VDAC1 Conversely Correlates with Cytc Expression and Predicts Poor Prognosis in Human Breast Cancer Patients

Fangfang Chen  
Wuhan University Renmin Hospital

Shuai Yin  
Jingzhou Central Hospital

Bin Luo  
Wuhan University Renmin Hospital

Xiaoyan Wu  
Wuhan University Renmin Hospital

Honglin Yan  
Wuhan University Renmin Hospital

Dandan Yan  
Wuhan University Renmin Hospital

Chuang Chen  
Wuhan University Renmin Hospital

Feng Guan  
Wuhan University Renmin Hospital

Jingping Yuan  
Wuhan University Renmin Hospital  
yuanjingping@whu.edu.cn  
Wuhan University Renmin Hospital  
https://orcid.org/0000-0001-7470-6899

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Abstract

**AIM:** The main objectives of this article were to evaluate the association of voltage-dependent anion channel 1 (VDAC1) expression with Cytochrome C (Cytc) protein, various clinicopathological features and prognosis in patients diagnosed with breast cancer (BC). Meanwhile, the correlation of Cytc expression with various clinical features and 5-year disease-free survival (5-DFS) of BC is also investigated.

**Methods:** Expression of VDAC1 protein and Cytc protein was conducted in 219 BC patients and 60 benign breast lesions by immunohistochemical (IHC) analysis.

**Results:** In our study, VDAC1 protein expression was significantly increased while Cytc protein was decreased in breast tumor tissues ($P=0.015$ and $P=0.029$, respectively). High expression of VDAC1 is conversely associated with Cytc expression in BC, especially in triple-negative breast cancer (TNBC) ($P=0.011$ and $P=0.004$, respectively). Interestingly, high expression of VDCA1 not only had a significant association with advanced TNM stage, histological grade, LNM, HER2 gene amplification and recurrence ($P<0.05$), but also displayed a poorer 5-DFS ($P<0.001$) in BC. The multivariate Cox proportional hazard model demonstrated VDAC1 as a novel independent poor prognostic factor in BC ($P=0.001$). Strikingly, our study also showed the prognostic significance of VDAC1 in triple-negative breast cancer in particular. Furthermore, low expression of Cytc was found to be correlated with histological grade, ER status, PR status and recurrence in BC ($P<0.05$). Kaplan-Meier analysis indicated that low Cytc expression was associated with poorer survival and high mortality rate ($P=0.007$). Cytc protein expression also served as a novel independent prognosis parameters in BC patients ($P=0.035$).

**Conclusion:** Our findings firstly revealed that VDAC1 was elevated in BC tissues and conversely associated with Cytc. Furthermore, high VDAC1 protein was associated with reduced 5-DFS and could be acted as an independent poor prognostic factor not only in breast cancer in general, but also in TNBC in particular. All in all, VDAC1 can be exploited as a potential prognostic marker and therapeutic target in BC, especially in TNBC.

Introduction

Breast cancer (BC) is by far the most common female malignant tumor in the world, with a high mortality rate, and is the leading cause of cancer-related death among women[1]. In China, 367900 new cases of BC were diagnosed in 2018, which accounts for 19.2% of all newly diagnosed female cancers[2]. Although breast conservation surgical resection, targeted therapy, endocrine therapy, or immunotherapy have made progress in the treatment of BC in recent years, the incidence of the disease has increased[3]. And only limited success has been achieved in cases of advanced cancer[4]. The burden of this disease is heavy and it remains a serious health threat for women relative to the large Chinese population[5]. Therefore, it is extremely important for new prognostic markers and early diagnosis of BC, so that patients can receive better treatments.
Cancer cells are characterized by a common set of properties, including high proliferation and resistance to apoptosis[6]. Cancer formation is correlated with cell metabolic reprogramming, such as enhanced aerobic glycolysis (Warburg effect)[7]. It is postulated that the "Warburg effect" is closely associated with the closure of Voltage-dependent anion channel (VDAC)[8, 9]. VDAC has been identified as a 31 kDa pore-forming protein, which is found in the outer mitochondrial membrane (OMM) of all eukaryotes[10]. Three homologous genes encode three VDAC isoforms: VDAC1, VDAC2, and VDAC3[11]. Among them, VDAC1 is the most abundantly expressed and best characterized one, which forms a channel for the entry and exit of mitochondrial metabolites including ATP and NADH across the OMM, thereby regulating mitochondrial activity[12]. VDAC1 plays a key role in regulating apoptosis by both interaction with pro-apoptotic (Bcl2 and Bcl-xL) and anti-apoptotic (Bax, Bak, and Bim) proteins[13, 14]. It has been proved that VDAC1 involved in several diseases, such as neurodegenerative, cardiac injury and neoplastic[15]. Several studies have demonstrated remarkable expression levels of VDAC1 in malignant tumors, such as uterine cervical cancer[16], hepatocellular carcinoma[17] and Cholangiocellular Carcinoma[18], pointing to its significance in high energy-demanding cancer cells. VDAC1 has also been reported to promote tumor growth and play a controversial role in the prognosis of different cancers[19]. Furthermore, some anticancer agents has taken VDAC1 as a novel pharmacological target[10]. However, the correlation of VDAC1 expression with tumorigenesis and progression of solid tumor of BC and the role of VDAC1 acts in the prognosis of BC patients is not yet well-documented.

