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Identification of the Genomics and Transcriptomics Regulators of the DNA Damage Response in Breast Cancer

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ABSTRACT

Breast cancer is one of the most prevalent malignancies and the most common cancer in female patients. In recent years, the clinical utilization of a class of drugs called poly (ADP-ribose) polymerase inhibitors has been observed to be detrimental to cells that harbor defective DNA damage repair mechanisms. Implementation of these drugs entails a series of unprecedented challenges, including the development of drug resistance to this treatment strategy. Thus, it is essential to gain a better understanding of the mechanisms that regulate the DNA damage response to maximize the treatment efficacy in breast cancer patients and minimize unwanted side effects. In this study, through the utilization of single-cell- and bulk-level transcriptional data, we set out to identify molecules and molecular circuits associated with DNA damage response in breast cancer patients. By identifying differentially expressed genes in single-cell cancer cell populations inherently different in DNA damage response, further clustering bulk RNA-sequencing samples based on the expression of these genes, and performing network and enrichment analysis at the bulk level, we have characterized breast cancer samples based on their DNA damage response. Moreover, we have been able to identify a central network module whose members can serve as treatment targets and yield further insights into the mechanisms of drug resistance and DNA damage response in breast cancer. Overall, this study contributes to the characterization of the transcriptional circuits involved in the heterogeneity of DDR in breast cancer and provides candidate avenues for the investigation of potential therapeutic interventions.

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Introduction

Breast cancer is among the most devastating cancer types worldwide. The complex inter- and intra-tumor heterogeneity, combined with the scarcity of feasible treatment targets, have contributed to a death toll of 685,000 in 2020 (Sedeta *et al*., 2023). Generally, breast cancer can be divided into four subtypes based on the presence of hormone receptors on cancer cells. These subtypes include estrogen receptor-positive, progesterone receptor-positive, human epidermal growth factor receptor-positive, and triple-negative breast cancer, which represents the lack of all the aforementioned hormone receptors (Burguin *et al*., 2021). Among these, triple-negative breast cancer is particularly heterogeneous and has up to 15% lower 5-year survival rate than that of the other subtypes of breast cancer (Howard and Olopade, 2021). In this light, identifying mechanisms by which

these tumors are regulated is of prime importance.

In recent years, it has been shown that the combination of cisplatin treatment, as a DNA damage-inducing factor, combined with poly (ADP-ribose) polymerase inhibitor (PARPi) drugs is a promising treatment avenue for patients that suffer from breast tumors harboring defunct homologous recombination DNA repair system (Wooten *et al*., 2023). Homologous Recombination Deficiency (HRD) is most commonly caused by loss of function mutations in the members of the DNA Damage Response (DDR) system (e.g., *BRCA1*, *BRCA2*, *RAD51*, etc.) (Herzog *et al*., 2023). Since mutations in the DDR genes are the most common direct cause of defects in the DDR system, studies concerned with this aspect of breast cancer are mostly focused on the utilization of genomic data to characterize this phenomenon (Belli *et al*., 2019; Toh and Ngeow, 2021). However, such studies are blind to the vast complexity added in the transcriptional level which, potentially, can explain a considerable portion of heterogeneity reported so far (P. Liu *et al*., 2023).

In this study, we set out to identify culprits in DNA damage in breast cancer to better understand the role of DNA damage in breast cancer. Toward this goal, we took advantage of publicly available single-cell and bulk RNA-seq data to characterize the transcriptional circuits involved in the heterogeneity of DDR in breast cancer.

Materials and Methods

Seven triple-negative breast cancer single-cell RNA-seq samples were retrieved from GSE176078 (Wu *et al*., 2021). Seurat framework (version 5.0.1) was used to analyze these samples (Hao *et al*., 2023). Only cells with a minimum of 200 features, a maximum of 5000 features, and a mitochondrial content of less than 5% were incorporated in the study. The individual samples were further filtered to include only breast cancer epithelial cells. The integration of the samples was performed using the Harmony algorithm (Korsunsky *et al*., 2019). The list of proteins involved in the DDR was retrieved from WikiPathways. It was used as the basis of the scoring criteria for the identification of cell populations that harbor higher DDR activity using the "AddModuleScore" function in Seurat (Agrawal *et al*., 2023). The differential expression analysis between the cells with a high DDR score and the cells with a low DDR score was assessed using the Wilcoxon test. The significance criteria were a log2 fold change equal to or greater than 0.5 and an adjusted p-value equal to or less than 0.05. Only the members upregulated in the group with the higher DDR activity were retained for further analysis.

