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Integrins linked kinase and focal adhesion kinase as the key signaling mediators of vascular mimicry in metastatic breast tumor cells

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Abstract

Objective In highly aggressive malignant cancers including breast cancer, vasculogenic mimicry (VM) is the potential of tumor cells to generate a vascular channel network for delivering blood to tumor cells. Detection of genes involved in this process is critical to designing targeted therapy against breast cancer metastasis. In this study, we evaluated the roles of FAK and ILK in the progression of VM in metastatic breast tumor cells.

Results Primary (4T1T), and highly metastatic (4T1B and 4T1L) breast tumor cells were isolated from cancerous mice. The potential of cancer cells to organize themselves into vascular-like structures (VM) has been evaluated with in vitro assessment. The expression of ILK and FAK were examined using real-time polymerase chain reaction. We confirmed the high ability of metastatic tumor cells in vascular-like structure formation. In molecular analysis, our data showed that ILK and FAK expression was significantly elevated in metastatic breast tumor cells. These results indicated that the higher potential of metastatic tumor cells in vascular-like structure formation may be related to higher expression of ILK and FAK. Analysis of molecular features of metastatic tumor cells could be utilized to create a targeted therapeutic strategy against metastasis in breast cancer.

Keywords Breast cancer, Metastasis, Vascular mimicry, Integrins linked kinase (ILK), Focal adhesion kinase (FAK)

Introduction

Breast cancer metastasis results in 90% of mortality from breast cancer in women worldwide [1]. Identification mechanism that can promote metastasis, invasion and cell migration is vital to designing targeted therapy against breast cancer [2]. One of the main mechanism that can facilitate metastasis and tumorigenesis in breast cancer is vascular mimicry (VM) [3].

VM is formation a tubular structure independent of endothelial cells in tumor cells for enhancing survival and metastasis [4]. VM is triggered through a wide range of genes with performance associated to tumor invasion, metastasis, hypoxia and angiogenesis [5]. Contribution

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of integrins linked kinase (*ILK*) and focal adhesion kinase (*FAK*) in tumor growth and VM formation of breast cancer have been reported [6, 7].

FAK is a non-receptor tyrosine kinase, performed as a key regulator of multiple signaling pathway through cell surface receptors consisting of integrins, growth factor and cytokine receptors [8]. *FAK* play a key role in the adjustment of cell spreading, adhesion, migration, invasion, survival, proliferation, differentiation, and angiogenesis: processes that are totally involved in the development of metastatic breast cancer [9]. These functional features recommend that FAK may mediate key roles in elevating breast cancer tumorigenesis and metastasis [10].

ILK is a protein kinase detected in a yeast by its correlation with the β1 subunit of integrins [11]. Structurally, *ILK* consist of three functional domains [12]. The C- terminus domain a protein kinase domain that mediate as a binding site for the β- integrin subunits [13]. Ankyrin (ANK) repeat domains exist at the N- terminus, which mediate protein–protein interactions [14]. In addition, the N-terminus regulates the localization of *ILK* within focal adhesion kinase. The communication between *ILK* and *FAK* in integrins signaling pathway performed through a groups of adaptor proteins that binds to the integrin-linked kinase (ILK) like a-parvin and b-Parvin [15]. The impacts of *ILK* and *FAK* in these signaling pathway may have critical implication in breast cancer metastases and tumorigenesis through induction vascular mimicry [11]. Therefore, in this study, we evaluated roles of *FAK* and *ILK* in VM of metastatic tumor cells. In our work; we revealed the high ability of metastatic tumor cells in vascular-like structures. In molecular level, our results showed that *ILK* and *FAK* expression was significantly elevated in metastatic breast tumor cells.

Materials and methods

Isolation of primary and metastatic breast tumor cells

The primary and metastatic breast tumor cells were isolated according to our previous study [16]. Briefly, 35 days after the injection of 4T1 cell line (1×10^6) to flank area of BALB-c mouse, the mice were euthanized with anesthetic overdose of ketamine/xylazine. The mice were obtained from the Animal Laboratory of the royan institute, Tehran, Iran. The primary and metastatic breast tumor cells, were isolated from the primary tumor tissue, brain and lung metastatic lesions of mice respectively. After primary isolation, two groups of tumor cells (primary(4T1T) and metastatic (4T1B and 4T1L)) were cultured in the same condition (DMEM with 10% FBS, 100 U/ml Penicillin, and 100 ug/ml Streptomycin incubated at 37 °C in 5% CO2) (all from Gibco, USA).

