Original Article
FLAD1 is overexpressed in breast cancer and is a potential predictor of prognosis and treatment

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Abstract: Flavin adenine dinucleotide synthetase 1 (FLAD1) is an important protein-coding gene involved in flavin metabolism and oxidative-reductive process. The expression of FLAD1 is vital to cell survival. The significance of FLAD1 expression has not yet been explored in breast cancer (BC). We used the Oncomine database to mine and analyze FLAD1 expression and found FLAD1 is elevated in BC tissues compared to normal ones. We then investigated its correlation with clinicopathological features using the Breast Cancer Gene-Expression Miner. The results revealed FLAD1 correlated with negative receptor status, high histological grade, and a subtype of worse prognosis. To further clarify the relationship between FLAD1 and the outcome of BC patients, we used the Kaplan-Meier plotter to generate a survival curve. FLAD1 was associated with poor outcome, especially poor recurrence-free survival (RFS) in luminal A, B, and basal-like subtypes. FLAD1 also indicated a worse outcome for grade 2 and lymph node negative BC. For patients with systematic treatment, FLAD1 indicated poor RFS in luminal A, B and basal-like subtypes, as well as in grade 2 and lymph node positive BC. Taken together, our work suggests FLAD1 could be an independent prognostic factor for BC. Further elucidation of FLAD1’s function and pathway may provide new strategies for future BC assessment and treatment.

Keywords: FLAD1, breast cancer, prognosis

Introduction
Breast cancer (BC) is the most common malignant tumor in women. In 2015, it was estimated that 1,384,155 new cases occurred worldwide and approximately 459,000 died from the disease [1]. Nearly 1 in 33 women is likely to be diagnosed with in-situ BC throughout her lifetime [2]. The high incidence and mortality rates have made BC a major public health concern. As BC is a heterogeneous disease with various histopathologic subtypes, genetic characteristics and prognoses, it is necessary to distinguish among different types to fully understand and develop strategies against them, respectively. The gene expression profile comprising hormone receptors (HR), namely estrogen receptor (ER) and progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) status can categorize BC into at least four intrinsic subtypes with distinct clinicopathological characteristics, prognoses and treatment schemes, i.e. luminal A (ER/PR positive, HER2 negative), luminal B (ER/PR positive, HER2 positive), HER2-enriched (ER/PR negative, HER2 positive), and basal-like (ER/PR negative, HER2 negative). Studies have shown this subtype model could predict the 10-year outcome regardless of the systemic treatment administered and the residual risk of distant recurrence after 5 years of endocrine therapy [3]. Although ER, PR, and HER2 are useful biomarkers in the diagnosis and treatment of BC, new biomarkers associated to BC can help build a much deeper and more comprehensive understanding and also provide new treatment targets. Apart from the molecular subtypes, many other clinical factors, such as age at diagnosis [4] and expression of other molecular biomarkers [5] may also impact the prognosis of BC.
FLAD1 codes for flavin adenine dinucleotide (FAD) synthetase, a key enzyme in the FAD biosynthesis process [6]. It is involved in phospho-adenosine phosphosulfate metabolism, FMN biosynthesis and the oxidative-reduction process. Human FLAD1 is ubiquitously expressed in the thyroid, lymph node, and 25 other tissues as four transcription variants, isoform 1-4 [7]. Mutation of the FLAD1 gene might result in multiple acyl-CoA dehydrogenase deficiencies (MADDs), a severe metabolic disorder with mitochondrial respiratory-chain deficiency [8]. Due to its significance in the oxidation-reduction chain, FLAD1 is closely related to the survival of tumor cells and thus the metastasis and prognosis of cancer. Previous studies have identified FLAD1 as a set of potential biomarkers to assess patients with non-small cell lung cancer (NSCLC) for their outcomes, combined with other genes [9]. It has been targeted for potential anti-tumor treatment by target-identification phenotypic screening and competitive affinity-based proteome profiling [10]. However, currently, there is no study available to illustrate the relationship between FLAD1 and BC; the role of FLAD1 in BC stratification remains to be explored.

