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Prognostic and immune correlation of IDO1 promoter methylation in breast cancer

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Indoleamine 2,3-dioxygenase 1 (IDO1) plays an important role in the initiation and progression of breast cancer. DNA promoter methylation status has the potential to be used as a biomarker for predicting the response to immunotherapy. This study aimed to investigate the biological and clinical significance of IDO1 promoter methylation in breast cancer. We analyzed IDO1 promoter methylation and its relationship with survival, patient prognosis, immune cell infiltration, immune-related pathways, and the expression of key immunomodulators via bioinformatics methods in The Cancer Genome Atlas (TCGA) breast cancer cohort (779 samples). Furthermore, the IDO1 promoter methylation status and expression of the IDO1 gene in the basal subtype of breast cancer were investigated in vitro via a methylation-specific PCR (MSP) assay and quantitative polymerase chain reaction (qPCR). The IDO1 promoter was significantly hypomethylated in the basal subtype of breast cancer tissues compared with normal adjacent tissues, and this effect was correlated with high expression of IDO1, resulting in abundant immune cell infiltration, activation of immune-related pathways, and upregulation of key immunomodulators. The influence of IDO1 promoter hypomethylation on the prognosis of patients with breast cancer was also investigated. The promoter hypomethylation of IDO1 in the basal subtype of breast cancer and its correlation with high expression of IDO1 were also investigated in vitro. Our results showed that IDO1 promoter methylation is vital for regulating its expression, which leads to the development of a tumor microenvironment in breast cancer. IDO1 promoter methylation and expression are associated with prognosis, immune cell infiltration, immune-related pathways, and immunomodulator expression in breast cancer. Our findings provide evidence for the validation of IDO1 promoter methylation as a promising biomarker to predict responses to immune checkpoint inhibitors in patients with breast cancer.

Keywords DNA methylation, Epigenetics, Biomarker, IDO1, Breast cancer, Tumor immune microenvironment

Abbreviations

IDO1	Indoleamine 2,3-dioxygenase 1
TCGA	The Cancer Genome Atlas
CPTAC	Clinical Proteome Tumor Analysis Consortium
HER2	Human epidermal growth factor receptor 2
CTLA4	Cytotoxic T-lymphocyte antigen 4
LAG3	Lymphocyte-activation gene 3
PD-1	Programmed cell death protein 1
PD-L1	Programmed cell death ligand 1
TIL	Tumor-infiltrating lymphocytes
L-Trp	L-tryptophan
DCs	Dendritic cells
Treg	Regulatory T cell
L-Kyn	L-kynurenine

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AhR	Aryl hydrocarbon receptor
breast cancer	Breast cancer
APC	Antigen-presenting cells
PD-L2	PD-L1 ligand 2
TIGHT	T cell immunoglobulin and ITIM domain
TNFRSF9	Tumor necrosis factor receptor superfamily member 9

Breast cancer accounts for 31% of all newly diagnosed cancer cases among women in the United States, according to estimates from cancer statistics in 2022[1], and it is the most prevalent type of cancer in women. Breast cancer is a diverse disease, and on the basis of the gene expression profile of tumor cells, it can be categorized into luminal A, luminal B, HER2-positive (nonluminal), and basal-like intrinsic molecular subtypes. In clinical practice, surrogate subtypes obtained through immunohistochemistry are often utilized. Notably, most triple-negative subtype tumors overlap with basal-like subtype tumors[2,3]. Among all breast cancer subtypes, triple-negative breast cancer, especially in its metastatic stage, has the lowest survival rate[4]. Unfortunately, the efficacy of chemotherapy in treating metastatic triple-negative breast cancer is limited[5]. Therefore, there is an urgent need for more effective treatments. One of the most extensively studied approaches for breast cancer immunotherapy involves immune checkpoint inhibitors, such as CTLA-4, PD-1, and PD-L1 antagonists. Current research on CTLA-4 antagonists has focused primarily on triple-negative breast cancer[6]. PD-1 or PD-L1 antagonists have shown promising and enduring therapeutic effects against metastatic triple-negative breast cancer, particularly in PD-1 or PD-L1 positive breast cancer patients[7]. Moreover, immune checkpoint inhibitors also exhibit potential therapeutic effects on other subtypes of breast cancer[8,9]. To optimize the selection of patients who respond well to immunotherapy, researchers are investigating potential biomarkers, such as PD-L1 expression and tumor-infiltrating lymphocytes (TILs)[10]. The identification of additional biomarkers capable of predicting the response to immunotherapy will significantly contribute to the advancement of immunotherapy for breast cancer.

Indoleamine 2,3-dioxygenase 1 (IDO1) plays a crucial role in the initial rate-limiting step of L-tryptophan (L-Trp) degradation via the kynurenine pathway. In 1998, it was discovered that IDO1 is responsible for maintaining the embryo's inhibitory effect on T cell activity and preventing maternal immune rejection of the embryo[11]. When dendritic cells (DCs) produce IDO1, it leads to the inhibition of effector T cell activity and the enhancement of regulatory T cell (Treg) activity, creating an immune microenvironment with inhibitory properties[12]. Additionally, IDO1 catalyzes the conversion of L-Trp to L-kynurenine (L-Kyn). L-Kyn activates aryl hydrocarbon receptor (AhR), which, in turn, promotes the expression of IDO1 in DCs[12]. Apart from its role in embryonic immune tolerance, IDO1 also allows tumor cells to evade T cell-mediated rejection[13]. As a result, the IDO1-Kyn-AhR pathway has emerged as a potential therapeutic target for cancer treatment. IDO1 inhibitors have shown promise in treating various types of cancer, including glioma, colon cancer, pancreatic cancer, and breast cancer[14–17]. While the effectiveness of combination therapy requires further demonstration, clinical trials investigating IDO1 inhibitors in combination with immune checkpoint inhibitors for the treatment of malignant tumors, including breast cancer, are underway[18,19]. Such research holds potential for advancing cancer therapies and improving patient outcomes.

During cancer development, DNA methylation modification plays a significant role, leading to the demethylation of many regions of the genome and the hypermethylation of CpG islands in tumor suppressor gene promoters[20]. In addition to its impact on tumor cells, epigenetic regulation, such as methylation, influences the function of cancer immune cells, thus exerting a crucial influence on the immune microenvironment[21]. Promoter methylation directly affects the expression of immune checkpoints and their ligands. Combining epigenetic modifiers with immune checkpoint inhibitors may prove beneficial in restoring a more physiologically balanced immune microenvironment and thereby reestablishing an effective response of the immune system against cancer cells[22]. This approach may address the issue of immunotherapy resistance in breast cancer. Additionally, DNA methylation status can be used as a biomarker to predict the response of patients with cancer to immune checkpoint inhibitors[23]. By identifying patients likely to respond favorably to immunotherapy, this biomarker can aid in tailoring treatment strategies and improving patient outcomes.

While IDO1 inhibitors have demonstrated promise in breast cancer treatment, further investigations into the relationship between IDO1 promoter methylation and the tumor microenvironment across various breast cancer subtypes are needed. The aim of this study was to clarify the associations among IDO1 promoter methylation, prognosis, and the immune microenvironment in different subtypes of breast cancer.

Materials and methods

Data and resources

TCGA cohort

Data on invasive breast cancer gene expression, methylation, and related clinical characteristics were obtained from The Cancer Genome Atlas (TCGA, http://cancergenome.nih.gov/). The Infinium HumanMethylation450 BeadChip data were processed via the 'ChAMP' R package. The clinicopathological data of the patients are presented in Table S1.

Methylation analysis

The methylation data for TCGA breast invasive carcinoma cohort were acquired from TCGA database. The beta value was considered to be approximately equivalent to the percent methylation. The 'limma' R package was used to conduct differential analysis of methylation sites, with thresholds of log (fold change) > 0.05 and adjusted P value < 0.05.

Gene set variation analysis (GSVA)

To analyze immune cell infiltration and biological pathways via GSVA enrichment, the 'GSVA' R package was used. GSVA estimates variation in pathway activity across a sample population in a nonparametric, unsupervised manner. GSVA uses an enrichment scoring algorithm that bypasses the traditional approach of explicitly modeling phenotypes[24]. Gene sets representing other biological processes were obtained from the study by Powles[25].

