Pathology - Research and Practice xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Pathology - Research and Practice

journal homepage: www.elsevier.com/locate/prp

Original article

Knockdown of PINCH-1 protein sensitizes the estrogen positive breast cancer cells to chemotherapy induced apoptosis

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ARTICLE INFO

Keywords:
Estrogen
Breast cancer
Apoptosis
PINCH-1
Chemotherapy

ABSTRACT

Introduction: PINCH-1 is a ubiquitously expressed protein belonging to the focal adhesion protein group which has a role in cell survival, spreading, adhesion and migration. It has been implicated in pathogenesis of several cancers. In the present study we aimed to investigate the role of this protein in estrogen positive and negative breast cancer subtypes.

Materials and methods: PINCH-1 expression was studied in two estrogen positive(T47D and MCF-7) and one estrogen negative cell lines before and after treatment with six drugs (Cyclophosphamide, Celecoxib, Doxorubicin, Paclitaxel, Etoposide and Tamoxifen). Then the protein was knocked down using siRNA against PINCH-1 and change in percentage of apoptotic cells was analysed by flow cytometry.

Results: We observed increased but differential expression of PINCH-1 in the three breast cancer cell lines with a higher expression in estrogen positive cell lines. Knocking down of PINCH-1 led to a significant (p-value $<$ 0.05) enhancement in apoptosis in T47D cells in response to 4/6 (cyclophosphamide, celecoxib, paclitaxel, doxorubicin) drugs. Though an increase in apoptosis was observed in MCF-7 cells also, it was not found to be significant.The MDA-MB-231 cells however, did not show significant apoptosis upon PINCH-1 knockdown.

Conclusion: The results suggest that PINCH-1 may be playing an important role in etiopathogenesis of both subtypes breast cancer. However, enhanced apoptosis observed only in estrogen positive and not in estrogen negative cells upon PINCH-1 knockdown point towards participation of some other protein with redundant functions in the later subtype which needs to be investigated.

1. Introduction

Particularly interesting new cysteine-histidine-rich (PINCH) proteins are 5 LIM domain–only adaptor proteins that function as key components of the integrin signaling pathway and play crucial roles in multiple cellular processes. Two PINCH proteins, PINCH1 and PINCH2, have been described in mammals and share 80–90% homology. Both PINCH1 and PINCH2 are ubiquitously expressed in most tissues and organs, including myocardium, liver and kidney.

PINCH-1 protein is well known to play a role in different physiological processes of cells like cell survival, spreading, adhesion and migration. However, the exact mechanisms involved in the above are still under exploration [1,2]. Due to above properties, its deregulation has been associated with different pathologies like CNS diseases and cancer. It has previously been shown that PINCH-1 imparts apoptotic resistance to cancer cells by regulating ERK/Bim pathway [3]. Further, its overexpression has also been linked to radio- and chemo-therapy resistance in malignancies. Eke et al. in their study demonstrated that loss of PINCH-1, reduced cell survival in mouse embryonic fibroblasts and human colon, lung, cervix, skin and pancreatic carcinoma cell lines in response to radiotherapy and chemotherapeutic agents like cisplatin, gemcitabine, and 5-FU. However, in their work they have used much higher clinically irrelevant doses of radiation. The studies on PINCH −1 expression in cancer patients have mainly been carried out in colorectal cancer where a relation between radio-resistance and protein expression has been demonstrated [4]. However, less work has been done on role of PINCH-1 in breast cancer or relationship of its expression to chemotherapeutic response.

Keeping in mind the above, in the present work, we aimed to check the expression of this protein in estrogen positive (ER+) and negative (ER^-) breast cancer cell lines, knockdown the cytoplasmic component of PINCH-1 through siRNA mediated transfection and to study its effect on apoptosis induced in response to chemotherapeutic drugs routinely used in therapy of breast cancer.

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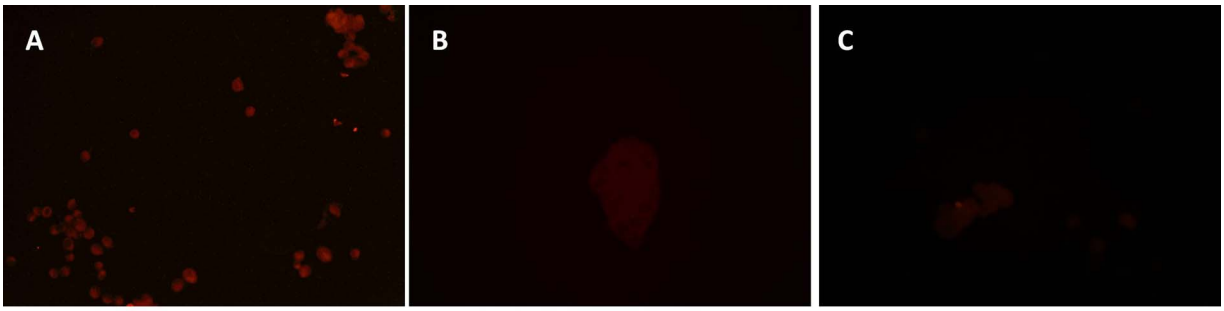
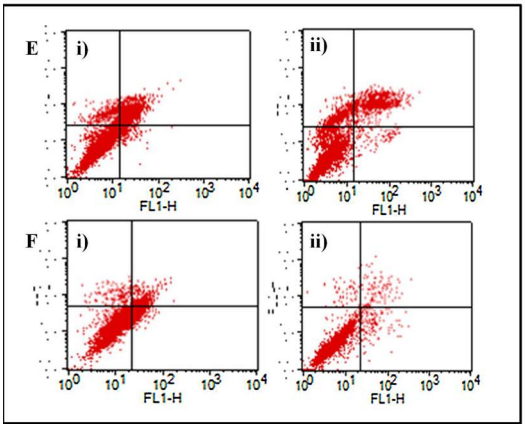
http://dx.doi.org/10.1016/j.prp.2017.09.026
Received 24 August 2017; Received in revised form 18 September 2017; Accepted 27 September 2017



