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Bioinformatic analysis of prognostic value of ARAP3 in breast cancer and the associated signaling pathways

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Abstract. – OBJECTIVE: In this study, we tried to pool previous annotated genomic data to assess the association between ARAP3 expression and metastatic relapse (MR) risk in patients with breast cancer. Moreover, we also investigated the signaling pathways in which ARAP3 might be involved in breast cancer.

MATERIALS AND METHODS: The raw microarray data (GDS5666) that compared gene transcriptional profiles of 4T1 derived lung-aggressive explant and primary tumor explant were reanalyzed to identify the dysregulated genes. ARAP3 mRNA expression, its association with MR-free survival and its co-upregulated genes in breast cancer, were studied by data mining in TCGA database and Breast Cancer Gene-Expression Miner Version 4.0 (bc-GenExMiner 4.0).

RESULTS: ARAP3 is a significantly upregulated gene in the metastatic breast tumor cells. The ER- patients with high ARAP3 expression had significantly increased the risk of MR, regardless of the nodal status. Patients in ER-/Nm group with high ARAP3 expression had the highest risk of MR (HR: 1.25; 95%CI: 1.10-1.41, $p<0.001$). In patients with basal-like tumors, High ARAP3 level is associated with significantly worse MR-free survival (HR: 1.63, 95%CI: 1.22-2.19, $p=0.001$). ARAP3 might be closely related to the NOTCH4 and CDH5 signaling pathways in breast cancer.

CONCLUSIONS: The expression level of ARAP3 might be a useful indicator of the metastatic likelihood of the basal-like breast tumors. ARAP3 might be involved in NOTCH4 and CDH5 related signaling pathways, but the underlying mechanism should be further studied.

Key Words:
ARAP3, Prognosis, Breast cancer, NOTH4, CDH5.

Introduction

Metastasis is one of the most important prognostic factors affecting the survival of breast cancer patient and is also a major cause of cancer-related death[1,2]. Metastasis involves multiple complex processes through which the cancer cells migrate from the primary tumor site to distant organs in the body[3]. Currently, the metastatic potential of a primary breast tumor is usually estimated by pathological characterization of tumor grade and stage[4,5]. Therefore, it is meaningful to explore the potential biomarkers to predict the metastatic likelihood of the primary tumor.

ARAP3 (ArfGAP with RhoGAP domain, ankyrin repeat, and PH domain 3) is a gene encoding a phosphoinositide binding protein containing ARF-GAP, RHO-GAP, RAS-associating, and pleckstrin homology domains[6]. Functionally, it can be recruited to the plasma membrane and act as a PI3K effector regulating both Arf and Rho GTPases[6]. In papillary thyroid carcinoma, ARAP3 showed a tumorigenic role in enhancing proliferation and tumor metastasis[7]. In breast cancer, one recent study reported that ARAP3 is involved in NEDD9 mediated MMP14 enzymatic recovery/recycling through the late endosomes, which enable disengagement of tissue inhibitor of matrix metalloproteinase 2 (TIMP2) and tumor invasion[8]. In fact, NEDD9 is an established marker of epithelial-mesenchymal transition (EMT) and invasion in aggressive breast cancer[9,10]. However, the prognostic value of ARAP3 in metastasis of breast cancer is still unexplored.

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In this study, we tried to pool previous annotated genomic data to assess the association between ARAP3 expression and metastatic relapse (MR) risk in patients with breast cancer. We also investigated the signaling pathways in which ARAP3 might be involved in breast cancer.

Materials and Methods

Microarray Reanalysis

The raw microarray data (GDS5666)[11] that compared gene expression profiles among the parental mammary tumor cell line 4T1 and 4T1 derived metastatic populations isolated from liver, lung or bone were downloaded from GEO datasets. The raw data of lung-aggressive explant and primary tumor explant were reanalyzed to identify the dysregulated genes.

Bioinformatic Analysis of ARAP3 Expression

ARAP3 mRNA expression in breast cancer cohort in TCGA database was assessed using the cBioPortal for Cancer Genomics (http://www.cbioportal.org/)[12]. To explore the expression of ARAP3 mRNA in different subtypes of breast cancer, data mining was performed by using Breast Cancer Gene-Expression Miner Version 4.0 (bc-GenExMiner 4.0), a database of published annotated genomic data including 5609 breast cancer patients[13,14].

