D**). Additionally, most of the PRF1-L/*C3*-L group samples were primary SKCMs, while the PRF1-H/*C3*-H group samples were dominated by metastatic SKCMs (**Supplementary Figure 3**).

Validation of *PRF1* and *C3* in independent set

In the TCGA SKCM dataset, the potential synergy between *PRF1* and *C3* was observed that may contribute to a good prognosis of this disease. Then, we verified this relationship in an independent GEO dataset. Due to their small sample sizes, three GEO datasets including GSE22155 (n= 29), GSE54467 (n= 70) and GSE65904 (n= 206) were integrated into one set by employing Z-score method. The combined dataset showed no batch effect after Z-score normalization (**Supplementary figure 4**). The expression of *PRF1* was positively associated with *C3* in the validation set (**Figure 5A**), which was in line with that in TCGA dataset. Four groups divided by median values of *PRF1* and *C3* expressions also presented a significant difference in prognosis, with PRF1-H/*C3*-H group having the best prognosis and PRF1-L/*C3*-L group the worst prognosis (**Figure 5B**).

Over-expression of *PRF1* in melanoma cell lines

The expression profiles of Cancer Cell Line Encyclopedia (CCLE) revealed that *PRF1* and *C3* were almost not expressed in SKCM cell lines. As a result, we attempted to overexpress *PRF1* on the lung metastasis cell line, MALME-3M, and quantify the *C3* expression to investigate their possible relationship. A recombinant vector containing the full fragment of *PRF1* was first constructed based on the recombination clone technology (**Supplementary figure 5**). Then, the empty vector, 0.1ug and 1ug recombinant vector were transfected to MALME-3M cells. The relative expressions of *PRF1* and *C3* were quantified by qPCR with *GAPDH* as the control gene. Results indicated that the *PRF1* expression was significantly up-regulated in cells transfected by recombinant vectors, and the expression of *C3* was also increased (**Figure 6A**). The relative expression values of both were associated with the concentration of transfected recombinant vectors (**Supplementary Table 5**). Moreover, the relative expressions of *PRF1* and *C3* also showed a strong positive correlation (**Figure 6B**).

The association of PRF1/C3 with immunotherapy

PRF1 is well-known as the marker of immune cytolytic activity, and *C3* is related to the inflammatory response of complement system. In the expression profiles of melanoma patients received adoptive T cell therapy, we investigated the expressions of *PRF1* and *C3* in four immune response groups including complete responders (CR, n=5), partial responders (PR, n=5), stable disease (SD, n=10), and progressive disease (PD, n=5). The PD group showed the lowest expressions of *PRF1* and *C3* in comparison with the other three groups (**Figure 7A**). A strong correlation between *PRF1* and *C3* was observed among the four group samples (**Figure 7B**).

Discussion

Although studies of CYT defined by *PRF1* and *GZMA* have been reported in SKCM, the related biological pathways remain unclear, especially the relationship between CYT and metastatic SKCM prognosis is rarely involved. Most of the previous studies are focused on the impact of dysfunctional *PRF1* caused by genomic variations on diseases [22]. In the current study, we firstly investigated the alterations of *PRF1*-related biological processes between primary and metastatic SKCMs, and then identified *C3* as the potential hub gene of the PPI network constructed by *PRF1* co-expression genes. Further investigations indicated that the *PRF1*/*C3* axis was associated with favorable immunotherapy response and prognosis. Finally, the relationship between *PRF1* and *C3* was verified in an independent validation set and malignant melanoma cell lines.

Almost all of the co-expression genes (96.91%) in primary SKCMs were also found in metastatic SKCMs and more metastasis-specific co-expression genes (62.31%) were identified, indicating that a few of new biology processes were stimulated in metastatic SKCMs, except for the pathways shared by both [12]. These results were confirmed by functional enrichment analysis. Both of the primary and metastatic co-expression genes were enriched in the regulation of lymphocyte activation, T cell activation, and leukocyte activation. However, the specific co-expression genes of both sides showed dramatic differences in BP terms, reflecting the extensive CYT-related biological processes involved in the metastasis of melanoma cells from local to distal. Although the metastasis of melanoma is a highly complex event such as the genomic mutations, the interaction between extracellular matrix and tumor cells, etc. [23, 24], our findings provide a new insight into the perspective of CYT.

Component 3 that was recognized as the hub gene of the PPI network, showed a positive correlation with *PRF1*, suggesting a possible synergistic interaction between them in metastatic SKCMs. The subsequent prognostic analysis revealed that *PRF1*-H/*C3*-H group had the best prognosis and the *PRF1*-L/*C3*-L group the worst prognosis, which was verified in a validation set. Overexpression of *PRF1* in the malignant melanoma cell lines increased the expression of *C3*. These results demonstrated the potential interaction between *PRF1* and *C3*. Indeed, *C3* is one of the well-known pro-inflammatory molecules that are up-regulated in most inflammatory diseases [25, 26]. The CYT-related modules are positively correlated with inflammatory response, suggesting a stronger inflammatory response in tumors compared to normal

tissues. Besides, it has also been reported that *C3* is required for the metastasis of lung and breast cancer cell lines to the soft meningeal space [27], which is line with our observations that *C3* was up-regulated in metastatic SKCMs.

