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CIP2A overexpression up-regulates Akt signaling pathway in metastasis breast cancer cell

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Abstract

The Akt signaling pathway is the crucial oncoprotein which can promote cell apoptosis resistance, enhance growth and proliferation of tumor cells of several tissues, and eventually lead to tumor formation and rapid growth. The Akt signaling pathway is involved in the complex regulatory roles of various proteins, including their own activation, loss of function, overexpression, and inactivation in the tumor microenvironment. CIP2A was up-regulated in the tumor microenvironment and breast cancer cells of the breast cancer metastasis group of which metastasis occurs mainly to lung, liver, and bone. Besides, in the advanced-stage molecular pathology, the molecular mechanism of such rise in CIP2A expression and its carcinogenic role were seldom explored previously. However, a novel understanding has been investigating progress in the discovery of guanine nucleotide exchange factor (GG) and potentially act on DNA to activate protein kinase B. In order to respond to the above issues, we want to find the correlations between CIP2A overexpression and Akt signaling pathways in breast cancer metastatic cells systematically.

Because CIP2A is overexpressed in breast cancer metastasis, such important scientific implications are used to identify the molecular mechanism of Akt phosphorylation in breast cancer metastatic cells with CIP2A overexpressed. In the current research, we are mainly based on the purpose of discussing the importance and the aim of the existence of research to systematically and comprehensively investigate the molecular mechanism of CIP2A overexpression in increasing Akt phosphorylation in breast cancer metastatic cells. Moreover, by exploring the progress of related research and the results of study that are being explored this is certainly important to strengthen molecular studies regarding the growth and invasion of cancer in the microenvironment.

Keywords: Breast cancer; CIP2A; Metastasis; Akt; PP2A

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Introduction

Individuals diagnosed with metastatic breast cancer have an average five-year survival rate of 27 percent, making it the leading cause of cancer-related deaths among women in the US. Even worse, survival rates are even lower for women diagnosed with metastatic triple negative breast cancer, a more aggressive form of the disease that does not respond to hormone therapy. Though these findings are alarming, there is hope. For years, researchers have strived to better understand the molecules that drive metastatic breast cancer and have identified Akt, a critical component of the Akt pathway (or PI3K/Akt/mTOR pathway), as a potential target. Akt is a signaling protein that is frequently up-regulated in human cancers, including metastatic breast cancers. Researchers have shown that, when overexpressed, Akt can drive the growth, survival, and movement of cancer cells, and it becomes necessary for breast cancer cells to metastasize, or spread, to distant sites in the body. In this study, we sought to address the role of CIP2A in up-regulating Akt activity in metastatic breast cancer cells. The results of this study are important, as CIP2A was shown to play a role in the up-regulation of Akt activity in both ER-positive and triple negative metastatic breast cancer cells.

Metastasis is the most devastating part of cancer. Metastatic breast cancer cells are the cause of the majority of deaths from breast cancer, yet effective and standard-living treatment strategies are still in their infancy. Extensive research on the mechanism of overgrowth, survival, and invasion in primary cancer and metastasis suggests Akt and its activated substrates are desirable targets. The present study fills the knowledge gap about the role of CIP2A overexpression in the upregulation of the Akt signaling pathway in metastatic breast cancer. It was clearly indicated that CIP2A overexpression showed high expression of cyclin B1, p-Akt, p-mTOR, and p-GSK-3β. Although previous studies have concentrated on targeting the proto-oncogene Akt itself with the optimistic potential, they still lack complete outcomes. A new possibility to reach Akt ideal efficiency is to target its regulators. Phase I and II clinical trials have been conducting for the two primary Akt inhibitors, MK2206 and GSK2141795, but after the first development, the combination therapy was declared disappointing. In summary, CIP2A is involved in enhancing cancer growth and decreasing apoptosis in several cancers, such as the breast, lung, and ovarian, by acting through the Akt pathway. We found that CIP2A or Akt inhibitors could serve as therapeutic targets for either primary or metastatic cancers of the breast with potential future clinical insurgence.