In vitro assessment of vascular mimicry

Experiments were performed on cultured cells with a density of 70 to 80%. 24 well plates were pre-coated with ice-cold growth factor-reduced Matrigel (Corning) and then allowed to set at 37 C for at least 2 h. 4T1T, 4T1B and 4T1L were trypsinized, counted and seeded onto the pre-coated Matrigel plates at 120,000 cells per well. After the time of the experiment (within 24 h of incubation at 37 °C), photos were taken using a digital camera connected to an inverted microscope. 3–5 images were taken per well and the average number of tube-like structures formed are measured.

Primer design

Primers were designed by AlleleID version 6 software.

Genes	FW (5′ – 3)	Rev (5′ – 3)
ILK	TGA ATG AGC ACG GCAATG	TCC AGA ATG TGT CCTTGTATG
FAK	AAC TTG GAC GCT GTATTGG	CGC ATT GTT AAG GCTTCTTG
GAPDH	CCTGGAGAAACCTGCCAAGTA	GGCATCGAAGGTGGAAGAGT

Quantitative real time PCR (RT-qPCR)

RNA extraction (*Trizol* reagent) and cDNA synthesis ((Easy cDNA Synthesis Kit for RNA or mRNA to cDNA - parstous)) were performed from primary and metastatic tumor cells. Real-time PCR procedure was performed according to the manufacturer's instruction: 1 cycle of 95 °C for 15 min, 40 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. (SYBR Green Real-time PCR Master Mix (Amplicon A/S, Denmark) using StepOnePlus™ Real-Time PCR System).

The exact mRNA expression was normalized to the expression level of GAPDH. Gene expression of each target was calculated by using the 1/ΔCT method.: (CT of Kinas (FAK or ILK)) – (CT of housekeeping gene).

Statistical analysis

Data analysis was performed using GraphPad Prism statistical software 6.0 (GraphPad Software, La Jolla, CA, USA) by Paired Samples t-test. $P<0.05$ was considered statistically significant.

Results

Vascular mimicry results

To determine the tumor cell's ability in VM formation, all tumor cells (including primary and metastatic) were cultured on Matrigel matrix with DMEM for 24 h. When compared to 4T1T cells, 4T1B and 4T1L cells showed increased ability of tube formation in both groups (Fig. 1).

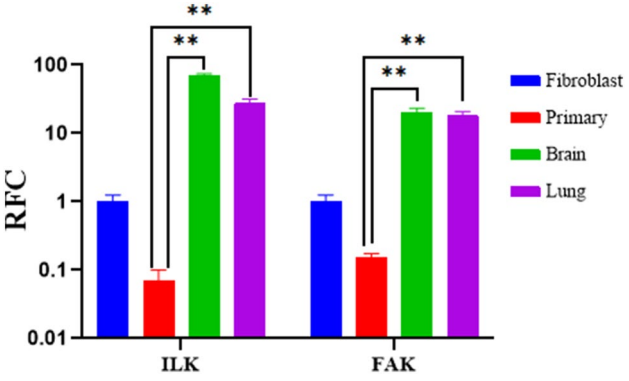

Fig. 1 Up-Regulation of ILK and FAK in metastatic breast tumor cells. Relative to primary breast tumor cells (4T1T) as control sample, ILK and FAK were significantly upregulated in metastatic breast tumor cells

Up-regulation of ILK, FAK in metastatic tumor cells compare to primary tumor cells

Firstly, to evaluate the role of *ILK*, *FAK* in induction of metastasis in tumor cells, we assessed the level of these genes in metastatic breast cancer (4T1B, 4T1L) and primary breast cancer (4T1P). The result indicated that the level of *ILK*, *FAK* was significantly increased in metastatic breast cancer relative to primary breast tumor cells (Fig. 2).

