Expression Patterns of Immune Checkpoints In Breast Cancer Patients

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Abstract

Background: immunotherapy with immune checkpoint inhibitors (ICIs) for solid tumors had significantly improved overall survival (OS). Positive response to PD-1/PD-L1 blockades was observed in the treatment of solid tumors. Breast cancer (BC) patients are no exception. However, the efficacy of immunocheckpoint therapy in BC patients remains poor. A particularly important factor is the lack of studies on the expression patterns of immune checkpoints in BC patients.

Results: It was found that increased expression of PD-1, PD-L1, STAT1, CTLA-4 was associated with poor OS in BC patients. In addition, co-expression of PD-L1 with PD-1, STAT1 or CTLA-4 and co-expression of PD-1 with CTLA-4 was related to poor OS. We analyzed associations between the proportionate expression of PD-L1 and PD-1, PD-L1 and STAT1, PD-1 and CTLA-4, PD-1 and LAG3, PD-L1 and CTLA-4 in BC patients, there was significance in correlation in both of the BC patients. The expression of STAT1 in BC patients was compared with that of HC, and it was found that STAT1 was highly expressed in BC patients.

Conclusions: our results suggest that transcriptome-based co-expression of STAT1 and PD-L1 is a predictor for poor OS in BC patients, which might provide novel insight into designing combinational targeted therapy for BC.

Background

Over the past 5 years, immunotherapy with immune checkpoint inhibitors has revolutionized the management of various cancers [1]. The occurrence and development of cancer is closely related to immune escape and abnormal immune monitoring. A familiar cell surface receptor, called programmed cell death protein 1 (PD-1), may allow T cells to escape anti-tumor immunity when the cells associated with cancer cells are upregulated. Ligand for the PD-1 receptor, programmed death ligand 1 (PD-L1), is expressed in a variety of epithelial cancers [2]. In addition, other immune checkpoint molecules such as CTLA-4, LAG3, and FGL1 have also been studied [3, 4]. The PD-1/PD-L1 signaling pathway mainly causes the body to be in the state of immunosuppression [5]. Recently, Cancer immunotherapy employing PD-1/PD-L1 inhibitors has resulted in drastic improvements in clinical outcomes in multiple malignant tumors [6, 7]. Moreover, an anti-PD-1 mAb have shown good safety to multiline systemic therapy in patients with advanced refractory TNBC [8]. Previous reports have shown that PD-1 and PD-L1 correlated gene expression profiles and their association with clinical outcomes of BC [9]. In addition, PD-L1 expression is closely related to the positive response of PD-1/PD-L1 blockade in solid tumor therapy [10]. These findings suggest that PD-1/PD-L1 blockade may be a novel immunotherapeutic strategy for BC. However, different types of BC patients have different clinical responses to PD-1/PD-L1 blockade, clinical use of triple negative breast cancer (TNBC) is more [11, 12]. We believe that in addition to PD-1 and PD-L1, there are other factors that may aggravate its immunosuppression, affect its immunotherapeutic effect, and lead to poor prognosis in patients with other types of BC.

STAT1 exists in cells in the form of inactive monomer in primarily. After phosphorylation by tyrosine kinases, STAT1 is activated to form homologous or heterodimer. Even after the formation of polymers and oligomers, STAT1 can also play a physiological role, especially in the interferon (IFN) system [13]. At the same time, STAT1 is defined as a growth inhibitor factor and a apoptosis factor due to its characteristics of promoting apoptosis and inhibiting cell growth and differentiation, and it plays an important role in inhibiting the occurrence and development of tumors. First of all, the overexpression of STAT1 is often observed in the pathogenesis of solid tumors, which indicates that STAT1 is indeed closely related to tumors [13]. Recently, STAT1 has been identified as a potential therapeutic target for malignancies, including breast cancer, mainly through the JAK/STAT1 signaling pathway that affects the body's normal immune system [13, 14]. The expression of phospho-STAT1 (p-STAT1) in the breast cancer microenvironment is a potential biomarker. Anti-PD-1/anti-PD-L1 monoclonal antibodies have been used in immunotherapy of cancer patients based on the expression of p-STAT1 and PD-L1 tumor cells [14]. In addition, PD-L1 is downstream of STAT1, and STAT1 inhibition facilitates the anti-tumor immune response in BC patients by suppressing PD-L1 expression [15]. Therefore, it is important to understand the correlation between STAT1 and PD-1/PD-L1 signal channel in BC patients.