Cytochrome C (Cytc) has been proposed to be a pro-apoptotic factor which is located in the inner membrane of the mitochondria. It is irreplaceable in mitochondrial electron transport and intrinsic type II apoptosis[20, 21]. Cytc release forms an essential step in the apoptotic cascade, upon binding with apoptosis protease-activating factor 1 (Apaf-1), dATP, and procaspase-9[22, 23]. Some studies have shown that Cytc can induce apoptosis of cancer cells[24, 25]. Jamsheed[26] et al. demonstrated that in non-small cell lung cancer patients, Cytc level was lower than healthy individuals, and the lower expression of Cytc were associated with advanced stages, high grade histological differentiation and shorter overall survival. Rahul[27] et al. explored that in prostate cancer, Cytc deficiency contributed to tumor invasiveness and therapeutic resistance, and leaded to faster recurrence. It has been widely reported that VDAC1 participates in the release of Cytc, which is able to signal Cytc to initiate the mitochondrial-mediated cell death cascade[28]. Conversely, VDAC1 has also been reported to interact with anti-apoptotic proteins such as Bcl-2 and hexokinase (HK) to control the release of Cytc[29]. In several melanoma and prostate cancer cell lines, a correlation has been demonstrated between the expression level of VDAC1 and the induction of Cytc release, which provides more possibilities for the pharmacological treatment of tumors [30]. However, the association of VDAC1 expression with Cytc in BC remains elusive and is rarely addressed, thereby indicating the need for further investigation.

The main objectives of this article were following: (i) evaluate the association of VDAC1 expression with Cytc in BC; (ii) investigate the correlation of various clinical features and 5-year disease-free survival (5-DFS) of BC with VDAC1 and Cytc respectively.
2. Material And Methods

2.1. Patient Tissue Samples.

A total of 219 formalin-fixed, paraffin-embedded primary invasive breast cancer tissue samples were obtained from the patients diagnosed with BC through histopathologic evaluation on surgical tissue specimens. Sixty cases of benign breast lesions were collected as controls. All the patients underwent surgical treatment at Renmin Hospital of Wuhan University between August 2009 and December 2010. There were no any previous chemotherapies, radiotherapies, or other treatments before surgery in these patients. The patients' written informed consent was obtained before the operation and the study was approved by the Ethics Committee of Renmin Hospital of Wuhan University. Patients were all followed up for 5 years. The follow-up data was calculated as the period from the date of surgery to the end of follow-up or death. We followed-up all the patients by telephone interviews or outpatient clinic visits.

2.2. Immunohistochemistry (IHC)

A tissue array was used which included two tumor samples from each patient. The paraffin-embedded tissues were cut into 4 µm thick sections, then deparaffinized, and dehydrated following standard procedures. Subsequently, paraffin sections were rinsed with PBS (3 × 5 min) and then blocked by treating with 3% hydrogen peroxide at 37°C for endogenous peroxidase ablation for 10 min. Antigen retrieval was conducted by microwave heating with citrate buffer (pH 6.0) for 20 min. Then the samples were exposed to normal goat serum at 37°C for 20 min to reduce nonspecific antibody binding. The tissue sections were incubated overnight at 4°C with the primary antibody (anti-VDAC1, 1:1000, ab15895, Abcam, UK; anti-Cytc, 1:50, sc-13561, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). After rinsing in PBS, the tissue sections were incubated with horseradish peroxidase-labeled anti-rabbit antibodies at 37°C for 20 min. Then, the tissue sections were rinsed with PBS for 4 times and then dripped with freshly prepared 3,3-diaminobenzidine (DAB). Microscopically, the staining was terminated when the tissue sections were brown or brown-yellow. Subsequently, all the tissue sections were restained with hematoxylin for about 1 minute. Finally, the slices were dehydrated with ethanol and toluene and then sealed with neutral gum. The sections with PBS, replacing the primary antibody, were used as negative controls.