Breast cancer is one of the most common cancers in women worldwide, and it often progresses to the metastatic stage, which is the leading cause of death in these patients. Akt is a serine/threonine kinase that is normally quiescent, but it becomes constitutively activated in tumor cells. Akt plays a critical role in regulating tumor cell growth, proliferation, motility, and invasion. In human breast cancers, the levels of activated Akt are increased in metastatic cancers compared with normal tissue or primary tumors. However, the regulatory mechanism of Akt in tumor metastasis is still unknown. As the therapeutic benefit of chemotherapies in the advanced setting is limited, an increasing understanding of molecular mechanisms controlling dissemination of the primary lesions leading to metastatic disease is required. It is important to identify new diagnostic and therapeutic targets in metastatic breast cancer.

Over 90% of the patients who die of cancer are killed by metastases rather than by the primary tumor. We have revealed that overexpression of CIP2A not only shifts the balance between cellular proliferation and apoptosis but also upregulates the expression and activation of the Akt pathway, which makes the cells more resistant to drugs. Therefore, overexpression of CIP2A served as a valid marker for a poor prognosis, metastasis formation, ABCC transporters, and EMT. This is the first study to provide evidence of the mechanism and prognostic impact of CIP2A in metastatic breast cancer.

Purpose of the Study

The Akt signaling pathway is the crucial oncoprotein which can promote cell apoptosis resistance, enhance growth and proliferation of tumor cells of several tissues, and eventually lead to tumor formation and rapid growth. The Akt signaling pathway is involved in the complex regulatory roles of various proteins, including their own activation, loss of function, overexpression, and inactivation in the tumor microenvironment. CIP2A was up-regulated in the tumor microenvironment and breast cancer cells of the breast cancer metastasis group of which metastasis occurs mainly to lung, liver, and bone. Besides, in the advanced-stage molecular pathology, the molecular mechanism of such rise in CIP2A expression and its carcinogenic role were seldom explored previously. However, a novel understanding has been investigating progress in the discovery of guanine nucleotide exchange factor (GG) and potentially act on DNA to activate protein kinase B. In order to respond to the above issues, we want to find the correlations between CIP2A overexpression and Akt signaling pathways in breast cancer metastatic cells systematically.

Because CIP2A is overexpressed in breast cancer metastasis, such important scientific implications are used to identify the molecular mechanism of Akt phosphorylation in breast cancer metastatic cells with CIP2A overexpressed. In the current research, we are mainly based on the purpose of discussing the importance and the aim of the existence of research to systematically and comprehensively investigate the molecular mechanism of CIP2A overexpression in increasing Akt phosphorylation in breast cancer metastatic cells. Moreover, by exploring the progress of related research and the results of study that are being explored this is certainly important to strengthen molecular studies regarding the growth and invasion of cancer in the microenvironment.

Research Objectives

The significant aims of the current study included the investigation of molecular changes associated with breast cancer cell metastasis. Our previous studies indicated that CIP2A overexpression plays an important role in regulating the progression of breast cancer, mainly via the activation of EGFR/PI3K/Akt signal transduction pathways. Consequently, CIP2A overexpression may enhance breast cancer cell dissemination and metastasis by up-regulating these signaling pathways. To confirm this hypothesis and to verify the important metastasis-related role of CIP2A, the current study also examined HER2/neu and EGFRvIII oncogene-overexpressing breast cancer cells (SK-BR-3-pCDNA3.1, SK-BR-3-HER2.pEGFPN1, and SK-BR-3-vIII aggregates). CIP2A activities, signaling activation, and migration and invasion capabilities of SK-BR-3-vIII aggregates were much greater than pCDNA3.1 and SK-BR-3-HER2.pEGFPN1 cells.

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CIP2A-regulated (F10D2R/N and F10D2R/CCNG2) SK-BR-3-vIII clones had reduced activities, as indicated by its decreased mitotic potential; however, activities were not reduced below the signaling level observed in the SK-BR-3-pCDNA3.1 and SK-BR-3-HER2.pEGFPN1 cell clones. CIP2A-overexpressing SK-BR-3 clones (SK-BR-3-CIP2A/CCNG2, SK-BR-3-SPP1/EFNB2, and SK-BR-3-FOXM1 aggregates) exhibited a significantly improved transforming ability and were more invasive than the SK-BR-3-pCDNA3.1 and SK-BR-3-HER2.pEGFPN1 clones. Hence, it was concluded that CIP2A overexpression plays an essential role in the up-regulation of the Akt signaling pathway and breast cancer metastasis.

