Protocol of Immunohistochemistry used in the Manuscript

(a) **Tissue Handling:** Breast cancer tissue samples were collected from patients diagnosed with TNBC and non-TNBC subtypes. Both pre-and post-therapeutic samples were included to assess changes in DARC and c-Myc expression. Normal breast tissue samples were used as controls. Subsequent slide preparations were conducted at the Department of Biochemistry, AIIMS, New Delhi, using standard operating protocols, where FFPE blocks were used to cut 5m sections onto glass slides. Control samples were collected from non-cancerous tissues.

(b) **Staining Procedure:**

Primary Antibodies: Sections were incubated with primary antibodies against DARC (Cat No:- #DF 13554 Affinity), CCL-8 (Cat No:- E-Ab-62908, Elabscience), ALDH1(Cat No:- E-Ab-60451, Elabscience) and c-Myc (Cat No:- #AF 3054, Affinity).

Secondary Antibodies: HRP-conjugated secondary antibodies were used for visualization (Bright-Vision, 2-step detection system goat anti-mouse/rabbit HRP + DAB Cat No:- UBVB110HRP).

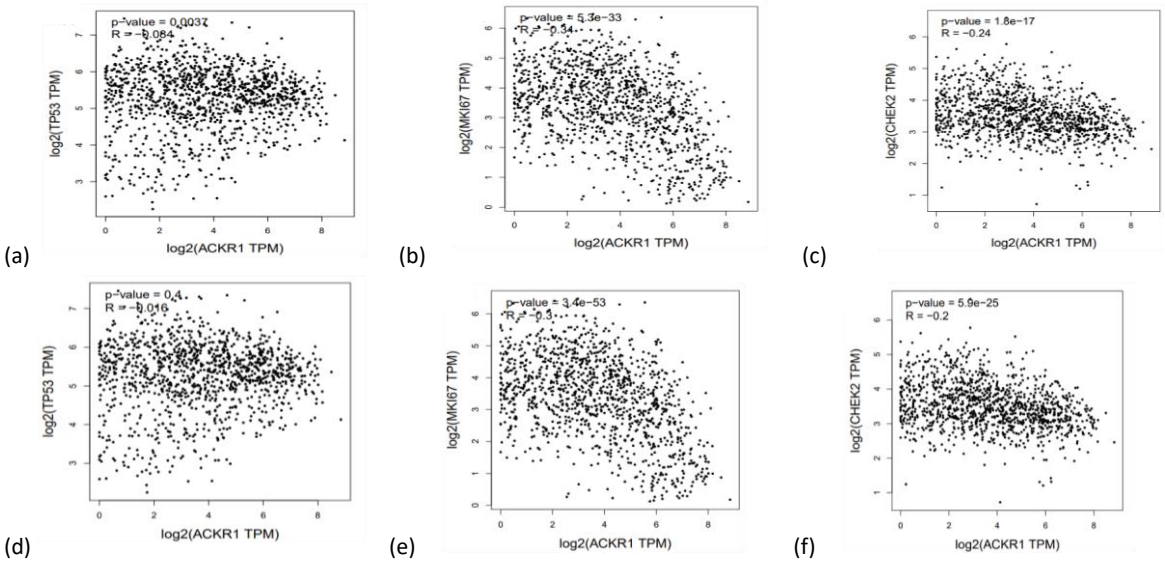
The samples were processed using a standard indirect two-step immunohistochemistry protocol [41]. Following deparaffinization and rehydration, endogenous peroxidase activity was blocked by incubating the sections in 5% H_2O_2 for 20 minutes at room temperature. Heat-induced epitope retrieval enhanced antigen detection was carried out (similar to Vinod et al. [42] with required changes). After blocking with Protein Block for 30 minutes at room temperature, the sections were incubated with the primary antibody for 1 hour at room temperature. Detection was performed using the Bright-Vision, 2-step detection system goat anti-mouse/rabbit HRP + DAB. Tris buffer (pH 7.6) was used for washing steps. Hematoxylin was used to counterstain the nuclei, and finally, the samples were dehydrated and mounted.

(c) **Image Analysis:**

Microscopy: Stained sections were examined under a confocal microscope (TI-E,601869, Nikon Laser Scanning Confocal Microscope) for visualization.

Quantification: Image analysis software (Image J) was employed to quantify the staining intensity and localization of DARC and c-Myc.

Supplementary Figures:-



Suppl Figure S1: Correlation Between ACKR1 and Tumor Suppressor Genes in Breast Cancer:-

The figures demonstrate that ACKR1 expression is inversely correlated with the expression of tumor suppressor genes TP53 and MK167, suggesting a potential role for ACKR1 in suppressing tumor suppressor activity. However, the relationship with CHEK2 is less clear, and further research is needed to elucidate the functional implications of these correlations.

1 (a-c) represent Pearson correlation and 1 (d-f) represent Kendall correlation

1 (a): The figure shows a weak negative correlation between ACKR1 and TP53 expression, suggesting that higher ACKR1 levels might be associated with lower TP53 expression. However, the correlation is not very strong, and other factors may also influence the relationship between these two genes. [X-axis: Log2(ACKR1 TPM) - Represents the expression level of ACKR1, Y-axis: Log2(TP53 TPM) - Represents the expression level of TP53, Scatter points: Show the relationship between ACKR1 and TP53 expression levels in individual samples, Correlation coefficient (R): -0.084, indicating a weak negative correlation, P-value: 0.0037, suggesting a statistically significant correlation.]

1 (b): The figure shows a moderate negative correlation between ACKR1 and MK167 expression, suggesting a stronger inverse relationship between these two genes. This might indicate that higher ACKR1 levels are associated with lower MK167 expression, potentially influencing cell proliferation and growth. [X-axis: Log2(ACKR1 TPM) - Represents the expression level of ACKR1,Y-axis: Log2(MK167 TPM) - Represents the expression level of MK167,Scatter points: Show the relationship between ACKR1 and MK167 expression levels in individual samples, Correlation coefficient (R): -0.24, indicating a moderate negative correlation, P-value: 5.2e-33, suggesting a highly statistically significant correlation.]

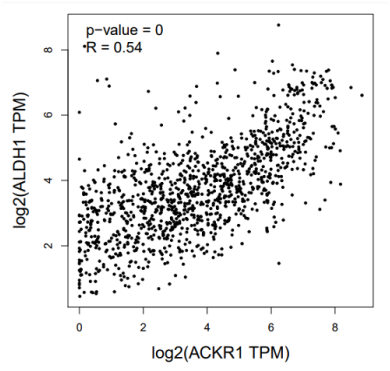
1 (c): The figure shows a weak negative correlation between ACKR1 and CHEK2 expression, suggesting a possible inverse relationship between these two genes. However, the correlation is not very strong, and further investigation is needed to understand the functional implications of this relationship.[X-axis: Log2(ACKR1 TPM)

- Represents the expression level of ACKR1,Y-axis: Log2(CHEK2 TPM) - Represents the expression level of CHEK2, Scatter points: Show the relationship between ACKR1 and CHEK2 expression levels in individual samples, Correlation coefficient (R): -0.22, indicating a weak negative correlation, P-value: 1.8e-17, suggesting a statistically significant correlation.]