2.3. Evaluation of immunohistochemical Staining

The slides were viewed via Olympus BX53(Tokyo, Japan) microscope. IHC staining was evaluated independently by two pathologists under the double blind condition. VDAC1 was mainly expressed in membrane of tumor cells. VDAC1 immunohistochemical staining in tumor cells was evaluated semiquantitatively as follows: (1). Staining intensity: 0 (no staining), 1 (weak staining), 2 (moderate staining), and 3 (strong staining); (2). The extent of staining: 0 (0%), 1 (1–20%), 2 (21–50%), 3 (> 50%). Five most representative fields of high magnification (400×) were selected to calculate the final score. The final score was multiplied by both of staining intensity and extent, theoretically from 0 to 9. Scores
less than or equal to 3 were defined as low expression, and scores greater than or equal to 4 were described as high expression.

Cytc protein was predominantly located in cell membrane and cytoplasm of tumor cells. The staining intensity was classified as four grades as follows: 0 (no staining), 1 (light yellow), 2 (brown yellow), 3 (dark brown). The percentage of positive cells was classified as five grades as follows: 0 (0%), 1 (≤ 30%), 2 (31–50%), 3 (51–80%), 4(≥ 80%). Five most representative fields of high magnification (400×) were selected to calculate the final score. The final score was multiplied by both of staining intensity and extent, theoretically from 0 to 12. Scores less than 4 were defined as low expression, and scores greater than or equal to 4 were described as high expression.

2.4. Statistical analysis

SPSS software version 17.0 was used to carry out all the statistical analyses. Comparison of VDAC1 and Cytc protein expression between BC tissues and benign breast lesions were analyzed by using Wilcoxon signed-rank tests. Statistical associations of VDAC1 and Cytc protein expression with clinicopathological parameters were assessed by using the chi-square test. The association of the expression of VDAC1 protein with Cytc and other clinicopathological parameters was performed by Spearman’s rank correlation analysis. The survival curves were disposed by using the Kaplan-Meier method and log-rank test. Cox proportional hazard regression model was conducted to evaluate univariate and multivariate analysis of survival as well as the independent prognostic values in BC patients. Hazard ratios (HRs) and their 95% confidence intervals (CIs) were calculated for both univariate and multivariate analyses. Two-tailed p values of < 0.05 were considered statistically significant.

3. Results

3.1. The characteristics of patients

The patients were composed of 219 females with a median age of 50 (age range, 29–78) years. Among the 219 cases, 165 (75.34%) were classified into G1 and G2, 54 (24.66%) were defined with G3 on the basis of histological differentiation. 153 (69.86%) patients were classified as stage I and II, 66 (30.14%) as III on the basis of tumor node metastasis (TNM) stage. Other basic clinicopathological characteristics, including age, menopausal status, lymph node metastasis (LNM), estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2) and recurrence, were presented in Table 1.
| Characteristic        | N (%)       |
|----------------------|------------|
| Age (years)          |            |
| ≤ 50                 | 135 (61.64%)|
| > 50                 | 84 (38.36%) |
| Menopause            |            |
| Before               | 125 (57.08%)|
| After                | 94 (42.92%) |
| TNM stage            |            |
| I/II                 | 153 (69.86%)|
| III                  | 66 (30.14%) |
| Histological grade   |            |
| G1/G2                | 165 (75.34%)|
| G3                   | 54 (24.66%) |
| Lymph node metastasis|            |
| No                   | 95 (43.38%) |
| Yes                  | 124 (56.62%)|
| ER status            |            |
3.2. Expression of VDAC1 is significantly higher in BC tissues than in benign breast lesions.

To examine the expression level of VDAC1 protein, we performed IHC on 219 cases of BC tissues and 60 cases of the benign breast lesions. As shown in Fig. 1, VDAC1 protein was mainly expressed in the membrane of tumor cells in BC tissues. Table 2 showed the results of IHC staining of VDAC1 protein. Of 219 BC samples, 162 (73.97%) showed high expression of VDAC1 protein and 57 (26.03%) showed low expression. In benign breast lesions, 35 (58.33%) showed high expression of VDAC1 protein and 25 (41.67%) showed low expression. The expression of VDAC1 was significantly higher in BC tissues ($\chi^2 = 6.606, P = 0.015$) as determined by chi-square test.
Table 2
The expression of VDAC1 and Cytc protein in breast cancer and benign breast lesions

|                | VDAC1 |            | Cytc |            |
|----------------|-------|------------|------|------------|
|                |       | P-value    | High expression | Low expression | P-value | High expression | Low expression |
| breast cancer tissue (n = 219) |       | 0.015      | 162 (73.97%)      | 57 (26.03%)    | 0.029   | 65 (29.68%)     | 154 (70.32%)   |
| benign breast lesions (n = 60)  |       | 0.35      | 35 (58.33%)      | 25 (41.67%)    | 0.003   | 23 (38.33%)     | 37 (61.67%)    |