Up-regulation of ILK, FAK in metastatic breast cancer compare to fibroblast cell line as control sample (3T3 cell line)

To compare expression level of these genes with control samples, we examined the level of *ILK*, *FAK* in metastatic breast cancer (4T1B, 4T1L) and fibroblast cell line as a control sample (3T3 cell line). The result showed that the level of *ILK*, *FAK* was increased in metastatic breast cancer (Fig. 1).

Down-regulation of ILK, FAK in primary breast cancer compare to fibroblast cell line as control sample (3T3 cell line)

Interestingly, examination the expression level of *ILK*, *FAK* in primary breast cancer relative to fibroblast cell line (control sample) showed a decreasing trend in these genes (Fig. 1).

Discussion

As a specific perfusion mechanism, VM is related to tumor progression, metastasis and poor cancer clinical features [17]. Because of VM's significant role in breast cancer tumorigenesis and metastases, examination genes related to this process at the molecular level could be suitable for metastatic breast cancer treatment strategies [18]. In the preset study, we reported a significant

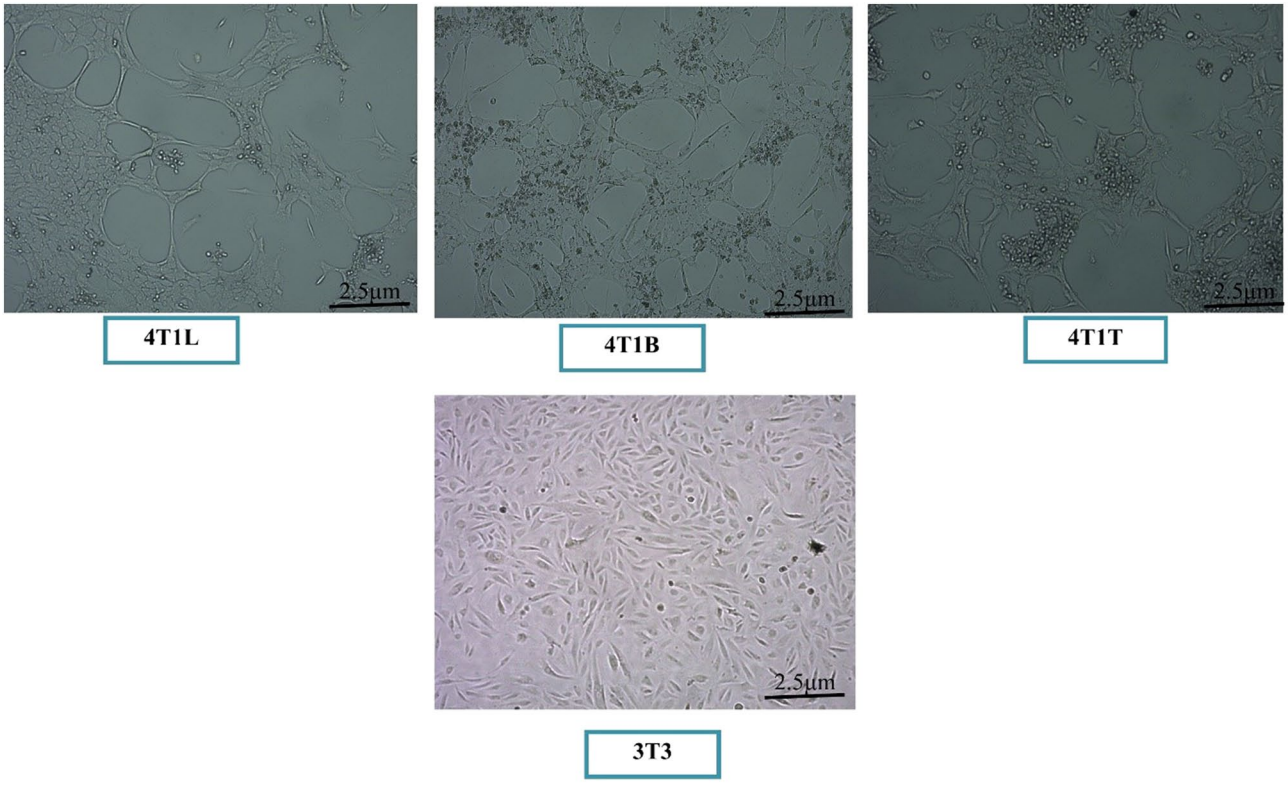

Fig. 2 Representative Matrigel network formation assay images of murine 4T1T 4T1B and 4T1L: compared to 4T1T cells, 4T1B and 4T1L cells showed increased ability of tube formation in both group

up-regulation of *ILK*, *FAK* in metastatic breast cancer relative to primary breast cancer and fibroblast cell line as a control sample.