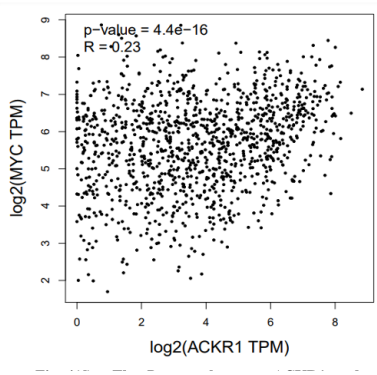
1 (d) : The figure shows a very weak negative correlation between ACKR1 and TP53 expression, suggesting that there is little or no association between these two genes. [X-axis: Log2(ACKR1 TPM) - Represents the expression level of ACKR1, Y-axis: Log2(TP53 TPM) - Represents the expression level of TP53,Scatter points: Show the relationship between ACKR1 and TP53 expression levels in individual samples, Correlation coefficient (R): -0.016, indicating a very weak negative correlation, P-value: 0.4, suggesting no statistically significant correlation.

1 (e): The figure shows a moderate negative correlation between ACKR1 and MK167 expression, suggesting a stronger inverse relationship between these two genes. This might indicate that higher ACKR1 levels are associated with lower MK167 expression, potentially influencing cell proliferation and growth. [X-axis: Log2(ACKR1 TPM) - Represents the expression level of ACKR1,Y-axis: Log2(MK167 TPM) - Represents the expression level of MK167,Scatter points: Show the relationship between ACKR1 and MK167 expression levels in individual samples, Correlation coefficient (R): -0.3, indicating a moderate negative correlation, P-value: 3.4e-53, suggesting a highly statistically significant correlation.]

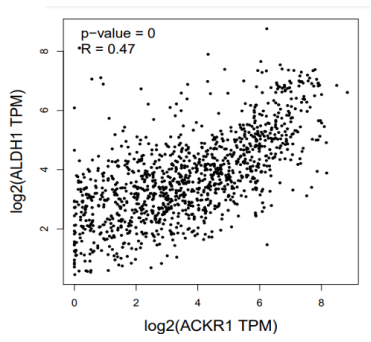
1 (f): The figure shows a weak negative correlation between ACKR1 and CHEK2 expression, suggesting a possible inverse relationship between these two genes.[X-axis: Log2(ACKR1 TPM) - Represents the expression level of ACKR1,Y-axis: Log2(CHEK2 TPM) - Represents the expression level of CHEK2,Scatter points: Show the relationship between ACKR1 and CHEK2 expression levels in individual samples, Correlation coefficient (R): -0.2, indicating a weak negative correlation, P-value: 5.9e-25, suggesting a highly statistically significant correlation.]



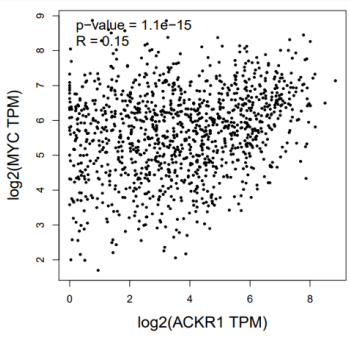
(a)



(b)



(c)



(d)

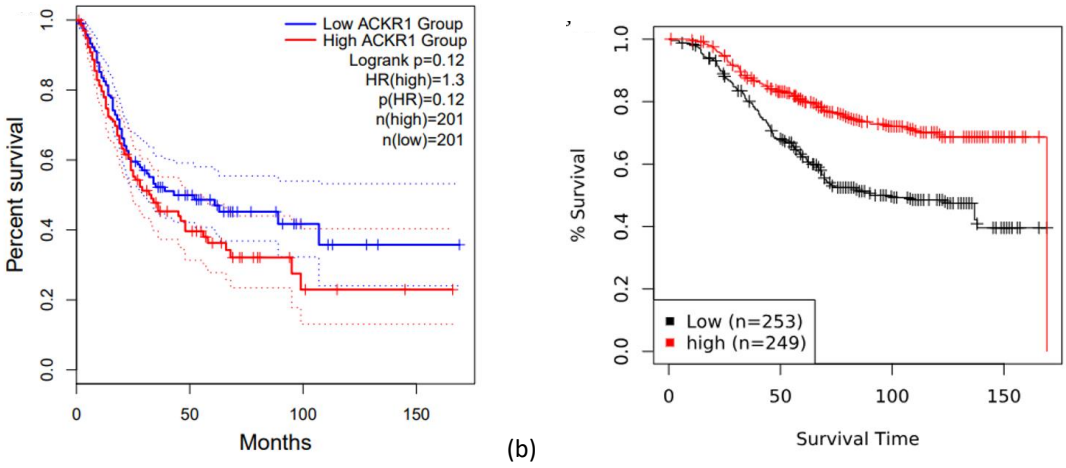
<u>Supply Figure S2: Correlation Between ACKR1 and Tumor Suppressor Genes in Breast Cancer</u>:-The data highlights the importance of ACKR1 (DARC) in breast cancer, emphasizing its connections with ALDH1 and c-MYC.

2 (a) :- The figure shows a moderate positive correlation between ACKR1 and ALDH1 expression, suggesting that higher levels of ACKR1 are associated with higher levels of ALDH1. This finding is consistent with the potential role of both genes in breast cancer progression, as ALDH1 is a marker of cancer stem cells and ACKR1 has been implicated in tumor growth and metastasis.[X-axis: Log2(ACKR1 TPM) - Represents the expression level of ACKR1,Y-axis: Log2(ALDH1 TPM) - Represents the expression level of ALDH1, Scatter points: Show the relationship between ACKR1 and ALDH1 expression levels in individual samples, Correlation coefficient (R): 0.54, indicating a moderate positive correlation, P-value: 0, suggesting a highly statistically significant correlation.]

2 (b):- The figure shows a weak positive correlation between ACKR1 and c-MYC expression. While this correlation is less strong than the one between ACKR1 and ALDH1, it still suggests a potential association between these genes. Further investigation is needed to elucidate the functional implications of this relationship. [X-axis: Log2(ACKR1 TPM) - Represents the expression level of ACKR1,Y-axis: Log2(c-MYC TPM) - Represents the expression level of c-MYC, Scatter points: Show the relationship between ACKR1 and c-MYC expression levels in individual samples, Correlation coefficient (R): 0.23, indicating a weak positive correlation, P-value: 4.46e-16, suggesting a highly statistically significant correlation.]