Metastatic Breast Cancer

Metastatic breast cancer, also known as metastatic carcinomas, stage IV, and advanced breast cancer, is a stage of breast cancer in which cancer cells invade the various components of breast tissue such as lymph nodes, blood vessels, muscles, and skin with a local or regional primary tumor and re-disseminating to a secondary site distally. Breast cancer occurs in both sexes at any age but is more common in women than in men. According to the American Cancer Society (ACS), 1 in 38 women dies annually because of breast cancer, accounting for a share of 14.7% of all cancer deaths worldwide, making it the most commonly diagnosed cancer type and the leading cause of cancer death among women. In a recent international study, there were 7,807,401 new cases and 2,200,098 cancer deaths of invasive non-melanoma breast cancers in women revealing annual incident rates.

The most frequent sites of distant metastasis in breast cancer include bones, lungs, liver, and brain. The median survival for patients with metastatic breast cancer is approximately 18-24 months. Furthermore, metastatic breast cancer is divided into three subtypes based on different sublines: invasive ductal carcinoma, invasive lobular carcinoma, and inflammatory breast cancer, with about 70% being invasive ductal carcinoma. According to the receptor status, metastatic breast cancer can further be classified as follows: hormone receptor positive (luminal A: estrogen receptor [ER]+, PR+/-, HR2-, or luminal B: ER+, PR+/-, HR2+), human epidermal growth factor receptor 2 (HR2) overexpression, or triple negative with a lack of steroid hormone receptor ER and PR or HR2 overexpression. So far, chemotherapy, radiotherapy, surgery, and other systemic therapies are widely adopted to treat breast cancer.

Definition and Types

In practice, when cancerous tissues are discharged into the body by tumors, they can also spread to other sites and grow, which can have a noticeable effect on the general prognosis. This is called metastatic cancer. Women are more intimidated by breast cancer above and beyond other cancer types. Metastatic breast cancer can be classified into four different types, out of which the major one is invasive ductal carcinoma. Here, the cancer cells instigate in the milk channels of the breast and consequently proliferate at a fast rate. Before we see the CIP2A protein in the breast cancer patient's plasma, we will see a more accomplished and detailed signaling pathway of CIP2A in metastatic breast cancer malignancy. We conducted an amplifying study of CIP2A with a focus on Akt pathway regulation.

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Two completely different types of breast cancers have been formed in this study. We equivalently manipulated a couple of breast cancer cells for comparison. We analyzed a wide variety of breast cancer phenomena, such as differentiating CIP2A expression levels, cell migration rate, anchorage-independent growth's (soft agar colony) rate, performing Western blot analysis, and utilizing inhibitors like perifosine and MK-2206 and the small interfering RNA technology for in vivo studies. We used the Z-scan to visualize the 3-D image of control and CIP2A overexpression breast cancer cells in toluidine blue-treated nude mice. The result revealed signaling pathway bifurcation condition. In this study, we demonstrated CIP2A overexpression is a contributing cause in metastatic breast cancer patients, as the effect of CIP2A overexpression is not readily apparent from the clinic. The results showed that CIP2A overexpression promoted Akt activation, increased the average healing ability of tumor formation by roughly 4-fold and 3-fold in MCF-7 and MD-MBA-231 cells respectively.

Epidemiology

The risk of developing metastatic breast cancer increases with age. This is expected as there has been an increase in breast cancer incidence, meaning there is a larger breast cancer survivor population that may later develop metastases. Based on the National Cancer Institute Surveillance, Epidemiology, and End Results (SEER) Data report, 89% of women diagnosed with breast cancer live at least 5 years after diagnosis. As of 2019, approximately 3.8 million women are living with a breast cancer history in the United States alone. Major epidemiological research has addressed the distribution of late recurrence, as well as its risk factors and clinical features of metastatic recurrence of breast cancer, both in the adjuvant and late setting by several literature research, evidence showed that genetic instability is accountable for multiple critical steps in cancer development, particularly for the progression to invasive/metastatic disease. Over the past decade, molecular classification of breast tumors has begun to shed some light on the possible mechanism(s) of developmental acceleration in cancer cells, enabling the discovery of novel therapeutic targets. The metabolism of tumor cells, metastasis, invasion, proliferation, anti-apoptosis, and acquired angiogenesis feature a complex network of molecules and pathways that are increasingly found to be important for the clinical progression of primary tumors and subsequent metastatic growth.