Interestingly, we observed a down regulation of these genes in primary breast cancer relative to fibroblast cell line. In our work, we used a fresh primary (4T1T) tumor cell (isolated from flank area of mouse models) than use cell line as a control sample. Our assumption study is that overexpression of *ILK*, *FAK* could be leads to permanent endothelial cell adhesion to the extracellular matrix and nonstop activation other downstream proteins related to integrin signaling pathway cause tumor progression and metastasis through induction vascular mimicry.

Lately, Ryota Kawahara and colleagues also found that integrin β1 (ITGB1) plays an important role in VM formation by communication with *ILK* and *FAK*. They recommended prevention of ITGB1 may be a novel therapeutic target for malignant cancer [11].

Marc Delcommenne and colleagues revealed that upregulation of *ILK* in epithelial cells also interfered cell–cell adhesion, which resulted in anchorage-independent growth and survival breast cancer metastasis [19]. Gregory E. Hannigan and colleagues also showed, overexpression of ILK in the mammary epithelia contribute to mammary gland hyperplasia, which related to promotion levels of Akt, GSK-3 and MAPK phosphorylation. This process is an important molecular mechanism involved in VM formation [12].

Therefore, we revealed the gross alteration of *ILK*, *FAK*, expression in metastatic tumor cells compare to primary tumor cells for the first time. More investigation needs to understand the prominent role of these genes in integrin signaling. In addition, proteomic analysis of these genes and siRNA-mediated silencing could be suitable for better understanding role of these genes in VM formation in breast cancer metastases and tumorigenesis.

Conclusion

These results proposed that significant ability of metastatic tumor cells in vascular-like structures formation may be related to higher expression of *ILK* and *FAK*. Analysis of molecular features of metastatic tumor cells could be used to create a targeted treatment strategy against metastasis.

Furthermore, *ILK*, *FAK* could be a critical target to development of effective therapy in combat to this deadly cancer in the world.

Limitation

Economical limitation for further investigation in proteomic level and gene knockout study is the major limitation of this project.

Abbreviations

ILK	Integrin linked kinase
FAK	Focal adhesion kinase
4T1T	Primary breast tumor cells
4T1B	Brain metastatic tumor cells
4T1L	Lung metastatic tumor cells

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Author contributions

MKF: Conceptualization, Methodology, Performing the Experiments (Animal Study, Cell Culture), Supervised the Experimentators; VK: Performing the Experiments (Real Time PCR); SD: Performing the Experiments, Writing-Original draft preparation; AA: Performing the Experiments (Primer design and Real Time PCR); All authors read and approved the final version of the manuscript.

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Data availability

The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

The Ethics Committee of Shahroud University of Medical Sciences approved this study for ethics in animal research (registration number: IR.SHMU.REC.1401.004). All studies were carried out in compliance with the ARRIVE guidelines (https://arriveguidelines.org) for the reporting of animal experiments. All methods were performed in accordance with the relevant guidelines and regulations.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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References