In this study, the expression of STAT1 in breast cancer was compared between the HC group and BC patients, as well as between the expression of different types of BC, to illustrate the correlation between STAT1 and clinical outcomes of BC patients, and to reveal how STAT1 regulates the PD-1/PD-L1 signaling pathway. This result was further verified by high-throughput sequencing data from GEO, TCGA, GTEx databases. Furthermore, to investigate the effects of PD-1, PD-L1, STAT1, IDO, CTLA-4, LAG3 and FGL1 on clinical outcomes of BC, and to determine its potential as a biomarker for prognosis in BC patients.

Materials And Methods

Microarray Data and Differentially Expressed Gene Analysis

Microarray dataset GSE10810 was downloaded from the Gene Expression Omnibus (GEO) database and collected using the GPL570 platform (Affymetrix Human Genome U133 Plus 2.0 Array). This microarray dataset formed the study of “BC and the normal mammary tissue.” This study aims to find the differentially expressed genes (DEGs) of the BC [16], which is consistent with the design of our study. Difference analysis was performed by R script using limma (Linear models for microarray analysis) R package, $p < 0.05$ and $|\log FC| > 1$ as cutoff values for screening DEGs. The DEGs of the comparison group were shown as the volcano plot. The heatmap of the expression data was generated using SangerBox online tools⁵. The expression value of the DEGs was obtained from the GEO2R online tools.

GO and KEGG Enrichment Analysis

The GO and KEGG enrichment analysis was using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) database⁷, which is an integrated online biological knowledge base and analytical tool. In this study, the target genes were mapped into DAVID and to identify the biological

processes, cellular components, molecular function, and KEGG pathways of the predicted target genes involved. The map of the KEGG signaling pathway was obtained from the KEGG database⁸.

TCGA and GTEx Dataset

The RNA sequencing and mutation data of 657 BC patients was obtained from the TCGA (<https://cancergenome.nih.gov/>) database. The RNA sequencing and mutation data from 1085 BC patients and 291 HC were obtained from the GTEx database (<http://gepia2.cancer-pku.cn/>). These RNA sequencing data from the TCGA database comprised the validation cohort for OS analysis. The TCGA and GTEx dataset was used to validate the results of the training cohort. The clinical information included age, status of distant metastasis, neoadjuvant therapy history and pathologic (Supplementary Table 1). Because the TCGA and GTEx dataset is publicly available, no local ethics committee approval was required.

MCF-10A, MCF-10AT, MCF-7, MDA-MB-231 cell culture

MCF-10A (a non-tumorigenic epithelial cells), MCF-10AT (Breast cancer precancerous cells) and MCF-7, MDA-MB-231 (human breast cancer cells) (American Type Culture Collection ATCC, USA). The cells were maintained as monolayer cultures (25 cm² plastic culture flasks) in F12/DMEM and DMEM (1x) medium with 10% fetal bovine serum (Gibco South America) and Donor Equine Serum (HyClone, USA), Pen strep at 37°C in an incubator with an atmosphere containing 5% CO₂. The cells were cultured every 2 days.

Real-time quantitative PCR (RQ-PCR)

RNA was reverse transcribed into the first-strand cDNA with random hexamer primers. RQ-PCR using the SYBR Green I technique was used to examine the PD-L1, STAT1, IDO and IFN γ -R gene expression level in cDNA from MCF-10A, MCF-10AT, MCF-7, MDA-MB-231 cells strain with the β 2 microglobulin gene serving as an endogenous reference. The primers were purchased from Generay Biotech Co., Ltd. (Shanghai, China) (Supplementary Table 2). The relative mRNA expression level was calculated using the 2^{- Δ Ct}×100% method [17].

Statistical Analysis

All statistical analyses were performed using Statistical Product and Service Solutions (SPSS) (version 13.0). Comparisons between the different cells strain and differences in mRNA expression between two groups were analyzed by the Kruskal-Wilcoxon H test. A log-rank test was applied to compare difference between groups. Spearman rank correlations analyses were used to estimate the correlation. A two-tailed p value < 0.05 was considered statistically significant.