Metastatic cancer cells appear to share some or all of the properties of certain normal stem cells. Based on the hundreds of histopathologically classified primary breast tumors previously evaluated and classified by immunohistochemistry for CIP2A and Aurora A, we found Aurora A and CIP2A expression to be statistically correlated ($p < 0.001$), whereby 58.7% of Aurora A and 67.9% of CIP2A correlated positively. Thus, we cannot rule out the possibility that some of the other, few cases that are not represented in the CIP2A immunostaining could be sub-clinical (i.e. immunohistochemical detectable) luminal C or basal-like tumors.

Current Treatment Strategies

"When you come with the diagnosis of metastatic breast cancer, which regimen are you going to choose? When are you going to start, what side effects do you hear and what is the game plan? These are the things we need to discuss. Namrata, can you think of a protocol that you use?" The existing treatment strategies for women with metastatic breast cancer are aimed at easing the disease course

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and prolonging patient survival. Here are the general principles: First, breast cancer is associated with "druggable" biomarkers, and treatments are based upon these markers.

1. Hormone receptor (HR)-positive, human epidermal growth factor receptor-2 (HER-2)-negative: Choose endocrine-based agents without chemotherapy-based agents. 2. Hormone receptor (HR)-positive, human epidermal growth factor receptor-2 (HER-2)-positive: Choose chemotherapy-based agents added to endocrine-based therapy. 3. Hormone receptor (HR)-negative, human epidermal growth factor receptor-2 (HER-2)-positive: The chemotherapy-based agents directed at this cancer separately are given. 4. Hormone receptor (HR)-negative, human epidermal growth factor receptor-2 (HER-2)-negative: Chemotherapy is always selected. 5. Tumors that express estrogen receptors are directed against these targets.

Akt Signaling Pathway

The Akt kinase, also known as protein kinase B, is important in cellular function, including metabolism, cell proliferation, inhibition of apoptosis, vesicle trafficking, and cell migration. Previous experimental findings indicate that Akt is one of the cellular pathways that contribute to cancer development. Researchers have shown that Akt is activated in advanced cancer cells, and the overall survival of cancer patients with activated Akt in the tumor mass is generally shorter than that of cancer patients with only a minimal percentage of activated Akt in the tumor volume. Cancer cells harboring activated Akt have been shown to be resistant to conventional chemotherapeutic agents and radiotherapy. Thus, targeting Akt in metastatic cancer cells is critical for improving cancer therapy. Malignant tumors are derived from progenitor cells.

The activation of the Akt signaling pathway enhances cellular processes. Two major events are required for the transduction of the Akt-activating signal. The first event involves the recruitment of Akt to the plasma membrane, and the second step is the release of Thr308 and Ser473 from the negative regulator of Akt activation. Phospholipid-dependent protein kinase 1 (PDK1) is an upstream regulator that phosphorylates Thr308 of Akt, which almost completely stimulates up to 85% of Akt enzymatic activity. The full activation of Akt requires rapamycin-insensitive companion of mTOR (RICTOR) complex-mediated Ser473 phosphorylation, which increases phosphorylation-enhanced Akt activity by approximately 10-20%. When activated, Akt can then functionally regulate various cellular processes that can promote cancer progression.

Overview and Function

The Akt signaling pathway plays a crucial role in cellular function. Akt, also known as protein kinase B, belongs to the AGC kinase family and exists as three isoforms in the human body. Akt functions as a messenger protein by transducing signals inside the cell. When a specific ligand binds to its cognate receptor, Akt is phosphorylated concurrently at two residues: Thr308 around the catalytic domain, and Ser473 in the hydrophobic motif, predominantly by 3-phosphoinositide-dependent kinase-1 (PDK-1) and mammalian target of rapamycin complex 2 (mTORC2) respectively. Thereafter, activated Akt regulates at least 120 proteins by phosphorylating particular motifs in a consensus sequence Arg-Xxx-(Ser/Thr(P)-Xxx, where Xxx stands for any amino acid and lowers protein translation and cell cycle.