In this study, we mined online bioinformatics database for FLAD1 profiles in BC and analyzed the relationship between its expression and several clinicopathological parameters of BC. We believe such analysis will provide an option for the assessment, stratification, and treatment of BC patients.

**Material and methods**

**The Kaplan-Meier plotter**

The relationship between FLAD1 expression and the prognostic parameters of BC was analyzed using the Kaplan-Meier plotter (www.kmplot.com), which contains the assessment of prognostic parameters of cancer by various genes [13]. Samples were divided into two cohorts according to the median expression level of FLAD1 (FLAD1-high vs FLAD1-low group: expression level ranking the upper vs lower half). The survival curve of the two cohorts was plotted and then compared across subtypes, histological grades, systematic treatment, as well as these factors combined. The prognoses were evaluated by the following criteria: overall survival (OS), recurrence-free survival (RFS), post-progression survival (PPS), and distal metastasis-free survival (DMFS). The univariate and multivariate survival analyses were conducted using the Cox proportional hazard model. The number at risk and the log-rank p-value and hazard ratio (HR) with 95% confidence intervals (CI) of FLAD1 overexpression were calculated.

**Oncomine 4.5**

Oncomine (www.oncomine.org) is an online cancer microarray database that provides researchers with gene expression profiles as well as clinical and pathological analyses for major types and subtypes of cancer with respective normal tissues [11]. Currently, Oncomine has more than 700 independent data sets. In this study, the expression level of FLAD1 was acquired and compared to normal breast tissue. All datasets were extracted and analyzed from October 2017 to December 2017. Student’s t-test was performed to verify FLAD1 overexpression. The threshold value of this study was defined as: 2.0 fold change, p-value < 0.05, and top 10% gene rank.

**Breast cancer gene expression miner**

Breast Cancer Gene-Expression Miner v4.0 (bc-GenExMiner v4.0), an online database which provides the effect of gene expression on clinical outcomes [12] was used to compare the expression level of FLAD1. The clinicopathological features of BC analyzed in this study included age, HR and HER2 status, nodal status, histological grades, subtypes, etc. The correlations between FLAD1 expression and the clinicopathological parameters of BC patients were assessed using a chi-square test. The FLAD1 mRNA level was compared and plotted according to the above parameters, and the Dunnett-Tukey-Kramer’s test was conducted.

**Results**

**FLAD1 was associated with a poor prognosis of BC patients**

We used the Kaplan-Meier plotter to plot and investigate the prognosis of BC patients with elevated FLAD1, including their OS, RFS, PPS and DMFS. The results indicated that up-regulated FLAD1 expression is correlated with worse OS (HR = 1.41, P = 0.0015), RFS (HR = 1.34, P < 0.001), DMFS (HR = 1.37, P = 0.033), and PPS (HR = 1.3, P = 0.0016) (Figure 1). We then examined FLAD1’s impact on survival of
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the four subtypes and discovered no obvious correlation between FLAD1 expression and OS (Figure 2A-D), but RFS was closely related to the FLAD1 expression level except in the HER2-enriched group (luminal A HR = 1.42, P = 0.0074; luminal B HR = 1.54, P = 0.0011; HER2-enriched HR = 0.77, P = 0.34; basal-like HR = 1.47, P = 0.034) (Figure 2E-H).

Further efforts were made to clarify whether FLAD1 expression could stratify prognosis in different histological grading and lymph node status, as well as the effectiveness of treatment. In the end, FLAD1 showed a capability in distinguishing prognosis in grade 2 BC (OS HR = 1.65, P = 0.023; DMFS HR = 1.69, P = 0.0033) (Figure 3B, 4B), while having a minor influence in grade 1 or 3 BC. As for nodal status, FLAD1 expression is a better prognostic factor in the LN negative group than it is in the LN positive group, indicating poor OS (HR = 1.52, P = 0.028) and DMFS (HR = 1.4, P = 0.014) (Figure 5E-G). Finally, to determine whether there was deviation caused by systematic treatment, we compared the outcomes of treated patients across subtypes, histological grades, and nodal statuses, respectively. As a result, RFS was still different in luminal A, B and basal-like BC among all subtypes (luminal A HR = 1.42, P = 0.0074; luminal B HR = 1.54, P =
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Figure 2. The relationship between FLAD1 expression and the OS/RFS of the different subtypes of BC. The survival curve was generated using the Kaplan-Meier Plotter. The red curve represents patients with high FLAD1 expression; the black curve represents patients with low FLAD1 expression. A-D: OS of luminal A, luminal B, HER2-enriched and basal-like BC. E-H: RFS of luminal A, luminal B, HER2-enriched and basal-like BC.
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**Figure 3.** The relationship between FLAD1 expression and the OS/RFS of different grades of BC. The survival curve was generated using the Kaplan-Meier Plotter. The red curve represents patients with high FLAD1 expression; the black curve represents patients with low FLAD1 expression. A-C: OS of grade 1~3 BC. D-F: RFS of grade 1~3 BC.