D. Percentage PINCH-1 positivity and intensity of staining in breast cancer cell lines

	T47D	MCF-7	MDA-MB-231
Mean% ± SD of Pinch- 1 positive cells	63±1.88	91±4.16	50±3.33
Intensity of staining	Weak	Strong	Moderate



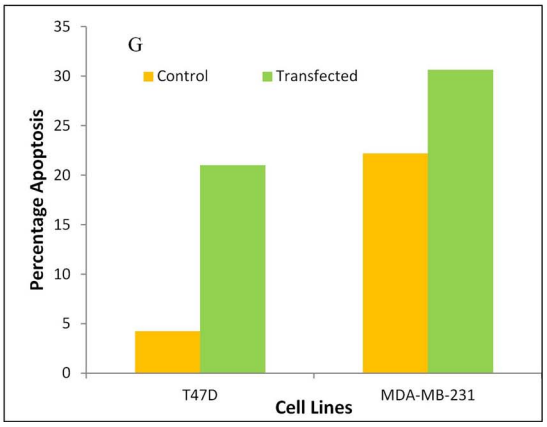


Fig. 1. (A-C) Microphotographs showing PINCH-1 expression in (A) MCF-7 (B) MDA-MB-231 and (C) **T47D** cell lines respectively after staining with rhodamine labelled PINCH-1 antibody. **D)** Table showing percentage of cells showing PINCH-1 staining in 3 cell lines and intensity of staining **(E-G)** Flow cytometric analysis showing percentage of apoptotic cells after 10 nM siRNA transfection in **(Ei)** Control **(Eii)** Transfected MDA-MB-231 and **(Fi)** Control **(Fii)** Transfected T47D cells respectively. **G)** Bar chart showing mean percentage apoptosis in the above cell lines after PINCH-1 knockdown.

2. Materials and methods

Three breast cancer cell lines T-47D, MCF-7 (ER+) and MDA-MB-231(ER$^-$) procured from a commercial source (NCCS, Pune) were used for the experimental work. They were maintained in nutrient media (MCF-7: MEM supplemented with 0.1 mg/ml calf insulin, T-47D: RPMI 1640 with glucose 4.5 g/l and 0.2IU/ml insulin, MDA- MB-231: L-15 medium) supplemented with 10% FCS at 37 °C in an atmosphere of 5% CO2. For analysis of PINCH-1 protein the cells were fixed on Poly L Lysine coated glass slides and permeabilized with 0.25% Triton X-100 followed by staining with anti PINCH-1 antibody (Santa Cruz, 1:100) and rhodamine tagged secondary antibody(Santa Cruz, 1:200). The stained slides were observed under fluorescent microscope using green filter for rhodamine. Pinch-1 knockdown was carried out using SiRNA against PINCH-1(Santa Cruz) and lipofectamine (Life technologies) as the transfection reagent. Annexin V antibody (BD Biosciences) was used for analysis of apoptosis. Trypan blue and MTT assays were used for analysis of cell viability and proliferation respectively. The cancer cells were incubated with chemotherapeutic drugs like doxorubicin, paclitaxel, etoposide, tamoxifen and cyclophosphamide(Sigma Aldrich) and celecoxib both before and after PINCH-1 knockdown. The number of apoptotic cells were then analysed by flow cytometry.

3. Results

3.1. Enhanced PINCH-1 expression in three breast cancer cell lines

All three cancer cell lines showed expression of PINCH-1 protein on immunocytochemistry. The percentage of PINCH-1positive cells was highest (91 ± 4.16%) in MCF-7 cell line followed by T47D (63 ± 1.88%) and MDA-MB-231(50 ± 3.33%) respectively. The staining was also more intense in MCF-7 cells as compared to T47D (Weak) and MDA-MB-231(Moderate) (Fig. 1(A–C)).

3.2. siRNA mediated knockdown of PINCH-1 in T47D and MDA-MB 231 cells

For PINCH-1 knockdown, the cells were exposed to PINCH-1 siRNA at 10 nM concentration for 48 h. The outcome was measured in terms of increase in percentage of apoptotic cells. An increase in percentage of apoptotic cells was noted both in T47D (control 4.21 ± 1.01%, SiRNA 21.02 ± 3.72%) and MDA-MB-231(control 22.18 ± 4.14%, SiRNA 30.62 ± 0.69%) upon transfection (Fig. 1(E–G)).

3.3. Effect of chemotherapeutic drugs on breast cancer cell lines

The cancer cells were treated with drugs like Etoposide (10, 20, 30, 50 μM), Tamoxifen (0.5, 2.5, 10, 20 nM), Paclitaxel (2, 20, 35, 50 nM), Doxorubicin (10, 20, 50, 70, 100 nM) and cyclophosphamide (0.5, 1, 2, 4 and 8 μg/ml) and cell viability and proliferation were analysed at 24 and 48 h respectively using MTT assay in T47D and MDA-MB-231 cell lines.

A significant cytotoxic response was noted with Cyclophosphamide and celecoxib at higher doses at 24 and 48 h in both the cell lines. IC50 was obtained at the interval of 4–8 μg/ml in cyclophosphamide whereas, it was observed at a concentration of 100–200 μM with