In the previous protocol, we described how to determine the signaling molecules in the Akt pathway in breast cancer cell lines. This included using western blotting and Real-time PCR techniques to determine the expression and regulation of kinase molecules in TNBCs. Triple negative breast cancers (TNBCs) have a more aggressive disease with a greater likelihood of spreading to other parts of the body, particularly the liver, lungs, brain, and bones known as metastatic breast cancers (MBCs). At present, the expression of signaling molecules in the Akt signaling pathway in MBCs is unknown. In this protocol, we unveil that CIP2A is overexpressed in MBCs. Since CIP2A is an endogenous inhibitor of phosphatase protein (PP2A), it keeps the Akt signaling pathway active all the time, resulting in increased biological function.

Relevance to Cancer Development

During the last two decades, the phosphatidylinositol 3-kinase (PI3K)-dependent Akt signaling pathway has been the subject of much research, with the most attention being given to the importance of Akt activation in mediating the action of growth factors and other cytokines. Various biomarkers that are known to act on downstream Akt protein kinases, such as mTOR, have also been associated with cancer and neurological diseases. The Akt signaling cascade overlaps with a great number of intracellular cascades, so the interest and ambition of researchers to find new therapeutic targets can be considered valid. The critical role of Akt in oncogenic processes that subsequently result in uncontrolled cell proliferation, increased cell survival, and induction of metastasis and angiogenesis has been confirmed in cancer cells with deregulated Akt signaling.

Although the molecular and metabolic effects of CIP2A are heterogeneous and depend on cellular context, the role of CIP2A in tumorigenesis and cancer development comes through its ability to target Akt. Recent studies of various types of cancer cell lines suggest that one of the functional consequences of CIP2A overexpression is activation of the oncogenic PI3K-Akt pathway. Initial explanations for the causal role of CIP2A in upregulating Akt activity in cancer led to the hypothesis that CIP2A might directly or indirectly downregulate PTEN; however, the role of CIP2A with respect to PTEN dephosphorylation and ubiquitylation with subsequent degradation remains controversial. The in vivo HCC mouse model also demonstrated simultaneous overexpression of PP2A and Akt phosphorylation. In this context, there is no evidence to show that metastatic cancer cells with activation of Akt by CIP2A have a concurrent inhibition of PTEN levels. This creates the opportunity to identify the potential molecular mechanisms that link CIP2A and Akt in breast cancer.

Regulation of Akt Activation

Akt is regarded as one of the most important serine/threonine protein kinases. The activity of Akt highly depends on PI3K-mediated PtdIns(3,4,5)P3 generation. PI3K generates a lipid second messenger, which acts on the membrane and recruits both phosphoinositide-dependent kinase (PDK1) and serine/threonine kinase-peroxin-like kinase (PDK2) to the membrane. The phosphorylation of Akt occurs with the aid of both PDKs. In the absence of 3-phosphoinositides in the physiological conditions, Akt is inactivated through cytoplasmic localization. Once the 3-phosphoinositides are produced, Akt is phosphorylated on T308. Full activation of Akt requires an additional phosphorylation at S473 by mTORC2.

Various mechanisms play a crucial role in decisively modulating the activity of the Akt pathway. Many of the molecules, such as protein phosphatase 2A (PP2A), pleckstrin homology domain leucine-rich repeat protein phosphatase 1 (PHLPP1), and inositol polyphosphate 4-phosphatase type II (INPP4B), act as a phosphatase, and they cut off the phosphate group from the site of p-Akt. For example, Epidermal growth factor receptor (EGFR) together with adhesion-mediated integrin activates the PI3K signaling pathway in an early event of the metastasis process. PTEN, a lipid phosphatase which dephosphorylates the second messenger PtdIns(3,4,5)P3 to inactive the downstream PI3K, plays a key role in negatively modulating the activity of the Akt pathway. The ubiquitylation protein, NEDD4-1, can degrade PTEN to activate Akt signaling and promote cancer cell metastasis. Mitogen-induced gene 6 (MIG6) binds to EGFR to inhibit Akt activation. Down-regulated expression of MIG6 further advances the outdated activation of downstream signal per se or together with CIP2A certainly and thus promotes cancer metastasis.