**Figure 4.** The relationship between FLAD1 expression and the DMFS/PPS of the different grades of BC. The survival curve was generated using the Kaplan-Meier Plotter. The red curve represents patients with high FLAD1 expression; the black curve represents patients with low FLAD1 expression. A-C: DMFS of grade 1~3 BC. D-F: PPS of grade 1~3 BC.
Figure 5. The relationship between FLAD1 expression and the outcome of different nodal status. The survival curve was generated using the Kaplan-Meier Plotter. The red curve represents patients with high FLAD1 expression; the black curve represents patients with low FLAD1 expression. A-D: OS, RFS, DMFS and PPS of lymph node positive BC. E-H: OS, RFS, DMFS, and PPS of lymph node negative BC.
Figure 6. The relationship between FLAD1 expression and the OS/RFS of different subtypes of BC after systematic treatment. The survival curve was generated using the Kaplan-Meier Plotter. The red curve represents patients with high FLAD1 expression; the black curve represents patients with low FLAD1 expression. A-D: OS of luminal A, luminal B, HER2-enriched and basal-like BC. E-H: RFS of luminal A, luminal B, HER2-enriched and basal-like BC.
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0.0011; basal-like HR = 1.47, P = 0.034) (Figure 6E-H) and in grade 2 BC among all histological grades (HR = 1.53, P = 0.019) (Figure 7); meanwhile, OS was different in lymph node positive BC (HR = 0.53, P = 0.027) (Figure 8A). Taken together, these observations implied systematic treatment did have an impact on the deviation of outcome, and the response to treatment was different with FLAD1 expression levels in some groups.

The expression level of FLAD1 was elevated in BC patients

To investigate whether FLAD1 overexpression can account for the poor prognosis, we compared the expression levels of FLAD1 in BC and normal breast tissues in the Oncomine database. Based on the cross-comparison of 19 studies, we found that the expression of FLAD1 is significantly elevated in BC tissue with a median rank of 567.0 and p-value = 0.007 (Figure 9). We also investigated FLAD1 expression in different histopathologic subtypes of BC and found that FLAD1 expression was up-regulated in ductal breast carcinoma in-situ (P < 0.001), invasive ductal breast carcinoma (P < 0.001), medullary breast carcinoma (P < 0.001), and invasive lobular breast carcinoma (P < 0.001) (Figure 10).

The relationship between FLAD1 mRNA expression and clinicopathological features

Further attempts were made to clarify the relationship between FLAD1 overexpression and clinicopathological features such as age, receptor status, histological grade, subtypes, and nodal invasion. The results suggested no statistical difference between the FLAD1 mRNA levels in patients > 51 years and < 51 years (P = 0.1582, Figure 11A). However, other clinical parameters were closely related to the FLAD1 mRNA level. To begin with, FLAD1 mRNA levels were different in patients with different receptor status: BC with negative PR (P < 0.001) and ER (P < 0.001) had higher levels of FLAD1 mRNA, while the difference was insignificant in regard to HER2 express statuses (P = 0.1816) (Figure 11B-D); high mRNA levels of FLAD1
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Figure 8. The relationship between FLAD1 expression and the OS/RFS of different nodal statuses after systematic treatment. The survival curve was generated using the Kaplan-Meier Plotter. The red curve represents patients with high FLAD1 expression; the black curve represents patients with low FLAD1 expression. A: OS of lymph node positive BC. B: OS of lymph node negative BC. C: RFS of lymph node positive BC. D: RFS of lymph node negative BC.

