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Original Article

Activation of major vault protein by TGF-beta is associated with advanced malignancy and poor prognosis in breast cancer

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Abstract: Emerging studies have demonstrated that major vault protein (MVP) may play critical roles in tumor development and progression. However, the biology functions of MVP in breast cancer have not yet been evaluated. The present study is to investigate the expression level and the significance of MVP in breast cancer and to explore the mechanism underlying the deregulation of MVP. We examined the expression of MVP and the relationship with clinicopathological features. Cell proliferation and transwell assays were performed to explore the effect of MVP in breast cancer cell lines. The results indicated that the expression levels of MVP mRNA and protein were significantly higher in breast cancer tissues and the overexpression of MVP was associated with lymph node invasion and advanced tumor stage. We also identified that MVP could be an independent prognostic factor. In addition, knockdown of MVP showed that deficiency of MVP retarded cell growth and invasion ability. Furthermore, we noticed that TGF-β signaling activation was involved in regulating MVP expression as MVP expression could be reduced by TGF-β signaling inhibitor. In conclusion, we found that abnormal activation of TGF-β signaling could induce MVP expression and high expression level of MVP promoted breast cancer cells proliferation and invasion, indicating that MVP could serve as a potential target for therapy of breast cancer.

Keywords: MVP, TGF-β, invasion, prognosis, breast cancer

Introduction

Breast cancer is one of the most common malignance and causes of cancer mortality worldwide. About 1/3 of female cancers was diagnosed in America and over 40,000 women died from breast cancer in 2011 [1]. In spite of earlier diagnoses and improved clinical treatments which are helpful to decline the disease-related mortality, the incidence of breast cancer is still elevating in decade and the 5-year survival rate is less than 50% because of relapse and metastasis [2]. Thus, the understanding of aggressive phenotypes of breast cancer is urgently needed. More importantly, the heterogeneity of breast cancer makes it more difficult to define whether the treatment is completed and how to evaluate risk factors of tumor development and progression [3, 4].

Metastasis is a hallmark of cancer and also the major cause of tumor recurrence and mortality. Breast cancer initiates in local area. However, along with the progression, tumors infiltrate lymph nodes and cause distal metastasis [5]. To accurately predict a patient's risk of metastasis is still limited in current days. About 80% of the patients would accept adjuvant chemo- and radio-therapy and 40% of them will relapse, and died because of metastasis [6]. However, the mechanisms are rarely known. Therefore, the study to improve the survival rate of patients with breast cancer metastasis is of great significance. It is urgent to identify the precise mechanisms and new markers for evaluating the diagnosis.

Major vault protein (MVP), also known as lung resistance-related protein (LRP) is a new molecular target for cancer therapy [7, 8]. MVP is a coactivator that participates in forming the outer vault complex which is comprised of multi-subunit ribonucleoprotein structures and involved in nucleo-cytoplasmic transport [9].

MVP was initially found overexpressing in non-small cell lung cancer cell line with multi-drug resistance, but failed to express P-glycoprotein [10]. For its biological functions, MVP plays important roles in multiple cellular processes by regulating the MAPK, JAK/STAT and PI3K/AKT signaling pathways [11]. For instance, proteomic analysis indicated that MVP and Bcl2-associated athanogene 3 took part in the resistance to apoptosis induced by chemotherapy by activating ERK1/2 signal in breast cancer [12]. Also, MVP expression could be altered by low density lipoprotein (LDL) and high-density lipoprotein (HDL) to enhance the doxorubicin-resistant property in uterine sarcoma [13]. Recently, it has been found that stabilization and up-regulation of specificity protein 1 by JNK inducted MVP expression under hyperosmotic conditions in colon cancer cells [14]. Besides, MVP is also found to mediate resistance to EGFR inhibitor gefitinibin hepatocellular carcinoma cells while the expression of ABC-transporter proteins ABCB1 and ABCC1 showed no association with drug resistance in these criteria [15]. However, the expression significance and crucial roles of MVP in breast cancer remain unclear.