3.3. Expression of Cytc is lower in BC tissues than in benign breast lesions

We examined the expression of Cytc by IHC in 219 cases of BC tissue and 60 cases of the benign breast lesions. As shown in Fig. 2, Cytc protein was mainly expressed in cell membrane and cytoplasm of BC tumor cells. The results of IHC staining of Cytc proteins were summarized in Table 2, which showed that the rate of high Cytc expression was 29.68% (65/219) and the rate of low Cytc expression was 70.32% (154/219) in all BC samples, while in benign breast lesions, the rate of high Cytc expression was 38.33% (23/60) and the rate of low Cytc expression was 61.67% (37/60). Thus, the expression of Cytc was significantly lower in BC tissues than benign breast lesions ($\chi^2 = 5.083, p = 0.029$).

3.4. VDAC1 protein expression correlates with Cytc protein expression and clinicopathological parameters in BC tissues, but not in benign breast lesions.

In the benign breast lesions, there was no significant association between VDAC1 expression and Cytc protein expression ($\chi^2 = 0.727, P = 0.432$. Table 3). Conversely, Table 4 showed that high expression of VDAC1 protein was inversely associated with Cytc protein expression in BC tissues ($\chi^2 = 7.423, r = -0.184, P = 0.017$), which was also shown in Fig. 3. Furthermore, Table 5 showed that high expression of VDAC1 protein was detected in 73.97% (162/219) of BC tissues, which was significantly associated with advanced TNM stage ($\chi^2 = 7.534, P = 0.007$), higher histological grade ($\chi^2 = 4.68, P = 0.033$), recurrence ($\chi^2 = 24.532, P < 0.001$), HER2 gene amplification ($\chi^2 = 6.949, P = 0.008$) and lymph node metastasis ($\chi^2 = 5.109, P = 0.03$), but not with other examined clinicopathological parameters, including age ($\chi^2 = 0.348, P$
= 0.636), ER status ($\chi^2 = 0.729, P = 0.44$), PR status ($\chi^2 = 0.499, P = 0.535$) and menopause ($\chi^2 = 0.028, P = 0.878$).

### Table 3
Correlation analysis of the expression of VDAC1 with Cytc in benign breast lesions

|            | High-VDAC1 expression (n = 35) | Low-VDAC1 expression (n = 25) | $P$-value |
|------------|-------------------------------|-------------------------------|-----------|
| Cytc expression |                               |                               |           |
| High (n = 23)   | 15 (65.22%)                   | 8 (34.78%)                    | 0.432     |
| Low (n = 37)    | 20 (54.05%)                   | 17 (45.95%)                   |           |

### Table 4
Correlation analysis of the expression of VDAC1 with Cytc in breast cancer patients

|            | High-VDAC1 expression (n = 162) | Low-VDAC1 expression (n = 57) | $P$-value |
|------------|-------------------------------|-------------------------------|-----------|
| Cytc expression |                               |                               |           |
| High (n = 65)   | 40 (61.54%)                   | 25 (38.46%)                   | 0.011     |
| Low (n = 154)    | 122 (79.22%)                  | 32 (20.78%)                   |           |
Table 5
Correlation between the expression of VDAC1, Cytc and clinicopathologic parameters