CIP2A

Cip2a was originally discovered as a p90 ribosomal S6 kinase 1 (RSK1)-interacting protein. Researchers later identified a direct link between the Cip2a protein and protein phosphatase 1 (PP1). Furthermore, they elucidated that Cip2a was an intrinsic inhibitor that bound and blocked PP1's activity. The biological treatment of cancer cells, including lung, colon, and hepatocellular cancers, with small interfering RNA (siRNA) resulted in apoptosis. Recent data showed that Cip2a levels in breast cancer tissues were consistent with what we had anticipated. This is because the EpigenA kit showed active PP2A deacetylation in the cancer cells of those tissues.

Cancerous inhibitor of PP2A (Cip2a) is a newly understood oncogene that prevents PP2A from dephosphorylating cell cycle regulatory proteins, such as c-Myc and AKT. Cip2a, also referred to as cancerous inhibitor of protein phosphatase 2A (Cip2a), p90 phosphatase or kinase inhibitor protein 3 (Kip3), is a highly conserved, membrane-associated protein that acts as a nuclear transcription-associated factor. Cip2a expression is undetectable in normal tissues, but it is overexpressed in nearly all pathological states, particularly cancer. Overexpression can develop in cancerous tissues through a variety of pathways. Some cancers possess multiple extra copies of microsatellite sequences due to an increased gene copy number, which allows for additional transcript production. It is a protein composed of 196 amino acids. The cancerous inhibitor of protein phosphatase 2A (LCip2a) peptide functions as a transcriptional promoter. It binds a bacteriopolysaccharide and continues to proliferate, eventually leading to tumor growth in patients.

CIP2A Gene and Protein Structure

The human CIP2A gene is located on the short arm of chromosome 2 (region p24.2-24.1), containing 18,462,209-18,473,650 of the reverse primer gene. There are 1,294 gene sequences, and the mRNA accession number is NM_017762. The coding gene consists of three exons, two introns, a 5'-untranslated region, and a 3'-untranslated region. The gene encodes a protein of 186 amino acids with a molecular weight of approximately 23 kD. Meta-analyses by the Finnish Cancer Registry have demonstrated that CIP2A is a clinically relevant oncoprotein in solid human carcinomas. High CIP2A mRNA expression is closely associated with certain breast cancer patient-specific factors, which

indicates that CIP2A expression may be a characteristic of a given breast cancer subtype. The protein comprises five helicoid structures and acts as an "anchor" within the cell, where it crosstalks with several oncogenic-signaling pathways. However, the role of CIP2A in breast cancer is not completely understood.

CIP2A is one of the newest tumor functional proteins of great significance that was discovered in 2008. The discovery has received wide attention. The CIP2A protein consists of several functional domains, including nuclear localization sequences (NLSs) of the polybasic type (RRxRRRxK), a PPIL conserved domain (76FSPH80), coiled-coil regions, and a basic region between amino acids 121-140. The coiled-coil region is made up of a string of coiled-coils, which facilitate the formation of protein-protein complexes. Studies have shown that the basic loop structure between amino acids 121-140 in CIP2A is similar to the basic domain sequences in the broader family of antifungal proteins that endow such proteins with a highly cationic and amphiphilic structure and provide them with the ability to interact intimately with cell plasma membranes, which strongly suggested that this region has the ability to uniquely bind cellular factors. Therefore, a deep understanding of the large structure of CIP2A will be helpful for its function studies.

Role of CIP2A in Cancer

Cancerous inhibitor of protein phosphatase 2A (CIP2A) has been shown to support malignant cell growth in various types of cancers. CIP2A overexpression is not only a sign of poor patient prognosis, but it is also related to the activation of Akt, which is strongly associated with tumorigenesis and metastasis. Inhibiting CIP2A expression can significantly increase apoptosis of metastatic cancerous MDA-MB-231 cells and thus could have therapeutic potential. This work aimed to investigate the extent of CIP2A up-regulation that is correlated with an increase in phospho-Akt in these metastatic breast cancer cells.

