論文の内容の要旨

論文題目　KLF15 suppresses mammalian cancer cell growth via interaction with PKM2

（KLF15 は PKM2 との相互作用を介して乳がん細胞の増殖を抑制する）

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Introduction

Breast cancer is one of the most common malignant diseases in women and the second leading cause of cancer related mortalities in women worldwide. Based on current incidence rate, a woman has 1 in 8 chance of being diagnosed with breast cancer during the lifetime. According to the expression of estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor-2 (HER-2), and Ki-67, breast cancer is classified into five different subtypes: luminal A-like, Luminal B-like (HER2 negative), Luminal B-like (HER2 positive), HER2-enriched, basal-like (triple negative). Hormonal therapy and targeted therapies are two of major treatments for breast cancer. Approximately 70% of invasive breast cancers are classified as ER and/or PR positive, which can be a predictor that cases will likely benefit from hormonal therapy. However, a large number of patients cannot benefit from hormone therapy because of intrinsic resistance or acquired resistance. Patients with Her2-positive breast cancer would benefit from Her2-targeted therapies, but not from hormonal therapies. Triple-negative breast cancer (TNBC) is believed to originate from mammary stem cells (MSCs) and is associated with poor clinical outcome. At present, it's difficult to develop effective targeted therapies for metastatic TNBC. Further characterization of molecular mechanism in tumorigenesis and cancer progression would provide valuable target factors for developing novel therapeutic strategy for breast cancer patients.

It is becoming clear that not only those hormone receptors but also other cellular components may critically modulate the biological characteristics of breast cancer cells. For example, PKM2 and β-catenin signalings have increasingly been highlighted in breast tumor biology. Enhanced activity of β-catenin signaling links with recurrent or metastatic breast cancer. High expression of PKM2 indicates poor prognosis and high rate of recurrence. Acting as a transcriptional coativator, nuclear PKM2 upregulates β-catenin/c-Myc/polypyrimidine tract-binding protein 1 (PTBP1) signaling, contributing to tumorigenesis and poor prognosis.

KLF15, a member of the Krüppel-like family of nuclear transcription factors, was first identified in 2000, as a repressor of the kidney-specific chloride channel gene CLC-K1. KLF15 has been revealed as a metabolic regulatory factor in liver, muscle and adipose tissues, acting as a central component for coordinating physiologic flux of glucose, amino acids, and lipids. Functions of KLF15 in regulating cell proliferation have been revealed.

KLF15 inhibited mesangial cell proliferation by regulating the expression of cell cycle regulation proteins, including E2F1, cyclin D1 and CDK2. Overexpression of KLF15 reduced proliferation of human airway smooth muscle (HASM) cells. Additionally, KLF15 was identified to inhibit growth and transformation induced by oncogenic V-Ki-ras2 Kirsten rat sarcoma viral oncogene homologue (KRAS) in human pancreas adenocarcinoma (BxPC-3) cells. Moreover, KLF15 showed an anti-proliferative action in estrogen-induced epithelial cells, Ishikawa cells (human endometrial adenocarcinoma cell line with ER and PR expression), and ER-positive T47D breast cancer cells by downregulating MCM2 protein level. Anti-proliferative action of KLF15 in T47D breast cancer cells triggered our interest in the functions of KLF15 in breast cancer, and we aimed to clarify the role of KLF15 in breast cancer cell proliferation and growth by identification of KLF15-interacting factors.

Methods

1. Expression plasmids of KLF15 or PKM2, and short hairpin (sh) RNA against KLF15 or PKM2, were constructed, and stably transfected into cells for comparing the effects of KLF15 and PKM2 in vitro and in vivo;
2. Cells (mouse C2C12 myoblasts and human breast cancer T47D cells) infected with Flag tagged-rat KLF15 expressing adenovirus, or transfected with Flag-tagged human KLF15 expressing plasmid, were used for identifying the novel interacting partners of KLF15 by immunoprecipitation assay, SDS-PAGE/Silver staining, western blotting and mass spectrometry analysis.
3. Western blotting analysis was used for the confirmation of KLF15-PKM2 interaction, and protein expression levels of KLF15- or PKM2- relevant factors.
4. Luciferase reporter gene assay was used for determining β-catenin activity.
5. qRT-PCR was used to detect mRNA expression levels of KLF15 or PKM2 in various cell lines.
6. Cell proliferation assay and wound healing assay were used to determine the effects of KLF15 or PKM2 on cell proliferation and migration in vitro.
7. Flow cytometric analysis was used to detect cell cycle distribution of stably transfected breast cancer cells.
8. Immunocytochemical and immunohistochemical analysis was used to explore the subcellular localization and the association of KLF15 and PKM2 in MCF7, MDA-MB-231 cells and human breast tissues.
9. Xenograft transplantation in NOD-SCID mice was used to investigate the effects of KLF15 or PKM2 on tumor formation and growth in vivo.

Results

Using in-solution digestion and subsequent shot-gun mass spectrometry, 11 molecules were identified as candidates of KLF15 binding proteins. Among others, we focused on PKM2, an important tumor promoter. Interaction between KLF15 and PKM2 was observed in not only T47D but also in other breast cancer cell lines.