|                      | n   | VDAC1       |   | Cytc        |   |
|----------------------|-----|-------------|---|-------------|---|
|                      |     | High (n = 162) | Low (n = 57) | High (n = 65) | Low (n = 154) |
| Age                  |     | 0.636       | 0.763         |
| ≤50                  | 1   | 98          | 37            | 39          | 96           |
| >50                  | 8   | 64          | 20            | 26          | 58           |
| Menopause            |     | 0.878       | 0.455         |
| Before               | 1   | 93          | 32            | 40          | 85           |
| After                | 9   | 69          | 25            | 25          | 69           |
| Recurrence           |     | 0.000       | 0.004         |
| No                   | 1   | 85          | 51            | 50          | 86           |
| Yes                  | 8   | 77          | 6             | 15          | 68           |
| TNM stage            |     | 0.007       | 0.895         |
| I/II                 | 1   | 105         | 48            | 45          | 108          |
| III                  | 6   | 57          | 9             | 20          | 46           |
| Lymph node metastasis|     | 0.003       | 0.905         |
| No                   | 9   | 63          | 32            | 28          | 67           |
|                          | n   | VDAC1  | P-value | Cytc  | P-value |
|--------------------------|-----|--------|---------|-------|---------|
|                          |     | High   |         |       |         |
|                          |     | Low    |         |       |         |
|                          |     | (n = 162) | (n = 57) | (n = 65) | (n = 154) |
| Yes                      | 1   | 99     | 25      | 37    | 87      |
| Histological grade       |     | 0.0    | 0.0     | 0.33  | 0.41    |
| G1/G2                    | 1   | 116    | 49      | 55    | 110     |
|                          | 2   |         |         |       |         |
|                          | 4   |         |         |       |         |
| G3                       | 5   | 46     | 8       | 10    | 44      |
| HER2 gene                |     | 0.0    | 0.0     | 0.08  | 0.50    |
| Non-amplication          | 1   | 121    | 33      | 70    | 120     |
|                          | 2   |         |         |       |         |
|                          | 4   |         |         |       |         |
| Amplification            | 4   | 41     | 5       | 12    | 34      |
| ER status                |     | 0.4    | 0.0     | 0.40  | 0.37    |
| Negative                 | 1   | 93     | 29      | 29    | 93      |
|                          | 2   |         |         |       |         |
|                          | 4   |         |         |       |         |
| Positive                 | 9   | 69     | 28      | 36    | 61      |
| PR status                |     | 0.5    | 0.0     | 0.50  | 0.00    |
| Negative                 | 1   | 94     | 30      | 29    | 95      |
|                          | 2   |         |         |       |         |
|                          | 2   |         |         |       |         |
| Positive                 | 9   | 68     | 27      | 36    | 59      |

3.5. Cytc expression is also correlated with various clinical features in BC tissues
As is shown in Table 5, low expression of Cytc was correlated with higher histological grade ($\chi^2 = 4.278, p = 0.041$), ER status ($\chi^2 = 4.609, P = 0.037$), PR status ($\chi^2 = 5.424, P = 0.025$) and recurrence ($\chi^2 = 8.629, P = 0.004$), but not age ($\chi^2 = 0.106, p = 0.763$), TNM stage ($\chi^2 = 0.018, p = 0.895$), lymph node metastasis ($\chi^2 = 0.003, p = 0.953$), HER2 gene amplification ($\chi^2 = 0.36, P = 0.591$) or menopause ($\chi^2 = 0.751, P = 0.455$) in tumor samples.

