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摘要

ErbB-3 是屬於 ErbB family 的一種有 tyrosine kinase 的受體，大多被報導位於細胞膜上。ErbB-3 的表現增加在包括攝護腺癌、乳癌與卵巢癌的許多腫瘤上都曾經被報告過。在攝護腺癌的標本上，我們發現當攝護腺癌轉移到骨頭時會有表現增加的現象。有趣的是，儘管 ErbB-3 被認為是一種細胞膜上的受體，但是卻有 75 %轉移到骨頭的攝護腺癌細胞，顯示 ErbB-3 在細胞核中表現。在這些轉移到骨頭的攝護腺癌細胞中，染色的結果顯示細胞核中 ErbB-3 具有 extracellular 與 cytoplasmic domains 兩者。我們進一步研究兩種由轉移到骨頭攝護腺癌衍生而來的細胞 MDA PCa 2b 與 PC-3。在體外培養的狀況下，ErbB-3 主要存在於細胞膜與細胞漿。如同人體標本一樣，當這些細胞注射到老鼠骨頭時，ErbB-3 會在細胞核中表現。相反地，當這些細胞注射到老鼠皮下時，ErbB-3 會在細胞膜或細胞漿表現。同時愈接近骨頭 ErbB-3 會在細胞核中表現愈明顯。這種現象顯示骨頭的微環境可以調控 ErbB-3 在細胞核中的表現。這篇研究提供 ErbB-3 會在細胞核中表現的現象。同時也讓我們想到是否 ErbB-3 在細胞核中表現在攝護腺癌轉移到骨頭的過程中是否扮演重要的角色。

Abstract

ErbB-3 is an ErbB family receptor tyrosine kinase and has been found to mainly localize in the plasma membrane. Increased in ErbB-3 expression has been shown in several malignancy including prostate, breast and ovarian cancers. In prostate cancer specimens, we found that expression of ErbB-3 is up-regulated in metastatic prostate cancer cells in bone. Interestingly, although ErbB-3 is a membrane receptor, among those prostate cancer specimens that expressed ErbB-3, 75% of them show ErbB-3 localization in the nucleus. In these specimens, nuclear ErbB-3 also stained positive with antibody against extracellular domain, suggesting that nuclear ErbB-3 may contain both the extracellular and cytoplasmic domains. We further examined the expression and localization of ErbB-3 in PCa cell lines MDA PCa 2b and PC-3, which were derived from PCa patients with bone metastasis. ErbB-3 was found predominantly in the plasma membrane and cytoplasm of both cell lines under in vitro culture conditions. As nuclear localization of ErbB-3 was mainly observed in metastasis specimens, we studied the effect of in vivo tumor microenvironment on ErbB-3 localization. PC-3 and MDA PCa 2b cells were injected into mouse subcutaneously or intrafemorally. We found that ErbB-3 was mainly localized in plasma membrane or diffusely in cytoplasm in MDA PCa 2b or PC-3 tumors generated subcutaneously. In contrast, ErbB-3 was found localized to the nucleus of PC-3 and MDA PCa 2b cells in the tumors growing in the bone. Interestingly, nuclear localization of ErbB-3 was apparent in tumors proximal to bone and become less obvious in tumors distal to bone, suggesting that the bone microenvironment may provide factors that regulate the nuclear/cytoplasmic translocation of ErbB-3. This report provides the first evidence for the nuclear expression of ErbB-3 in human prostate cancer

specimens and raises the possibility that nuclear ErbB-3 may have a role in metastatic progression of prostate cancer.

Introduction:

Microenvironment is critical for metastatic progression of cancer in distant sites. Such an environmental influence is especially critical in prostate cancer in bone, which is the most common sites of distant metastases. These observations suggest that interaction between the metastatic PCa cells and tumor microenvironment is important in the progression of PCa in bone.

ErbB family membrane proteins are receptor tyrosine kinase that mediates cell growth and differentiation through binding of its ligands. Elevated ErbB3 mRNA levels were detected in human mammary tumor cell lines, and overexpression of ErbB3 is associated with a poorer prognosis in patients with endometrioid carcinoma of the ovary. These observations suggest that increased ErbB3 expression may play a role in human malignancies.