In the present study, we found the MVP mRNA was elevated in breast cancer tissues compared with adjacent non-tumorous tissues according to the previous investigation in public database and the enhanced expression was confirmed by immunohistochemistry in our study. We demonstrated that high level of MVP was associated with advanced clinical stage and lymph node invasion. Using multivariate analysis, we found that MVP could be an independent diagnostic factor. We then performed in vitro experiments analysis and showed that MVP promoted cell proliferation and invasion. Furthermore, we explored that potential mechanism that TGF-β was involved in regulating MVP expression.

Materials and methods

Cell culture

Human mammary epithelial cell line MCF10A and breast cancer cell lines MCF7, MDA-MB-468, MDA-MB-231, MDA-MB-453, T47D were obtained from Cell Bank, Chinese Academy of Sciences and American Type Culture Collection (ATCC). Cells were maintained in RPMI-1640 medium (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), 100 IU/ml penicillin and 100 μg/ml streptomycin. All cells were incubated at 37°C in a 5% CO_2 humidified atmosphere.

Immunohistochemical staining

Two tissue microarrays containing 150 cases of breast cancer specimens and adjacent non-tumorous tissues with pathological parameters were obtained from Shanghai Outdo Biotech Company. The tissues microarray slides were deparaffinized, and then incubated in 3% hydrogen peroxide for 10 minutes. Antigen retrieval was performed and followed by blocking with 5% BSA for 1 hour to reduce the nonspecific backgrounds. MVP primary antibody (1:200) was left overnight in the wet box at 4°C. Next day, after PBS washed, the slides were incubated with peroxidase-conjugated secondary antibody for 30 minutes at room temperature. Immunostaining was performed using diaminobenzidine reaction. Then slides were counterstained with hematoxylin [16].

MVP expression was determined independently by two pathologists and scored based on the percentage and intensity of positive staining. Briefly, the percentage was divided from 1 to 4: 1, 0%-5%; 2, 6%-50%; 3, 51%-75%; 4, 76-100%. The intensity was classified as 1 to 4:1, no staining; 2, weak staining; 3, moderate staining; 4, strong staining. A final score of MVP expression ranged from 1 to 16 was calculated by multiplying percentage and intensity score. For each sample, it was indicated in negative (≤8) or positive (>8). PBS was used instead of MVP antibody for the negative control.

Silencing of MVP expression by small interfering RNAs (siRNAs)

The siRNAs targeting MVP were purchased from Santa Cruz Co. A scrambled sequence was used as a control. Cells were transfected with a mixture of three siRNAs using the lipofectamine 2000 reagent (Invitrogen) as protocol described.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted using TRIzol (Invitrogen) according to the manufacturer's instructions and cDNA was synthesized using PrimeScript RT-PCR kit (Takara). qRT-PCR was performed

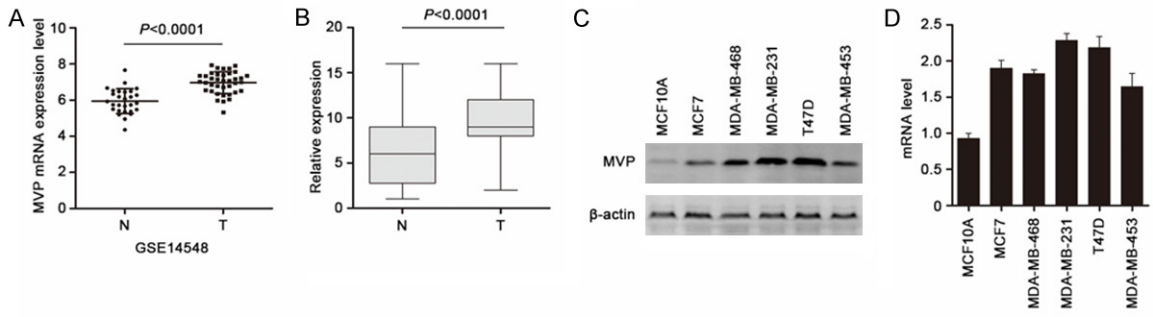

Figure 1. MVP was overexpressed in breast cancer tissues and cell lines. A. Data from Oncomine database indicated MVP mRNA level in breast cancer samples (T) and non-tumorous tissues (N). B. Relative score of MVP immunochemistry staining for breast cancer tissues. C. The protein level of MVP was tested in breast cancer cell lines (MCF7, MDA-MB-468, MDA-MB-231, T47D and MDA-MB-453) and human mammary epithelial cell line (MCF10A). D. The mRNA level of MVP was tested in these cell lines. Data were representative as the mean ± SEM of triplicated independent experiments.