Estimation of tumor microenvironment immune cell infiltration

To quantify immune cell infiltration into the tumor microenvironment, an algorithm known as single-sample gene set enrichment analysis (ssGSEA) was used. A gene set corresponding to immune cell types was acquired from the study by Zhou[26] (Table S3). ssGSEA provides enrichment scores that represent the relative abundance of specific immune cells infiltrating the tumor microenvironment. Table S4 provides additional information on the relative abundance of cells in the tumor microenvironment in each TCGA sample. The immune cell infiltration status was assessed in all the tumors and in the tumors of the different subtypes.

Correlations between IDO1 expression/promoter methylation and immune-related pathways

For analysis of the correlation between IDO1 promoter methylation and expression, a total of 779 breast cancer samples with DNA methylation and transcriptome data from TCGA were used. Previous studies have constructed a gene set panel consisting of genes involved in various biological processes relevant to the tumor immune microenvironment[25–28] (Table S5). Table S6 presents supplementary information for the relative pathway enrichment scores for each sample in TCGA cohort. In addition to measuring enrichment scores for each biological process in all tumors, enrichment scores were assessed for tumors of the different subtypes.

Cell culture

The SUM-159PT, MDA-MB-231, HCC 549, HCC 1806, JIMT-1, MCF-7, T47D, and ZR75 cell lines were purchased from Pricella, Wuhan, China. The cell lines were cultured at 37 °C in a 5% CO_2 atmosphere. SUM-159PT, MDA-MB-231, HCC 549, HCC 1806, and JIMT-1 cells were grown in Dulbecco's modified Eagle's medium (Cat# PM150210, Pricella, Wuhan, China). T47D and ZR75 cells were cultured in RPMI-1640 medium (Cat# PM150110; Pricella, Wuhan, China). MCF-7 cells were cultured in minimum essential medium (Cat# PM150411B; Pricella, Wuhan, China). All media were supplemented with 10% fetal bovine serum (Pricella, Wuhan, China) and 1% streptomycin and penicillin (Cat# PB180120; Pricella, Wuhan, China).

All the cell lines were authenticated in the past 3 years. The identity of the cells was confirmed via short tandem repeat (STR) analysis before the experiment. Mycoplasma was detected via a Mycoplasma Stain Assay Kit (Cat# MA0344; MeilunBio, China).

DNA and RNA extraction

Genomic DNA was extracted from the cell lines with a Blood/Cultured Cells DNA Kit (Cat# 3002050, Simgen, Hangzhou, China). Total RNA was extracted with an RNA-Quick Purification Kit (Cat# RN001-50 Rxns; Shanghai Yishan Biotechnology, China). The concentrations of the DNA and RNA samples were measured via spectrophotometry (Tnano-700, Tuohe, Shanghai, China). All the samples were stored at − 80 °C.

Quantitative polymerase chain reaction

The RNA was reverse transcribed with Hifair III 1st Strand cDNA Synthesis SuperMix for qPCR (Cat# 11137ES10; Yeasen, China). Quantitative polymerase chain reaction (qPCR) was performed with a Hieff qPCR SYBR Green PCR Master Mix kit (Cat# 11202ES50, Yeasen, China) on a real-time PCR system (Gentier 96R, Tianlong, Xi'an, China). The relative expression of IDO1 was estimated via the 2(− Ct) method, and all the assays were performed in triplicate. GAPDH was used as a loading control. The primers are shown in Supplementary Table 7.

Methylation-specific PCR

A methylation-specific PCR (MSP) assay was performed with bisulfite-converted DNA. Bisulfite conversion was performed with an EZ DNA Methylation Lightening Kit (Cat# D5030, Zymo Research, USA). Completely methylated (methylated gDNA) or completely unmethylated (unmethylated gDNA) genomic DNA was used as the samples. *ZymoTaq*™ DNA Polymerase (Cat# E2001, Zymo Research, USA) was used for PCR. The primers are shown in Supplementary Table 7. The PCR conditions were 10 min at 95 °C and then 35 cycles of 30 s at 95 °C, 35 s at 50 °C, and 45 s at 72 °C. 6 × DNA loading buffer (Cat# CW0610S, CWBio, China) was added to the PCR products, which were then visualized under UV illumination (ChampChemi610, Saizhi, Beijing, China). A 100-bp ladder was obtained from CWBio (Cat# CW0636S, CWBio, China).

Statistics

Various statistical software packages, including SPSS version 23.0, GraphPad Prism version 8, and R version 4.0.3, were used for the analyses. The correlation coefficients were calculated via Spearman's rank correlation coefficient. Differences between two groups were compared via either the t test or the Mann–Whitney test. For comparisons of multiple groups, one-way ANOVA was used to analyze the differences. The 'survminer' R package was employed to determine the optimal cutoff values for survival analysis. In invasive breast carcinoma, the correlation among IDO1, its promoter methylation, and the overall survival of patients was analyzed via Kaplan–Meier survival analysis. Two-tailed statistical analyses were used in all analyses, and a P value of less than 0.05 was considered statistically significant. The significance levels are denoted as follows: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

Results

The IDO1 promoter is abnormally methylated in breast cancer

IDO1 can be carcinogenic or tumor suppressive, depending on the type of cancer involved. To explore the function of IDO1 in breast cancer, an analysis using TCGA database was conducted to examine the IDO1 mRNA expression levels across various subtypes of breast cancer. The IDO1 mRNA expression was greater in the basal subtype (Fig. 1A). A similar analysis using the CPTAC database revealed higher IDO1 protein expression in both the basal and HER2 subtypes than in the luminal subtype (Fig. 1B). Further survival analysis indicated that HER2-subtype patients with high IDO1 expression had a better prognosis, while there was no significant difference in survival between patients with high IDO1 expression and those with low IDO1 expression in the basal and luminal subtypes (Fig. 1C–F). An increasing number of studies have indicated that DNA promoter methylation is critical for tumor grading and patient prognosis. The UCSC Xena browser (http://xena.ucsc.edu) was used to download methylation data from TCGA breast cancer cohort (Infinium HumanMethylation450 BeadChip). The Infinium HumanMethylation450 BeadChip annotation indicated that 3 probes are located in the IDO1 gene, with cg10262052 positioned in TSS1500, while cg08465774 and cg24188163 are located in the gene body of IDO1. Subtype analysis was conducted, which revealed that the methylation levels of the cg08465774 and cg10262052 sites were significantly lower in the basal subtype than in the HER2 and luminal subtypes. Conversely, the methylation level of the cg24188163 site was greater in the basal subtype (Fig. 2A–C). Additionally, there were no significant differences in the methylation levels of these three sites among the different clinical stages (Fig. 2D–F). These results indicated that the IDO1 promoter is abnormally hypomethylated in the basal subtype of breast cancer.

IDO1 methylation predicts the prognosis of breast cancer patients

To elucidate the prognostic potential of IDO1 methylation in breast cancer, the correlations between methylation at three CpG sites and overall survival and disease-free survival were analyzed. The hypomethylation of cg10262052 was indicative of poor prognosis in patients with breast cancer (including all subtypes). Specifically, in the basal subtype, hypomethylation of this site suggested a favorable prognosis. In the luminal subtype, decreased methylation of this site was associated with better OS and worse DFS. However, in the HER2 subtype, the methylation level of this site was not significantly correlated with prognosis (Fig. 3A). In addition, the methylation status of the cg08465774 site exhibited no discernible correlation with the prognosis of patients with breast cancer (Fig. 3B). The hypomethylation of the cg24188163 site was associated with worse DFS in patients with breast cancer, and this association was also confirmed within the luminal subtype (Fig. 3C). These findings indicated that promoter methylation may serve as a potential prognostic biomarker in breast cancer; however, the predictive value of IDO1 promoter methylation needs to be explored in a subtype-dependent manner.

IDO1 methylation correlates with IDO1 expression in breast cancer

Gene promoter methylation plays a pivotal role in the regulation of gene transcription. To elucidate the influence of IDO1 methylation on IDO1 expression, the correlation between three distinct methylation sites and IDO1 expression was evaluated. The cg10262052 and cg08465774 sites were negatively correlated with the expression of IDO1, whereas the cg24188163 site was positively correlated with IDO1 expression. Furthermore, these findings remained consistent across different subtypes of breast cancer (Fig. 4). The above results showed that hypomethylation at the IDO1 promoter in breast cancer may be the primary cause of upregulated IDO1 expression.