ErbB3 may be one of the molecules that are involved in PCa progression. Myers et al. reported expression of erbB-3^{p160} in prostate cancer progression. They found that ErbB3 is upregulated in tumor cells found in the prostate. They also found that 9 out of 11 lymph nodes metastatic cases had ErbB3 staining on the membranous or cytoplasmic area of tumor cells. However, the study on the expression of ErbB3 in the bone metastasis of prostate cancer is relatively limited.

Unlike EGFR and ErbB-2, whose expression patterns include many mesenchymal tissues, erbB3 are not expressed in fibroblasts and their expression in epithelial cells is limited to specific organs. On other hand, mesenchymal cell are the major producers of ligands (heregulin) for erbB3, implying that ligand-receptors interaction may play a role in mesenchyme-epithelial interaction.

In previous study, they show increased HRG-alpha (total 72%) according to increased grade, but no significant was found. High percentage of tumor cells also has erbB-3 (RTJ.2) expression but not related to grade. They proposed that activation of HRG-alpha/erbB3 receptor system may have an adverse effect on clinical outcome. Heterodimerize with erbB-2 and EGF receptors, resulting in transphosphorylation and signal transduction.

In this study, we would approach the ErbB3 expression during metastatic progression of prostate cancer. In addition to be located in membrane, we would test the translocation of ErbB-3 during the metastasis and try to verify the effect of micro-environment on the ErbB-3 translocation.

Materials and Methods:

Immunostaining of prostate cancer specimens

Formalin-fixed, paraffin-embedded tissue samples representing a spectrum of localized and metastatic prostate cancer, including radical prostatectomy specimens, and bone specimens with prostate cancer metastases, were selected. A mouse monoclonal antibody against the cytoplasmic domain of ErbB3 (RTJ.2) (Santa Cruz, CA, USA) and a polyclonal antibody against extracelluar domain of ErbB3 (Ab-10) (Neomarker, CA, USA) were used for immunohistochemical studies. The monoclonal antibody against Heregulin is also used for immunohistochemical studies.

Nuclear and cytoplasmic fractionation

Prostate cancer cell line PC-3 were purchased from American Type Culture Collection. MDA PCa 2b was generated from bone metastasis of a prostate cancer patient as described. PC3 cells were maintained in DMEM with 10% fetal calf serum and MDA PCa 2b cells were maintained in BRFF-HPCI medium (AthenaES, Baltimore, MD) supplemented with 20% fetal calf serum. Separation of nuclear and cytoplasmic fraction of prostate cancer cells was done by nuclear and cytoplasmic extraction reagents (NE-PER Pierce, Rockford, IL) as described by manufacturer.

Immunoprecipitation and Western blotting

Cell lysates of MDA2b and PC-3 were incubated with anti-ErbB3 antibody (2F12; Neomarker) for 16 h at 4 °C. Immune complexes were collected by incubation (2 h, room temperature) with protein G-agarose (SantaCruz), separated on a 10% SDS-PAGE, analyzed by Western blotting with an anti-ErbB3 antibody (C-17, Santa Cruz, CA), and the signal detected by using the enhanced chemiluminescence system.

Immunostaining of prostate cancer cell lines

For immunostaining of ErbB-3, cells were washed with PBS, fixed in methanol and acetone for 3 min at -20℃. The cells were blocked in 5% normal horse serum in PBS for 30 min at room temperature, and incubated with RTJ.2 (2 ug/ml in 5% normal horse serum) overnight at 4°C. The cells were washed several times with PBS, incubated with FITC-conjugated goat anti–mouse antibody (1:100 in 5% horse serum/PBS) for 30 min at room temperature, mounted with Vectashied (Vectashied with DAPI, Vector Laboratories, Inc., Burlingame, CA), and evaluated in an IX71 microscope.

Subcutaneous and intra-femur injection of prostate cancer cells

All procedures were performed in compliance with the Institutional Animal Care and Use Committee. To generate subcutaneous tumors, PC-3 (1 x 10^6 cells) or MDA PCa2b (4x10^6 cells) were injected subcutaneously into SCID mice. The tumors were excised and fixed in formalin.