on an ABI7500 real-time PCR machine (Applied Biosysterm) with SYBR Green methods. The gene expression level was measured by ΔΔCt and β-actin was acted as the internal control. The primers used for detecting MVP and β-actin were as followed: MVP, forward, 5'-TCTAGATGCTCCCCAGGTTCA-3' and reverse 5'-GAACTCTTCAGTTGCCATGGTG-3'; β-actin, forward, 5'-GCCGCCAGCTCACCAT-3' and reverse 5'-CACGATGGAGGGGAAGACG-3'.

Western blot

Cell lysates were prepared in RIPA buffer and total protein concentration was detected by BCA methods (Thermo Scientific) according to the manufactures' protocol. A total of 60 μg protein was loaded and separated in 10% SDS-PAGE as described previously. Anti-MVP (sc-23917) and anti-collagen I (sc-8783) were from Santa Cruz Co (Santa Cruz Biotechnology). Anti-phosphor-smad3 (#9520), anti-smad3 (#9523) andanti-β-actin (#3700) were from Cell Signaling Technology Co (CST).

Cell proliferation assay

Cell proliferation assay was performed using Cell Count Kit-8 (CCK-8, Dojindo). Briefly, cells were seeded in 96-well plates in complete medium at approximately 2×10^3 cells/well. 10 μl CCK-8 reagent was added to each well at day 1, 2, 3, 4 and 5, respectively. After two hours of incubation at 37°C, the optical density value (OD) was read at 450 nm on the microplate reader. The experiments were repeated at three times.

Matrigel transwell invasion assay

For transwell invasion assay, 50 μl Matrigel (BD Bioscience) was plated into the top chamber of transwell for 30 minutes at 37°C. Cells were starved in serum free medium for 24 hours and added to the top chamber. The bottom chamber was filled with completed medium. After 48 hours incubation, the non-invading cells which remaining in the top chamber were removed carefully and the lower membrane surface was fixed with 4% paraformaldehyde and staining with crystal violet. Cells adhering to the lower membrane of the well were counted and imaged in 10 fields under ×200 magnification and presented as mean ± SD. The assay was repeated at three times.

Statistical analysis

All statistical analyses were performed using SPSS 21.0 software. Statistical analysis of clinical parameters and MVP expression were conducted with Chi-squared test, and the Student's t was used for cell proliferation and transwell assays. The Kaplan-Meier method was employed to determine the overall survival distribution (OS) and Cox regression model for multiple-factor analysis. A *P* value less than 0.05 was considered statistically significant.

Results

MVP protein is overexpressed in breast cancer tissues and cell lines

To determine the expression level of MVP in clinical specimens, we firstly explored the

Table 1. Correlation between MVP expression and clinicopathological parameters of breast cancer

Parameters	Case number	MVP expression		χ^2	*P*
		Low	High		
Overall	150	65	85		
Age (years)					
≤50	72	27	45	1.919	0.166
>50	78	38	40		
Tumor size (cm)					
≤3	91	36	55	1.341	0.247
>3	59	29	30		
Lymph node invasion					
Yes	61	33	28	4.852	0.028*
No	89	32	57		
Tumor differentiation					
Well	42	12	30	5.352	0.069
Moderate	103	51	52		
Poor	5	2	3		
T classification					
I	34	17	17	0.796	0.372
II-IV	116	48	68		
Clinical Stage					
1	13	9	4	6.053	0.048*
2	93	42	51		
3	44	14	30		
ER					
Negative	47	28	19	7.352	0.007*
Positive	103	37	66		
PR					
Negative	58	33	25	7.084	0.008*
Positive	92	32	60		
HER2					
Negative	97	42	55	0.000	0.991
Positive	53	23	30		

*$P<0.05$.