IDO1 expression and promoter methylation are correlated with immune cell infiltration in breast cancer

Because previous studies have shown that IDO1 significantly influences the function and regulation of immune cells[12,13,17], we hypothesized there may be a correlation between IDO1 expression and the infiltration of immune cells within the tumor microenvironment of breast cancer. Therefore, we examined the RNA-seq signatures of 23 distinct immune cell types and their associations with IDO1 expression and promoter methylation. Our r findings demonstrate a positive correlation between increased IDO1 expression and increased infiltration of immune cells, as well as hypermethylation of the cg24188163 site. Conversely, the cg10262052 and cg08465774 sites were negatively correlated with immune cell infiltration (Fig. 5A). Specifically, high expression of IDO1 was significantly associated with increased infiltration of immune cells (Fig. 5B). Hypermethylation at the cg24188163 site was consistent with the relationship between elevated IDO1 expression and increased infiltration of immune cells. In contrast, hypermethylation at the cg10262052 and cg08465774 sites exhibited the opposite pattern (Fig. 5C–E). We also obtained similar results in the analysis of the breast cancer subtypes (Fig. S1A–C). Although further in vitro and in vivo experiments are needed, these findings suggested a positive correlation between elevated IDO1 expression and promoter hypomethylation with immune cell infiltration.

IDO1 expression and promoter methylation are correlated with immune-related pathways in breast cancer

To explore the impact of IDO1 promoter methylation and its expression on immune-related pathways in breast cancer, we analyzed their associations with 17 distinct biological pathways. Our findings revealed elevated IDO1 expression was correlated with increased scores in several pathways, including the CD8+T effector, antigen processing machinery, cytolytic activity, and MHC-HLA signature (Fig. 6A). These pathways are indicative of a rich infiltration of immune cells in the tumor microenvironment. Moreover, high IDO1 expression and signatures related to coinhibition of APCs and T cells were significantly associated with increased immune checkpoint activity (Fig. 6B). These findings demonstrated that tumors exhibiting high levels of IDO1 expression displayed significant infiltration by immune cells while simultaneously exhibiting heightened immune

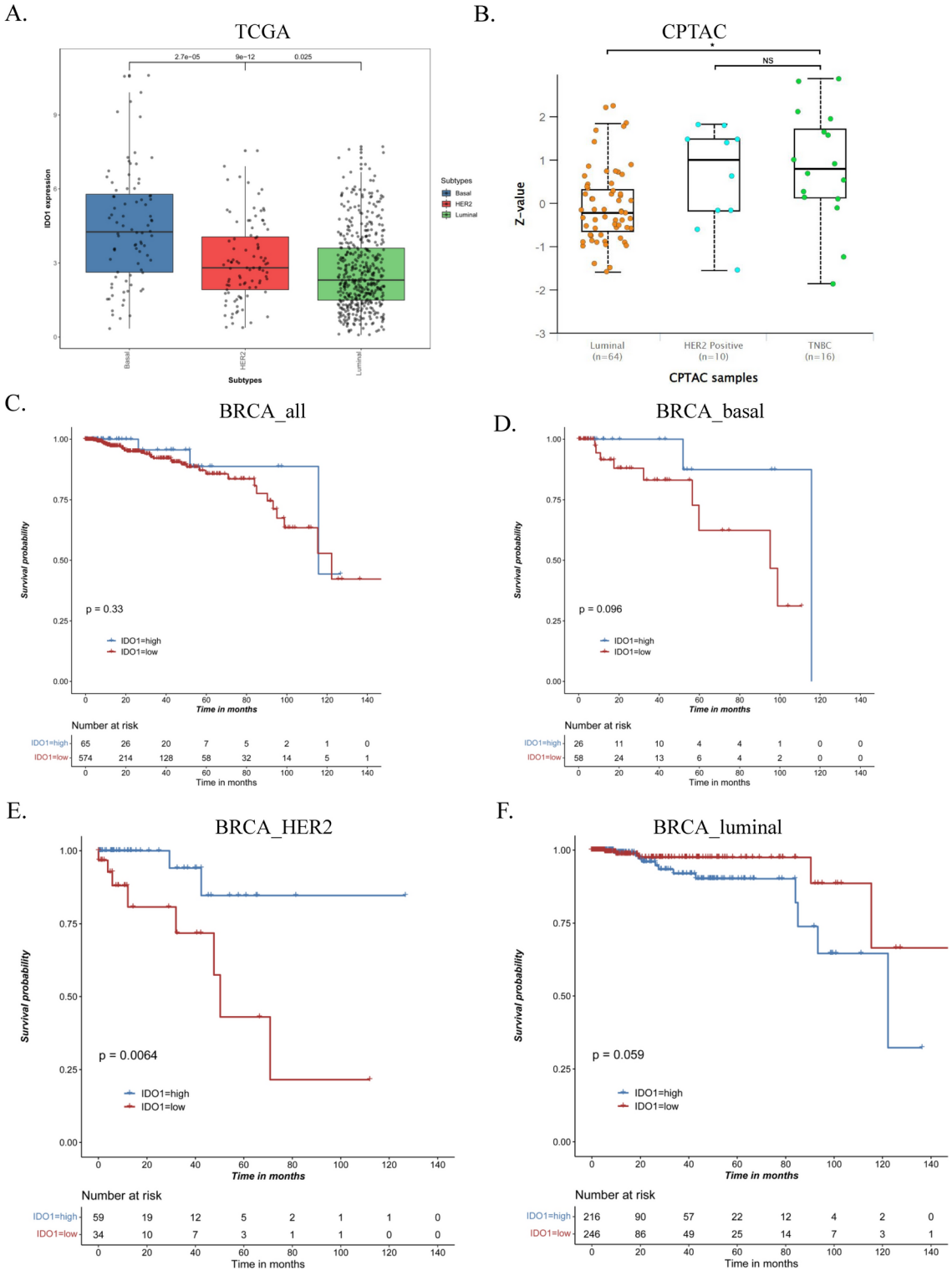

Fig. 1. The expression of IDO1 varies among different subtypes of breast cancer. (**A**) Relative mRNA expression of IDO1 between different subtypes of breast cancer using the data from TCGA cohort; (**B**) relative expression of IDO1 between different subtypes of breast cancer using the data from CPTAC cohort; (**C**–**F**) Kaplan–Meier survival analysis of IDO1 expression for overall survival of patients from TCGA breast cancer cohort.