3.6. Correlation analysis of the 5-DFS with the expression of VDAC1, Cytc protein and other parameters in BC.

Kaplan-Meier analysis showed that low VDAC1 protein expression in BC predicted a better survival and lower mortality rate (Fig. 4A, $P < 0.001$). Similarly, high expression of Cytc also predicted a better outcome in BC patients (Fig. 4B, $P = 0.007$). Univariate analysis of predictive factors for the 5-DFS in BC patients was performed by Cox proportional hazards regression model (Table 6). In univariate analysis, histological grade ($P < 0.001$), TNM stage ($P < 0.001$), ER status ($P = 0.033$), PR status ($P = 0.001$) and lymph node metastasis ($P < 0.001$) were also significantly correlated with the 5-DFS of BC patients. However, age, HER2 gene amplification, menopause had no significant correlation with 5-DFS in BC patients ($P > 0.05$) (Table 6). In order to analyze whether the above univariate was an independent prognostic factor, a multivariate Cox proportional hazard model for 5-DFS was performed. The results indicated that both the expression of VDAC1 and Cytc were independent prognostic parameters for 5-DFS of BC patients [hazard ratio (HR): 3.982 (1.723–9.207), $P = 0.001$; hazard ratio (HR): 0.542 (0.307–0.959), $P = 0.035$, respectively. Table 6]. Concurrently, TNM stage, PR status, lymph node metastasis and histological grade were also independent predictors regarding the 5-DFS of patients.
|                | n   | Univariate |          |          | Multivariate |          |
|----------------|-----|------------|----------|----------|--------------|----------|
|                |     | P-value    | Hazard ratio, 95% CI | P-value | Hazard ratio, 95% CI |
| VDAC1 expression |     |            |          |          |              |          |
| High           | 162 | 0.000      | 5.636 (2.454–12.942) | 0.001  | 3.982 (1.723–9.207) |
| Low            | 57  |            |          |          |              |          |
| Cytc expression |     | 0.007      | 0.463 (0.265–0.811)  | 0.035  | 0.542 (0.307–0.959) |
| High           | 65  |            |          |          |              |          |
| Low            | 154 |            |          |          |              |          |
| Age            |     | 0.718      | 1.084 (0.699–1.683)  |        |                |
| ≤ 50           | 135 |            |          |          |              |          |
| ≥ 50           | 84  |            |          |          |              |          |
| TNM stage      |     | 0.000      | 3.879 (2.512–5.989)  | 0.028  | 1.772 (1.063–2.956) |
| I/II           | 153 |            |          |          |              |          |
| III            | 66  |            |          |          |              |          |
| Lymph node metastasis |     | 0.000      | 4.717 (2.692–8.265)  | 0.003  | 2.690 (1.401–5.164) |
| No             | 95  |            |          |          |              |          |
| Yes            | 124 |            |          |          |              |          |
| Histological grade |     | 0.000      | 5.399 (3.489–8.356)  | 0.000  | 2.998 (1.899–4.731) |
| G1/G2          | 165 |            |          |          |              |          |
| G3             | 54  |            |          |          |              |          |
| HER2 gene      |     | 0.075      | 1.568 (0.956–2.574)  |        |                |
| Non-amplification | 173 |            |          |          |              |          |
| Amplification  | 46  |            |          |          |              |          |
|         | n     | Univariate |             | Multivariate |             |
|---------|-------|------------|-------------|--------------|-------------|
|         |       | P-value    | Hazard ratio, 95% CI | P-value    | Hazard ratio, 95% CI |
| ER status |       | 0.033      | 0.613 (0.390–0.962) |             |             |
| Negative | 122   |            |              |              |              |
| Positive | 97    |            |              |              |              |
| PR status |       | 0.001      | 0.442 (0.275–0.711) | 0.016      | 0.553 (0.341–0.896) |
| Negative | 124   |            |              |              |              |
| Positive | 95    |            |              |              |              |
| Menopause |     |            |              |              |              |
| Before   | 125   | 0.184      | 1.339 (0.870–2.060) |             |             |
| After    | 94    |            |              |              |              |

3.7. High expression of VDAC1 is associated with the poor prognosis of HER2-negative breast cancer

From Table 5, the expression of VDAC1 was significantly correlated with HER2 gene, but not with ER and PR status. As targeted therapy can be implemented for HER2-positive breast cancer, we next analyzed the correlation of VDAC1 protein expression with the prognosis of HER2-positive breast cancer patients and HER2-negative breast cancer patients, respectively. Kaplan-Mayer analysis showed that VDAC1 expression had no association with 5-DFS in HER2-positive breast cancer patients \( (P = 0.159) \). However, a significant relevance has been found between high VDAC1 expression and shorter 5-DFS \( (P < 0.001) \) in HER2-negative breast cancer patients (Fig. 4C). As triple-negative breast cancer (TNBC) is a special molecular subtypes of HER2-negative breast cancer, which is defined by the absence of the ER, PR and HER2 genes, and there is no standard treatment for TNBC at present. We then explored the association of VDAC1 expression with the prognosis of TNBC. Interestingly, VDAC1 was also associated with reduced 5-DFS in TNBC \( (P = 0.001) \) (Fig. 4D), predicting a poorer survival and higher mortality rate. To further validate the prognostic significance of VDAC1, Cox proportional hazards model analysis was also performed in TNBC. As presented in Table 7, VDAC1 was an independent predictor of poor 5-DFS [hazard ratio (HR): 4.018 (1.415–11.407), \( P = 0.009 \)]. Meanwhile, VDAC1 protein expression was inversely associated with Cytc in TNBC \( (\chi^2 = 9.102, P = 0.004) \) (Table 8). In summary, high expression of VDAC1 is associated with the prognosis of TNBC, and inversely correlates with Cytc.
Table 7
Univariate and multivariate analyses of predictive factors for disease free survival in TNBC