Bulk RNA-seq data were obtained from the BRCA dataset of TCGA project (Cancer Genome Atlas Network, 2012) from the GDC data portal (https://portal.gdc.cancer.gov/). The clustering was performed using k-medoid clustering and differential expression analysis between clusters was carried out using the DESeq2 package in the R programming environment (Love *et al*., 2014). Enrichment analysis was performed using Metascape (Zhou *et al*., 2019) and TRRUST (Han *et al*., 2018), and the samples were compared in terms of their mutational status through cBioportal (de Bruijn *et al*., 2023; Zhou *et al*., 2019). The network analysis was carried out in Cytoscape, and the MCODE plugin was used to identify the highly connected components of the network.

Results

Single-cell RNA-seq is a powerful method to deconstruct the heterogeneity common to breast cancer samples. Through the capacities of single-cell RNA-seq and further filtering of the detected cells to retain only cancer cells, we have tried to evaluate the DDR status precisely in malignant cells. Overall, after filtration, 4,216 cells from the single-cell dataset were kept for further analysis. These cells were scored based on the activity of the 120 genes in the WikiPathway DDR genes (Fig. 1A). This was followed by a differential expression analysis between cells that had a high score and those that had a low score, which resulted in a list of 55 genes (Table 1). The enrichment analysis indicates that these genes are involved in the DNA metabolic process and DNA replication (Fig. 1B). This list of 55 genes was further used as the starting point for the bulk RNA-seq analysis.

To perform the bulk RNA-seq portion of this study, 1,082 samples available in the BRCA project of TCGA were used. We used the 55-membered gene list obtained from the single-cell analysis to perform k-medoid clustering on the bulk RNA-seq samples. The purpose of this step was to divide the samples into two groups inherently different in their DDR status.

Table 1. The list of 55 differentially expressed genes between the cells with high- and low DDR activity

Gene symbol	Log2 fold change	Gene symbol	Log2 fold change
PCNA	2.4	ORC6	1.9
TYMS	2.6	CHEK1	1.8
RRM2	3.7	MCM3	1.5
FEN1	2.2	RAD23A	0.7
RFC2	1.7	RPA3	1.0
ASF1B	3.0	TMPO	1.3
FAM111B	3.0	SMC4	1.4
CLSPN	2.7	GINS2	1.8
DEK	1.0	HMGB2	1.3
RNASEH2A	1.7	WDR34	1.1
UBE2T	1.5	SLBP	1.3
ZWINT	2.4	CDCA4	1.6
USP1	1.3	DNAJC9	1.1
PARP1	0.8	TK1	1.1
PKMYT1	2.3	TMSB15A	1.0
XRCC5	0.9	RPA2	1.3
POLD2	0.8	UBE2C	1.1
NASP	1.1	SAC3D1	1.1
POLD3	1.7	BARD1	1.1
DNMT1	1.1	KIF22	1.1
GMNN	1.2	SMC3	0.8
H2AFX	1.4	RFC1	1.0
MCM7	1.1	PRKDC	1.0
RFC4	1.8	BTG3	1.1
ATAD2	2.1	TEX30	1.1
RAD51C	1.5	APEX1	0.7
POLE4	1.0	XRCC6	0.4
CENPU	1.8	-	-

K-medoid clustering resulted in 624 samples clustered in Cluster 1 and 458 samples clustered in Cluster 2. These clusters were compared against each other to find mutations enriched in either of the clusters. It was observed that mutations in critical genes, including TP53 and MYC, were highly enriched in cluster 1 (Fig. 2A).

In order to investigate the transcriptional landscape of the identified clusters, differential expression analysis was carried out. The criteria of significance were adjusted p-values of 0.05 or less and absolute log2 fold change of 1 or more. With this criteria, 3312 genes were differentially expressed between the clusters (Fig. 2B). However, due to the large size of this gene list, only the top 200 genes were selected for enrichment analysis. Enrichment analysis was carried out using Metascape. The results of this step are shown in Fig. 2C. Interestingly, the enrichment of these 200 members in TRRUST indicates that the top three transcription factors governing the expression of these genes are E2F1, E2F4, and TP53 (Fig. 2D).