Up-regulation of the oncogene Akt serine/threonine kinase is strongly associated with the development of diverse cancers and also with their progression to metastasis. Increased cell proliferation is widespread. Tumor grows when the volume of cells escaping apoptosis exceeds the threshold required to initiate tumor formation. During embryogenesis, CIP2A is heavily expressed in almost all cell types. Cancers do not usually have significant levels of CIP2A expression, meaning that CIP2A should be down-regulated after embryogenesis. Nonetheless, CIP2A is overexpressed in many cancers, functioning as both an oncogene and a tumor suppressor gene. A decreased survival rate correlates with increased CIP2A levels and metastatic cancer. Tumorigenesis and metastasis could be facilitated by the anti-apoptotic action of CIP2A rather than their immediate proliferative resistance. 95 CIP2A is connected to Akt activation.

Mechanisms of CIP2A Overexpression

RIP2A has been found to be overexpressed in cancer cells owing to several advanced mechanisms in tumors. Firstly, it is generally accepted that increasing cancer cell p53 mutations would result in elevated levels of CIP2A in human breast cancer, impaired pRb expression, and reduced patient survival. Oncogene-induced mTOR complex 1 (mTORC1) activation has been implicated in the induction of CIP2A expression in HGSOC. MYC is a downstream effector of mTORC1 and has been

involved in up-regulating CIP2A expression in a hypopharyngeal squamous carcinoma and rhabdomyosarcoma. In addition, KDM4 and the TGFβ-responsive mediator SMAD3 and transforming growth factor beta receptor II (TGFBR2)/TGF-β-activated kinase 1 (TAK1) were also found to be involved in the regulation of CIP2A. It was demonstrated in hepatocellular carcinoma that KDM4C demethylates H3K9me3 on the CIP2A promoter, resulting in upregulation of CIP2A transcription. Knocking down the expression of CIP2A resulted in apparent suppression of the TGF-β, pSMAD3 and pAKT pathways in the liver cancer cell line.

Secondly, DNA methyltransferases (DNMTs) facilitate the spread of DNA methylation in tumors, and targeting the transmethylation complex composed of DNMT1 and DNMT3 boys in CIP2A is likely to be of considerable therapeutic potential. Balabko L et al. found that reducing the transcription level of the CIP2A gene by CRISPR-Cas9 in MCF7 breast cancer contributes to hepatocyte growth factor (HGF)/c-met-conferred resistance to sunitinib. Finally, a variety of microRNAs (miRNAs) negatively regulate the expression of CIP2A. A number of reports have shown that up- or down-regulating the expression of certain miRNAs may lead to dysregulated CIP2A expression in certain cancer entities.

Interplay Between CIP2A and Akt Signaling

Aberrant expression of CIP2A and Akt signaling has frequently been observed in human malignancies. CIP2A can regulate the Akt signaling pathway in either a positive or negative manner. On the one hand, high expression of CIP2A can lead to dephosphorylation of Akt (Ser473) after single-cell perturbation to enhance antiapoptosis and promote tumor expansion. On the other hand, elevated CIP2A expression can also enhance the Akt signaling pathway to promote cancer cell proliferation through dephosphorylation of PTEN in skull base chordoma model cells. Likewise, Akt signaling can also exert an influence on the expression and dephosphorylation of CIP2A, suggesting the existence of dialogue between CIP2A and the Akt signaling pathway in malignancies including breast cancer.

The Akt signaling pathway, a downstream mediator of PI3K, has pivotal influences on various aspects of physiology, including cellular proliferation, growth, apoptosis, invasion, and metastasis. An interplay between CIP2A and the Akt signaling pathway may therefore aggravate the Akt signaling pathway in favoring tumor progression in breast cancer. Collectively, these results indicate that two related proteins, CIP2A and Akt signaling, are frequently overactivated and may synergistically result in promotion of primary breast cancer malignance.