2 (c):- The figure shows a moderate positive correlation between ACKR1 and ALDH1 expression, suggesting that higher levels of ACKR1 are associated with higher levels of ALDH1.[X-axis: Log2(ACKR1 TPM) - Represents the expression level of ACKR1,Y-axis: Log2(ALDH1 TPM) - Represents the expression level of ALDH1, Scatter points: Show the relationship between ACKR1 and ALDH1 expression levels in individual samples, Correlation coefficient (R): 0.47, indicating a moderate positive correlation, P-value: 0, suggesting a highly statistically significant correlation.]

2 (d):- The figure shows a weak positive correlation between ACKR1 and c-MYC expression.[X-axis: Log2(ACKR1 TPM) - Represents the expression level of ACKR1,Y-axis: Log2(c-MYC TPM) - Represents the expression level of c-MYC, Scatter points: Show the relationship between ACKR1 and c-MYC expression levels in individual samples, Correlation coefficient (R): 0.15, indicating a weak positive correlation, P-value: 1.1e-15, suggesting a highly statistically significant correlation.]



<u>Supply Figure S3:- ACKR1 Expression and Survival in Different Breast Cancer Subtypes:-</u> The figures demonstrate a significant association between ACKR1 expression and survival in breast cancer patients. High ACKR1 expression is linked to poorer survival outcomes, particularly in patients with expression levels above the median.

3 (a):- Overall Survival:- The figure demonstrates a significant difference in survival rates between patients with low and high ACKR1 expression levels ($P < 0.001$ by log-rank test). Patients with high ACKR1 expression have a higher risk of death (HR = 1.3), suggesting that ACKR1 expression is associated with poorer survival outcomes.[Y-axis: Percent survival, X-axis: Survival time (in months), Lines: Represent the survival probability over time for patients with low and high ACKR1 expression levels, Labels: Indicate the number of patients in each group, Hazard Ratio (HR): Quantifies the relative risk of death for patients with high ACKR1 expression compared to those with low expression, P-value: Indicates the statistical significance of the difference in survival between the two groups.]

3 (b) :- Survival Divided by Median Expression Level:- The figure further confirms the association between ACKR1 expression and survival. Patients with high ACKR1 expression (above the median) have significantly poorer survival compared to those with low expression (below the median). This suggests that ACKR1 expression is a robust predictor of survival in breast cancer patients [Y-axis: Percent survival, X-axis: Survival Time (in months), Lines: Represent the survival probability over time for patients with low and high ACKR1 expression levels, divided by the median expression level, Labels: Indicate the number of patients in each group.]

(a)

Spearman's ρ : positive correlation (p<0.05, ρ>0)

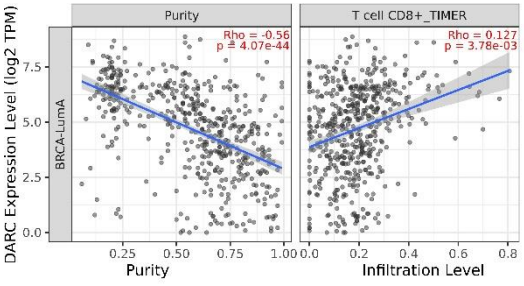
Spearman's ρ : negative correlation (p<0.05, ρ<0)

Spearman's ρ : not significant (p>0.05)

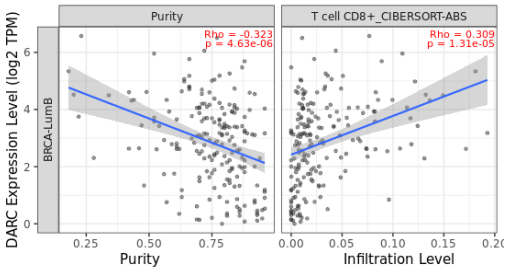
Search: BRCA

cancer	T cell CD8+ TIMER	T cell CD8+ EPIC	T cell CD8+ MCP-COUNTER	T cell CD8+ CIBERSORT	T cell CD8+ CIBERSORT-ABS	T cell CD8+ QUANTISEQ	T cell CD8+ XCELL	T cell CD8+ naive XCELL	T cell CD8+ central memory XCELL	T cell CD8+ effector memory XCELL
BRCA-LumB (n=219)	-0.061	0.158	0.263	0.274	0.309	0.202	0.188	0.047	0.173	0.099
BRCA-Her2 (n=82)	-0.096	0.351	0.523	0.511	0.557	0.534	0.433	0.084	0.523	0.173
BRCA-Basal (n=191)	0.041	0.129	0.139	0.041	0.234	0.161	0.184	0.113	0.193	-0.017
BRCA (n=1100)	0.092	0.083	0.275	0.308	0.366	0.26	0.108	0.083	0.182	0.019
BRCA-LumA (n=568)	0.127	0.133	0.503	0.449	0.539	0.46	0.23	0.156	0.335	0.058

(b)



(c)



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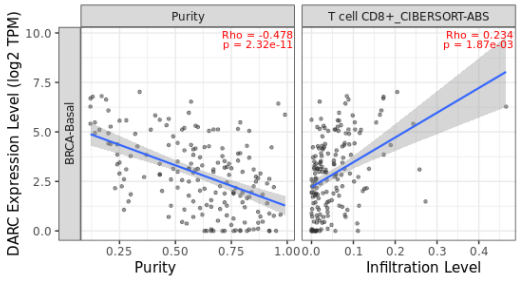


Suppl Figure S4: Correlation of CD8+ with immune infiltration in (a) Different types of Breast Cancer (b) LumA type breast cancer (c) LumB type breast cancer (d) Basal type Breast Cancer

Spearman's ρ : positive correlation (p<0.05, ρ>0)

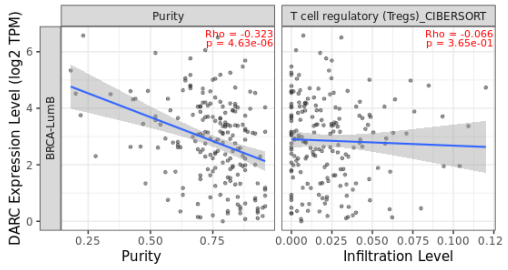
Spearman's ρ : negative correlation (p<0.05, ρ<0)

Spearman's ρ : not significant (p>0.05)

