論文の内容の要旨

論文題目 ： Identification and characterization of *BIG3* and *C12orf32* as novel molecular targets for breast cancer therapy
(乳癌新規治療標的分子 *BIG3*・*C12orf32* の同定及びその機能解析)

氏名 ： **金 正元**

Breast cancer is the most common cancer in women worldwide. Incidence of breast cancer is increasing in most countries including the USA and Japan. Development of molecular-targeted therapeutic drugs, such as tamoxifen, aromatase inhibitors, and trastuzumab have contributed to a reduction of mortality rate and improving the quality of life of women diagnosed with breast cancer. However, these drugs have been shown the adverse reactions like the increases in the risk of endometrial cancer with long-term tamoxifen administration and severe cardiac toxicity with trastuzumab treatment. Therefore, it is necessary to search for novel anticancer-drugs with the minimum risk of adverse reactions.
Gene-expression profiles obtained by cDNA microarray analysis have been proven to provide a detailed characterization of individual cancers, and such information should contribute to select more appropriate clinical strategies for individual patients through the development of novel drugs and providing the basis for personalized treatment. Toward such goals, my laboratory had established genome-wide gene expression profiles of 81 breast tumors and 29 normal human tissues by means of cDNA microarray representing 23,040 cDNAs or ESTs, and identified dozens of molecules that were over-expressed in a great majority of breast cancers and were undetectably expressed in normal human organs, especially heart, lung, kidney and liver. Among them, I focused on the identification and characterization of novel genes, brefeldin A-inhibited guanine nucleotide-exchange protein 3 (*BIG3*) and chromosome 12 open reading frame 32 (*C12orf32*) as therapeutic molecular targets for breast cancer.

<BIG3>
We confirmed up-regulation of *BIG3 gene* in nine out of 12 clinical breast cancer specimens, compared with normal breast ductal cells as well as mammary gland by semiquantitative RT-PCR. Subsequent northern-blot analysis also confirmed overexpression of its 15-kb transcript in breast cancer cell lines, but its undetectable expression in normal human organs except the brain as concordant to the results of cDNA microarray analysis. To examine a possible biological role of *BIG3* in mammary

carcinogenesis, we knocked down the expression of endogenous *BIG3* using a mammalian vector-based RNA interference technique, and found that knocking-down of *BIG3* expression drastically suppressed the growth of breast cancer cell lines, SK-BR-3 and BT-474.

Since the biological functions of BIG3 are totally unknown, we searched for a protein(s) interacting with BIG3 by immunoprecipitation and mass spectrometry analyses, and identified Prohibitin 2/repressor of estrogen receptor activity (PHB2/REA) as a binding partner of BIG3. Subsequent co-immunoprecipitation experiments and immunoblot assays confirmed an interaction of Flag-tagged BIG3 with endogenous PHB2/REA protein in SK-BR-3 cells. Since PHB2/REA was reported to selectively repress the transcriptional activity of ERα through its interaction with ERα in the nucleus, I investigated the direct interaction between BIG3 and ERα, but failed to indicate their interaction.

Because PHB2/REA was reported to be localized mainly at the cytoplasm and to be translocated to the nucleus in ERα-positive breast cancer cells after the estradiol (E2)-treatment, I hypothesized that BIG3 might interact with PHB2/REA in the cytoplasm and interfere with its nuclear-translocation. To examine my hypothesis, I investigated the subcellular distribution of PHB2/REA protein in the presence or absence of BIG3. PHB2/REA was localized in the cytoplasm of MCF-7 cells, in which BIG3 protein was overexpressed, with or without treatment of E2. Moreover, I confirmed that endogenous PHB2/REA was translocated into the nucleus of T47D cells, in which BIG3 was expressed at a very low level after treatment with E2. On the other hand, PHB2/REA remained in the cytoplasm even with E2 treatment when BIG3 was exogenously introduced into T47D cells. Moreover, I confirmed that intracellular-localization of PHB/REA was mostly consistent with the cytoplasmic localization of BIG3 protein in breast cancer tissue by immunohistochemistry. These findings suggest that BIG3 interacted with PHB2/REA and interfered with its nuclear translocation in breast cancer cells.