mRNA level on public database Oncomine (www.oncomine.org). The mRNA level of MVP in breast cancer tissues was significantly higher than that in adjacent non-tumorous tissues (**Figure 1A**). Then we analyzed the protein expression of MVP in 150 breast cancer specimens by immunohistochemistry. In adjacent non-tumorous tissues, weak expression of cytoplasmic was observed, but strong positive cytoplasmic MVP staining was detected in breast cancer tissues (**Figure 1B**). These data indicated that MVP expression was significantly elevated in breast cancer. Meanwhile, we assessed the expression of MVP in human mammary epithelial cell line MCF10A and 5 breast cancer cell lines by qRT-PCR (**Figure 1C**) and western blot (**Figure 1D**). We found that MVP expression was also higher in breast cancer cell lines than normal epithelial cells.

High expression of MVP is associated with tumor malignance and poor prognosis

To explore the significance of overexpressed MVP protein in breast cancer patients, we analyzed the correlation between MVP expression and clinical parameters. As shown in **Table 1**, the inappropriate overexpression of MVP was associated with lymph node invasion, advanced stage, positive expression of ER and PR. While there was no relationship between MVP expression and age, tumor size, grades or HER2 status.

Furthermore, survival analysis was performed depending on the collected follow-up data. The results indicated that patients with high level of MVP expression had significantly shorter survival rates than those with low level of MVP expression group (**Figure 2**). By Cox regression analysis, we recognized the prognostic factors for the 150 breast cancer patients. Using a univariate analysis, OS was significantly associated with ER status, PR status and MVP expression. Multivariate analysis demonstrated that positive MVP expression remained a significantly independent prognostic factor for breast cancer survival (**Table 2**).

MVP depletion inhibits tumor cell proliferation and invasion

As the expression of MVP was detected in breast cancer cell lines, we found a relatively high mRNA and protein expression in MDA-MB-231 and T47D cells. In order to investigate the biological function of MVP in breast cancer cells, siRNA-mediated knockdown was performed in MDA-MB-231 cells. As shown in **Figure 3A**, the protein level of MVP was significantly decreased after the mixture of 3 siRNAs

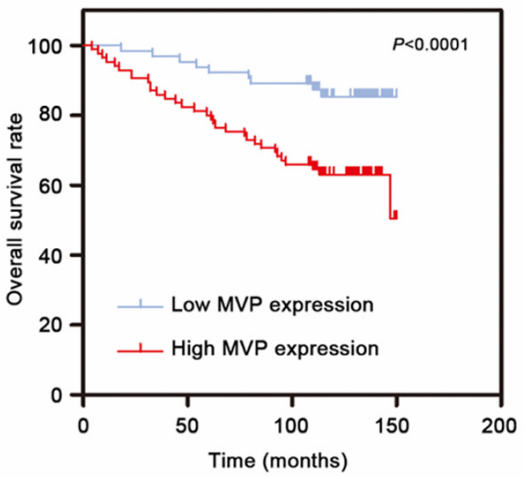

Figure 2. High expression of MVP indicates poor prognosis. Kaplan-Meir curves for overall survival from 150 breast cancer patients according to MVP expression in tumor tissues. Log-rank regression was used to test the significance.

transfection. The role of MVP in cell proliferation was assessed by CCK-8 assay. As a result, MDA-MB-231 cells treated with MVP siRNAs exhibited a notably slower growth rate compared to control cells (**Figure 3B**).

As the MVP expression was associated with lymph node invasion, we performed in vitro assays to examine the role of MVP on cell invasion.As shown in **Figure 3C**, depletion of MVP could significantly abrogate cell invasion ability. These results together demonstrated that MVP was important for breast cancer cells proliferation and invasion.