Furthermore, these top 200 differentially expressed genes were imported to Cytoscape, and the interaction information was retrieved from the STRING database (Confidence cutoff = 0.7). The MCODE plugin was used to find the top-performing highly connected subnetwork. This module comprises 16 genes, many of which are important in various DNA damage response mechanisms (Fig. 2E).

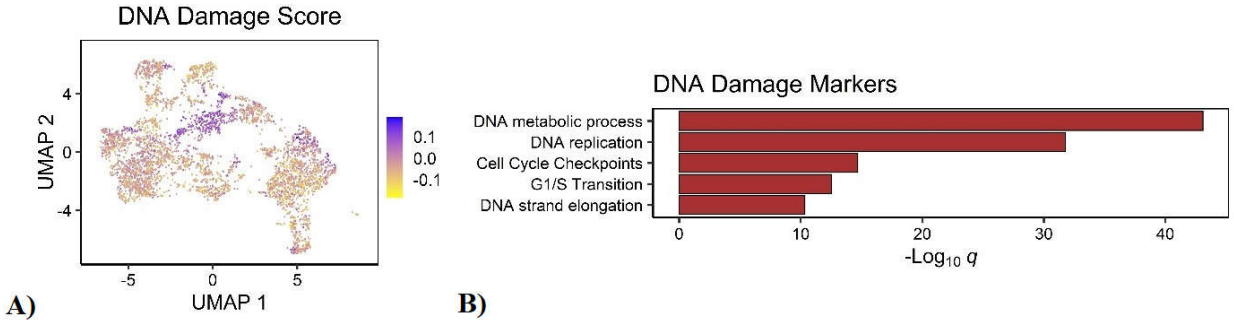

Fig. 1. Identification of the markers of DNA damage response (DDR) at single-cell resolution: A) Uniform manifold approximation and projection (UMAP) map of the single-cells scored based on the activity of the 120 genes involved in the DDR. The color of the cells represents the collective activity of these genes in the respective cells; B) The enrichment analysis of the 55 markers of DDR identified using the differential expression analysis between the cells with high and low activity of the DDR genes shows that these genes are enriched in pathways such as the DNA metabolic process and DNA replication.

Discussion

DNA damage response has been implicated in breast cancer treatment outcomes and patient prognosis (Lei *et al*., 2022). In the current study, by taking advantage of transcriptional data in bulk and single-cell resolutions, we have been able to identify several culprits in DNA damage response in patients diagnosed with breast cancer. Our analysis indicates that E2F1 is highly associated with DNA damage response pathways. Indeed, it has been shown that the activity of E2F1 is tightly regulated by cell cycle cues (Fouad *et al*., 2021). In this line, in the malignant state, regulation of E2F1 is commonly perturbed. This usually takes place as a downstream effect of perturbations in oncogenic signaling pathways aiming to maximize tumor proliferation (Dubrez, 2017). E2F1 is ubiquitously expressed in cancer cells and is responsible for the replication of many cell-cycle genes (Sheldon, 2017). It has been demonstrated that E2F1 is a necessary component for cell viability and RAD51-mediated DDR (Choi and Kim, 2019). Thus, it is by no accident that this study identifies RAD51 as one of the core components of the constructed network. The module identified through the MCODE algorithm includes RAD51 in addition to other genes related to DDR. Key genes in DDR, including RAD51, have already been implicated as important cellular components and have been associated with therapy (Wang *et al*., 2022). In this sense, further studying the network identified in this study, especially the genes present in our module, might yield insight into the association of DDR with cancer and result in the identification of therapeutic targets to improve patient outcomes. Moreover, an investigation of the mutational status of clusters retrieved based on the differential expression of cancer cells in the single-cell dataset indicated that mutations in critical genes, including TP53, MYC, and TRPS1, are highly enriched in cluster 1. TP53 has a very established role in most, if not all, of the hallmarks of cancer (Marei *et al*., 2021). Most importantly, TP53 is a regulator of DDR. Upon induction of DNA damage, TP53 binds to DNA and, through transcriptional regulation, plays a pivotal role in various pathways broadly categorized under cell fate decision, DDR, and cell cycle (Liu and Kulesz-Martin, 2001). Mutations in *TP53* have long been established as a driver of breast cancer (Walerych *et al*., 2012), and it is not surprising to observe a difference in carriers of these mutations while dividing the patients based on DDR. Most importantly, targeting the regulators of TP53 is being established as a possible treatment approach (Abuetabh *et al*., 2022). Therefore, it is crucial to recognize the significance of *TP53* mutations in regulating the observed differences in DDR, as highlighted by this study.