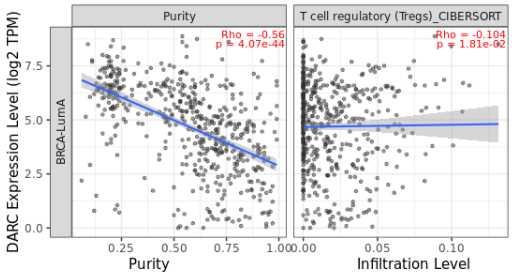
Search: BRCA

cancer	T cell regulatory (Tregs) CIBERSORT	T cell regulatory (Tregs) CIBERSORT-ABS	T cell regulatory (Tregs) QUANTISEQ	T cell regulatory (Tregs) XCELL
BRCA (n=1100)	-0.075	-0.008	0.067	-0.137
BRCA-Basal (n=191)	0.135	0.224	0.302	-0.074
BRCA-Her2 (n=82)	0.284	0.379	0.158	-0.147
BRCA-LumA (n=568)	-0.104	-0.02	0.08	-0.136
BRCA-LumB (n=219)	-0.066	0.024	0.001	-0.065

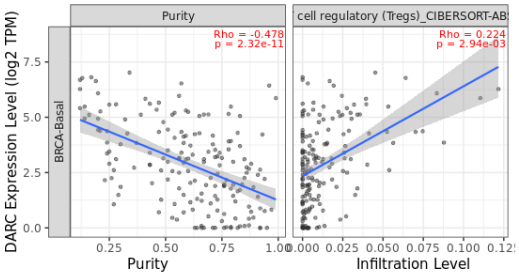
(a)



(b)



(c)



(d)

Suppl Figure S5: Correlation of Tregs with immune infiltration in (a) Different types of Breast Cancer (b) LumA type breast cancer (c) LumB type breast cancer (d) Basal type Breast Cancer

Spearman's ρ : positive correlation (p<0.05, ρ>0)

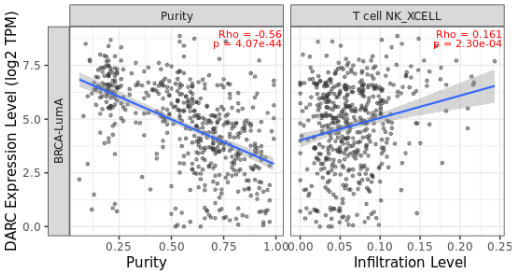
Spearman's ρ : negative correlation (p<0.05, ρ<0)

Spearman's ρ : not significant (p>0.05)

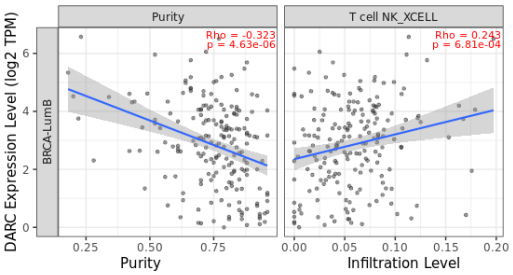
Search: BRCA

cancer	T cell NK XCELL
BRCA (n=1100)	0.199
BRCA-Basal (n=191)	0.046
BRCA-Her2 (n=82)	0.246
BRCA-LumA (n=568)	0.161
BRCA-LumB (n=219)	0.243

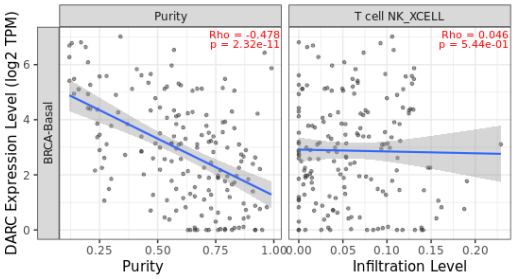
(a)



(b)



(c)



(d)

Suppl Figure S6: Correlation of T cell – NK cell with immune infiltration in (a) Different types of Breast Cancer (b) LumA type breast cancer (c) LumB type breast cancer (d) Basal type Breast Cancer

Spearman's ρ : positive correlation (p<0.05, ρ>0)

Spearman's ρ : negative correlation (p<0.05, ρ<0)

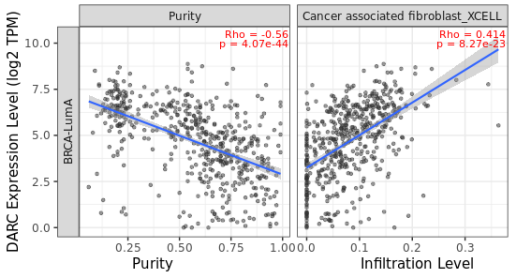
Spearman's ρ : not significant (p>0.05)

Search: BRCA

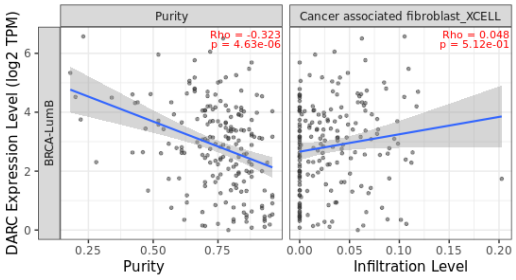
(a)

cancer	Cancer associated fibroblast EPIC	Cancer associated fibroblast MCP-COUNTER	Cancer associated fibroblast XCELL	Cancer associated fibroblast TIDE
BRCA (n=1100)	0.073	0.109	0.441	0.103
BRCA-Basal (n=191)	0.171	0.202	0.234	0.11
BRCA-Her2 (n=82)	-0.491	-0.48	0.065	-0.557
BRCA-LumA (n=568)	-0.022	0.018	0.414	-0.017
BRCA-LumB (n=219)	-0.194	-0.174	0.048	-0.195

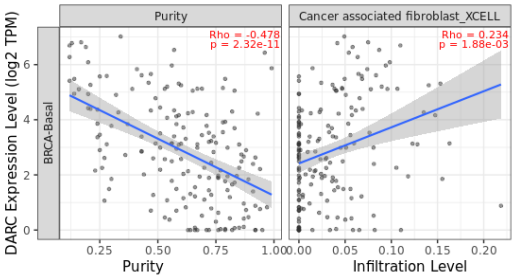
(b)