TGF-β signaling is involved in regulating MVP expression

Then we intended to explore the molecular mechanism that caused MVP overexpression in breast cancer tissues. TGF-β as a multi-functional factor plays key roles in breast cancer development and progression and TGF-β mediated tumor metastasis is already well recognized [17, 18]. So we analyzed whether the overexpression of MVP was due to the abnormal activation of TGF-β signaling. We found that TGF-β (10 ng/ml) could up-regulate the mRNA level of MVP in both MCF-7 and MDA-MB-453 cells (**Figure 4A**). Furthermore, we employed the TGF-β signaling inhibitor SB-431542 to further examine whether MVP overexpression was associated with TGF-β signaling activation. As shown in **Figure 4B** and **4C**, we confirmed that TGF-β induced MVP expression as phosphorylated-samd3 and TGF-β downstream target collagen I were also enhanced. However, with SB431542 inhibition effects, phosphorylated-samd3 and collagen I were both prohibited and MVP expression was decreased as well. The relative quantitation of western blot results indicated that SB431542 could abolish the MVP up-regulation mediated by TGF-β, suggesting that TGF-β signaling was involved in regulating MVP expression.

Discussion

MVP expression has been reported up-regulated in various cancer tissues. Ma et al explored the gene expression profile of tumor microenvironment during breast cancer progression and provided a comprehensive catalog of gene expression changes in both tumor and tumor-associated stroma [19]. In present study, we further deeply analyzed the data and found that MVP mRNA level was significantly higher in malignant than normal tissues. Consistent with mRNA expression level, we also demonstrated that MVP protein expression was elevated in most breast cancer tissues than their adjacent non-tumorous tissues by IHC, implying its oncogenic roles in breast cancer. Then we surveyed the relationship between MVP expression and clinicopathological features. With regard to malignant properties, enhanced MVP expression was associated with adverse prognosis, suggesting that MVP played crucial biological functions in breast cancer malignant phenotypes.

Breast cancer is a type of malignant tumors with strong heterogeneity. Based on the ER, PR and HER-2 status, breast cancer could be divided into 4 types which have different pathological features and clinical outcomes [20, 21]. MVP expression was found to be associated with ER and PR status, implying the hormone therapy strategy such as tamoxifen might be more effective when combination with reducing MVP expression. However, enhanced level of MVP had no relationship with HER-2 status and previous study indicated that high HER-2 expression might be associated with tamoxifen resistance [22]. So we hypothesized that MVP was also contributed to drug resistance in spite of HER-2 status. In accordance, silencing of MVP in breast cancer cells decreased ERK1/2

Table 2. Univariate and multivariate analysis for overall survival (Cox regression analysis)

Parameters	Univariate analysis			Multivariate analysis[a]		
	HR	95% CI	*P*	HR	95% CI	*P*
Age	1.671	0.885-3.156	0.114			
Tumor size	1.138	0.608-2.130	0.686			
Lymph node invasion	1.147	0.612-2.151	0.669			
Histological differentiation	1.614	0.854-3.050	0.140			
T classification	1.055	0.502-2.216	0.888			
Clinical stage	1.694	0.987-2.906	0.056			
ER	0.439	0.237-0.811	0.009*	0.361	0.131-0.994	0.049*
PR	0.509	0.275-0.943	0.032*	0.667	0.244-1.822	0.430
HER2	1.095	0.580-2.068	0.780			
MVP	3.151	1.503-6.604	0.002*	4.974	2.290-10.804	<0.001*

[a]Final multivariate analysis include only those covariates that were significantly associated with survival (*P*<0.05). **P*<0.05.

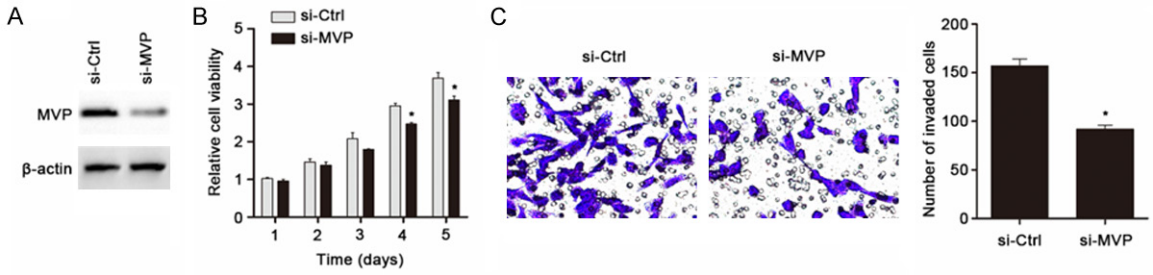

Figure 3. MVP knockdown attenuated breast cancer cells proliferation and invasion abilities. A. Western blot was used to validate the siRNA-mediated downregulation of MVP in MDA-MB-231 cells. B. Cell viability of cells with MVP knocking down was measured by CCK-8. C. Transwell assays were employed to analyze the invasion ability alternation after knockdown of MVP (magnification, ×200). Quantification of invasive cells was calculated from five randomly selected fields. Data were representative as the mean ± SEM of triplicated independent experiments (*P<0.05).