1. Hinton CV, Avraham S, Avraham HK. Contributions of integrin-linked kinase to breast cancer metastasis and tumourigenesis. J Cell Mol Medi. 2008;12(5a):1517–26.
2. Sun Y, Ding Y, Guo C, Liu C, Ma P, Ma S, et al. α-Parvin promotes breast cancer progression and metastasis through interaction with G3BP2 and regulation of TWIST1 signaling. Oncogene. 2019;38(24):4856–74.
3. Morales-Guadarrama G, García-Becerra R, Méndez-Pérez EA, García-Quiroz J, Avila E, Díaz L. Vasculogenic mimicry in breast cancer: clinical relevance and drivers. Cells. 2021;10(7):1758.
4. Luo Q, Wang J, Zhao W, Peng Z, Liu X, Li B, et al. Vasculogenic mimicry in carcinogenesis and clinical applications. J Hematol Oncol. 2020;13(1):19.
5. Wechman SL, Emdad L, Sarkar D, Das SK, Fisher PB. Vascular mimicry: triggers, molecular interactions and in vivo models. Adv Cancer Res. 2020;148:27–67.
6. Friedrich EB, Liu E, Sinha S, Cook S, Milstone DS, MacRae CA, et al. Integrin-linked kinase regulates endothelial cell survival and Vascular Development. Mol Cell Biol. 2004;24(18):8134–44.
7. Tsirtsaki K, Gkretsi V. The focal adhesion protein integrin-linked kinase (ILK) as an important player in breast cancer pathogenesis. Cell Adhes Migr. 2020;14(1):204–13.
8. Pan MR, Hou MF, Ou-Yang F, Wu CC, Chang SJ, Hung WC, et al. FAK is required for tumor metastasis-related fluid microenvironment in triple-negative breast cancer. J Clin Med. 2019;8(1):38.

9. Tancioni I, Miller NL, Uryu S, Lawson C, Jean C, Chen XL, et al. FAK activity protects nucleostemin in facilitating breast cancer spheroid and tumor growth. Breast Cancer Res. 2015;17(1):47.
10. The FAK inhibitor BI 853520 exerts. anti?tumor effects… Google Scholar [Internet]. [cited 2024 Feb 4]. https://scholar.google.com/scholar?hl=en&as_sdt=0%2C5&q=The+FAK+inhibitor+BI+853520+exerts+anti%02tumor+effects+in+breast+cancer&btnG=.
11. Kawahara R, Niwa Y, Simizu S. Integrin β1 is an essential factor in vasculogenic mimicry of human cancer cells. Cancer Sci. 2018;109(8):2490–6.
12. Qu Y, Hao C, Xu J, Cheng Z, Wang W, Liu H. ILK promotes cell proliferation in breast cancer cells by activating the PI3K/Akt pathway. Mol Med Rep. 2017;16(4):5036–42.
13. Mi Z, Guo H, Wai PY, Gao C, Kuo PC. Integrin-linked kinase regulates osteopontin-dependent MMP-2 and uPA expression to convey metastatic function in murine mammary epithelial cancer cells. Carcinogenesis. 2006;27(6):1134–45.
14. Lahlou H, Muller WJ. β1-integrins signaling and mammary tumor progression in transgenic mouse models: implications for human breast cancer. Breast Cancer Res. 2011;13(6):229.
15. Yousefi H, Vatanmakanian M, Mahdiannasser M, Mashouri L, Alahari NV, Monjezi MR, et al. Understanding the role of integrins in breast cancer invasion, metastasis, angiogenesis, and drug resistance. Oncogene. 2021;40(6):1043–63.
16. Kamalabadi-Farahani M, Karimi R, Atashi A. High percentage of Cancer stem cells in metastatic locations: Upregulation of cicBIRC6 in highly metastatic breast Cancer Subline. Mol Biol Rep. 2023;50(2):1303–9.
17. Song X. Targets Related to Vasculogenic Mimicry in Breast Cancer. In: SHS Web of Conferences [Internet]. EDP Sciences; 2022 [cited 2024 Feb 4]. p. 01001. https://www.shs-conferences.org/articles/shsconf/abs/2022/14/shsconf_stehf2022_01001/shsconf_stehf2022_01001.html
18. Shin SU, Cho HM, Das R, Gil-Henn H, Ramakrishnan S, Al Bayati A, et al. Inhibition of vasculogenic mimicry and angiogenesis by an anti-EGFR IgG1-human endostatin-P125A fusion protein reduces triple negative breast cancer metastases. Cells. 2021;10(11):2904.
19. Yang HJ, Zheng YB, Ji T, Ding XF, Zhu C, Yu XF, et al. Overexpression of ILK1 in breast cancer associates with poor prognosis. Tumor Biol. 2013;34(6):3933–8.

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