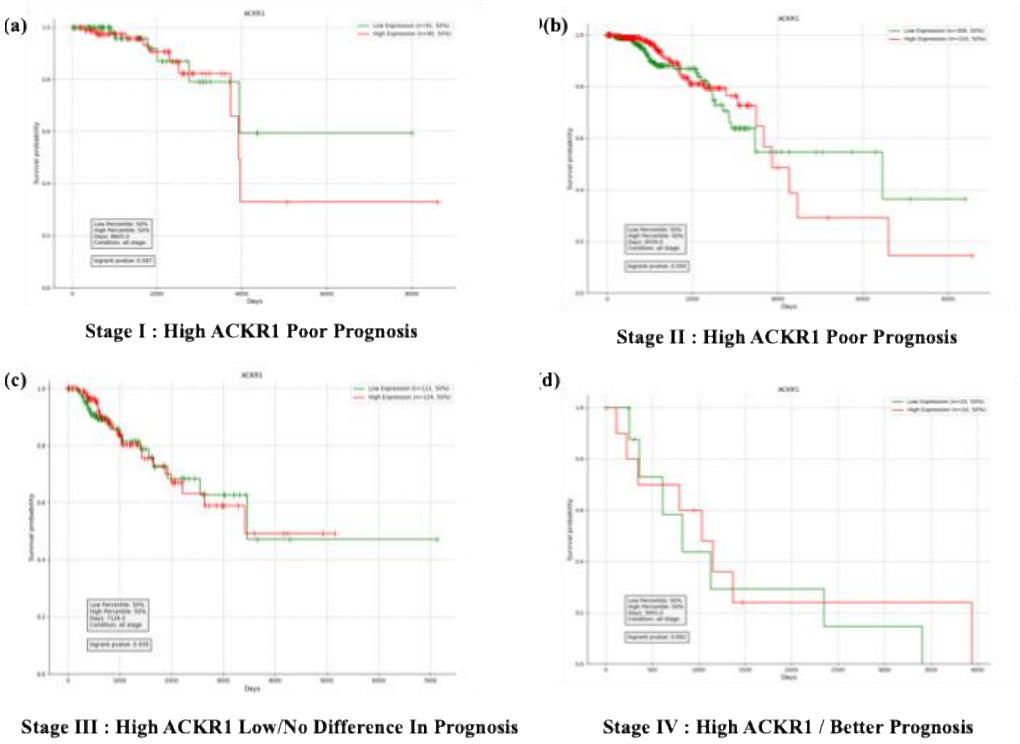
(c)



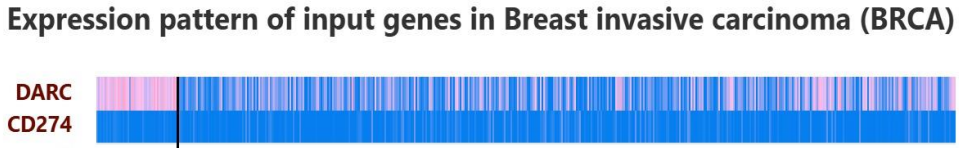
(d)



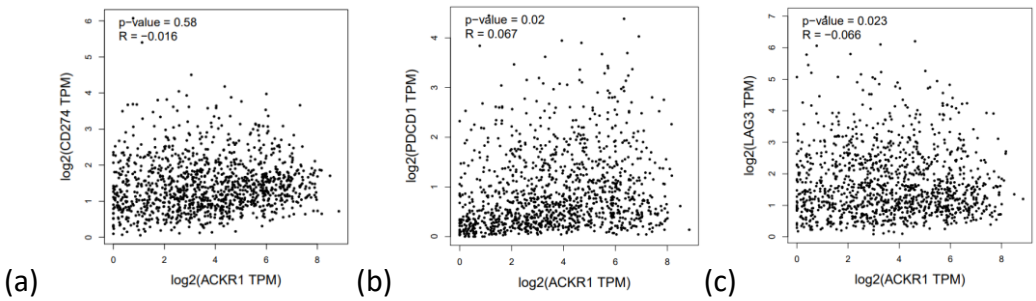
Suppl Figure S7: Correlation of Cancer Associated Fibroblasts with immune infiltration in (a) Different types of Breast Cancer (b) LumA type breast cancer (c) LumB type breast cancer (d) Basal type Breast Cancer

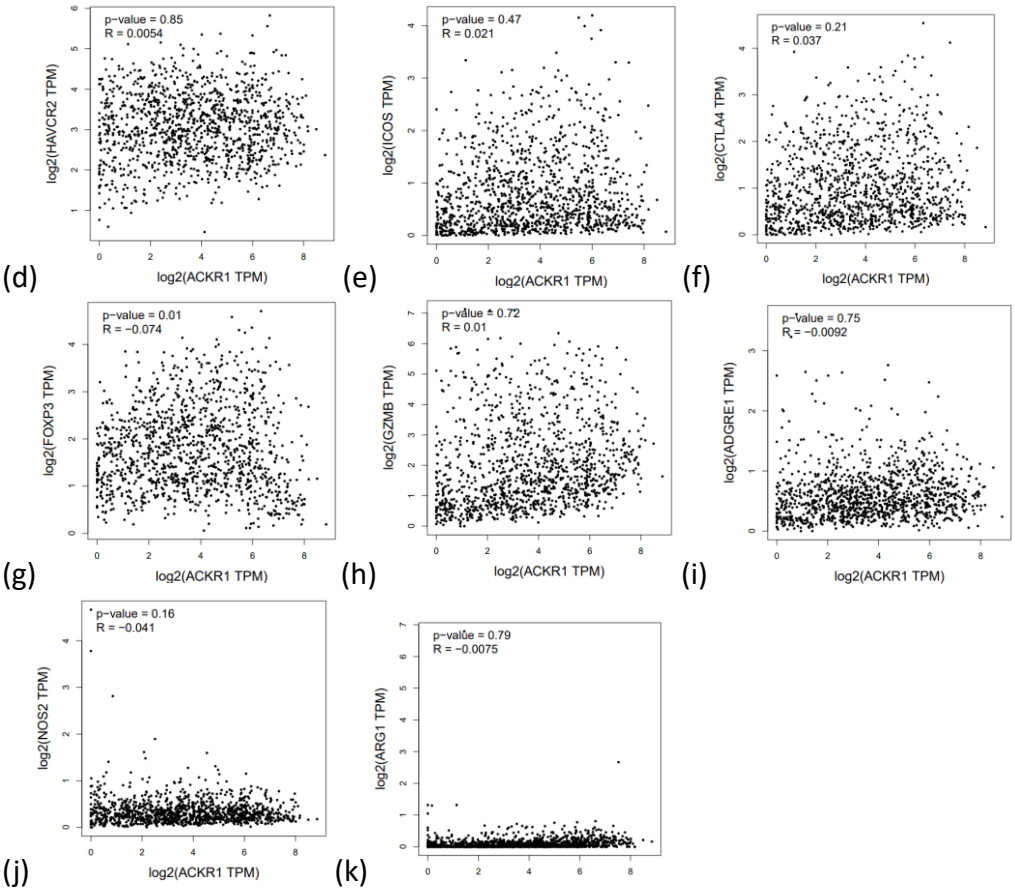


Supply Figure S8: Kaplan-Meier Survival Curves for ACKR1 Expression Levels Across Different Stages: The Kaplan-Meier survival curves display the survival probability over time for patients with low (blue line) and high (red line) expression levels of ACKR1 across different stages of the condition. The groups are divided into "Low Expression" and "High Expression," each constituting 50% of the sample population. The x-axis represents the time in days, and the y-axis represents the survival probability.