Results

Identification of Differentially Expressed Genes of BC Patients in datasets

Firstly, we downloaded the breast tissue genes expression dataset GSE10810 from GEO database to analyze the changes of BC related genes. Then, the expression of related genes in healthy control (HC) and BC groups was compared and analyzed. As shown in Figs. 1A, B, there were a total of 2371 differentially expressed genes with 747 upregulated and 1624 downregulated in BC patients. After analysis, this may be closely related to the progression and prognosis of BC.

Functional Enrichment Analysis for the BC Target Genes

In order to adequately find out the disparity of these target genes, we performed GO and KEGG enrichment analysis using DAVID database. A variety of GO enrichment terms were enriched, including 169 biological processes, 70 cellular components, and 46 molecular functions. The top 10 GO terms are shown in Fig. 1C. We found that the biological processes such as negative regulation of apoptotic process and neuronal migration, cellular components like nucleus and mitochondrion, and molecular function like chromatin binding and ATP binding, were enriched, which may be involved in the biological activity in the BC treatment process. In addition, 10 KEGG pathways were enriched (Fig. 1D), and the important genes were mainly distributed in the cell cycle signaling pathway. Although the JAK/STAT1 pathway did not make the top 10, the data suggest that the JAK/STAT1 signaling pathway is associated with the development of BC.

Expression of PD-L1, STAT1, PD-1, or CTLA-4 is associated with poor OS in BC

We performed OS analysis on 657 BC patients in TCGA database. And the results showed that the higher expression of PD-L1, PD-1, STAT1 and CTLA-4 in BC patients correlated with poor OS in the TCGA database analysis (20-year OS 0% vs 40.76%, 0% vs 35.09%, 0% vs 32.63% and 0% vs 32.69%, respectively, $P = 0.262$, $P = 0.002$, $P = 0.105$, $P = 0.446$) (Figures. 2A-C and F). But not all data were statistically significant. Expression of IDO1, IDO2, LAG3 and FGL1 in BC patients uncorrelated with poor OS in the TCGA database analysis (20-year OS 18.22% vs 33.55%, 28.16% vs 28.01%, 36.00% vs 17.05% and 18.39% vs 32.77%, respectively, $P = 0.496$, $P = 0.002$, $P = 0.997$, $P = 0.140$) (Figures. 2D-E and G-H). We further analyzed the correlation between PD-1, PD-L1 and STAT1 expression patterns of other important ICs. Then, with Spearman's correlation analysis, we found that the expression of PD-1 was positively associated with the expression of PD-L1 ($r = 0.420$, $P < 0.0001$), lymphocyte activation gene-3 (LAG-3) ($r = 0.713$, $P < 0.0001$), STAT1 ($r = 0.485$, $P < 0.0001$) and cytotoxic T-lymphocyte associated protein 4 (CTLA-4) ($r = 0.789$, $P < 0.0001$) in the TCGA group (Figures. 4B-D and F). Similarly, we found that the expression of PD-L1 was positively associated with the expression of STAT1 ($r = 0.624$, $P < 0.0001$) and CTLA-4 ($r = 0.553$, $P < 0.0001$) in the TCGA group (Figures. 4A and E). And it was statistically significant. But the expression of FGL1 was no correlation in the expression of LAG3 ($r = 0.313$, $P = 0.424$) in the TCGA group (Figures. 4G). In conclusion, the expression of PD-1, PD-L1 and STAT1 is related to the progression and prognosis of BC patients.

Co-Expression of PD-L1 and STAT1 or CTLA-4 is associated with poor OS in BC

Combination of IC inhibitors (ICIs) has the potential to improve responses. We analyzed expression patterns of ICs and found that pairwise combinations of PD-L1 and PD-1, STAT1 or CTLA-4 correlated with poor OS in BC patients (20-year OS: PD-1^{high} and PD-L1^{high} vs PD-1^{high} or PD-L1^{high} vs PD-1^{low}PD-L1^{low} 0% vs 34.96% vs 39.28%) (20-year OS: PD-L1^{high} and STAT1^{high} vs PD-L1^{high} or STAT1^{high} vs PD-L1^{low}STAT1^{low} 0% vs 46.48% vs 39.43%; PD-L1^{high} and CTLA-4^{high} vs PD-L1^{high} or CTLA-4^{high} vs PD-L1^{low}CTLA-4^{low} 0% vs 15.72% vs 42.26%) ($P = 0.113$, $P = 0.021$, $P = 0.014$) in the TCGA (Figures. 3A-C).