Bioinformatic Analysis of the Association Between ARAP3 Expression and Survival Outcomes

The association between ARAP3 mRNA expression and overall survival (OS) in breast cancer cohort in TCGA database was assessed using the cBioPortal for Cancer Genomics (http://www.cbioportal.org/)[12].

To pool previous annotated genomic data that assessed the association between ARAP3 expression and any event (AE, defined as any relapse or death)-free survival and metastatic relapse (MR)-free survival in breast cancer patients, a meta-analysis was performed by using bc-GenExMiner 4.0[13,14].

Bioinformatic Analysis of ARAP3 Co-upregulated Genes in Breast Cancer

The top 10 genes co-upregulated with ARAP3 in breast cancer cohort in TCGA database was identified using the cBioPortal for Cancer Genomics. UCSC Xena browser (http://xena.ucsc.edu/) was further used to get the expression heat map of ARAP3, NOTCH4, and CDH5 and to make regression analysis. The co-expression profiles of the top 10 genes in previous annotated genomic data were analyzed by using bc-GenExMiner 4.0.

Results

4T1-Derived Metastatic Lung Tumor had Increased Expression of ARAP3 Compared to Primary Tumors

By comparing the transcriptional profiles between 4T1-derived lung-aggressive cell populations and the primary tumor explant, we observed that ARAP3 is a significantly upregulated gene in the metastatic tumor cells (Figure 1A). Since metastasis is a poor prognostic indicator, we then investigated whether the expression of this gene is associated with survival of breast cancer patients by retrieving OS data in breast cancer cohort in TCGA database. The Kaplan-Meier curve showed that high ARAP3 expression is associated with unfavorable OS among the patients ($p<0.01$, N=1200) (Figure 1B). Then, we examined the expression profile of ARAP3 across PAM50 breast cancer subtypes by using bc-GenExMiner 4.0. The results showed that the basal-like tumors had significantly higher ARAP3 expression than HER2+, Luminal A and Luminal B subtypes (Figure 1C). The following two-group comparison also confirmed that the basal-like group had significantly higher ARAP3 expression than not basal-like group (Figure 1D).

High ARAP3 Expression is Associated with Increased risk of MR in ER- breast Cancer Patients

To assess the association between ARAP3 expression and MR risk across different subtypes of breast cancer, data mining was further performed in bc-GenExMiner 4.0. By using univariate Cox analysis, we found that in the patients with mixed ER status, the association between high ARPA3 expression and increased MR risk was only observed in nodal positive group (HR: 1.22; 95%CI: 1.06-1.41, p=0.006, N=735) (Table I), but not in nodal status mixed group (HR: 1.07; 95%CI: 1.00-1.16, p=0.062, N=3301) (Table I, Figure 2A). In ER+/Nm group, no association was observed (HR: 0.97; 95%CI: 0.89-1.07, p=0.55, N=2350) (Table I, Figure 2B). However, the ER- patients with high ARAP3 expression had significantly increased the risk of