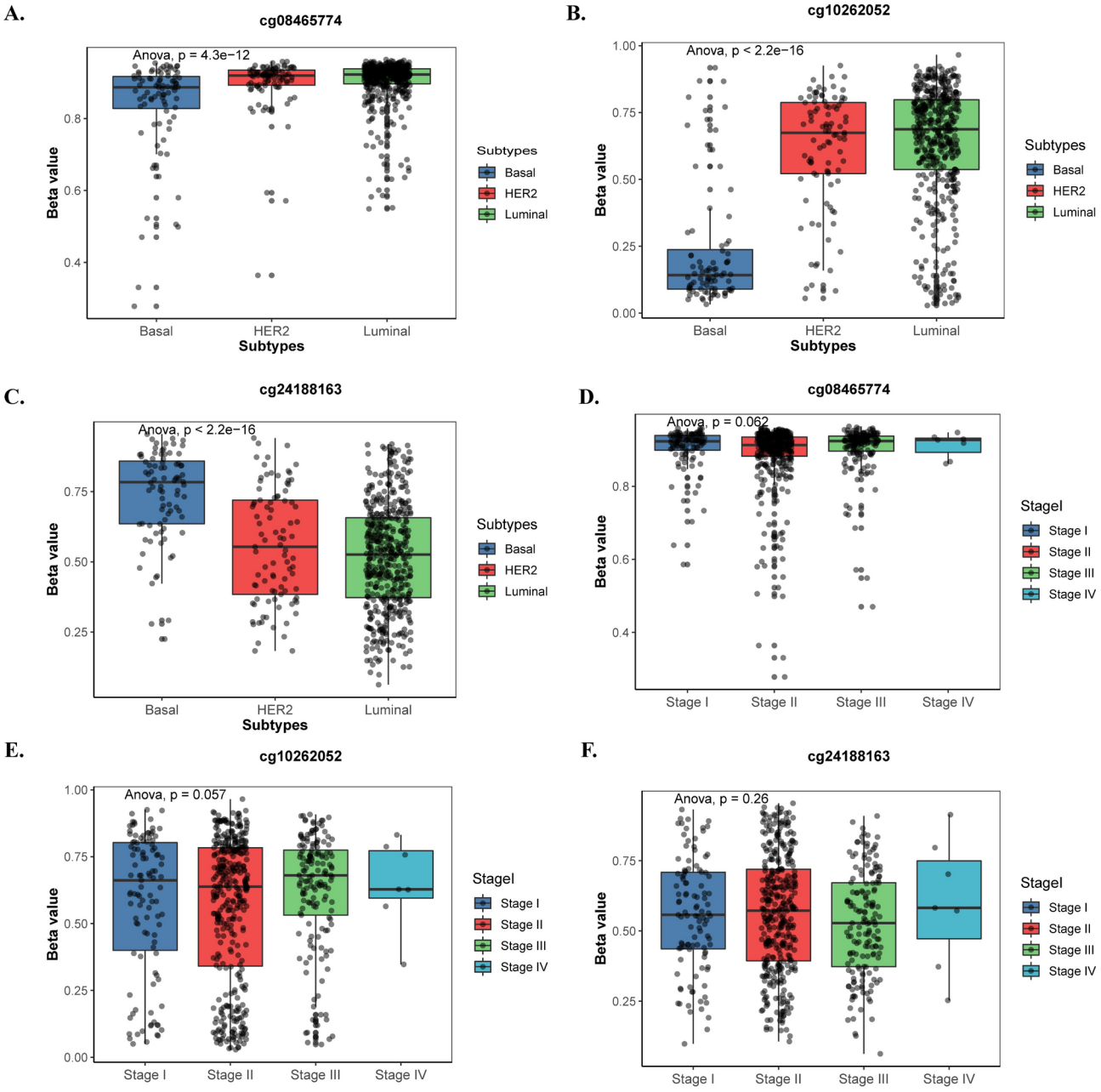

Fig. 2. IDO1 promoter is abnormally methylated in breast cancer. (**A**) Relative methylation of the three differentially methylated CpG sites (cg10262052, cg08465774 and cg24188163) located in IDO1 gene between tumor and adjacent normal tissues of TCGA cohort; (**B**–**G**) relative expression of methylation of cg10262052, cg08465774, and cg24188163 in different breast cancer subtypes and stages of patients from TCGA cohort.

checkpoint signatures, thereby contributing to the establishment of an immunosuppressive microenvironment. We subsequently investigated the relationships between IDO1 methylation and 17 immune-related pathways. Hypomethylation at the cg10262052 and cg08465774 sites correlated with elevated scores for CD8+ T effector cells, antigen processing machinery, cytolytic activity, and the MHC HLA signature, as well as increased immune checkpoint activity and coinhibitory signatures for APCs and T cells, whereas cg24188163 showed the opposite pattern (Fig. 6C–E). Moreover, analogous findings were obtained when analyzing the breast cancer subtypes (Fig. S2A–C). These findings indicated a positive correlation among elevated IDO1 expression, promoter hypomethylation, and the activation of immune-related pathways, suggesting the presence of immunoreactive "hot tumors". Moreover, these "hot tumors" exhibited significant enrichment of immune checkpoint molecules, making them potential candidates for immune checkpoint inhibitor therapy. Hence, IDO1 and its promoter methylation may serve as molecular biomarkers to predict the efficacy of immunotherapy.

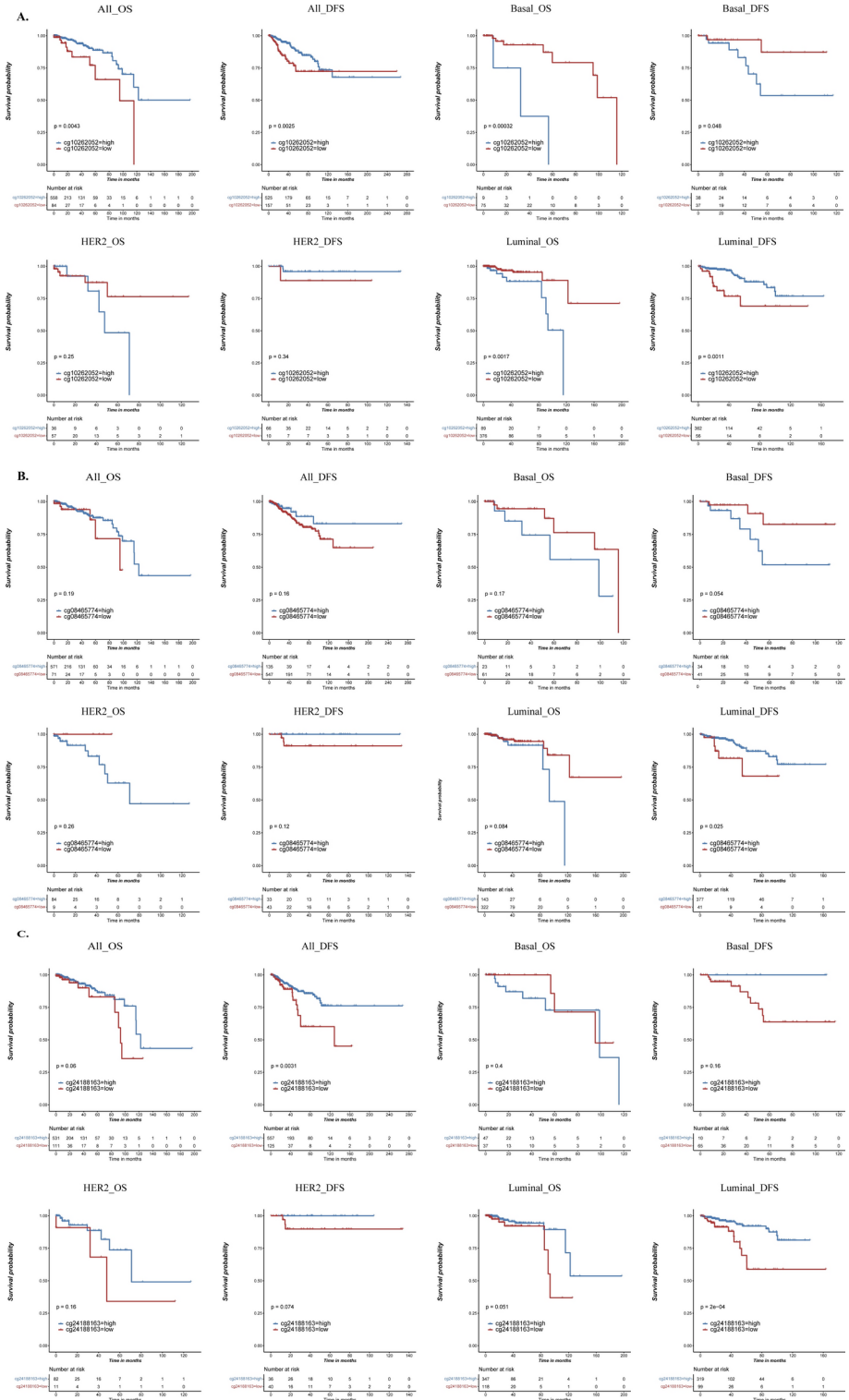

Fig. 3. IDO1 methylation predict the prognosis in breast cancer. (**A**–**C**) Kaplan–Meier survival analysis of cg10262052, cg08465774, and cg24188163 for overall survival and disease-free survival of patients from TCGA breast cancer cohort.

