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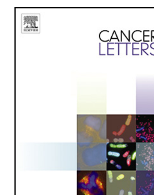

Original Articles

Cyclin D1b splice variant promotes αvβ3-mediated adhesion and invasive migration of breast cancer cells

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ABSTRACT

Cyclin D1b, a splice variant of the cell cycle regulator cyclin D1, holds oncogenic functions in human cancer. However, the mechanisms underlying cyclin D1b function remain poorly understood. Here we introduced wild-type cyclin D1a or cyclin D1b variant into non-metastatic MCF-7 cells. Our results show that ectopic expression of cyclin D1b promotes invasiveness of the cancer cells in a cyclin D1a independent manner. Specifically, cyclin D1b is found to modulate the expression of αvβ3, which characterizes the metastatic phenotype, and enhance tumor cell invasive potential in cooperating with HoxD3. Notably, cyclin D1b promotes αvβ3-mediated adhesion and invasive migration, which are associated with invasive potential of breast cancer cells. Further exploration indicates that cyclin D1b makes breast cancer cells more sensitive to toll-like receptor 4 ligand released from damaged tumor cells. These findings reveal a role of cyclin D1b as a possible mediator of αvβ3 transcription to promote tumor metastasis.
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Introduction

Cyclin D1, encoded by CCND1 and overexpressed in a broad range of solid malignancies including breast cancer, is an important cell cycle regulator that promotes G1/S phase transition by activation of Cdk4 and Cdk6 kinase activity [1,2]. Multiple studies indicate that cyclin D1 functions as an oncogene [3,4]. It is now apparent that cyclin D1 exists in two isoforms: the conventional cyclin D1 (referred to as cyclin D1a) and cyclin D1b. The cyclin D1b variant arises as a consequence of alternative splicing of the CCND1 transcript. This mutant transcript differs from cyclin D1a in the last 55 amino acids of the carboxy-terminus, thereby lacking the Thr-286 phosphorylation site required for nuclear export and subsequent proteasome-mediated degradation [5]. Although the levels of cyclin D1b represent <10% of the total cyclin D1 protein in a number of asynchronously proliferating cell lines, it seems that cyclin D1b is a highly oncogenic variant of cyclin D1 [6]. It has been discovered that a major function of cyclin D1b is to induce nuclear receptor-dependent transcriptional events, which results in markedly enhanced invasion and prometastatic potential activities [7]. These suggest that cyclin D1b may be a key event triggering tumorigenesis and metastasis.

In breast cancer, elevated cyclin D1b expression in breast cancer is independent of cyclin D1a and tightly associated with poor disease outcome, including recurrence, distant metastasis and decreased survival [8,9]. Previous findings show that cyclin D1a associates with and modulates activity of estrogen receptor α (ERα) while cyclin D1b can effectively promote resistance to ER antagonists, which could contribute to therapeutic failure in ER-positive breast cancer [6,10]. These studies suggest that cyclin D1b may play a unique role in breast cancer as opposed to the role of cyclin D1a in cell proliferation (pRb pathway). While cyclin D1b is induced during disease progression in many cancer types, the mechanisms underlying cyclin D1b function remain poorly understood.

Metastases, rather than primary tumors, are responsible for most cancer deaths [11]. The integrins are often up-regulated on metastatic tumors as well, leading to enhanced invasion, proliferation, and tumor survival [12]. In breast cancer, integrin activation controls metastasis and αvβ3 characterizes the metastatic phenotype [13]. In our previous study, we found that the breast cancer cells were metastatic by overexpression of αvβ3. Besides this, toll-like receptor 4 (TLR4) ligand released from damaged tumor cells promotes αvβ3-mediated adhesion and invasive migration of the survival cancer cells [14], which have a negative effect on antimetastasis therapy based on targeting αvβ3 [15]. For these reasons, the integrins and their regulatory mechanisms are attractive targets for the development of therapeutic drugs.

Here, we assessed the underlying mechanism through which cyclin D1b elicits metastatic phenotypes in the context of αvβ3-mediated adhesion and invasive migration of non-metastatic breast cancer cells.

Abbreviations: HoxD3, homeobox D3; DTC-Ms, molecules from damaged tumor cells; ECM, extra-cellular matrix; TLR, toll-like receptor.

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The present study establishes a previously unknown link between cyclin D1b and αvβ3 pathways, and identifies a key mechanism by which cyclin D1b promotes metastatic tumor phenotypes.