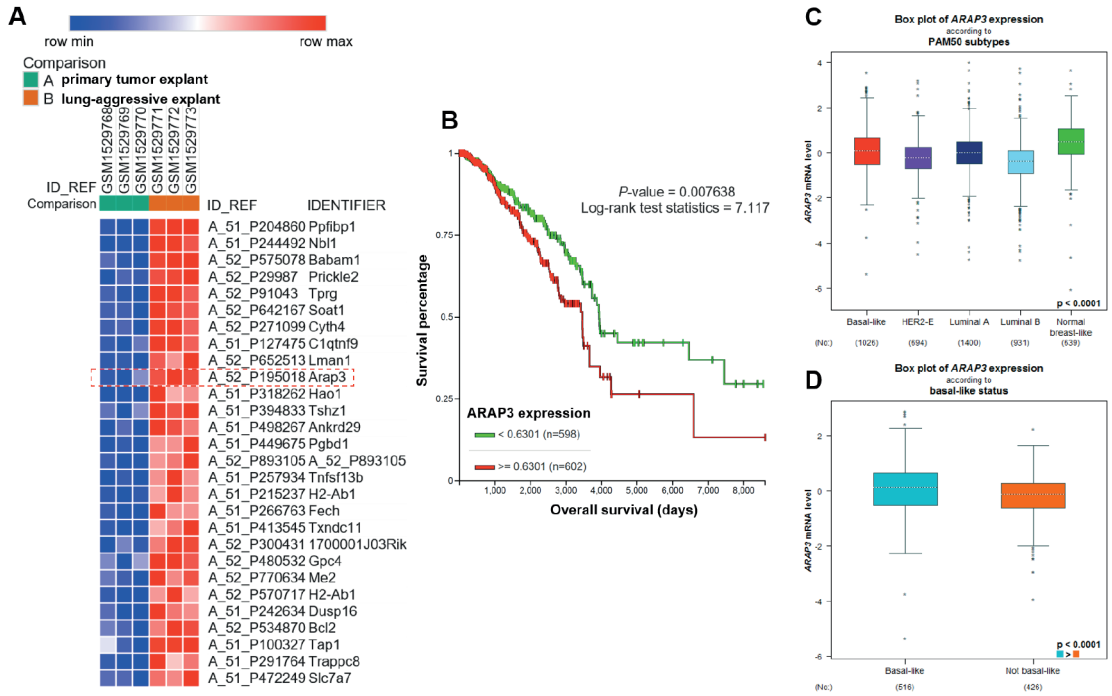

Figure 1. 4T1-derived metastatic lung tumor had increased expression of ARAP3 compared to primary tumors. ***A,*** Heat map of the 30 most upregulated genes in 4T1-derived lung-aggressive cell populations compared to the cells from primary tumor explant. Red: up-regulation. Blue: down-regulation. Image was obtained by re-analysis of the raw microarray data of GDS56661. ***B,*** The Kaplan-Meier curve of ARAP3 expression and OS in 1200 breast cancer patients in the TCGA database. Data was obtained using the UCSC Xena (http://xena.ucsc.edu/). ***C-D,*** Box plot of ARAP mRNA expression across PAM50 breast cancer subtypes (C) and in basal-like compared to not basal-like breast cancer patients (D) in bc-GenExMiner 4.0. The number of tumor samples is indicated at the bottom of the box plot.

Table I. ARAP3 univariate Cox analysis.

#	Population and event criteria			*p*-value	HR	95% CI	No. patients	No. events
1	Nm	ER-	MR	6E-04	1.25	1.10 - 1.41	937	290
2	Nm	ER-	AE	7E-04	1.19	1.07 - 1.31	1 362	519
3	N+	ER-	MR	0.002	1.46	1.15 - 1.86	251	101
4	N+	ER-	AE	0.006	1.29	1.08 - 1.54	394	189
5	N+	ERm	MR	0.006	1.22	1.06 - 1.41	735	232
6	Nm	ER+	AE	0.051	0.93	0.87 - 1.00	3 361	1 045
7	N-	ER-	MR	0.058	1.18	0.99 - 1.41	421	118
8	Nm	ERm	MR	0.062	1.07	1.00 - 1.16	3 301	830
9	N-	ER-	AE	0.085	1.14	0.98 - 1.33	577	186
10	N+	ERm	AE	0.11	1.09	0.98 - 1.21	1 250	510
11	N+	ER+	AE	0.331	0.94	0.82 - 1.07	851	320
12	N-	ER+	MR	0.401	0.94	0.83 - 1.08	1 182	266
13	N-	ERm	AE	0.48	1.03	0.95 - 1.13	2 156	638
14	Nm	ER+	MR	0.547	0.97	0.89 - 1.07	2 350	538
15	N-	ERm	MR	0.581	1.03	0.93 - 1.15	1 612	385
16	N-	ER+	AE	0.599	0.97	0.87 - 1.08	1 564	449
17	Nm	ERm	AE	0.62	1.01	0.96 - 1.07	4 756	1 571
18	N+	ER+	MR	0.813	1.02	0.85 - 1.23	480	130

95% CI: 95% confidence interval; HR: hazard ratio; N: nodal status; +: positive, -: negative, m: mixed; AE: any event; MR: metastatic relapse.