Suppl Figure S9: Expression pattern of PDL1 or CD274 and DARC in Breast Invasive Carcinoma (based on TCGA data)

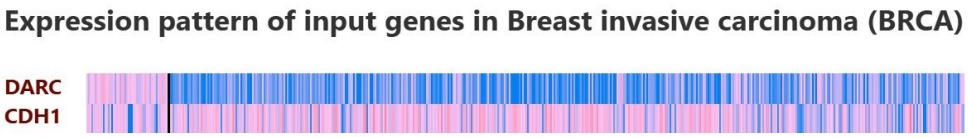


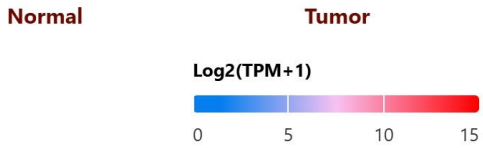


Suppl Figure S10: Correlation Between ACKR1 and Immune checkpoints (a) PDL1 (Statistically irrelevant) (b) PD1 (Statistically relevant slightly positive correlation) (c) LAG3 (Statistically relevant slightly negative correlation) (d) TIM3 (Statistically relevant highly positive correlation) (e) ICOS (Statistically relevant positive correlation) (f) CTLA4 (Statistically relevant positive correlation); Lymphocyte subsets (g) FOXP3 (Statistically relevant slightly negative correlation); Myeloid Markers (h) Granzyme B (Statistically irrelavant) (i) F4/80 or ADGRE1 (Statistically irrelevant) (j) NOS2 or iNOS (Statistically relevant slightly negative correlation) (k) Arginase 1 or ARG1 (Statistically irrelevant)

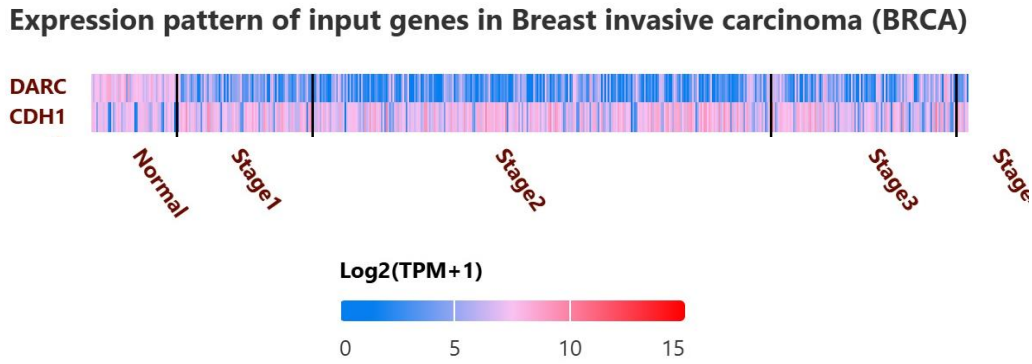
Correlation with EMT

(a)

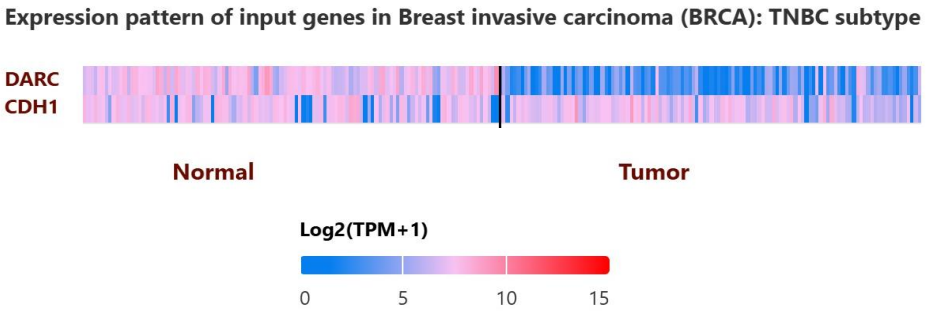



Normal
Tumor
Log2(TPM+1)
0
5
10
15

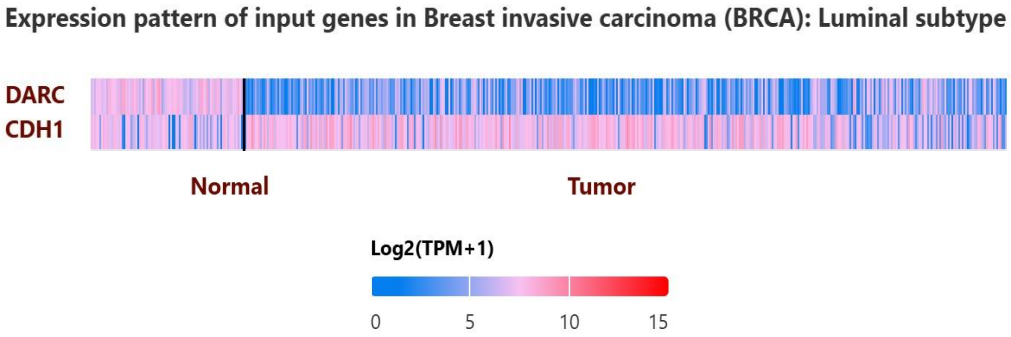
(b)


Expression pattern of input genes in Breast invasive carcinoma (BRCA)
DARC
CDH1
Normal
Stage1
Stage2
Stage3
Log2(TPM+1)
0
5
10
15

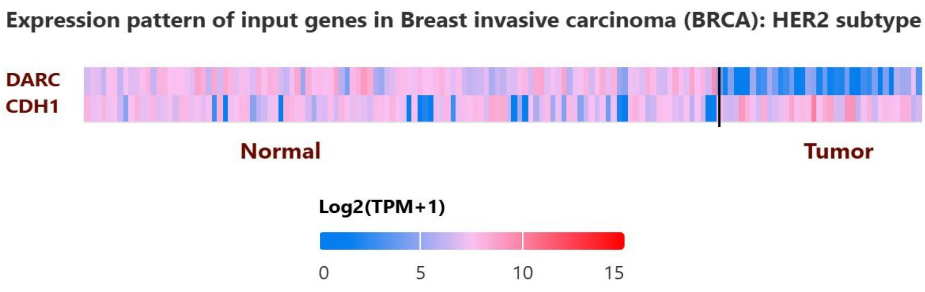
(c)


Expression pattern of input genes in Breast invasive carcinoma (BRCA): TNBC subtype
DARC
CDH1
Normal
Tumor
Log2(TPM+1)
0
5
10
15