To study tumor growth in bone, three to four week-old male SCID mice were used. Bone injections were performed under anesthesia. A 32-gauge needle was inserted 3 mm into the distal end of the right femur using a drilling motion and 3 ul of

the cell preparation containing $1x10^5$ cells was injected. The left femur was injected with saline. Animals were sacrificed when obvious tumor growth were found. After sacrifice, both the tumor-bearing and the sham-injected legs were harvested. All tumors were fixed in formalin and decalcified, then embedded in the paraffin for immunostaining.

PC-3 cells treated with heregulin

The PC-3 cell on coverglass in six wells culture plate. After starvation for 24 hours, the dose dependent (20ng/ml, 50 ng/ml and 100 ng/ml) or time course treatment (100ng/ml in 1 hour, 2 hour and 6 hours) for observed the subcellular localization of erbB3. The controlled wells with treated with DMEM (no serum) are observed for control. Immunofluorescent study of erbB3 and DAPI stain the nucleus for localized the nucleus.

Results:

Nuclear localization of ErbB3 in PCa bone metastasis

To examine the expression and localization of ErbB-3 in prostate cancer specimens, we use antibodies against the cytoplasmic domain (RTJ.2) to stain the specimens obtained from patients with localized or disseminated metastatic PCa. We found that epithelial cells in the prostate cancer specimens from patients with localized prostate cancer express very low or undetectable levels of ErbB-3. In contrast, metastatic PCa cells in bone showed increased expression of ErbB-3.

Among the 26 bone metastasis specimen, 12 specimens stained positive for ErbB-3. Although some specimens show membraneous/cytoplasmic localization as expected, some samples showed predominantly nuclear localization of ErbB-3 in the PCa cells (Fig. 1A) while some showed mixed nuclear and cytoplasmic localization (Fig. 1B). A total of 8 cases, representing 67% of total ErbB-3 positive cases, showed nuclear localization of ErbB-3.

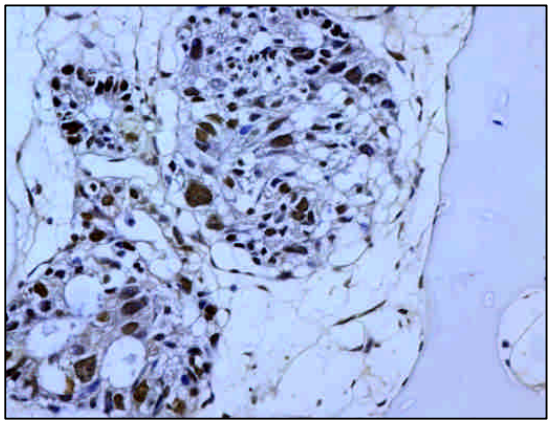

Figure 1A

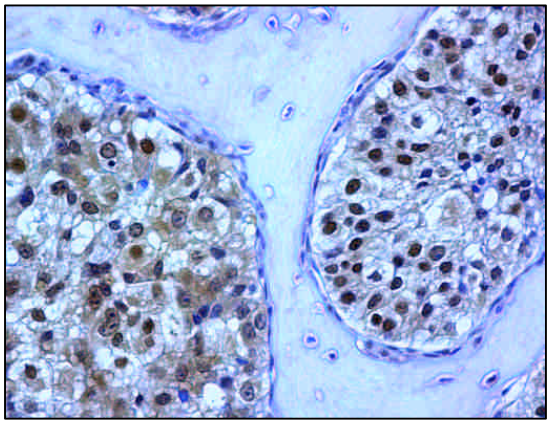

Figure 1B

Taken together, 26 human prostate cancer specimens from bone metastases

were examined and ErbB-3 was upregulated in 12 of the specimens. A total of 8 cases, representing 75% of total ErbB-3 positive cases, showed nuclear localization of ErbB-3. These observations suggest that nuclear localization of ErbB-3 occurs in bone metastasis.

Expression of heregulin in prostate cancer with bone metastasis

We found that 18 out of 26 cases with bone metastasis have heregulin expression within the cytoplasm of tumor cells.