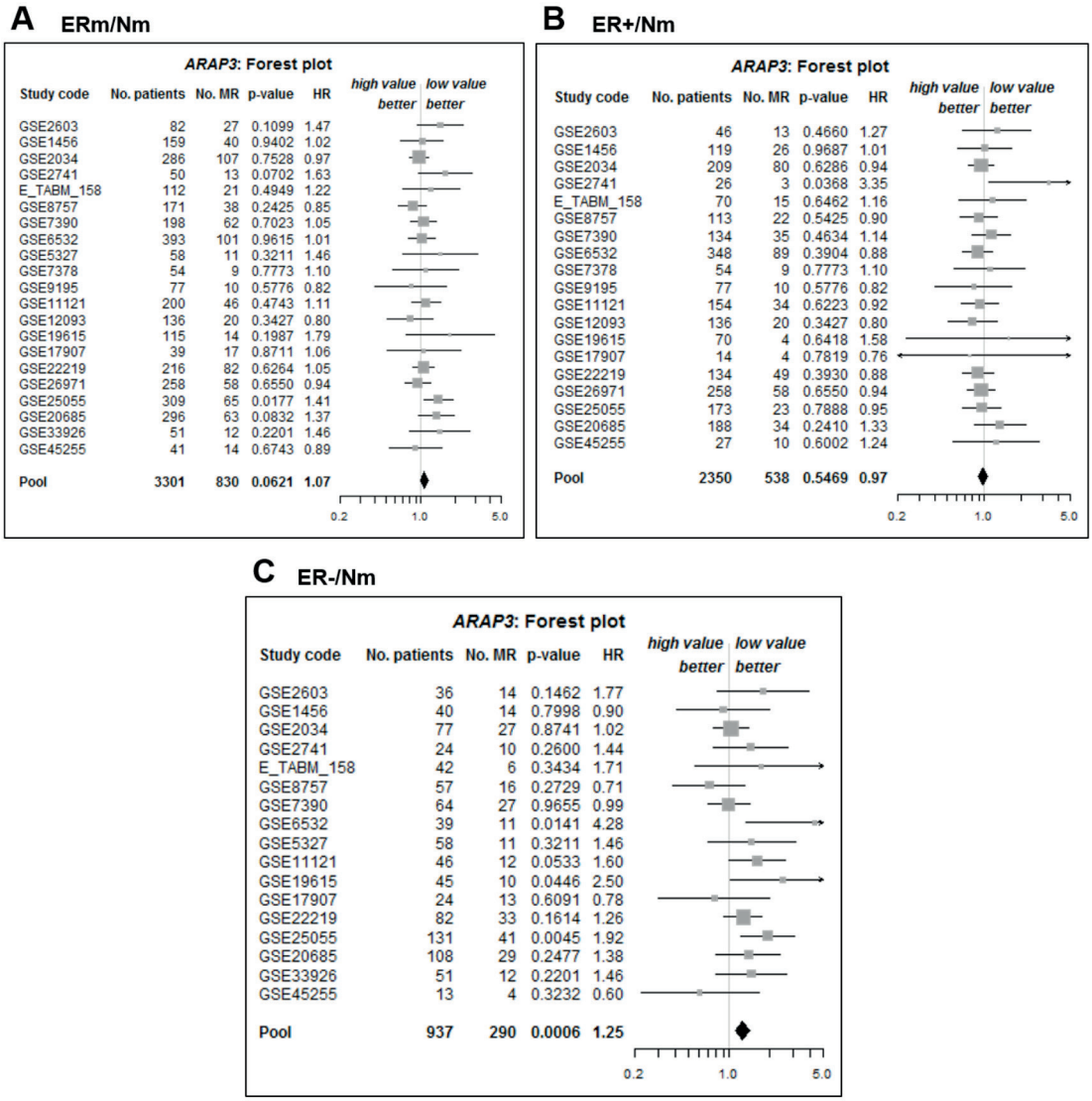

Figure 2. The forest plots of univariate Cox's analysis of ARAP3 expression and the risk of MR in ERm (mixed), ER+ and ER- breast cancer patients. *A-C.* Forest plots displaying univariate Cox's analysis of ARAP3 expression and the risk of MR in ERm (mixed) (A), ER+ (B) and ER- (C) breast cancer patients. Nm: nodal status mixed.

MR, regardless of the nodal status (Table I). Patients in ER-/Nm group with high ARAP3 expression had the highest risk of MR (HR: 1.25; 95%CI: 1.10-1.41, $p<0.001$, N=937) (Table I, Figure 2C).