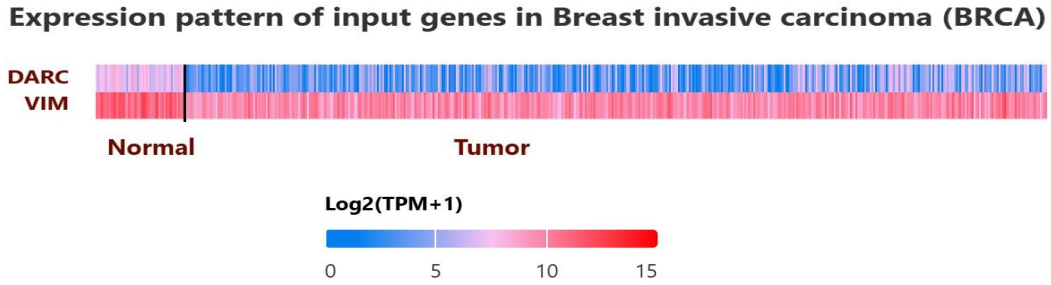
(d)


Expression pattern of input genes in Breast invasive carcinoma (BRCA): Luminal subtype
DARC
CDH1
Normal
Tumor
Log2(TPM+1)
0
5
10
15

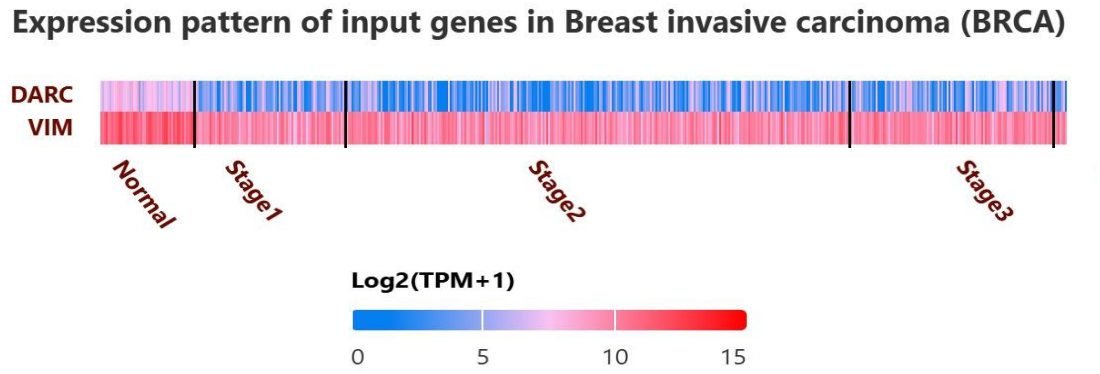
(e)


Expression pattern of input genes in Breast invasive carcinoma (BRCA): HER2 subtype
DARC
CDH1
Normal
Tumor
Log2(TPM+1)
0
5
10
15

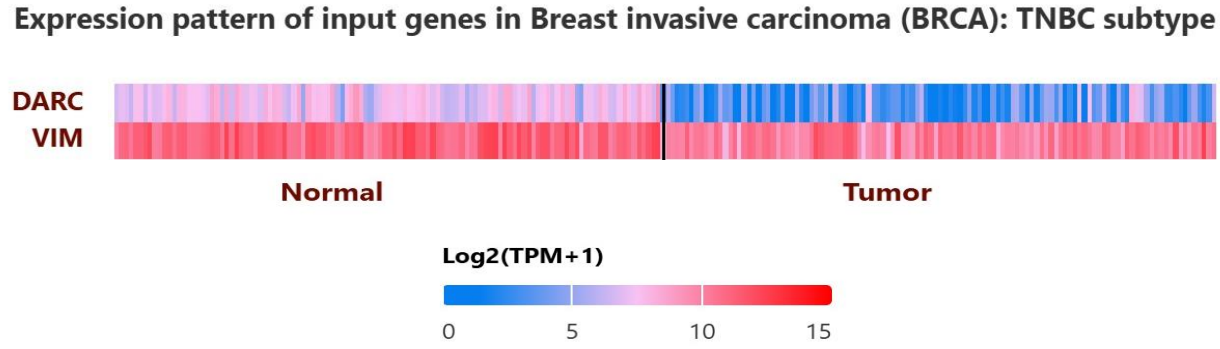
(f)


Expression pattern of input genes in Breast invasive carcinoma (BRCA)
DARC
VIM
Normal
Tumor
Log2(TPM+1)
0
5
10
15

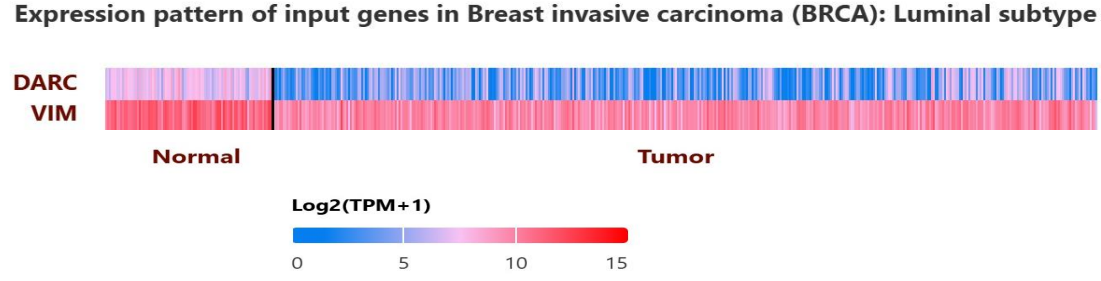
(g)


Expression pattern of input genes in Breast invasive carcinoma (BRCA)
DARC
VIM
Normal
Stage1
Stage2
Stage3
Log2(TPM+1)
0
5
10
15

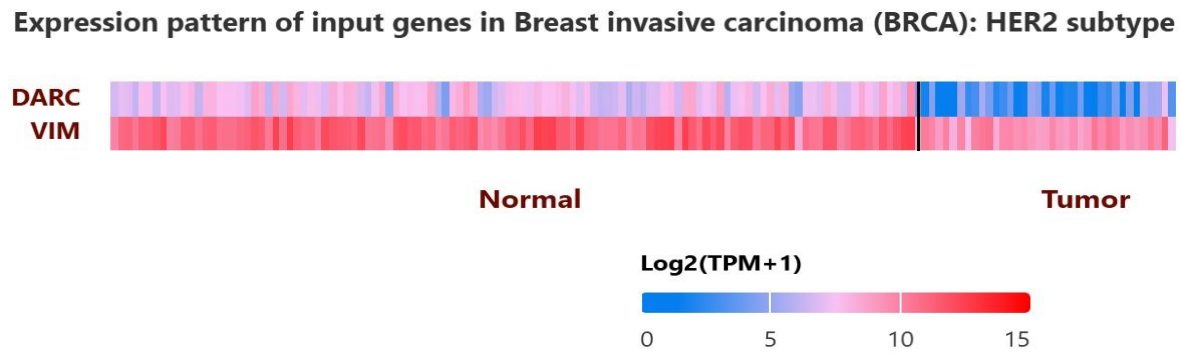
(h)