Discussion

FLAD1 (FAD1, FADS, PP591, or LSMFLAD) is the protein-coding gene for FAD synthetase. The human FLAD1 gene is located on the long arm of chromosome 1q21.3 and is expressed ubiquitously across all tissue types, with the highest expression level in lymph nodes, testis, and adipose tissue [7]. FAD-related enzymes constitute a considerable proportion of intracellular proteins [15]. Consistent with this, they possess cellular functions including FAD biosynthesis, oxidation-reduction, and riboflavin metabolism [6]. A defect of the FLAD1 gene

were found in triple-negative BC patients (Figure 11E). As for the Scarff Bloom & Richardson (SBR) grade, a higher FLAD1 mRNA level was related to a higher histological grade of BC (P < 0.0001; between-group P < 0.0001) (Figure 11F). Moreover, different subtypes of BC had different FLAD1 mRNA levels, in brief: luminal A type had a lower FLAD1 mRNA level than any of the other three types (P < 0.001), while the difference among the other three types was not significant (P > 0.10) (Figure 11G). The status of nodal invasion was not related to FLAD1 expression (P = 0.7714) (Figure 11H).
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Figure 9. FLAD1 expression is elevated in BC patients compared to normal breast tissue, according to heat map obtained from Oncomine database.

can result in metabolic disorders, such as MADDs [8]. Besides supporting the above cell functions, FAD synthetase has been identified as interacting with other genes, for example, ANXA7 [16], possibly unveiling a large-scale protein-protein interaction network.

FLAD1 has been found to correlate with not only metabolic syndromes but also the occurrence and development of cancers. Eeles et al. have identified FLAD1 as one of the common prostate cancer susceptibility loci [17]; Mitra et al. further investigated the relation between FLAD1 and non-small cell lung cancer and found significant differential expression of FLAD1 in recurrent tumors, suggesting that a high FLAD1 expression level might be a risk factor for tumor relapse [9]. Unfortunately, the expression of FLAD1 in BC has not been explored to date, leaving a gap in the research of BC biomarkers and prognosis. We first explored the Oncomine database and found that FLAD1 overexpression was correlated with a poor prognosis of BC. Moreover, our study suggested FLAD1 was overexpressed in BC and associated with the invasive clinicopathological features of BC.

We conducted survival analyses using FLAD1 levels and observed excellent stratifying power in terms of OS, RFS, DMFS, and PPS. We further subdivided the groups according to subtypes, histological grades, and nodal status to testify whether FLAD1 predicts poor prognosis in different subgroups. No obvious relationship between FLAD1 and OS was observed in the four intrinsic subtypes, respectively. However, a worse RFS was observed with high FLAD1 level in luminal A, B, and basal-like BC. As previously discussed, the subtypes were derived predominantly according to the gene-expression profile of HR and HER2; our finding implied a possible crosstalk between FLAD1 level and oncogene expression of the tumor cell; more importantly, it served to stratify risk within each subtype. First, among the four intrinsic subtypes, the luminal A type is associated with the most favorable outcome, but there still remains a
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Figure 10. FLAD1 expression was elevated in different histopathologic subtypes, including invasive ductal carcinoma, medullary breast carcinoma, ductal breast carcinoma in-situ, and invasive lobular breast carcinoma. The gene expression level was compared between a normal breast (left box) and cancer (right box).

demand for more accurate stratification [18]; FLAD1 can be a potential prognostic marker to spare low-risk luminal A type BC patients from unnecessary endocrine and targeted therapies. Secondly, the luminal B/HER2-enriched type responds well to an HER2 blockade combined with chemotherapy [3]; FLAD1 expression can possibly identify patients in need of further intervention. Finally, basal-like type B, with basal or myoepithelial markers, has the worst prognosis [19] and accounts for up to 15% of all BC [20]; it represents a heterogeneous group of BC that demands further classification. We confirmed FLAD1 could differentiate the outcome of basal-like type BC patients, but whether FLAD1 is an independent prognostic factor within each basal-like subtype remains to be explored.