High ARAP3 expression is Associated with worse MR-free Survival in Basal-like Breast Cancer Patients

To examine the association between ARAP3 expression and MR-free survival in breast cancer patients, Kaplan-Meier survival analysis was performed. The survival curves showed that there is no significant difference in MR-free survival between high and low ARAP3 groups in patients with Nm/ERm, Nm/ER+ and Nm/ER- breast cancer (Figure 3A-C). However, in patients with basal-like tumors, High ARAP3 level is associated with significantly worse MR-free survival (HR: 1.63, 95%CI: 1.22-2.19, $p=0.001$, N=699) (Figure 3D).

ARAP3 Might be Involved in NOTCH4 and CDH5 related Signaling Pathways

Since high ARAP3 expression is associated with worse MR-free survival in basal-like breast cancer, we decided to further investigate the signaling pathways in which ARAP3 might be

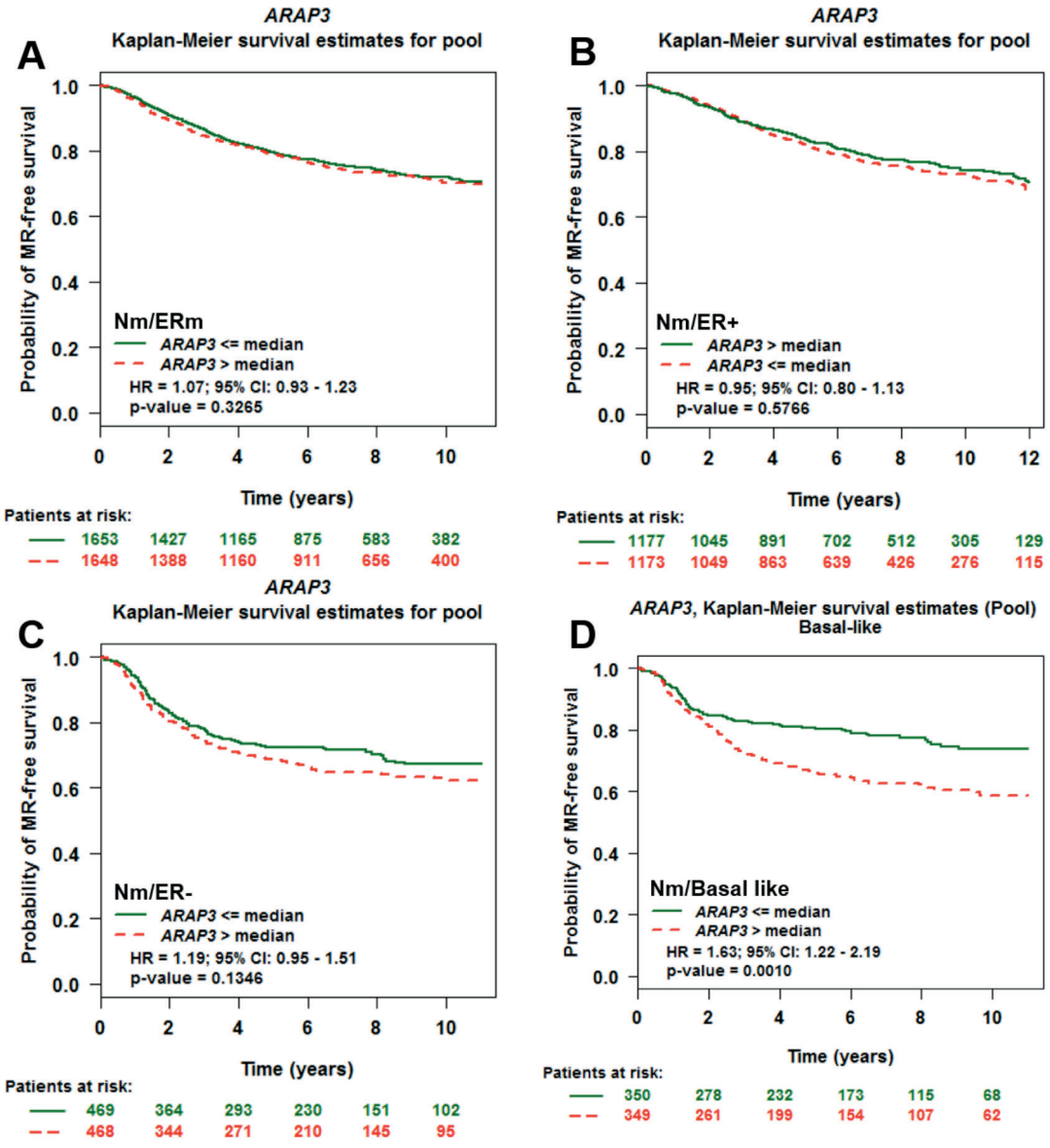

Figure 3. The relationship between ARAP3 expression and MR-free survival. ***A-D.*** Kaplan-Meier survival analysis showing the relationship between ARAP3 expression and MR-free survival in Nm/ERm (A), Nm/ER+ (B), Nm/ER- (C) and Nm/Basal like (D) breast cancer patients. Data was obtained and analyzed by using bc-GenExMiner 4.0. N: nodal status; +: positive, -: negative, m: mixed.