Materials and methods

Animals, cells and tissue specimens

BALB/c athymic nude (nu/nu) mice (6–8 weeks old) were purchased from SLAC Laboratory Animal Co. Ltd. (Shanghai, China). The mice were maintained in the accredited animal facility of Tongji Medical College, and used for studies approved by the Animal Care and Use Committee of Tongji Medical College. Human breast cancer cell lines (MCF-7, T47D, MDA-MB-468, MDA-MB-231 and MDA-MB-435S) were purchased from China Center for Type Culture Collection (CCTCC, Wuhan, China) and cultured according to their guidelines.

To detect cyclinD1a/b expression in human breast cancer, tumor samples were acquired by surgery from untreated cancer patients at Tongji hospital, which was approved by the Ethical Committee of the Medical Faculty of Tongji Medical College. Informed consent was obtained from all subjects. All samples included two matched pairs, namely one piece of tumor tissue and a separate piece of adjacent normal breast tissue (≥5 cm away from tumor).

Plasmids and cell transfection

Eukaryotic expression vectors p-cyclin D1a and p-cyclin D1b carrying the cDNAs encoding human cyclin D1a and cyclin D1b were constructed by insertion of cDNA into plasmid pcDNA3.1 (Invitrogen, Carlsbad, CA) in our laboratory. To express cyclin D1a/b in tumor cells, the cells were transfected with p-cyclin D1a or p-cyclin D1b using FuGENE 6 transfection reagent (Roche, Indianapolis, IN) according to the manufacturer's protocol. MCF-7 cells transfected with empty vector (pcDNA3.1) were used as a control. MCF-7 cells expressing exogenous cyclin D1a and cyclin D1b were named as MCF-7-D1a and MCF-7-D1b separately as follows. To down-regulate HoxD3 in tumor cells, the cells were transduced with HoxD3 shRNA(h) lentiviral particles or control shRNA lentiviral particles (Santa Cruz Biotech, Inc.) according to the manufacturer's protocol. The same protocol was used to down-regulate Sam68 in tumor cells. After selection with G418 or puromycin, the cells were used for further experiments.

Matrigel invasion assay

Matrigel invasion assay was performed using Boyden chambers (Transwell, Corning, Inc., Corning, NY). The transwell filter inserts were coated with Matrigel. The lower chambers were filled with DMEM medium containing 10% FBS. 1×10^5 tumor cells were placed in the upper compartment. After 6 h incubation at 37 °C in a humidified incubator with 5% CO_2, the non-invading cells were removed. The invasive cells attached to the lower surface of membrane insert were fixed, stained, and counted under a microscope from five randomly chosen fields in each membrane. The average number of cells per field was calculated. Experiments were repeated three times with triplicate samples in each group.

Immunoprecipitation and Western blot

For immunoprecipitation (IP), cells were rinsed with cold PBS, harvested and resuspended in lysis buffer. Nuclear extracts were prepared by using Chemicon's Nuclear Extraction Kit (Millipore, Bedford, MA), according to the manufacturer's protocol. Nuclear extracts was centrifuged at 16,000 g for 5 min at 4 °C. The supernatant was incubated with primary antibody overnight at 4 °C. The immunocomplexes were bound to protein-G sepharose for 2 h at 4 °C and washed three times with IP buffer. Proteins bound to the protein-G sepharose were eluted by adding Laemli-SDS sample buffer followed by boiling for 3 min. After centrifugation at 10,000 g for 2 min, the supernatant was analyzed by immunoblotting.

Immunoblotting was done as described previously [16]. Antibody against cyclin D1 (DSC6, reacts with both cyclin D1a and b) was purchased from Cell Signaling Technology. Other Abs were purchased from Santa Cruz Biotech, Inc.

Analysis of gene expression

Total RNA was extracted from cells with TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Expression of cyclin D1 isoforms and HoxD3 was assessed by regular RT-PCR and real-time RT-PCR assay. Expression of Sam68, αv and β3 was assessed by real-time RT-PCR procedure. The regular RT-PCR products were separated by electrophoresis in a 2% agarose gel containing ethidium bromide and then photographed. The relative quantity of mRNA was determined by real-time RT-PCR procedure. Briefly, 100 ng of total RNA was used for reverse transcription using Superscript II RNase H reverse transcriptase (Invitrogen) in a volume of 25 µl. Then, 2 µl of cDNA was amplified with SYBR Green Universal PCR Mastermix (Bio-Rad, Richmond, CA) in duplicate. Gene expressions in each sample were respectively normalized with the housekeeping gene GAPDH expression. Relative quantification of target gene expression was evaluated using the $2^{-\Delta\Delta CT}$ method. All the experiments were performed for at least three times independently.