The other five genes with highest degrees were all human leucocyte antigen (HLA) group genes (HLA-DRB1, HLA-DPA1, HLA-DRB5, HLA-DQA2 and HLA-DQA1). HLA group genes are encoded by human major histocompatibility complex (MHC) genes characterized by high polymorphism [28]. These molecules exert an important role in regulating the host immune response, mainly participated in the presentation of antigens to T-cell receptors (TCRs) [29]. Our results revealed that the five HLA genes were the central nodes of the PPI network, indicating their extensive interactions with other *PRF1*-related processes. These findings suggested a high immune activity in the immune microenvironment of metastatic SKCMs, which might contribute to a favorable prognosis of this disease.

In melanoma patients received the adoptive T cell therapy, we observed that high expression of *PRF1* and *C3* was associated with a good therapy response, and the two genes also showed a strong correlation, reflecting a synergistic interaction between them, which may benefit a positive response to adoptive T cell therapy. The relationship between T cell densities at the margins and centers of invasive tumor was investigated in colorectal cancer, and high granzyme density was found to be associated with a lower probability of tumor recurrence and prolong overall survival, implying that high activity of CYT can be a negative regulator of tumor recurrence [30]. The acquisition of cytolytic property is a key step for activated T cells (CD4 + T cell and CD8 + T cell) to exert cytotoxic function [31]. Cytolytic property plays an important role in the prognosis of melanoma patients, for patients with high cytolytic score having a better prognosis. Therefore, it is recognized as one of the functional effectors of toxic T cells [13]. In addition, the upregulated *PRF1* was also observed in anti-CTLA-4 and anti-PD-L1 immunotherapy response groups [32]. Previous studies and the current study imply that *PRF1/C3* may serve as a predictor of immunotherapy in melanoma patients.

However, some issues still need to be addressed in the future. Firstly, it remains unclear whether there is a causal relationship between *PRF1* and *C3*, because the current results only revealed a positive correlation between them. Although overexpression of *PRF1* increased the *C3* expression, more thorough molecular experiments are required to investigate their potential relationship. Second, the synergistic effect of *PRF1* and *C3* cannot tell us if there exists a direct or indirect interaction between them. Third, the prognosis of melanoma is affected by numerous complex factors, and some other pathways may interact with *PRF1/C3* axis to regulate the patient prognosis.

Conclusions

In summary, we observed vast variations between primary and metastatic SKCMs in the CYT-related biological processes. *C3* was identified as the hub gene in the PPI network by comparing the co-expression genes of *PRF1* in primary and metastatic SKCMs. The subsequent prognostic survival analysis and cytological experiments provide preliminarily evidence that *C3* may act synergistically with

PRF1 and thus contributed to the favorable prognosis of SKCM. We further observed a correlation between the PRF1/C3 expression and positive immunotherapy response, indicating their potential to serve as the predictors of immunotherapy.

Declarations

Ethics approval and consent to participate

All data used in this study are publicly accessible to any researcher, our research did not require the approval of an ethics committee.

Consent for publication

Not applicable.

Availability of data and materials

The gene expression profiles are publicly accessible in TCGA and GEO database. All the intermediate data and results are presented in the manuscript and supplementary materials.

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Author's contributions

Bingran Qi and Mengzhu Lv performed data preparation and interpretation. Mengzhu Lv and Chang Zheng established cell lines and conducted overexpression experiment. Kezhu li, Shifeng Jin and Yuqing Dong wrote the manuscript. Yinibg Shao and Shu Guo revised the study.

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We declare that submitted manuscript is not under consideration for publication elsewhere. The manuscript is truthful original work without fabrication, fraud or plagiarism.

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Figures

Figure 1

Correlation of PRF1 with SKCM prognosis. A: Overall survival time of primary and metastatic SKCMs. B: PRF1 expression in primary and metastatic SKCMs. C: Survival curves of PRF1 high and low groups. D: Correlation of PRF1 expression with lymphocyte infiltration score. Boxplot in the bottom-right showed the lymphocyte infiltration scores in primary and metastatic SKCMs.

Figure 2

Functional enrichment of the co-expression genes. A: The enriched biological process (BP) of primary and metastatic co-expression genes. B: The enriched BPs of primary and metastatic specific co-expression genes. C: The enriched pathways of primary and metastatic co-expression genes by GSEA. Only top 15 BP terms and pathways were showed.

Figure 3

PPI network analysis. A: PPI network constructed by metastatic specific co-expression genes. Nodes in the network represent genes. The potential hub gene C3 was showed. B: Number of directed edges of the nodes. C: Betweenness centrality and number of directed edges of the nodes. The top 5 genes with highest degrees were showed in B and C.

Figure 4

Relationship between PRF1 and C3. A: The expression of C3 in primary and metastatic SKCMs. B: Survival curves of C3 high and low expression groups. C: The correlation of PRF1 and C3 expressions. D: Survival curves of the four SKCM groups defined by PRF1 and C3 expressions.

Figure 5

Validation of the relationship between PRF1 and C3 in independent set. A: The correlation of PRF1 and C3 expressions. B: Survival curves of the four SKCM groups defined by PRF1 and C3 expressions.

Figure 6

Overexpression of PRF1 in melanoma cells. A: Relative expression of PRF1 and C3 in MALME-3M cells. B: The correlation of PRF1 and C3 relative expressions.

Figure 7

Association of PRF1/C3 with immunotherapy. A: The expressions of PRF1 and C3 in four immune response groups. B: The correlation of PRF1 and C3 expressions. PR: partial responders (n=5), SD: stable disease (n=10), CR: complete responders (n=5), PD: progressive disease (n=5).

Supplementary Files

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