Co-Expression of PD-1 and STAT1, CTLA-4 or LAG3 is associated with poor OS in BC

We analyzed expression patterns of ICs and found that pairwise combinations of PD-1 and STAT1, LAG-3 and CTLA-4 correlated with poor OS in BC patients (20-year OS: PD-1^{high} and LAG3^{high} vs PD-1^{high} or LAG3^{high} vs PD-1^{low}LAG3^{low} 0% vs 49.78% vs 32.59%) (20-year OS: PD-1^{high} and STAT1^{high} vs PD-1^{high} or STAT1^{high} vs PD-1^{low}STAT1^{low} 0% vs 50.99% vs 26.73%) (20-year OS: PD-1^{high} and CTLA-4^{high} vs PD-1^{high} or CTLA-4^{high} vs PD-1^{low}CTLA-4^{low} 0% vs 27.77% vs 34.71%) in the TCGA ($P = 0.032$; $P = 0.005$; $P = 0.086$) (Figures. 3D-F). And finally, what we found expression of FGL1 and LAG3 failed to correlate with OS in BC patients (20-year OS: LAG3^{high} and FGL1^{high} vs LAG3^{high} or FGL1^{high} vs LAG3^{low}FGL1^{low} 23.35% vs 30.25% vs 32.59%) ($P = 0.474$) in the TCGA (Figures. 3G).

Gene expression levels of immunofunction-related molecules in BC patients and patients with status in different clinical subgroups of BC

A total of 1085 BC patients (include Basal-like / Triple negative: 135, HER2 + non-luminal: 66, Luminal A: 415, Luminal B: 192) and 291 HC from the GTEx database were used analysis. We found that there was no significant difference in the gene expression levels of PD-L1, PD-1, CTLA-4, FGL1 and IDO2 between the BC group and the HC group ($P > 0.05$), and the Basal-like/Triple negative, HER2 + non-luminal, Luminal A, Luminal B had no significant difference ($P > 0.05$), it was not related to the clinical stage of BC (Figures. 5A, C, E, F, H). However, the expression levels of STAT1 and IDO1 genes in BC patients and HC patients were significantly different ($P \leq 0.05$). The expression of STAT1 in Basal-like/Triple negative, HER2 + non-luminal, Luminal A, Luminal B was statistically significant compared with that in HC ($P \leq 0.05$). But the expression of IDO1 in Basal-like / Triple negative, HER2 + non-luminal had significant difference ($P \leq 0.05$) (Figures. 5B and D). Besides, the expression levels of LAG3 genes in BC patients and HC patients were no significantly different ($P > 0.05$), but the expression in Basal-like/Triple negative in BC patients and HC patients were significantly different ($P \leq 0.05$) (Figures. 5G). Subsequently, using the difference in clinical stages of BC patients in GTEx database, it was found that the expression of PD-L1 and IDO1 was reduced in stage IV-X compared with stage I-III ($P = 0.004$; $P = 0.006$). Although the expression of PD-1, STAT1, CTLA-4, IDO2, FLG1 and LAG3 showed no significant difference in different stages of BC ($P =$

0.523, $P = 0.083$, $P = 0.124$, $P = 0.699$, $P = 0.99$, $P = 0.058$) (Figures. 5). But also confirmed that the key role of immune checkpoints BC progress.

The expression changes of target genes in the BC process

To further validate whether these four genes are related to BC pathology, we performed a serial of RQ-PCR quantification in the MCF-10A, MCF-10AT, MCF-7, MDA-MB-231 cells. As shown in Fig. 6, We found significant differences in PD-L1 gene expression levels in MCF-10A, MCF-10AT, MCF-7 and MDA-MB-231 (Figs. 6A, $P = 0.016$), and showed MCF-10A < MCF-7 < MCF-10AT < MDA-MB-231 trend. Similarly, STAT1 gene expression levels differed significantly among MCF-10A, MCF-10AT, MCF-7, and MDA-MB-231 (Figs. 6B, $P = 0.025$), and showed MCF-7 < MCF-10A < MCF-10AT < MDA-MB-231 trend, which were consistent with the GSE10810 dataset results. Moreover, the mRNA levels of IFN γ -R and IDO were no significantly in MCF-10A, MCF-10AT, MCF-7 and MDA-MB-231 (Figs. 6C and D, $P = 0.110$, $P = 0.248$). These results support that PD-L1 and STAT1 genes are closely related to the occurrence, development and prognosis of BC.