IDO1 expression and promoter methylation are associated with the expression of key immunomodulators in breast cancer

The tumor immune microenvironment is intricately regulated by a diverse array of membrane proteins and cytokines. To investigate the correlations among IDO1 expression, methylation, and key molecules involved in antitumor immune responses, the expression of 74 pivotal immunomodulators was analyzed in

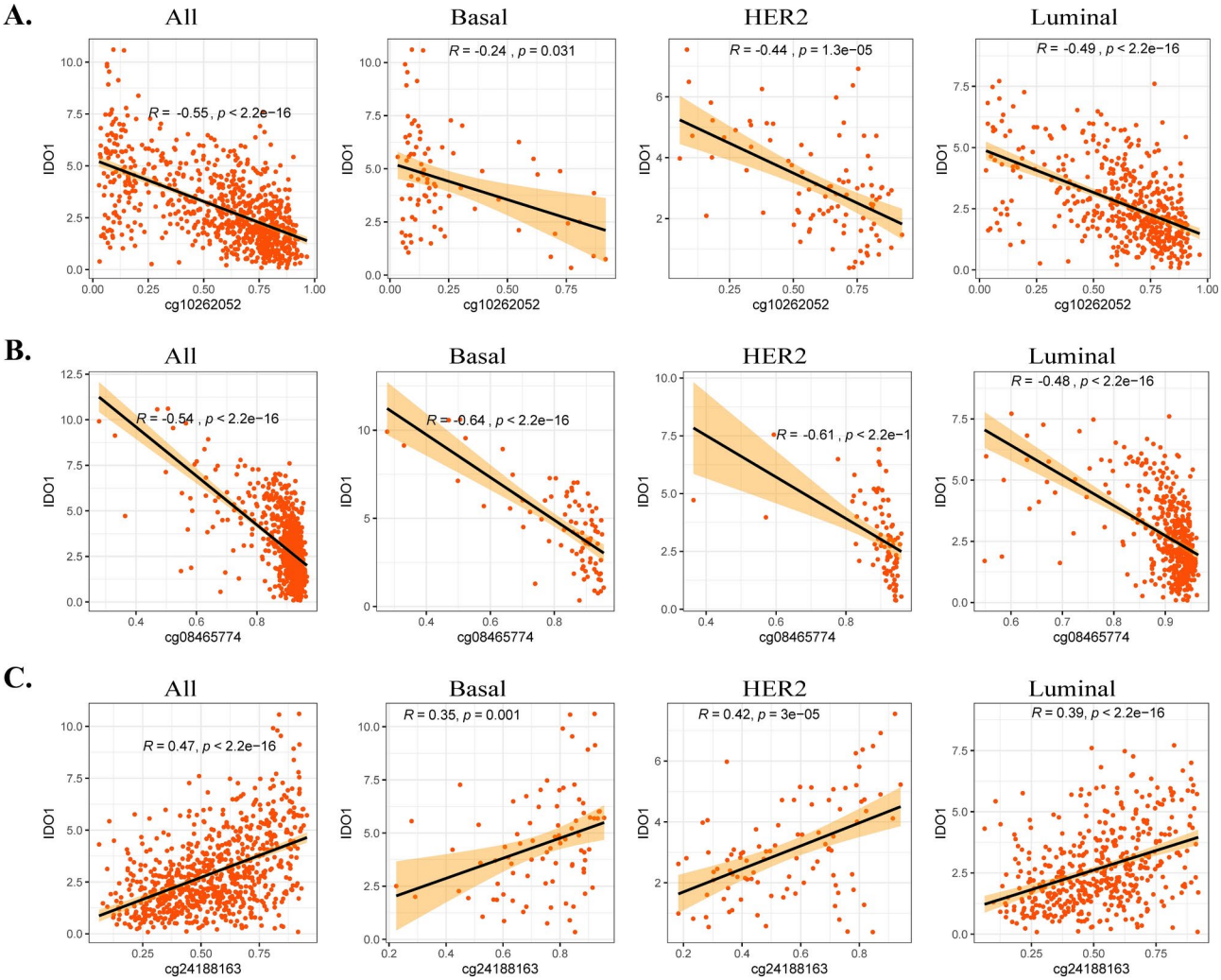

Fig. 4. The correlation of IDO1 methylation with IDO1 in breast cancer. (**A**–**C**) The correlation of cg10262052, cg08465774, and cg24188163 methylation with IDO1 expression in TCGA tumor tissues.

a cohort obtained from TCGA. Our study revealed correlations between high IDO1 expression, cg10262052 hypomethylation, cg08465774 hypomethylation, and cg24188163 hypermethylation with the expression of most of the key immunomodulators (Fig. 7A). Because of the potential efficacy of immune checkpoint inhibitors in different tumor types, we examined the correlation of IDO1 and its methylation at cg10262052/cg08465774/cg24188163 with immune checkpoint molecules, such as LAG3, PD1, PDL1, CTLA4, TIGIT, and PDL2. We found a significant positive correlation between IDO1 expression and the expression levels of these immune checkpoint molecules (Fig. 7B). Conversely, cg10262052/cg08465774 methylation was significantly negatively associated with the expression of these immune checkpoint molecules, whereas cg24188163 methylation was positively correlated with their expression (Fig. 7C–E). Additionally, comparable outcomes were obtained when examining the breast cancer subtypes (Fig. S3A–C). These results further supported the involvement of IDO1 and its promoter methylation in the immune suppressive tumor microenvironment of breast cancer.

IDO1 promoter hypomethylation and high expression in basal-subtype breast cancer in vitro

To further investigate the methylation status of IDO1 at cg10262052 and its correlation with IDO1 mRNA expression, quantitative polymerase chain reaction (qPCR) and methylation-specific PCR (MSP) were performed. qPCR results both with and without IFN-γ revealed high expression of IDO1 in SUM-159PT, HCC 1806, and JIMT-1 cells but low expression in T47D, MCF7, and ZR75 cells (Fig. 8A, B). HCC549 cells had high expression of IDO without IFN- but lower expression of IDO lower with IFN-γ. MDA-MB-231 cells expressed IDO at low levels without IFN-γ but expressed IDO at much higher levels with IFN-γ. These results demonstrated lower expression of IDO1 in cell lines corresponding to the basal subtype of breast cancer than in cell lines corresponding to the luminal subtype of breast cancer. The MSP results revealed that cg10262052 of IDO1 was relatively hypermethylated in MDA-MB-231, T47D and JIMT-1 cells, whereas it was relatively hypomethylated in MCF7, HCC 1806, and SUM-159PT cells (Fig. 8C–E). IDO1 hypomethylation in HCC549, HCC 1806, and SUM-159PT cells was consistent with high IDO1 expression in these cells. However, the MSP results for