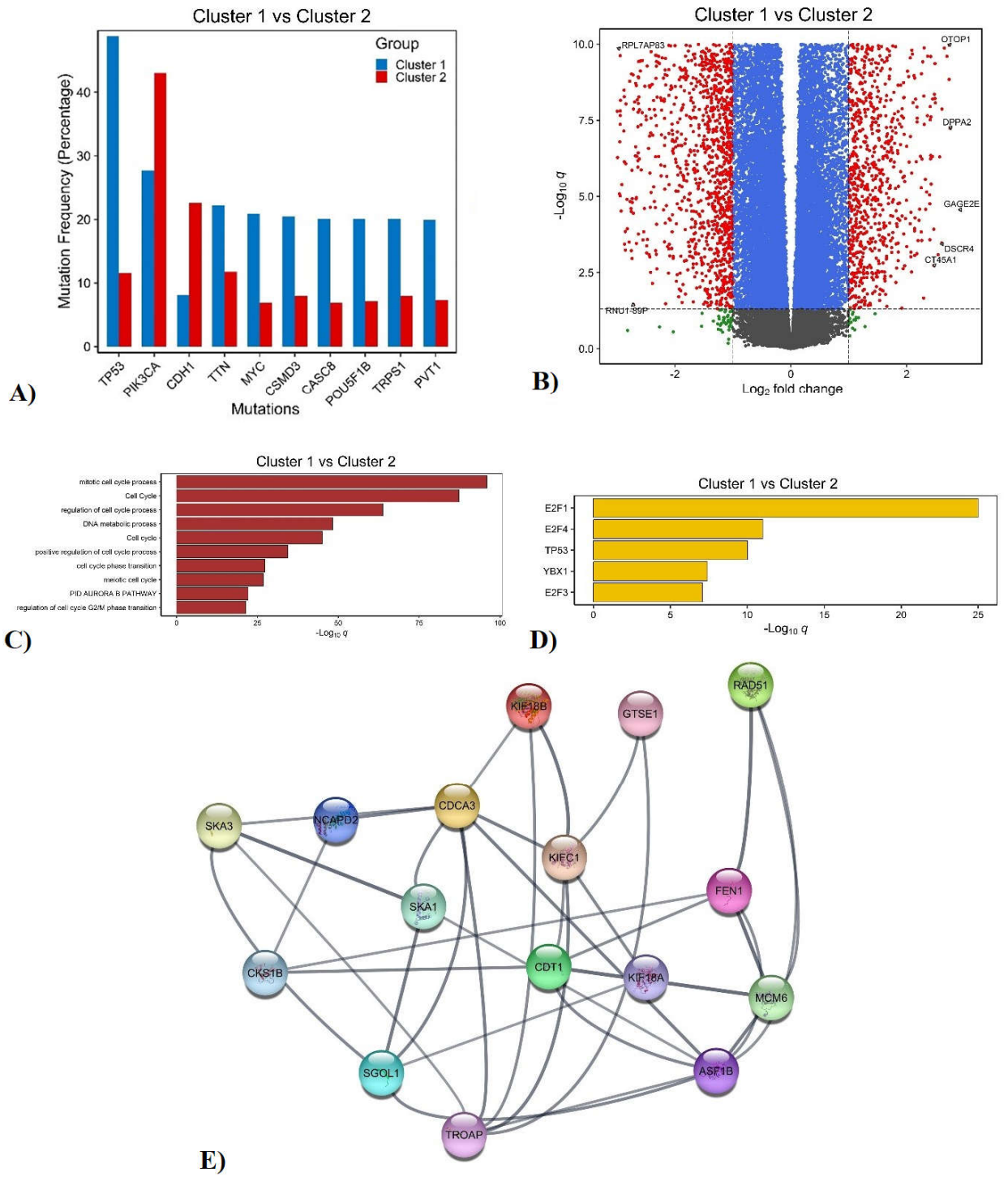

Fig. 2. Genomics and transcriptomics comparison of the two clusters of samples with inherent differences in their DNA damage response (DDR): A) Comparison of the mutations between the two clusters reveals statistically significant differences in the frequency of mutations in genes such as *TP53*, *MYC*, and *TRPS1* in Cluster 1; B) The volcano plot of the differential expression of the genes between the identified clusters; C) The enrichment analysis of the top 200 genes differentially expressed genes between the clusters shows very significant differences in pathways related to the cell cycle and DNA metabolic process between the two clusters; D) Querying the TRRUST database, it was revealed that the differentially expressed genes are regulated by transcription factors such as *E2F1*, *E2F4*, and *TP53*; E) The network analysis of the top 200 differentially expressed genes identified a significantly connected subnetwork of molecules central to the DDR. These groups of proteins represent candidate targets for therapeutic interventions.

MYC is another transcription factor with a critical role in cellular development (Hurlin, 2013). It is evident from the observations indicating that MYC expression is dysregulated in about 70% of tumors that this transcription factor has a pivotal role in regulating cancer cells (Madden *et al*., 2021). MYC is a critical element in tumorigenesis and tumor maintenance. It is known that many hallmarks of cancer require MYC activation and its activity is essential for tumor suppression evasion (Gabay *et al*., 2014). From the perspective of DDR, MYC has a paradoxical role. On the one hand, MYC is induced as a result of DNA damage and exhibits a tumor-suppressive role. On the other hand, it reduces replication stress and thus is an essential factor in the survival of cancer cells (Campaner and Amati, 2012). In the case of breast cancer, MYC becomes especially important. Regularly, *BRCA1*, one of the most important genes mutated in breast cancer, inhibits the transcription of *MYC* (Xu *et al*., 2010). However, *MYC* amplification is significantly higher in *BRCA1*-mutated samples (Brambillasca *et al*., 2016). This trade-off becomes vastly important when one takes into account that BRCA1 is an essential component of DDR (Mylavarapu *et al*., 2018). Currently, diagnostic approaches that lead to the selection of PARPi drugs are based on the presence of deleterious mutations in *BRCA1* and/or other essential members of DDR in breast cancer patients (Tung and Garber, 2022). The current study indicates that there is a difference in *MYC* mutation enrichment when samples are clustered based on DNA damage-associated genes. This is especially important to consider when addressing questions currently faced in the clinics, such as the challenge of rapid treatment resistance in patients treated with PARPi drugs or the lack of response to these drugs in patients with a *BRCA1* mutation (Pham *et al*., 2021).

TRPS1 expression has been observed to be high in all four types of breast cancer (Ai *et al*., 2021). This observation has prompted the possibility of utilization of this protein as a diagnostic marker in breast cancer patients. Interestingly, TRPS1 is associated with treatment resistance due to its regulatory role on the *MGMT* gene (Liu *et al*., 2018). MGMT is involved in DDR through a DNA damage reversal phenomenon (Bai *et al*., 2023).

Conclusion

Overall, in this study, through the implementation of transcriptomics analysis in bulk and single-cell RNA-sequencing datasets, we have been able to yield insights into the culprits involved in DDR mechanisms in breast cancer. The main limitation of the current study is the lack of further experimental validation. We have been able to identify a subnetwork possibly involved in the regulation of DDR in breast cancer patients. Hopefully, future studies will shed light on specific interactions between these molecules and explore the possibility of using the identified module and its specific members as treatment targets.

Author Contributions

Conceptualization, M.S., M.R.K. and A.H.K.; methodology, M.S., M.R.K. and A.H.K.; software, M.S., M.R.K. and A.H.K.; validation, M.R.K. and A.H.K.; formal analysis, M.S., M.R.K. and A.H.K.; investigation, M.S., M.R.K. and A.H.K.; resources, M.R.K. and A.H.K.; data curation, M.S., M.R.K. and A.H.K.; writing—original draft preparation, M.R.K., A.H.K. and M.S.; writing—review and editing, M.S., M.R.K. and A.H.K..; visualization, M.S., M.R.K. and A.H.K.; supervision, M.S.; project administration, M.S.; funding acquisition, M.S.. All authors have read and agreed to the published version of the manuscript.

Data availability statement

All of the datasets used in this study are accessible from the GDC data portal (https://portal.gdc.cancer.gov/) and the Gene Expression Omnibus database (under the accession GSE176078).

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Conflict of Interest

The authors declare no conflict of interest.

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