We studied the expression of those factors in various cell lines. At mRNA and protein levels, KLF15 showed significant cell-to-cell variation, but PKM2 did less variation. Immunocytochemistry analysis in MCF7 and MDA-MB-231 cells demonstrated that KLF15 is preferentially expressed in the nucleus, but PKM2 is observed in both nucleus and cytoplasm. This observation was also supported by immunohistochemical analysis in human breast carcinoma tissues.

In order to confirm the minimal region crucial for the KLF15-PKM2 interaction, we performed domain deletion analysis. Our study implied the most critical interaction region resides at the N-terminal region (amino acids 1-377) of PKM2, and amino acids 45-351 of KLF15 (the region spanning β-catenin repression domain). Reporter gene assay revealed that KLF15 full-length and domains interacting with PKM2 showed inhibitory effect on β-catenin-dependent gene expression, however KLF15 mutants lacking PKM2 binding did not.

In order to test the effect of KLF15 and PKM2 on breast cancer cell growth, we established MCF7 cell lines that are genetically engineered to express KLF15 and PKM2 at various levels. In stably transfected MCF7 cells, protein levels of PKM2/β-catenin-downstream factors (c-Myc, PTBP1), and cell cycle regulators (MCM2 and CDK2) appeared to be reduced in KLF15-overexpressing and PKM2-knocked down cells. In contrast, those protein levels appeared to be enhanced when KLF15 was knocked down or PKM2 was overexpressed.

Cell cycle distribution of those cell lines was explored. Either Flag-KLF15 or shRNA-PKM2-expressing cells show G0/G1/S phase-dominant distribution with less cell distribution in G2/M phase when compared to either shRNA-KLF15- or Flag-PKM2-expressing cells. As expected, cell proliferation and wound healing rate were decreased in Flag-KLF15- or shRNA-PKM2-expressing cells, but rather increased in shRNA-KLF15- or Flag-PKM2-expressing cells compared to host MCF7 cells. We transplanted those cells into NOD-SCID mice. Tumor volume and mass appeared to be positively modulated by PKM2 but negatively by KLF15.

Finally, we performed immunohistochemical analysis of KLF15 and PKM2 in pathological specimens obtained from patients with breast carcinoma. Although, at this moment, we studied only 12 patient samples, a negative association between the expression of KLF15 and PKM2 can be observed in those cases.

Discussion

In the present study, we focused on KLF15 as a candidate of such factors that show influence on breast cancer tumorigenesis and maintenance. We found that KLF15 interacts with an identified tumor promoter PKM2. Our study provided evidence that KLF15 downregulated β-catenin/c-Myc/PTBP1 signaling levels, suppressed MCF7 breast cancer cell growth in vitro and in vivo. These observations indicate a potential role of KLF15-PKM2 interaction in regulating cancer cell proliferation.

However, our results also raise an important issue that KLF15 and PKM2 exhibited mutually exclusive expression in human breast carcinoma. In terms of expression levels of KLF15 and PKM2, breast cancer might be divided into two categories: KLF15-high/PKM2-low tumors might not require PKM2-mediated process for tumorigenesis; KLF15-low/PKM2-high tumors might dependent on PKM2-mediated cellular machinery.

Despite its widespread known role as a tumor promoter, exceptions about the role of PKM2 in tumor are also raised. It has been suggested that PKM2 is not essential for the growth of some tumors. Analysis of large-scale human tissue proteome via " The Human Protein Atlas" revealed PKM2 exhibits uneven expression in human breast carcinoma, even though high RNA level has been identified in MCF7 breast cancer cells. So far, multiple transcription variants of human PKM2 have been reported. Furthermore, previous investigation demonstrated the existence of loss-of function mutation as well as lower enzyme activity mutation of PKM2 in breast cancers. We, therefore, think that further characterization of the biological significance of the KLF15-PKM2 interaction would contribute to clarifying the role of KLF15 and PKM2, and the development of a novel therapeutic approach in breast cancer.

Previous investigation suggested the role of ER in the function of KLF15. But, in the present study, we did not find the correlation between expression of ER and KLF15-PKM2 interaction, in immunoprecipitation experiments, KLF15 co-precipitated PKM2 irrespective of whether the presence of ER in detected breast cancer cell lines.

Of course, we cannot rule out such possibility that KLF15 acts as a tumor suppressor in PKM2-independent fashion. Indeed, KLF15-overexpressing MCF7 cells exhibited downregulation in protein levels of factors that link with decreased cell population in G2/M phase and cell proliferation rate, such as MCM2 and CDK2. KLF15 also inhibited growth and transformation of human BxPC-3 cells via suppressing the effect of oncogenic KRAS. Moreover, KLF15 showed the inhibitory effect on NF-κB signal. These findings implied that the potential anti-proliferative effect of KLF15 might be mediated by diverse ways, which may not limit to via interaction with PKM2.

Conclusion

KLF15 might be a potential breast tumor suppressor. The functions of KLF15 in tumor biology may partly mediate by, but not limits to, the interaction with PKM2 and inhibition of PKM2/β-catenin/c-Myc/PTBP1 feedback loop signaling network.