Furthermore, I investigated the subcellular localization of endogenous PHB2/REA in MCF-7 cells in which endogenous BIG3 expression was knocked down using the siRNA oligonucleotides targeting BIG3. The cell population of nuclear translocated-PHB2/REA was significantly increased in si-BIG3-transfected MCF-7 cells with E2 treatment, compared with those in si-BIG3-transfected MCF-7 cells without E2 treatment. To further examine whether BIG3 protein can enhance the ERα transcriptional activity in breast cancer cells, I performed a reporter assay after knocking–down of BIG3 expression in MCF-7 cells. The depletion of BIG3 expression showed the significant decrease of ERα transcriptional activity. These findings suggest that the presence of BIG3 protein is likely to enhance the ERα transcriptional activity through the inhibition of nuclear translocation of PHB2/REA in breast cancer cells.

<C12orf32>

I confirmed up-regulation of *C12orf32* in five out of 11 breast cancer specimens, compared with normal breast ductal cells as well as mammary gland by semi-quantitative RT-PCR. Subsequent northern-blot analyses revealed that two transcripts of *C12orf32* (approximately 1.8-kb and 1.5-kb) in breast cancer cell lines were up-regulated, whereas the expression of both transcripts was hardly detectable in normal human organs except testis, prostate, ovary, thymus and small intestine in concordant with the results of cDNA microarray analysis. According to sequencing analysis of these two transcripts, they share the same open reading frame encoding a 238 amino acids peptide, seemed to correspond to the two bands observed in northern-blot analyses.

To assess a growth-promoting role of *C12orf32* in breast cancer cells, we knocked down the expression of endogenous *C12orf32* in breast cancer cell lines, HBC4 and T47D, which expressed a high-level of *C12orf32*, by means of the mammalian vector-based RNA interference (RNAi) technique. Knock-down of *C12orf32* expression showed significant decrease of cell growth compared with a control si-EGFP-transfected cells. Moreover, I confirmed the results of specificity to its knockdown effects by using mismatched shRNA that contained 4-base replacement in the effective shRNA sequence. Furthermore, I identified an increase in the population of sub-G1 cells in the cells with siRNA-oligonucleotides targeting C12orf32, although no increase of sub-G1 population was observed in those transfected with si-EGFP as a control, indicating that inhibition of C12orf32 expression might induce apoptosis.

To further investigate expression level of endogenous C12orf32 protein in breast cancer cells, I generated a polyclonal antibody to C12orf32 protein (α-C12orf32), and then performed western-blot analysis using cell lysates from seven breast cancer cell lines. Unexpectedly, I observed the smaller size band (16-kDa) in most of breast cancer cell lines examined, although I observed the predicted size of C12orf32 protein (34kDa) in the C12orf32 construct exogenously-expressed-COS7 cells. To investigate whether this smaller band corresponds to the endogenous C12orf32 protein, I knocked down the C12orf32 expression by siRNA in breast cancer cells. I found that the expression of 16-kDa protein was also decreased at protein level as well as transcriptional level, suggesting that this smaller-size protein is corresponding to the endogenous C12orf32 protein in breast cancer cells.

Furthermore, I investigated the subcellular-localization of endogenous C12orf32 protein in breast cancer cells by immunocytochemistry using α-C12orf32. It was observed the cell-cycle-dependent localization of endogenous C12orf32 protein in T47D breast cancer cells. The endogenous C12orf32 was mainly localized in the nucleus of interphase cells, but was observed diffusely from prophase to anaphase. Finally, this protein was concentrated at the contractile ring of cells in telophase. Subsequently, I investigated the effects on each cell-cycle phase by FACS analysis when knocked down of C12orf32

expression in T47D cells by si-C12orf32 after synchronization at the G1/S boundary by aphidicolin treatment. Compared with si-EGFP-treated cells (control), depletion of C12orf32 expression resulted in the increase of cell population of G1 phase, whereas significant reduction of cell population of S phase, suggesting that depletion of C12orf32 might induce the inhibition of G1/S transition. These results suggest that C12orf32 might have an important role to G1/S transition.

In conclusion, the results of my study about *BIG3* and *C12orf32* clearly suggest that *BIG3* and *C12orf32* are specifically and frequently overexpressed in breast cancers, and downregulation of these two molecules by treatment with shRNA significantly suppresses the growth of breast cancer cells, indicating their crucial role in the growth of breast cancer cells. Taken together, these findings should contribute to a better understanding of mammary carcinogenesis, and imply that *BIG3* and *C12orf32* are promising molecular targets for breast cancer treatment.