Discussion

STAT1 plays a significant role in the progression and prognosis of various tumors such as gastric cancer, colorectal cancer, ovarian cancer and other tumors [18–20]. We all know that the combined of PD-1 and PD-L1 has a negative regulatory effect on the immune system in cancer patients. However, little is known about the prognostic value of STAT1 in BC patients and how STAT1 affects the expression of PD-L1 in tumor cells [21]. This study investigated the relationship between STAT1 expression and OS in patients with BC. We found that overexpression of STAT1 predicts poor OS in BC, STAT1 was positively correlated with PD-L1. Therefore, STAT1 inhibitors can be used in combination with PD-L1 monoclonal antibodies to achieve optimal therapeutic efficacy. More importantly, immune checkpoints can be used as biomarkers to predict the prognosis of patients with BC. The expression of immune checkpoint molecules on T cells is considered to be one of the important regulatory mechanisms for immune cells to regulate their own antigen response [22, 23]. Some studies have shown that immune checkpoints such as PD-1, CTLA-4 and LAG-3 are upregulated on the surface of T cells in breast tumor tissues. The epigenetic modifications behind their upregulation are depends on the methylation of DNA and the distribution of inhibitory histone [24]. Therefore, the combination of targeted drugs and STAT1 blocking function is a new strategy for the treatment of tumors. Recent studies have shown that combination therapy of ICB with other targeted agents has the potential to enhance the anti-tumor response of BC [25, 26]. Besides that, PD-L1 has been proved to be a immediate target for STAT1-mediated gene expression [14]. In this study, through the analysis of GEO database, we found that the expression of STAT1 in all BC patients was increased. Then, it was also found that the expression of STAT1 was positively correlated with PD-1 and PD-L1. More importantly, STAT1 co-expression with PD-L1 and PD-1 also predicted OS in BC patients. In addition, compared with HC, STAT1 was overexpressed in BC patients regardless of type. These findings may provide accurate and valuable OS prediction for BC. Using data from GTEx, we first clarified the expression status of PD-L1 and IDO1 in different subtypes and clinical stages of BC patients. PD-L1 and

IDO1 expression was decreased in stage IV -X compared with stage I-III in BC patients. On the basis of molecular typing, basal-like BC subtype showed highest expression of STAT1, IDO1, LAG3, which suggest that different subtypes possess various immune checkpoints status. In addition, the expression of PD-1 and PD-L1 was associated with recurrence and difficulty in BC patients [9]. Both PD-1 and IDO are highly expressed in the tumor microenvironment of BC patients, while LAG3 and PD-1 were highly expressed in T cells of BC patients, suggesting poor prognosis [27, 28]. Which not only confirm the critical role of immune checkpoint in BC progression and prognosis, but also associated with immune evasion in numerous. In summary, we detected PD-L1 and STAT1 in different cell lines such as MCF-10A, MCF-10AT, MCF-7 and MDA-MB-231, and the expression of PD-L1 and STAT1 was different in various types of breast cell lines. Especially in TNBC, PD-L1 and STAT1 are both highly expressed, while in conventional BC, PD-L1 and STAT1 are simultaneously low expressed, which may be related to the poor treatment of PD-L1/PD-1 inhibitors in some BC. Surprisingly, PD-L1 and STAT1 are also overexpressed in precancerous breast cancer cells. This suggests that the expression of immune checkpoint may be different in different subtypes of breast cancer or precancerous lesions. This may provide a new direction for our treatment strategy.