Localization of ErbB-3 in Prostate Cancer Cell Lines

To study the possibility that tumor microenvironment may have an effect on ErbB3 localization, we first determined the subcellular localization of ErbB-3 in two prostate cancer cell lines, i.e. MDA PCa 2b and PC-3, which were derived from patients with bone metastasis of prostate cancer. Cell fractionation was used to separate nuclei from non-nuclear fractions. These fractions were then subjected to immunoprecipitation followed with western blot for the detection of ErbB3. We found that ErbB3 was expressed in both MDA PCa 2b and PC-3 cells and mainly localized in the membrane/cytoplasmic fraction of MDA PCa 2b and PC-3 cells (Fig. 2A). PARP (Poly(ADP-ribose) Polymerase and tubulin were used as markers for nuclear and cytoplasmic fractions, respectively.

We further examined the expression of ErbB3 in MDA PCa2b and PC-3 cells by immunofluorescent staining. Consistent with those observed by cell fractionation, immunostaining of MDA PCa2b and PC-3 cells showed that ErbB3 was localized in the membrane/cytoplasm of these cell lines in culture (Fig. 2B)

RTJ.2

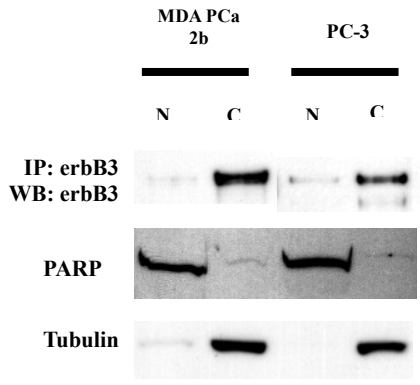

Figure 2A

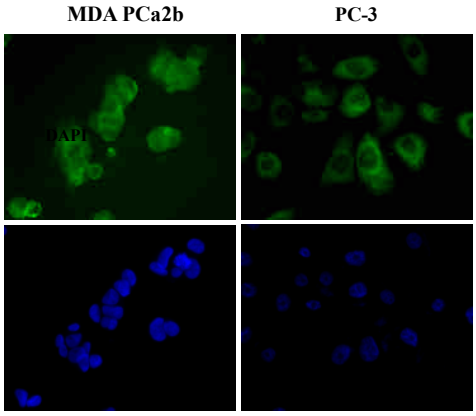

Figure 2B

Regulation of Nuclear Translocation of ErbB-3 by bone microenvironment

In prostate cancer, the interaction between the tumor cells and the tumor microenvironment was critical in the development of bone metastasis. To examine whether nuclear translocation of ErbB-3 is influenced by the bone environment, we generated PCa tumors by injecting PCa cells subcutaneously or into the femur bone of mice. Tumors that were grown from subcutaneous sites or in the bone were analyzed.

As shown in Fig. 3A, ErbB3 was localized in membrane in the MDA PCa 2b tumors grown in subcutaneous site. In contrast, growth of MDA PCa2b cells in mouse femur induced nuclear translocation of ErbB-3 (Fig. 3B). Similarly, ErbB3 was found to localize in membrane in PC-3 tumor grown subcutaneously (Fig. 3C) and in the nucleus of PC-3 tumors grown in bone (Fig. 3D). These observations suggest that factors present in the bone microenvironment have effects on the nuclear localization of ErbB3.

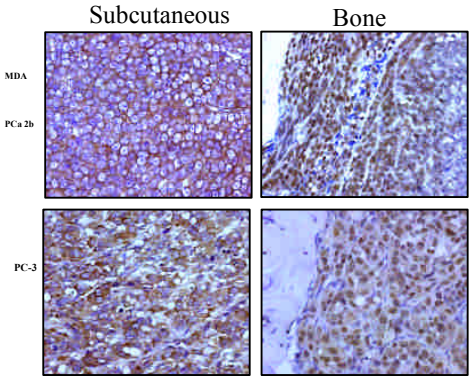

Figure 3: (A: right upper, B: left upper, C: right lower, D: left lower)

Nuclear Translocation of ErbB-3 not influenced by Heregulin

After starvation for 24 hours, the dose dependent (20ng/ml, 50 ng/ml and 100 ng/ml) or time course treatment (100ng/ml in 1 hour, 2 hour and 6 hours) for observed the subcellular localization of erbB3. The controlled wells with treated with DMEM (no serum) are observed for control. Immunofluorescent study of erbB3 and DAPI stain the nucleus for localized the nucleus. We could not find erbB3 translocation within these conditions.

