主論文の要旨

UBE2S is associated with malignant characteristics of breast cancer cells

（UBE2S は乳癌の悪性化に関与している）

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Introduction

Ubiquitination is a reversible biochemical process that attaches ubiquitin to substrate proteins to regulate multiple cellular functions. The process of ubiquitination is mediated by three types of enzymes: E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme and E3 ubiquitin ligase. Accumulating evidence has demonstrated that ubiquitination is associated with the progression of cancer. Mdm2, COP1 and Pirh1 are E3 ligases for p53 and they are overexpressed in several cancers. There are additional E3 ligases whose mutation or dysregulation are associated with cancer progression. For example, germline mutations of BRCA1 gene are predictors for the risk of ovarian and breast cancers. Somatic mutations of Von Hippel-Lindau (VHL) E3 ligase are related to the development of clear cell renal carcinoma. In addition to E3 ligases, E2 conjugating enzymes are associated with cancer progression. One of the most studied E2 is UBE2C because of its association with cancer. UBE2S, also known as E2-EPF, is essential for the elongation of ubiquitin chains to target substrate proteins to the 26S proteasome. Once UBE2C attaches ubiquitin onto the target proteins, UBE2S promotes the elongation of ubiquitin chains for the degradation. Recent studies have shown that UBE2S is also overexpressed in cervical, breast and kidney cancers. But the physiological role of UBE2S in cancer still remains uncertain. In this report, we show that UBE2S depletion suppressed migration, spreading and invasion of breast cancer cells.

Methods

We first generated purified anti-UBE2S antibody to check the expression of UBE2S in breast cancer cells by immunoblotting as well as in normal and breast cancer patient's tissue sample through immunohistochemistry. Tissues microarrays for breast cancer were obtained from US Biomax. We also depleted UBE2S expression in multiple breast cancer cell lines using two different siRNAs targeting different regions of the gene. siRNAs were transfected using RNAi/Max obtained from Invitrogen according to the manufacturer's protocol. Whether UBE2S knockdown can affect the malignant characteristics of breast cancer cells or not were checked by cell attachment, cell spreading, migration, invasion and colony formation assay.

Results

Immunoblot analysis with the antibody detected single bands at the expected molecular weight. Although UBE2S was highly expressed in breast cancer cell lines, a reduced expression of UBE2S was observed in human mammary epithelial

cells (MBE) (Fig. 1A). Immunostaining and cell fractionation analysis revealed that UBE2S was localized to both the nucleus and cytoplasm (Fig. 1B and 1C). We next examined the expression of UBE2S in breast cancer tissues using the antibody. The antibody specifically stained cells and UBE2S expression was assessed and scored .We found that UBE2S expression was increased in breast cancer tissues compared with normal tissues (Fig. 1D).

We next examined the effect of UBE2S depletion using siRNAs. Breast cancer cell lines were transfected with two siRNAs. We noticed that the cells became significantly round in the absence of UBE2S. We speculated that the morphological changes were induced by the disruption of actin cytoskeletal organization. Immunostaining for actin cytoskeleton and vinculin, a component of focal adhesions, demonstrated a disrupted organization of the actin cytoskeleton and focal adhesions by UBE2S knockdown (Fig. 2A). Focal adhesion kinase (FAK) is a cytoplasmic tyrosine kinase that is critical for the organization of focal adhesions and the actin cytoskeleton. We thus checked the expression and activity of FAK in the absence of UBE2S. To detect the activity of FAK, we used an antibody that specifically detects phosphorylation at Tyr397. UBE2S knockdown did not affect expression level of FAK but significantly reduced the phosphorylation of FAK on Tyr397 (Fig. 2B). Organization of the actin cytoskeleton and focal adhesions is essential for cell attachment to the extracellular matrix (ECM) and cell spreading. We found that cell spreading was suppressed by UBE2S knockdown (Fig. 2C).

We next investigated the migration and invasion of the cells in the absence of UBE2S using transwell chambers. Three luminal cell lines (MCF7, T47D, MDA-MB-453) and three basal cell lines (BT20, MDA-MB-231, Hs578t) were transfected with siRNAs and 72 h later, cells were placed on the upper surface of the filter and allowed to migrate to the bottom surface, which was coated with fibronectin. Twenty hours later, cells that migrated to the bottom surface were quantified. Cell migration was significantly suppressed by UBE2S depletion (Fig. 3A). To examine cell invasion, we used matrigel-coated transwell chambers. siRNA-transfected cells were seeded on the matrigel-coated chamber, and cells that invaded to the lower surface of the filter were counted. UBE2S depletion suppressed invasion of these cell lines (Fig. 3B). Anchorage-independent growth is one of the major characteristics of tumor cells. To determine whether UBE2S depletion affects the anchorage-independent growth of breast cancer cells, siRNA-transfected cells were cultured on agar; 2 weeks later, colony formation

was evaluated. The proliferation of cells on the agar was significantly reduced by UBE2S knockdown (Fig. 3C). Anoikis is a form of cell apoptosis that is induced by the detachment of cells from the extracellular matrix. We speculated that the suppression of colony formation by UBE2S knockdown was partly mediated by the promotion of anoikis. Cells transfected with siRNAs were cultured in suspension for 48 h, and apoptotic cells were then detected via TUNEL assay. As shown in Fig. 3D, UBE2S knockdown promoted anoikis of breast cancer cells.

Discussion

Consistent with previous findings, we found that the expression of UBE2S was increased in breast cancer tissues by immunohistochemical analysis. High expression of UBE2S has also been reported in other cancers. These previous reports and our study clearly indicate that UBE2S can be used as a marker for multiple cancers. In addition to its important functions in mitosis, we found that UBE2S is associated with regulating the actin cytoskeleton and focal adhesions. The actin cytoskeleton plays a critical role in numerous cellular functions, such as cell migration and spreading. We found that both migration and spreading were delayed in the absence of UBE2S. A number of proteins are associated with the regulation of actin cytoskeletal organization and cell migration. Among these proteins, FAK is one of the most studied proteins, and its inhibitors are being investigated in clinical trials for cancer treatment. FAK is also known to activate survival signals to prevent anoikis. We found that the phosphorylation of FAK at Tyr397 was reduced in the absence of UBE2S. Decrease in Tyr397 phosphorylation via UBE2S knockdown may suppress numerous signals for cell spreading, migration, invasion and anchorage-independent growth.

Conclusion

Our results provide a quantitative framework for the design of future experiment to investigate UBE2S function in vivo. Further investigation to identify target proteins of UBE2S may give novel insight into the regulation of actin cytoskeletal organization and cell migration.