As is known to all, the research of immune checkpoint is becoming more and more important in the field of cancer. Tumor cells use various tricks to scape of immune system, such as activating immune checkpoint pathways that induce immunosuppressive function. PD-1 and PD-L1, CTLA-4 and LAG3 have been extensively evaluated as putative markers of response to immunotherapy with PD-1/PD-L1 and CTLA-4 blockade, respectively [29, 30]. Abirami et al. demonstrations of clinical benefit of immunotherapy in female metastatic breast cancers (MBC) support the need for development and utilization of biomarkers to guide the use of immune checkpoint inhibitors (ICPIs) for these patients, such as PD-1, PD-L1, CTLA-4 and LAG3 [29]. In this study, both PD-1 and PD-L1 were positively correlated with STAT1 expression. Although not associated with short-term survival outcomes, it is significant for long-term survival, especially for PD-1 expression and co-expression PD-L1 or STAT1 expression. This may be due to inconsistencies in the subtypes of BC. In addition, we observed that PD-L1 was more expressed in TNBC. Therefore, in the near future, the application of molecular and biochemical markers in a personalized manner will improve optimal combination immunotherapy, providing customized treatments that may save the lives of patients with malignant breast cancer who have a poor prognosis.

Conclusions

In summary, we demonstrate that transcriptome-based co-expression of STAT1 and PD-1/PD-L1 could be a predictor of poor OS in BC patients. This finding will provide novel insights into designing combinational immuno-targeted therapy in BC patients.

Abbreviations

ICIs: immune checkpoint inhibitors

OS: overall survival

BC: Breast cancer

PD-1: programmed cell death protein 1

PD-L1: programmed death ligand 1

CTLA-4: cytotoxic T-lymphocyte-associated antigen 4

LAG3: Lymphocyte-activation gene 3

FGL1: fibrinogen-like protein 1

TNBC: triple negative breast cancer

p-STAT1: phospho-STAT1

MBC: metastatic breast cancers

ICPIs: immune checkpoint inhibitors

Declarations

Availability of data and materials

The data sets analyzed in the present study are publicly available data from The TCGA, GTEx and GEO database. The original contributions presented in the study are included in the article. Further inquiries can be directed to the corresponding authors.

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Authors' Contributions

MM, and GJZ contributed to the concept development and study design. DQ, XXY, XQX and JYC performed the laboratory studies. RRM, YQW and SYH collected the clinical data. DQ and XXY participated in the manuscript and figure preparation. MM coordinated the study and helped draft the manuscript. All authors read and approved the final manuscript.

Ethics declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Additional information

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Figures

Fig 1

Figure 1

Bioinformatics analysis of differentially expressed genes (DEGs) in the mammary tissue of health control (HC) and Breast cancer (BC) patients. (A) The volcano plot of DEGs in the mammary tissue between the HC group and the BC group. (B) Heatmaps of DEGs in the mammary tissue of the HC group and the BC group. Colors in the heatmaps indicate the row Z-score among the different datasets. High expression is shown by the red color, and low expression is shown by the blue color. (C) Gene ontology (GO) enrichment analysis of the BC target genes. (D) KEGG enrichment analysis of the BC target genes. The number of genes enriched in each KEGG term is shown as the circle size, the p-value shown as different colors.

Fig 2

Figure 2

Overall survival (OS) of ICs in BC patients. A-H: The OS probability in BC patients with high or low PD-L1, PD-1, STAT1, IDO1, IDO2, CTLA-4, FGL1 or LAG3 expression in TCGA group. The median was used to define the optimal cutoff value for gene expression levels for prognosis.

Fig 3

Figure 3

Relationship between PD-1, PD-L1 and other immune checkpoints in TCGA group.

Fig 4

Figure 4

The Spearman rank's coefficient with a P value < 0.05 for the correlation of two IC genes.

Figure 5

Differences in PD-1, PD-L1, STAT1, CTLA-4, IDO1, IDO2, FGL1 and LAG3 expression between different clinicopathological information. Differences in PD-1, PD-L1, STAT1, CTLA-4, IDO1, IDO2, FGL1 and LAG3 expression in molecular type. Differences in PD-1, PD-L1, STAT1, CTLA-4, IDO1, IDO2, FGL1 and LAG3 expression in stage.

Fig 6

Figure 6

The expression differences of PD-L1, STAT1, IFN-γ-R and IDO genes in MCF-10A, MCF-10AT, MCF-7 and MDA-MB-231, respectively.

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