Using the histological grade is an important method of describing tumor behavior. The Nottingham Grading System considers three aspects of tumor histology to grade BC: degree of differentiation, nuclear pleomorphism, and mitotic activity, with each given 1 to 3 points, adding up to a final score ranging from 3 to 9 (3-5 points: grade 1, 6-7 points: grade 2, 8-9 points: grade 3). This grading system is well established and recognized for BC risk stratification: grade 3 BC cancer has a high incidence of relapse and early distal metastasis; meanwhile, although the behavior of grade 2 BC is intermediate during early follow-up, there is an obvious deterioration of outcomes after long-term follow-up [21]. Our analysis suggested FLAD1 was associated with the OS and DMFS of grade 2 BC patients among all grades. While the underlying mechanism was unclear, it can potentially assist with the identification of high-risk grade 2 BC patients and facilitate attentive follow-up and prompt intervention.

The lymph node status has a significant impact on the prognosis of BC [22]; however, it should
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Figure 11. The FLAD1 mRNA expression level according to the clinicopathological features. Welch’s test was conducted for global differences and a p-value was generated for each group. Dunnett-Tukey-Kramer’s test was conducted where statistical differences existed (P < 0.05). A: FLAD1 mRNA expression level was not different in the > 51 and < 51 age groups. B-D: FLAD1 mRNA expression level was different with receptor status. E: FLAD1 mRNA expression level was different with triple-negative status. F: The FLAD1 mRNA expression level was different with each SBR grade. G: The FLAD1 mRNA expression level was different with each subtype. H: The FLAD1 mRNA expression level was not correlated to nodal status.
be emphasized that lymph node status is only one manifestation of tumor metastasis and can often shift from negative to positive throughout its progression. We found that a high FLAD1 level indicated a poor OS and DMFS for the lymph node negative subgroup, but the correlation was not prominent in the lymph node positive group, one plausible explanation being that lymph node status is time-dependent so there is often a lag time between molecular changes and lymph node invasion [21]. With FLAD1 showing more capacity in the lymph node negative group, it may help identify high-risk patients from other lymph node negative patients and buy them more time for further treatment.

After systematic treatment, the luminal A type had robust distinction on OS; FLAD1 was associated with a worse RFS for luminal A and basal-like BC. Regarding the histological grading, only grade 2 BC showed a statistical difference. Current endocrine therapy and chemotherapy mainly work by suppressing cell proliferation. FLAD1 promotes oxidation-reduction activity and is consistent with the energy demand during cell proliferation; therefore, FLAD1-high tumors are more likely to resist proliferation suppression. According to the results, FLAD1 is a potential monitoring item during the systematic treatment for luminal BC, basal-like BC, and any grade 2 BC.

Our investigation of expression identified FLAD1 overexpression in BC tissue. Specifically, FLAD1 expression was up-regulated across the board in ductal breast carcinoma in-situ, invasive ductal breast carcinoma, medullary breast carcinoma, and invasive lobular breast carcinoma. Interestingly, the FLAD1 mRNA level varied in the different subtypes of BC. Our analyses suggested an elevated FLAD1 mRNA level was associated with negative ER and PR; triple-negative BC patients had higher expression levels of FLAD1 mRNA. This was consistent with our observation that luminal A type BC had a significantly lower FLAD1 mRNA level than any of the other subtypes, especially the basal-like subtype. These results suggested a higher FLAD1 mRNA level was associated with lower HR expression, indicating a poor response to endocrine and molecular targeted treatment. Meanwhile, we observed that the FLAD1 mRNA level was positively correlated with the histological grade of BC, but the status of the nodal invasion was not relevant to the FLAD1 mRNA level. It is known that nodal invasion represents a tumor’s ability to metastasize, so we speculated that FLAD1 contributed to the development of BC predominantly by affecting differentiation and proliferation instead of migration.

One limitation of this study was the lack of mechanism investigations. As it remains to be seen whether the FLAD1 pathway exerts unique impact on tumor cells other than flavin metabolism, we were not able to explain but only hypothesize how FLAD1 was related to the poor prognosis of BC. Nevertheless, FLAD1 still offers a path forward towards a future BC risk assessment system.

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Disclosure of conflict of interest

None.

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