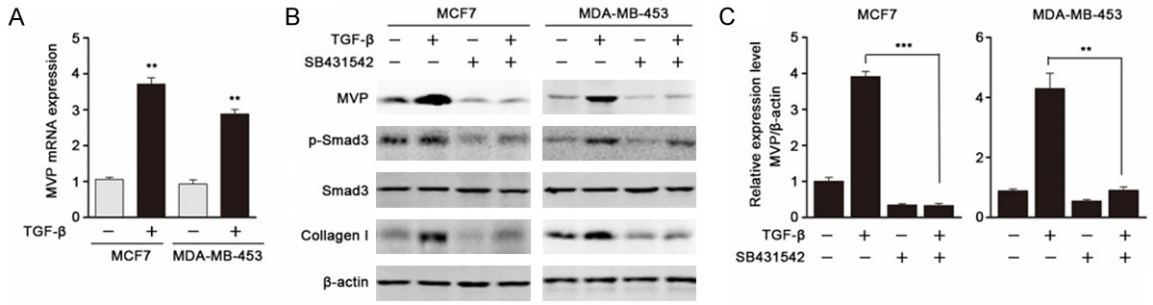

Figure 4. TGF-β signaling is involved in regulating MVP expression. A. MVP mRNA was measured by qRT-PCR after TGF-β treatment for 6 h in MCF7 and MDA-MB-453 cells. B. Western blot assay was performed to test the protein level of MVP after TGF-β and SB431542 treatments. C. Quantified analytical system was used to detect the relative MVP protein level according to the gray-scale value (**P<0.01, ***P<0.001).

activation and promoted apoptosis when adriamycin treated [12]. Also in nervous system, MVP mediated malignant transformation in glioblastoma and its expression was induced in epileptic brain areas and considered as a possible factor leading to failure of treatment with anti-epileptic drugs [23, 24]. Besides, MVP played critical roles in non-small cell lung cancer and related to poor prognosis [25].

Our present study suggested that MVP expression inducted cell growth and invasion by CCK-8

and transwell assays. Accordingly, these similar effects were also recognized in glioma cells. Concerning the oncogenic functions, it motivated us to investigate the causing of enhanced MVP expression. We here characterized that TGF-β signaling was partially involved in regulating MVP expression. TGF-β has long been recognized as a multifunctional cytokine that orchestrated a sophisticated signal network to modulate carcinogenesis and progression. Firstly, TGF-β was identified as tumor-suppression role which could induce cell cycle arrest and cause apoptosis [26]. However, at the advanced tumor stage, TGF-β is involved in the malignant phenotypes such as enhanced proliferation and metastasis, implying its oncogene potential. Additionally, we observed distinctly reduced MVP expression after treating with SB431542. These results indicated that TGF-β induced MVP expression. Collectively, our study suggested that MVP-mediated cell growth and invasion could be also the consequence of TGF-β activation.

In summary, our results demonstrated that MVP expression was significantly up-regulated in breast cancer compared to adjacent non-tumorous tissues. To the best of our knowledge, high level of MVP expression was associated with tumor aggressiveness. Also, patients with high expression of MVP had an unfavorable prognosis. Further data indicated that elevated expression of MVP promoted cell proliferation and invasion. In addition, TGF-β signaling was found to be at least partially accounted for the increased MVP expression, as suggested by our inhibitor reverse experiments. Although the detailed downstream mechanism of MVP remains to be clarified, our study provides a possible consideration of MVP overexpression as predictive markers for breast cancer therapy.

Disclosure of conflict of interest

None.

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