The primer sequences were as follows: cyclin D1a, sense 5′-CTACACCGACAACTCCATC-3′, anti-sense 5′-TCCAGCAGGGCTTCGATC-3′; cyclin D1b, sense 5′-CATCTACACCGACAACTCCATC-3′, anti-sense 5′-TGGGACATCACCCTCACTTAC-3′; HoxD3: sense 5′-CGGCAACTTCGTCGAGTCC-3′, anti-sense 5′-ATGAGGGTCGCAAGGTCCA-3′; αv, sense 5′-CTCGGGACTCCTGCTACCTC-3′, antisense 5′-AAGAAACATCCGGG AAGACG-3′; β3, sense 5′-CATCCTGGTGGTCCTGCTCT-3′, antisense 5′-GCCTCTTTA TACAGTGGGTTGTT-3′; GAPDH, sense 5′-TCATTGACCTCAACTACATGGTTT-3′, antisense 5′-GAAGATGGTGATGGGATTTC-3′.

Adhesion assay

Tumor cells were added to 6-well plates (5×10^5 cells per well) which were precoated with fibrinogen (Sigma). After 2 h incubation at 37 °C, non-adherent cells were harvested. Then, adherent cells were harvested by treatment with trypsin. The percentage of adherent cells was calculated. Each assay was tested in triplicate wells in three independent experiments.

Tumor cell proliferation assay

Tumor cells were labeled with CFSE and cultured for 3 days. The proliferation of the cells was analyzed by flow cytometry. Flow cytometric analysis included at least 1×10^4 events on a BD LSR II Flow Cytometer. The proliferation index was calculated in the responder population gate using the ModFit LT for Win32 software. Each sample was assayed in triplicate in three independent experiments.

Flow cytometric analysis

Tumor cells were stained with FITC-conjugated mouse-anti-human αvβ3 (Chemicon) or isotype control IgG1 for flow cytometric analysis. Parameters were acquired on a FACS Calibur flow cytometer (BD Biosciences) and analyzed with CellQuest software (BD Biosciences). Percent staining was defined as the percentage of cells in the gate (M1) which was set to exclude ~99% of isotype control cells.

Breast tumor metastasis in mice

For the mammary fat pad tumor model, 5×10^6 MCF-7 cells transfected with p-cyclin D1a or p-cyclin D1b were labeled with CFSE, and injected into the second pair of mammary glands of nude mice respectively (8 mice for each cell line). Axillary lymph nodes were harvested 24 h after the injection. Frozen sections were prepared and analyzed by fluorescence microscopy. Fluorescent spots were counted from 20 randomly chosen fields in sections of each mouse.

Unlabeled tumor cells were inoculated likewise (24 mice for each cell line). Tumor growth was monitored every 5 days and the volume of tumor (V) was determined as described previously [17]. On d30 after inoculation, 8 mice out of each group were sacrificed. Lungs as well as axillary lymph node were dissected. The expressions of cyclin D1a and cyclin D1b were identified by RT-PCR assay. On d60 after inoculation, another 8 mice were sacrificed. Lungs were surgically retrieved from mice. Tissues were embedded in paraffin, sectioned, stained with H/E. The mice left were all sacrificed on d90 after inoculation. Lungs were harvested and fixed in Bouin's solution. Tumor nodules at lung surface were counted.

To assay tumor cell arrest in lung during blood flow, MCF-7 cells transfected with p-cyclin D1a or p-cyclin D1b were labeled with CFSE, and injected into mice via tail vein (2×10^6 cells/mouse, n = 8 for each group). Lungs of mice were harvested 5 h or 24 h after the injection. Frozen sections were prepared and analyzed by fluorescence microscopy as described above.

All these experiments were repeated three times.

MMP assay by gelatin zymography

Tumor cells were cultured for 48 h in DMEM medium containing 1% FBS in the presence of pre-coated Matrigel. The assay of MMP-2 and MMP-9 in supernatants was performed as described previously [18].

Preparation of DTC-Ms from tumor cells

MCF-7 cells were washed with PBS and resuspended in PBS to a final concentration of 5×10^7/ml. After four-round frozen–thaw cycles followed by vortexing for 30 sec, the cells were removed by centrifugation. The supernatant contained a mixture of molecules from damaged tumor cells (DTC-Ms). The concentration of DTC-Ms was defined by the concentration of protein, which was determined using Coomassie Bradford reagent (Thermo Fisher Scientific Inc., Rockford, IL) according to the manufacturer's instructions.

Statistics

Results were expressed as mean value ± SD and interpreted by one-way ANOVA. Differences were considered to be statistically significant when $P < 0.05$.

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