|                     | n    | Univariate |                |            | Multivariate |                |
|---------------------|------|------------|----------------|------------|--------------|----------------|
|                     |      | P-value    | Hazard ratio,  |            | P-value      | Hazard ratio,  |
|                     |      |            | 95% CI         |            |              | 95% CI         |
| VDAC1 expression    |      | 0.001      | 4.616 (1.635–13.029) | 0.009      | 4.018 (1.415–11.407) |
| High                | 61   |            |                |            |              |                |
| Low                 | 26   |            |                |            |              |                |
| Age                 |      | 0.802      | 0.921 (0.484–1.753) |            |              |                |
| ≤ 50                | 49   |            |                |            |              |                |
| ≥ 50                | 38   |            |                |            |              |                |
| TNM stage           |      | 0.000      | 3.332 (1.757–6.317) | 0.003      | 2.663 (1.381–5.135) |
| I/II                | 61   |            |                |            |              |                |
| III                 | 26   |            |                |            |              |                |
| Lymph node metastasis | 0.002 | 3.186 (1.504–6.747) |            |              |                |
| No                  | 38   |            |                |            |              |                |
| Yes                 | 49   |            |                |            |              |                |
| Histological grade  |      | 0.000      | 3.149 (1.660–5.974) | 0.007      | 2.469 (1.28–4.762) |
| G1/G2               | 59   |            |                |            |              |                |
| G3                  | 28   |            |                |            |              |                |
| Menopause           |      | 0.590      | 0.839 (0.444–1.587) |            |              |                |
| Before              | 43   |            |                |            |              |                |
| After               | 44   |            |                |            |              |                |
### Table 8
Correlation analysis of the expression of VDAC1 with Cytc in TNBC

| Cytc expression | High-VDAC1 expression (n = 61) | Low-VDAC1 expression (n = 26) | P-value |
|----------------|-------------------------------|-------------------------------|---------|
| High (n = 19)  | 8 (24.24%)                    | 25 (75.76%)                   | 0.004   |
| Low (n = 68)   | 53 (77.94%)                   | 15 (22.06%)                   |         |

### 4. Discussion

In this study, we observed that VDAC1 was elevated while Cytc was decreased in breast carcinoma patients compared with benign breast lesions. VDAC1 protein expression was conversely correlated with Cytc in BC, especially in TNBC. Both high VDAC1 and low Cytc protein expression had significant positive correlation with poor prognosis. High VDAC1 expression also represented an independent predictor for poor prognosis of TNBC.

VDAC1 participates in cancer metabolism via its modulatory roles in the transport of various metabolites\[31\]. In many types of cancer, the interaction of VDAC1 with HK, especially HK II, directly accesses to mitochondrial ATP for phosphorylation of glucose to glucose-6-phosphate and contributes to the cancer cells unrestricted growth and the inhibiting of apoptosis\[19, 32\]. VDAC1 has been found to be involved in tumor proliferation, migration, metastasis and invasion\[11\], and acts as a controversial role in the prognosis of different malignant tumors. In uterine cervical cancer, VDAC1 was associated with exhibited deeper stromal invasion, larger tumor size, higher recurrence and poorer overall survival\[16\]. Conversely, in Cholangiocellular Carcinoma, VDAC1 levels were inversely correlated with cancer stage classification and lower VDAC1 was present in patients with lymph node involvement and reduced survival\[18\]. These conflicting consequences demonstrate the differential effects of VDAC1 expression in different kinds of cancer and may need further exploration. In the present study, 219 cases of primary invasive breast cancer tissues were collected and it was found that VDAC1 protein was primarily located in the membrane of tumor cells in BC tissues. The relative expression of VDAC1 protein in breast cancer solid tumors was significantly higher than that in benign breast lesions, and high expression of VDAC1 protein correlated with advanced TNM stage, higher histological grade, recurrence, lymph node metastasis and HER2 gene amplification, thereby suggesting that high VDAC1 expression may play a role in promoting tumorigenesis and progression of BC. Interestingly, our present results were consistent with the reports in pancreatic cancer\[33\] and colorectal cancer\[34\], which indicated VDAC1 expression was upregulated in tumor and promoted the growth and invasion of cancer cells. Furthermore, by the multivariate analysis, we found that overexpression of VDAC1 protein in BC tissues could be as an independent poor prognostic factor. Consistent with this result, Chih-Hsien and Eiran's studies also showed that high expression of VDAC1 was correlated with poorer prognosis in uterine cervical cancer.
and hepatocellular carcinoma [16, 17]. Therefore, these data suggest that VDAC1 has the potential of being a poor prognostic marker in BC. As TNBC showed more progressively malignant manifestation with worse clinical outcomes and is mostly insensitive to drug treatment, we next explored the correlation of VDAC1 expression with the prognosis of TNBC. Amazingly, our result demonstrated that high VDAC1 protein was also associated with reduced 5-DFS and acted as an independent predictor of poor prognosis in TNBC, suggesting more potential use of VDAC1 should be exploited in prognostic marker and therapeutic target.