Discussion

We examined the expression of ErbB3 in human bone metastasis specimens and found that ErbB3 is upregulated in prostate cancer cells found in bone and ErbB3 was present in the nucleus of PCa cells in about 75% of ErbB3 positive bone specimens. In the prostate cancer cell lines MDA PCa2b and PC-3, derived from human bone metastasis specimen, we showed that nuclear localization of ErbB-3 in PCa cells was induced by the tumor microenvironment. Thus, nuclear localization of ErbB3 is a dynamic process that responds to environmental factors and may have a role in the growth of PCa in the metastatic sites. This study provides the first evidence that nuclear translocation of ErbB-3 occurs in human prostate tissues and may be involved in metastatic progression of prostate cancer.

Nuclear localization of other ErbB family receptors were also observed and most of these studies are mainly on their localization in cell lines. ErbB1 was observed in the nucleus of many different tissues including mouse uterus, human mouth mucosa, human oral cancer sample, and MDA MB468 cell line. ErbB2 was shown to be present in the nucleus of NIH3T3 cells by transfection with rat homologue of ErbB2. Localization of ErbB4 in nucleus has been

documented extensively. The first report on nuclear localization of ErbB3 was by Offterdinger et al., in which they showed that ErbB3 was localized in several breast cancer cell lines and cell lines derived from normal breast or breast cancer tissues. Our finding that ErbB3 is localized in the nucleus of metastatic PCa cells in bone is the first report of nuclear localization of ErbB3 in prostate cancer tissues. We found that nuclear localization of ErbB3 mainly occurred in vivo and this may be the reason that it has not been reported before. Our observations raised the possibility that nuclear ErbB3 may play specific role in different pathological stages.

The mechanism of nuclear translocation for ErbB-3 is not clear. Various mechanisms of nuclear translocation have been shown for each ErbB family proteins. The best-studied member is ErbB4. After binding with its ligand HRG or activation by protein kinase C (PKC) through 12-o-tetradecanoyl phorbol-13-acetate (TPA), the ErbB4 ectodomain is cleaved by a metalloprotease. Subsequent cleavage by γ-secretase releases the ErbB4 intracellular domain from the membrane and facilitates its translocation to the nucleus. Thus, only the cytoplasmic domain of ErbB4 is translocated into the nucleus. In contrast, Lin et al. demonstrated that the entire ErbB1, including both the cytoplasmic and extracellular domain, was translocated into the nucleus. Similarly, Offterdinger et al. eported that both extracelluar and cytoplasmic domain of ErbB3 are detected in the nucleus of several breast cancer cell lines. We also found that nuclear ErbB-3 contains both the extracellular and cytoplasmic domain. The mechanism of nuclear translocation of membrane protein is not known.

The finding of nuclear localization of ErbB3 protein in prostate cancer specimens was unexpected and raised questions of its function in the nucleus. Because interactions between prostate cancer cells and their microenvironment plays an important role in the development of prostate cancer in distant sites, the high percentage of translocation of ErbB3 into nucleus in bone metastasis specimens suggests that nuclear ErbB3 may have an important role in prostate cancer progression in bone. However, the exact role of nuclear ErbB3 in tumor cells in bone is not clear. In the heregulin treatment study, we found that nuclear translocation of ErbB-3 doesn't happen with variable concentration of heregulin. This observation seems to suggest that nuclear ErbB-3 translocation influenced by a complex interactions between the tumor cells and microenvironment. Further study will be needed to approach the complex mechanism.

成果自評:

(1) 本篇成果與當初預期大致相符，唯 heregulin 對於 ErbB3 translocation 的影響在目前培養的狀況下，並沒有見到預期的結果。

(2) 經由這次研究，我們的發現 ErbB3 可以在攝護腺癌轉移到骨頭時進行表現增加的現象。本篇成果顯示 ErbB-3 表現於核的現象可以被骨頭的微環境所調控。不過於細胞培養的模式，無法僅以 heregulin 影響 ErbB-3 移動到核的現象。