involved in. By performing data mining in breast cancer cohort in TCGA database, we identified the top 10 genes positively correlated to ARAP3 expression, which include NOTCH4 and CDH5 (Figure 4A). In fact, NOTCH4 and CDH5 are two important genes involves in EMT and aggressive phenotypes of breast cancer[15,16]. To verify the correlation between ARAP3 and NOTCH4, CDH5, we also examined the RNA-seq data in breast cancer cohort in TCGA database by using CBioPortal. The heat map and regression analysis confirmed that ARAP3 is highly co-upregulated with NOTCH4 and CDH5 (Pearson's r=0.80 and 0.75 respectively) (Figure 4D-F). These findings suggest that ARAP3 might be closely related to the NOTCH4 and CDH5 signaling pathways in breast cancer. Then, we also examined the co-expression profiles among the 10 genes co-upregulated with ARAP3. Data mining in bc-GenExMiner 4.0 showed that CDH5 and TIE1, CDH5 and ESAM are co-upregulated (Figure 4G).

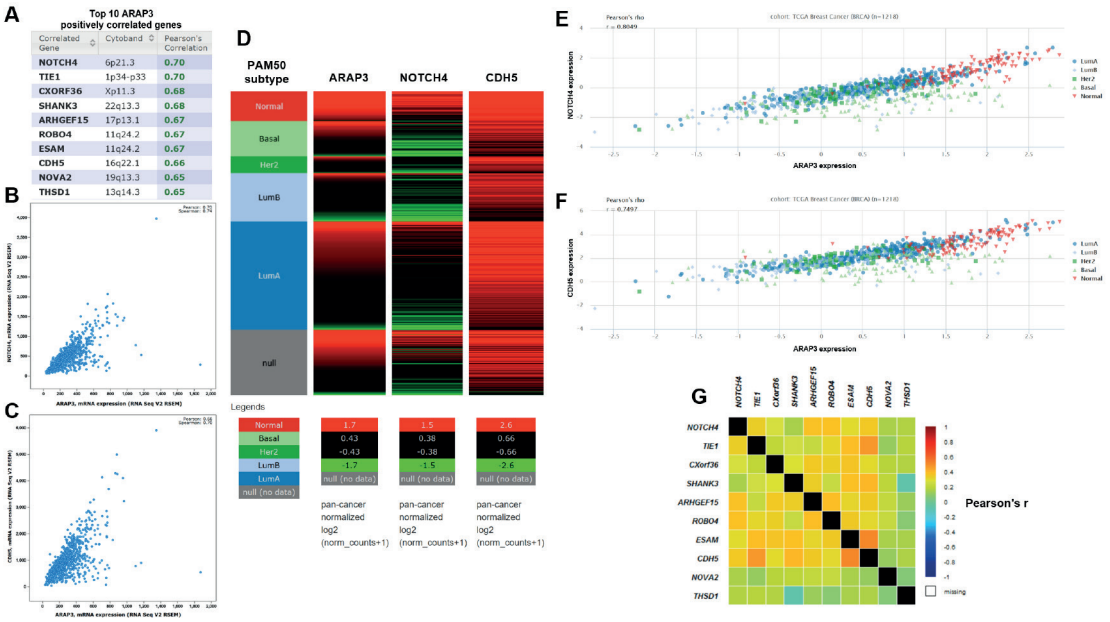

Figure 4. ARAP3 might be involved in NOTCH4 and CDH5 related signaling pathways. ***A,*** The top 10 genes positively correlated to ARAP3 expression in breast cancer cohort in TCGA database. ***B,*** The correlation between ARAP3 and NOTCH4. ***C,*** the correlation between ARAP3 and CDH5. Data was obtained using the cBioPortal for Cancer Genomics. ***D-F,*** The heat map (D) and regression analysis (E-F) of the correlation between ARAP3 and NOTCH4 (E) and between ARAP3 and CDH5 (F) in breast cancer cohort in TCGA database. Data was obtained using the UCSC Xena browser. *G.* Co-expression profiles among the 10 genes co-upregulated with ARAP3. Data mining was performed by using bc-GenExMiner 4.0.