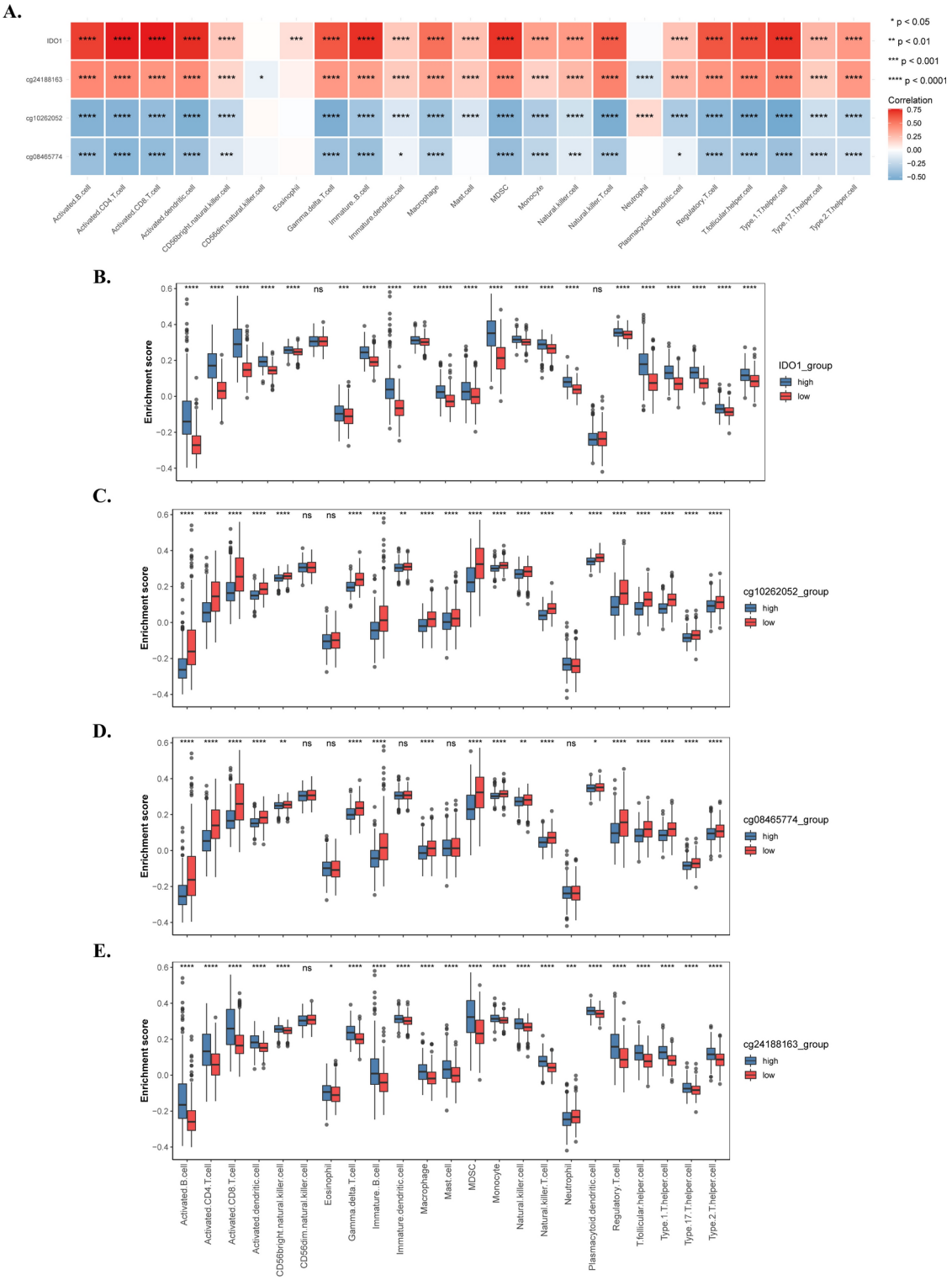

Fig. 5. IDO1 expression and methylation were correlated with immune cell infiltration in breast cancer. (**A**) The correlation heatmap of IDO1 expression and its differentially methylated sites with 23 types of immune cells in the TCGA breast cancer cohort, only statistically significant ($P < 0.05$) were shown in correlation coefficients. (**B**–**E**) Relative enrichment scores of 23 types of immune cells in the high and low groups according to the median of IDO1 expression and the methylation of cg10262052, cg08465774, and cg24188163, respectively.

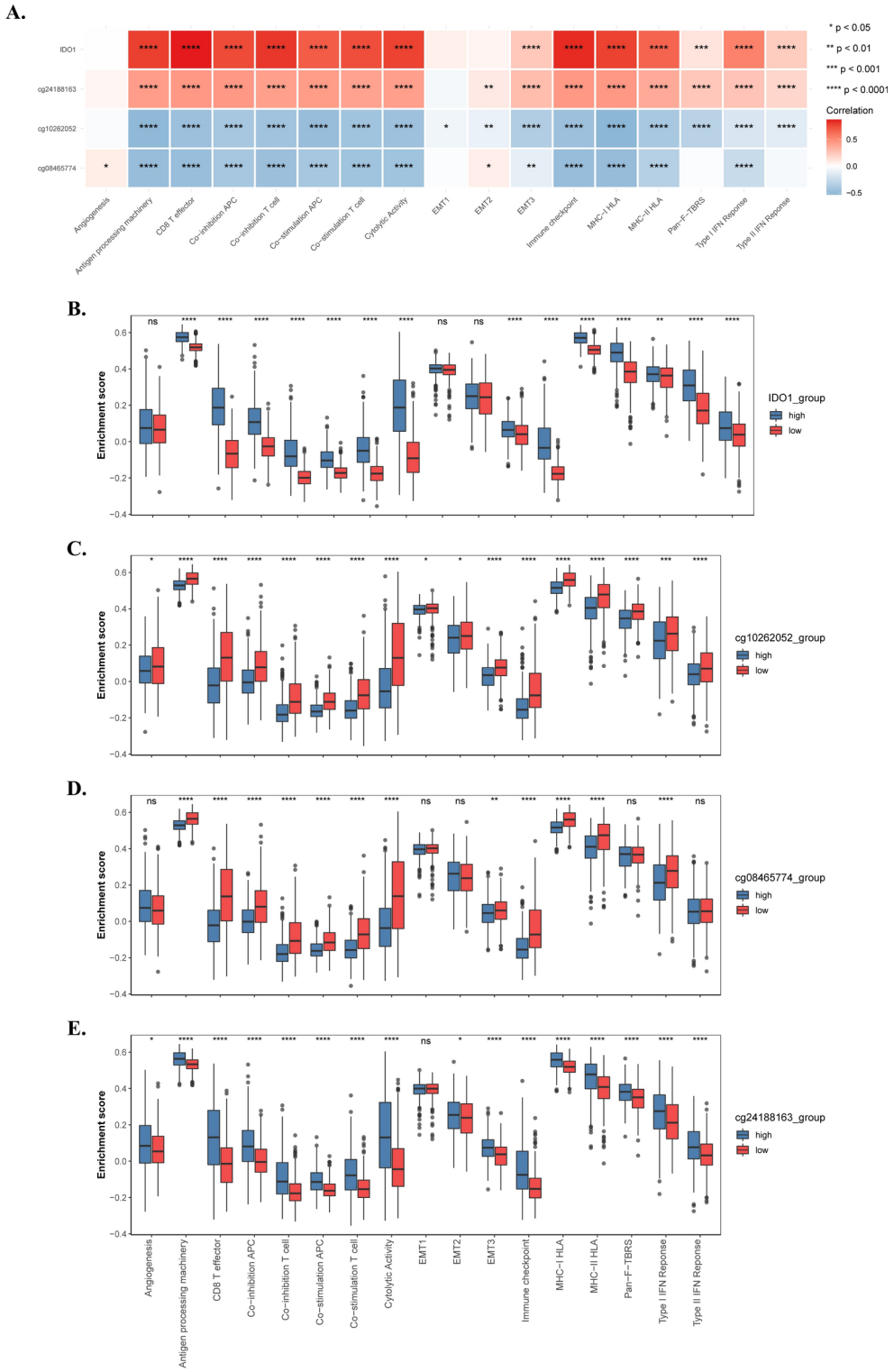

Fig. 6. IDO1 expression and methylation were correlated with immune-related pathways in breast cancer. (**A**) The correlation heatmap of IDO1 expression and its differentially methylated sites with 17 immune-related pathways in the TCGA breast cancer cohort, only statistically significant ($P < 0.05$) were shown in correlation coefficients. (**B**–**E**) Relative enrichment scores of 17 immune-related pathways in the high and low groups according to the median of IDO1 expression and the methylation of cg10262052, cg08465774, and cg24188163, respectively.