Cytc release from mitochondria is the driving force for apoptosome leading to apoptotic cell death in several malignant tumor[35], and VDAC1 has been widely reported to be interacted with pro- or anti-apoptotic proteins such as Bcl-2 and HK whereby mediating the release of Cytc[36]. In many types of cancer cells, when HK2 binds to VDAC1, the interaction between VDAC1 and Bcl-2 protein family will be blocked, resulting in a decrease in Cytc release, thus protecting tumor cells from apoptosis[19]. In our present study, we observed that VDAC1 protein expression was inversely associated with Cytc in BC, especially in TNBC. Furthermore, Cytc was lower expressed in BC compared with benign breast lesions and low expression of Cytc was correlated with higher histological grade, ER status, PR status and recurrence. A similar correlation was found in prostate cancer which Cytc deficiency contributed to tumor invasiveness and faster recurrence[27]. Importantly, we also found that decreased expression of Cytc was an independent prognostic factor in breast cancer solid tumors and played a pivotal role in the poorer 5-DFS of that cohort of BC patients. As a result, contrary to VDAC1, Cytc has the potential to be an improved prognostic marker in BC.

It should be acknowledged that there are also some limitations in this study. Firstly, due to the limited number of patients in this study, a larger cohort is required to explore the role and mechanism of VDAC1 and Cytc in the progression of breast cancer in the future. Secondly, more investigations need to be conducted to explore the mechanisms by which VDAC1 reduce Cytc release in BC. Thirdly, the prognostic significance of VDAC1 in breast cancer was discussed only at histological level. The exact role of VDAC1 in breast cancer, especially in TNBC, still needs to be evaluated in follow-up mechanistic investigations. Fourthly, our study evaluated prognosis by 5-DFS rather than OS. Since DFS sometimes not correlated with OS, the influence of VDAC1 expression on OS is still a topic for future research.

5. Conclusion

Our study showed for the first time that VDAC1 was elevated in BC tissues. Meanwhile, our findings firstly revealed that VDAC1 expression was conversely associated with Cytc, and promoted malignant behavior of BC. Furthermore, high VDAC1 protein was associated with reduced 5-DFS of BC tissues. By the multivariate analysis, we found that overexpression of VDAC1 protein could be acted as an independent poor prognostic factor not only in breast cancer in general, but also in TNBC in particular. All in all, VDAC1 can be exploited as a potential prognostic marker and therapeutic target in BC, especially in TNBC.

Declarations
Acknowledgements

Not applicable.

Authors' contributions

FC and JY made contributions to the conception and design of the study; CC and XW collected the samples and performed immunohistochemistry experiments; DY and HY made contributions to statistical analysis; BL and FG evaluated the immunohistochemical staining of all sections; SY made contributions to critical revision of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

Not applicable.

Ethics approval and consent to participate

This study was approved by the Ethical Committee of Renmin Hospital of Wuhan University (WDRY2019-K010). The written informed consents were obtained from all the patients.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures
Figure 1

Immunohistochemical staining of VDAC1 in breast cancer (BC) lesions and benign breast lesions. The staining of VDAC1 protein (brown) was mainly located in the membrane of BC tumor cells: (A) high expression of VDAC1 protein in BC; (B) low expression of VDAC1 protein in BC; (C) high expression of VDAC1 protein in benign breast lesions; (D) low expression of VDAC1 protein in benign breast lesions. Scale bar, 50\(\mu m\).
Figure 2

Immunohistochemical staining of Cytc in breast cancer (BC) lesions and benign breast lesions. The staining of Cytc protein (brown) was mainly located in the membrane and cytoplasm of BC tumor cells: (A) high expression of Cytc protein in BC; (B) low expression of Cytc protein in BC; (C) high expression of Cytc protein in benign breast lesions; (D) low expression of Cytc protein in benign breast lesions. Scale bar, 50\(\mu\)m.
Figure 3

Immunohistochemical staining for VDAC1 and Cytc in breast cancer tissues: high expression of VDAC1 protein (A) with low Cytc expression (B); low expression of VDAC1 protein (C) with high Cytc expression (D). Scale bar, 50 μm.
Figure 4

Kaplan–Meier survival analysis showing the correlation between VDAC1 expression (A), Cytc expression (B) and 5-DFS in breast cancer patients (log-rank test). C-D, Kaplan-Meier survival analysis showing the correlation between VDAC1 expression and 5-DFS in HER2-negative breast cancer (C), and triple-negative breast cancer (D) (log-rank test).
Figure 5

Immunohistochemical staining for VDAC1 and Cytc in triple-negative breast cancer lesions: high expression of VDAC1 protein (A) with low Cytc expression (B); low expression of VDAC1 protein (C) with high Cytc expression (D). Scale bar, 50μm.

Supplementary Files

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