Discussion

ARAP3 is co-expressed and co-localized with NEDD9 in breast cancer tissues, but not in normal breast tissues, suggesting that the cooperation between ARAP3 and NEDD9 could be critical for tumor progression[8]. In fact, NEDD9 can act as a cytoplasmic docking protein required for mesenchymal migration and invasion driven by extracellular matrix (ECM) proteolysis, and it has been characterized as a pro-metastatic gene that is upregulated in multiple different metastatic cancers[8,17]. In lung cancer, NEDD9 can promote cancer cell migration and invasion through the induction of EMT potentially via focal adhesion kinase activation[18]. In breast cancer, NEDD9 can trigger EMT via increasing of several EMT-inducing transcription factors and can also activate the ERK signaling[9]. Its upregulation is significantly associated with increased risk of metastasis and poor prognosis in triple-negative breast cancer patients[10].

Mechanistically, ARAP3 forms a complex with Arf6-GGA3 and interacts with NEDD9[8]. This complex is required to decrease the levels of active Arf6 and allow for trafficking of the MMP14/TIMP2 complex to late endosomes[8], which enable disengagement of tissue inhibitor of TIMP2 and tumor invasion[8]. Therefore, as an important co-regulator with NEDD9, ARAP3 might also play an important role in breast cancer invasion and metastasis. In this study, by comparing the transcriptional profiles between 4T1-derived lung-aggressive cell populations and the primary tumor explant, we observed that ARAP3 is a significantly upregulated gene in the metastatic tumor cells. The following data mining in the breast cancer patient cohort in TCGA database and meta-analysis of previous annotated genomic data suggest that high ARAP3 expression is associated with increased risk of MR in ER- breast cancer patients and indicates significantly worse MR-free survival among the patients with basal-like tumors. Based on these findings, we infer that the expression level of ARAP3 might be a useful indicator of the metastatic likelihood of the basal-like tumors.

Data mining in this work also found NOTCH4 and CDH5 are two genes highly co-upregulated with ARAP3 in breast cancer. Previous studies found that these two genes are important for the aggressive phenotype of breast cancer. The

NOTCH signaling pathway is a key regulator of EMT modulating migration and invasion of breast cancer cells[19], and previous researches[19,20] have revealed the link between EMT and the generation of cancer stem cells (CSCs), which are necessary for dissemination and formation of metastasis. Among the NOTCH genes, NOTCH4 signaling is necessary for the maintenance of breast cancer stemness[21]. Inhibition of NOTCH4 reduced breast cancer stem cell activity *in vitro* and reduced tumor formation *in vivo*[22]. NOTCH4 inhibition also resulted in reduced proliferation and invasiveness of TNBC cells[23]. CDH5 is a cell membrane glycoprotein, which is found at adherens junctions and functions as an adhesion receptor between non-proliferative endothelial cells[24] and emerged as a novel biomarker for metastatic breast cancer[25]. One previous paper[26] in a murine model of mammary carcinogenesis showed that the cells underwent EMT had increased cell surface CDH5. Mechanistically, CDH5 can enhance breast cancer progression via the transforming growth factor β (TGF-β) signaling pathway[27]. One recent study found that elevated CDH5 levels and the ratios of CDH5:HPA (Helix pomatia agglutinin) binding can distinguish breast cancer patients with metastatic disease from those that remained metastasis-free[16].

However, although we identified the co-upregulation profiles, we did not perform studies to investigate the underlying mechanisms of their co-upregulation, which should be further explored in future investigations.

Conclusions

The expression level of ARAP3 might be a useful indicator of the metastatic likelihood of the basal-like breast tumors. ARAP3 might be involved in NOTCH4 and CDH5 related signaling pathways, but the underlying mechanisms should be further studied.

Conflict of interest

The authors declare no conflicts of interest.

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