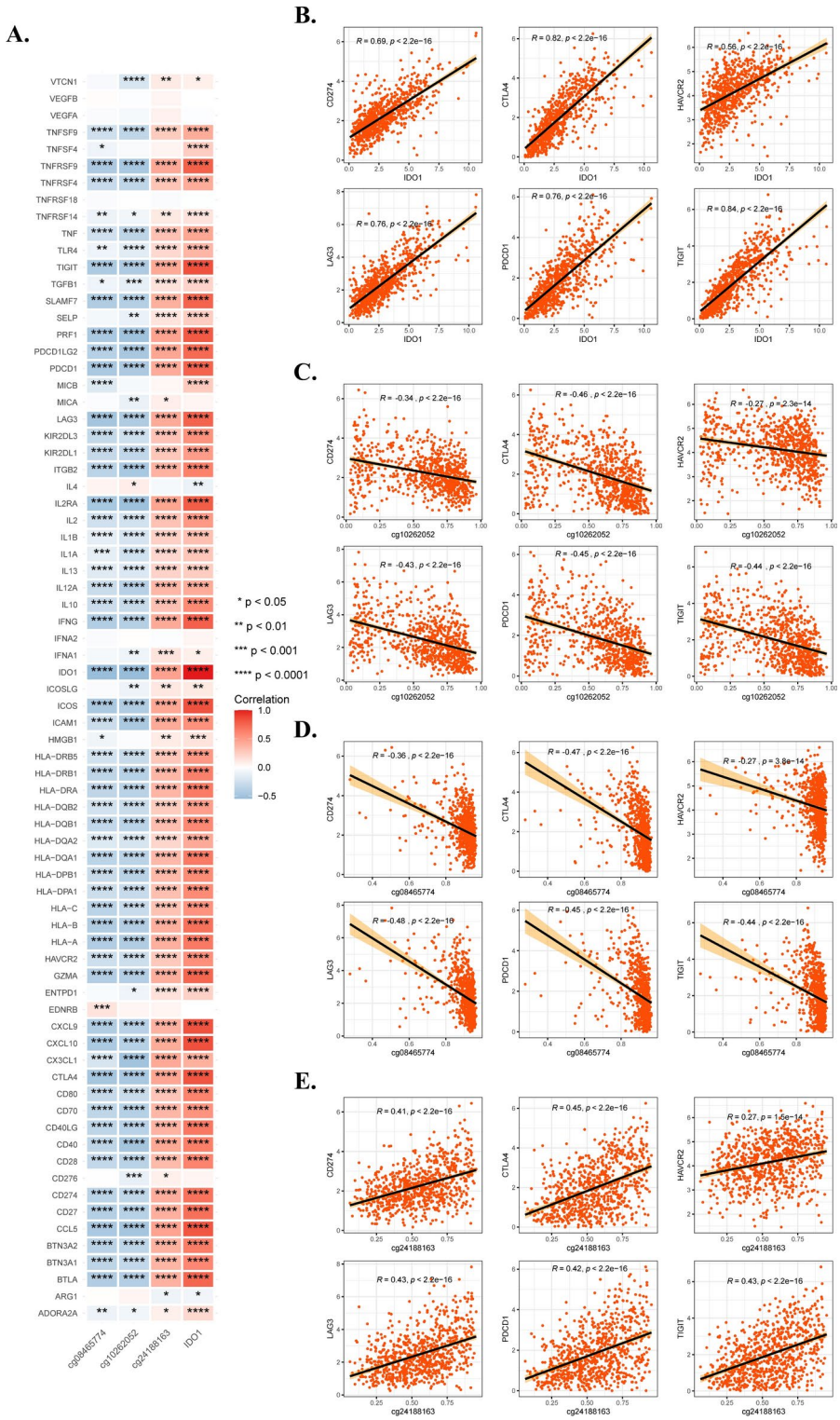

Fig. 7. IDO1 expression and its methylation were associated with the expression of the key immunomodulators in breast cancer. (**A**) The correlation heatmap of IDO1 expression and the three methylated CpG sites with the key immunomodulators in the TCGA breast cancer cohort, only statistically significant ($P < 0.05$) are shown correlation coefficients. (**B**–**E**) The correlation of IDO1 expression, cg10262052, cg08465774, and cg24188163 methylation with immune checkpoint molecules CD274, CTLA4, HAVCR2, LAG3, PACD1, TIGIT, respectively.

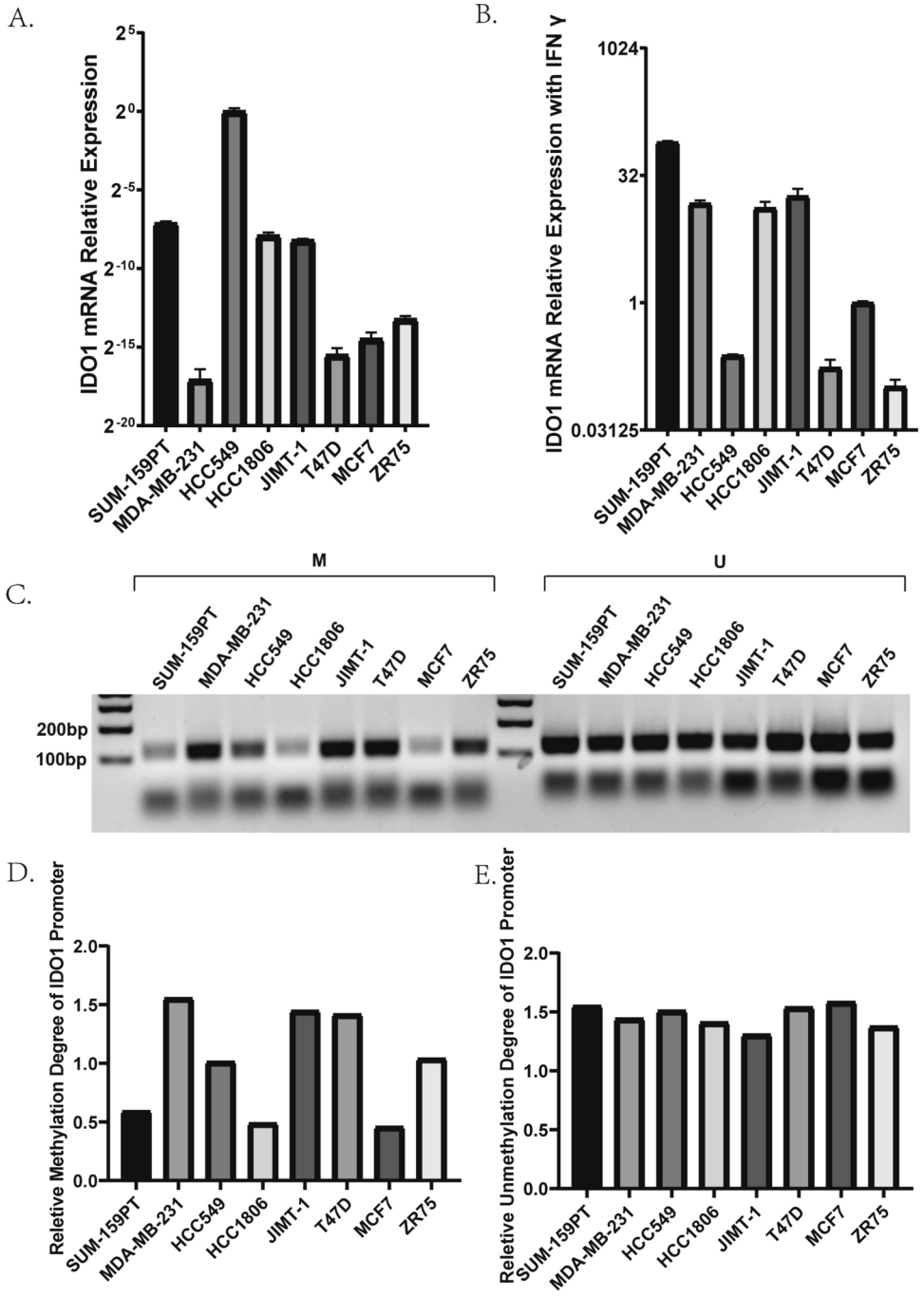

Fig. 8. IDO1 promoter methylation and relative expression in breast cancer in vitro. (**A**, **B**) The relative mRNA expression of IDO1, (**A**) without or (**B**) with IFNγ stimulation. (**C**–**E**) Methylation status of IDO1 promoter at cg10262052. Original blots/gels are presented in Fig. S4.

MDA-MB-231, MCF7 and JIMT-1 cells were not consistent with the mRNA expression results. Although there were some unexpected results, there was a low level of IDO1 promoter methylation and a high level of IDO1 mRNA expression in most of the breast cancer cell lines with the basal subtype, which was consistent with our previous analysis. However, the promoter methylation status and IDO1 expression in cell lines do not necessarily represent their status in patient tissues, and further experiments are needed to verify our results in different subtypes of breast cancer.

Discussion

Indoleamine 2,3-dioxygenase 1 (IDO1) is a heme enzyme responsible for the oxidation of L-tryptophan. Functionally, IDO1 plays a crucial role in facilitating cancer immune evasion by initiating kynurenine pathway catalysis[12]. Moreover, elevated IDO1 expression is linked to unfavorable outcomes in different types of

cancers[29–34]. IDO1 upregulation has also been reported to be associated with immune cell infiltration in breast cancer[35,36]. The present study demonstrated that IDO1 mRNA and protein expression levels were significantly greater in the basal subtype of breast cancer (Fig. 1A, B). Further survival analysis indicated that HER2-subtype patients with high IDO1 expression had a better prognosis, while there was no significant difference in survival between patients with high IDO1 expression and those with low IDO1 expression in the basal and luminal subtypes (Fig. 1C–F). The finding that HER2-subtype patients with high IDO1 expression had a better prognosis needs to be validated in external cohorts, and the intrinsic mechanism should be investigated in future studies.