Expression pattern of input genes in Breast invasive carcinoma (BRCA): TNBC subtype
DARC
VIM
Normal
Tumor
Log2(TPM+1)
0
5
10
15

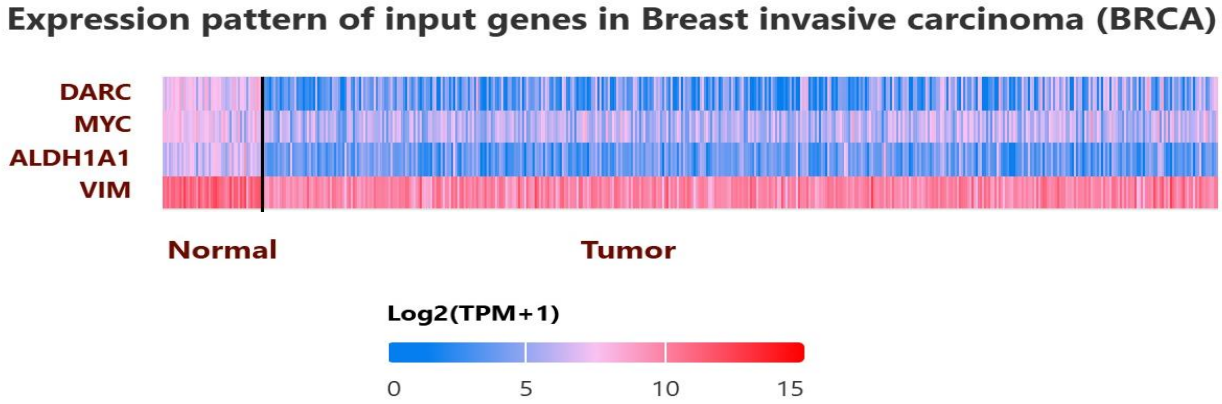
(i)


Expression pattern of input genes in Breast invasive carcinoma (BRCA): Luminal subtype
DARC
VIM
Normal
Tumor
Log2(TPM+1)
0
5
10
15

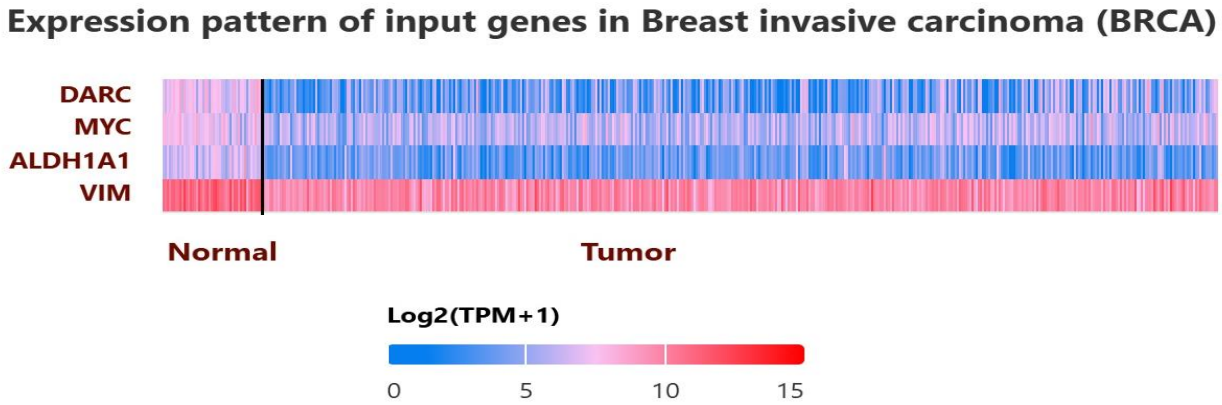
(j)



(k)



(l)



Suppl Figure S11: Expression pattern of EMT and stemness genes and DARC in Breast Invasive Carcinoma (based on TCGA data) (a) CDH1 with respect to Normal vs Tumor (overall) (b) CDH1 with respect to Normal vs Tumor (stage-wise) (c) CDH1 with respect to Normal vs Tumor (in TNBC specifically) (d) CDH1 with respect to Normal vs Tumor (in Luminal subtype Breast Cancer specifically) (e) CDH1 with respect to Normal vs Tumor (in Her2 subtype Breast Cancer

specifically) (f) VIM with respect to Normal vs Tumor (overall) (g) VIM with respect to Normal vs Tumor (stage-wise) (h) VIM with respect to Normal vs Tumor (in TNBC specifically) (i) VIM with respect to Normal vs Tumor (in Luminal subtype Breast Cancer specifically) (j) VIM with respect to Normal vs Tumor (in Her2 subtype Breast Cancer specifically) (k) MYC, ALDH1A1 and VIM with respect to Normal vs Tumor (overall) (l) MYC, ALDH1A1 and VIM with respect to Normal vs Tumor (in TNBC specifically)

Gene	Normal Avg FPKM	Stage	Avg FPKM	Fold Change	Q-value
ACKR1	97.933	I	23.720	4.129	5.811e-19
ACKR1	96.628	II	16.638	5.808	1.295-2i
ACKR1	96.883	III	27.181	3.564	1.359e-17
ACKR1	97.582	IV	16.449	5.932	8.826e-14

Table S1: Comparative Analysis of ACKR1 Expression Levels in Normal and Cancer Tissues Across Different Stages:- The table highlights the differential expression of ACKR1 across normal and cancerous tissues, demonstrating a significant downregulation in malignancy. Normal tissues exhibit high ACKR1 expression (~97 FPKM), which progressively declines across cancer stages. The most pronounced reductions occur in Stage II and Stage IV compared to normal . Although Stage III shows a slight increase over Stage II ,the overall trend indicates a consistent loss of ACKR1 expression with disease progression. The Q-values remain extremely low (e.g., 8.826e-14 for Stage IV), confirming the statistical significance of these findings.

Comparison	Statistical Significance
Normal-vs-Stage I	1.62436730732907E-12
Normal-vs-Stage II	1.62447832963153E-12
Normal-vs-Stage III	1.62447832963153E-12
Normal-vs-Stage IV	1.50569556822688E-11

Table S2: Statistical Significance of Transcript Levels Between Normal Tissues and Various Cancer Stages:- The provided table presents the **statistical significance of differential expression comparisons** between normal tissues and various cancer stages. The **p-values** indicate a highly significant difference in **ACKR1 expression** between normal tissues and all cancer stages. These findings validate previous observations of **ACKR1 downregulation in malignancy** and further support its **potential role as a biomarker for cancer progression**. The strong statistical significance across all stages indicates a robust association between **ACKR1 loss and tumor development**, warranting further investigation into its functional implications in cancer biology.