Abnormalities in DNA methylation are important features in the occurrence and development of various cancerous diseases. DNA methylation participates in the regulation of the expression of numerous oncogenes and tumor suppressor genes, and it has become a promising biomarker for cancer diagnosis, prognostic analysis, and immunotherapy. Therefore, the role of IDO1 methylation was investigated in breast cancer. Among all three investigated methylation probes, only cg10262052 is located in the IDO1 promoter. Therefore, cg10262052 methylation was the main focus in the present study. Methylation was significantly lower in the basal subtype than in the HER2 and luminal subtypes (Fig. 2B). Further analysis revealed that cg10262052 methylation was negatively correlated with the expression of IDO1 (Fig. 4A). Thus, hypomethylation at the IDO1 promoter may play a significant role in the increased expression of IDO1 in the basal subtype of breast cancer. To further elucidate the predictive value of the IDO1 promoter, the associations of cg10262052 with overall survival and disease-free survival were analyzed, which revealed that the hypomethylation of cg10262052 was indicative of poor prognosis in patients with breast cancer (including all subtypes). Specifically, in the basal subtype, hypomethylation of this site suggested a favorable prognosis. In the luminal subtype, decreased methylation of this site was associated with better OS and worse DFS. However, in the HER2 subtype, the methylation level of this site was not significantly correlated with prognosis (Fig. 3A). The prognostic role of IDO1 promoter methylation may need to be validated separately in different subtypes. For cg08465774 (located in the gene body of IDO1), methylation was also significantly lower in the basal subtype than in the HER2 and luminal subtypes (Fig. 2A), and it was also negatively correlated with the expression of IDO1 (Fig. 4B). However, the methylation status of the cg08465774 site exhibited no discernible correlation with the prognosis of patients with breast cancer (Fig. 3B). Methylation of cg24188163 (located in the gene body of IDO1) was significantly greater in the basal subtype than in the HER2 and luminal subtypes (Fig. 2C). Methylation of this gene was positively correlated with the expression of IDO1 (Fig. 4C). Hypomethylation of the cg24188163 site was associated with worse DFS in patients with breast cancer and in luminal subtypes (Fig. 3C). However, disease-free survival (DFS) records in TCGA were truncated in advance, which may have created bias. Thus, further studies are needed to verify the present results.

The tumor microenvironment consists of multiple types of immune cells and other cell types, and it is pivotal in the immune response to tumor cells. To test whether IDO1 expression and methylation are associated with immune cell infiltration within the tumor microenvironment, we analyzed the RNA-seq signatures of 23 distinct immune cell types and their associations with IDO1 expression and promoter methylation. Our study revealed that hypermethylation of cg24188163 was positively correlated with increased IDO1 expression and increased infiltration of immune cells. In contrast, hypermethylation of the cg10262052 and cg08465774 sites was negatively associated with IDO1 expression and immune cell infiltration (Fig. 5A, B). The relative enrichment scores of the three methylation sites supported the above results (Fig. 5C–E). Because cg10262052 is the site in the promoter of the IDO1 gene, this finding suggested that IDO1 promoter hypomethylation may influence the prognosis of breast cancer patients by modulating immune cell infiltration and the tumor microenvironment. However, as a highly complex system, the tumor microenvironment is regulated by numerous molecules, and IDO1 is only one of the potential players. Despite the promising results, the status of immune cell infiltration in tissues and the effect of IDO1 on the immune status of patients require further validation.

To investigate the possible mechanisms by which IDO1 promoter methylation is correlated with immune cell infiltration, we investigated the relationships between IDO1 and its methylation and immune-related pathways. Our results revealed high IDO1 expression and hypomethylation at the cg10262052 and cg08465774 sites were correlated with higher scores for many immune-related pathways (Fig. 6A–E). These characteristics suggested that the tumor microenvironment is rich in infiltrating immune cells. Increased immune checkpoint activity and coinhibitory features contribute to an immunosuppressive microenvironment. The cg24188163 site had opposite results (Fig. 6C–E). Similar findings were found in the breast cancer subtypes (Fig. S2A-C). These findings suggested that there is a positive correlation among IDO1 promoter hypomethylation, high IDO1 expression, and the activation of immune-related pathways. In addition, because of the increase in immune checkpoint activity, immune checkpoint inhibitor therapy may be potentially therapeutic for these patients. Therefore, IDO1 promoter methylation may serve as a molecular biomarker to predict the efficacy of immunotherapy.

Previous studies have confirmed that DNA methylation biomarkers are associated with immune cell infiltration, patient response to immunotherapy, and patient prognosis[37–41]. Because immunomodulator molecules play important roles in the regulation of the tumor microenvironment, the present study investigated the relationship between IDO1 promoter methylation and these molecules in breast cancer by analyzing the correlation between IDO1 methylation and the expression of 74 key immunomodulators. High expression of IDO1, low methylation of cg10262052, low methylation of cg08465774, and high methylation of cg24188163 were positively correlated with upregulated expression of most key immunomodulators (Fig. 7). These findings suggested that under the influence of IDO1 promoter methylation, IDO1 expression is likely related to the potential efficacy of immune checkpoint inhibitors in breast cancer. This result was also comparable across the breast cancer subtypes (Fig. S3A–C). These results verified that the aforementioned "hot tumors" with high IDO1 expression are significantly enriched in immune checkpoint molecules. Therefore, IDO1 expression and IDO promoter methylation may be used to predict the tumor response to immune checkpoint inhibitors.

qPCR and MSP analyses were performed using the SUM-159PT, HCC 1806, T47D, and ZR75 breast cancer cell lines. Hypomethylation of the IDO1 promoter at cg10262052 in SUM-159PT and HCC 1806 cells, as well as the corresponding expression of IDO1 were greater than those in the other two cell types (Fig. 8A–D). This finding further demonstrated promoter hypomethylation of IDO1 in the basal subtype of breast cancer and that promoter hypomethylation is correlated with high expression of IDO1.

The present study had several limitations. The present study used TCGA methylation data, which were acquired from the Infinium HumanMethylation450 BeadChip. According to the annotation of the Infinium HumanMethylation450 BeadChip, there are only three probes within the IDO1 gene, and only one probe is located in the promoter of the IDO1 gene. In addition to these three sites, other IDO1 methylation sites may also play important roles in breast cancer diagnosis, prognosis, and response to immunotherapy. Because the Infinium HumanMethylation850 BeadChip includes more CpG sites in the human genome, subsequent studies should consider using data from the Infinium HumanMethylation850 BeadChip, which may identify additional IDO1 methylation sites. Although it was established that IDO1 promoter methylation is associated with immune cell infiltration and immune-related pathways and that it has the potential to predict the tumor response to immunotherapy, immune cell infiltration in the tumor microenvironment was deconvoluted from RNA-seq data. Therefore, this conclusion needs to be validated with clinical cohorts of patients who have received immune checkpoint inhibitor therapy. Finally, our in vitro studies were performed only to determine the promoter methylation status and expression of IDO1. Thus, the correlations of IDO expression and IDO promoter methylation status with immune cell infiltration and immune-related pathways need to be further studied.

IDO1 is highly expressed in various types of cancers, and it is associated with a poor prognosis[29–34]. IDO1 inhibitors have been studied for the treatment of various cancers, including breast cancer[15–17]. The present study revealed that IDO1 and its methylation are correlated with immune cell infiltration, immune-related pathways, and the expression levels of immune checkpoint molecules in breast cancer. IDO1 expression may lead to differences in patient outcomes by interacting with the tumor microenvironment of breast cancer. These findings suggest that IDO1 promoter methylation has potential as a marker of the response to immunotherapy in breast cancer patients. Several IDO1 inhibitors have been under clinical trials, primarily in combination with immunotherapy[18,19,42]. One study has reported the use of an IDO1 inhibitor combined with a taxane in patients with HER2-negative metastatic breast cancer, but the results revealed no difference in progression-free survival[43]. Many types of IDO1 inhibitors have emerged, but further tests and progress are still needed to determine the potential of IDO1 inhibition treatment[44].

Overall, the regulatory effect of IDO1 promoter methylation on the expression of immune checkpoint molecules needs to be further confirmed, but combination therapy with an IDO1 inhibitor and an immune checkpoint inhibitor has shown promise for the future.

Conclusions

Overall, our study demonstrated that IDO1 methylation is associated with IDO1 expression and prognosis in breast cancer, immune cell infiltration, immune-related pathways, and the expression levels of immune checkpoint molecules. The results of this study support the conclusion that IDO1 methylation may be used as a biomarker to predict the prognosis and response to immunotherapy in patients with breast cancer. This new biomarker may aid in the precision treatment of breast cancer.

Data availability

All processed data generated or analyzed in this study are included in the additional files.

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Author contributions

Q.L., S.D., Q.Z. and W. Y. conceived and designed the project. S.D., R.Y., X.G., Y.H., and J.M. analyzed and interpreted the data. R.Y., Q.L., J.X., L.Q., and J.J. wrote the main manuscript text. S.D. prepared figures. All authors contributed to the article and approved the submitted version.

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Competing interests

The authors declare no competing interests.

Ethics approval

The use of the public data was approved by TCGA.

Consent for publication

Not applicable.

Additional information

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