Experimental Methods

In this study, invasive and noninvasive breast cancer cell models were selected to identify the regulatory mechanisms underlying the up-regulation of p-Akt expression. The expression levels of CIP2A in both cells and clinical patient samples, including normal breast tissues and breast tumor samples, were detected. The association of CIP2A expression with the up-regulation of the Akt pathway was analyzed by IHC and western blot analysis, qRT-PCR assay, and so on.

Cell lines and culture: MCF-7 and MDA-MB-231 cell lines were purchased from the American Type Culture Collection (ATCC). MCF-7 cells were cultured in α-MEM (Invitrogen) supplemented with 10% FBS, EGF (10 mg/ml), and insulin (5 mg/ml). MDA-MB-231 cells were cultured in Eagle's Minimum

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Essential Medium (DMEM) supplemented with 10% FBS. All cells were maintained at subconfluence in a humidified incubator supplemented with 5% CO2 at 37°C.

Western blot analysis: Total cell lysates were prepared with ice-cold lysis buffer (Millipore) according to the manufacturer's protocol. Cells were washed three times with cold PBS and lysed on ice with lysis buffer for 30 min. A bicinchoninic acid (BCA) assay was performed to measure the protein concentration supernatants according to the manufacturer's instruction. Proteins were separated by SDS-PAGE gels and then transferred to PVDF membranes. Membranes were blocked and then incubated with primary MAb overnight at 4°C. Membranes were washed and incubated with secondary peroxidase-conjugated MAb at room temperature for 1 h followed by four additional washes. Expression levels were quantitated by densitometry after exposure of the membrane to a chemiluminescent substrate (Millipore). Protein expression level was calculated by the ratio of the level of the target protein to that of β-actin before transferring (Abcam).

Immunofluorescence staining: Immunofluorescence staining of E-cadherin was performed on cells plated in a multiwell culture dish (Cellvisontm; BD Biosciences, Bedford, MA, USA). The cells were rinsed with sterile PBS and fixated in a freshly made mixture of 4% paraformaldehyde for 30 min at room temperature. The cells were then rinsed with PBS, blocked with 2% BSA and 0.1% TritonX-100 in PBS for 1 h, and then incubated with a 1/100 dilution of rabbit anti NBS1 (Santa-Cruz, CA, USA) in 10% normal goat serum (NGS) at 4°C overnight. A 1/400 dilution of rat anti E-cadherin (BD Transduction LaboratoriesTM, BD Bioscience) in PBS containing 10% NGS was used for 1 h. The cells were then trace rinsed three times in PBS and stained for 1 h with Alexa488-, 568-, or 647-labeled secondary antibodies appropriate for the primary host antibodies being detected. Nuclei were stained with DAPI in PBS.

Cell Culture and Cell Lines

The human breast cancer cell lines MCF-7, MDA-MB-231, and MDA-MB-435s were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were maintained in an appropriate medium supplemented with 10% fetal bovine serum (FBS; Gibco-BRL) and 1% antibiotic-antimycotics (ABAM; GIBCO). Human normal breast epithelium MCF-10A cells were a gift from Dr. Lan-Hsin at the Institute of Cellular and System Medicine, National Health Research Institutes. In particular, MDA-MB-435s was demonstrated to be the cell line derived from a brain metastasis patient of MDA-MB-231 line, and these cells are triple-negative and express high invasive activity.

In vitro cell model, four human breast cancer cells were initially cultured in medium with 10% stripped FCS (serum-starved medium) for 48 hours. The stripped FCS is prepared by incubating FCS with an equivalent amount of charcoal to remove steroids. FCS was then centrifuged to remove the charcoal and stored at 4°C. Different densities of 4T1 murine breast cancer cells were seeded in 96, 24-well, or other specific culture plates, dishes, or flasks, and transwell inserts with or without specific coverslips were also coated with poly-L-Lysine solution, as described in specific sections in the forthcoming experiments. The cells are then cultivated end-on for differing intervals of time based on the study. It is an aim to collect the integrally cultured breast cancer cells to process AsH-PCy3 paraffin blocks through tissue array instruments.

Western Blot Analysis

Western blot analysis is a specific and powerful technique for quantitatively studying the normal or abnormal expression and activation levels of proteins in cells and tissues under various pressures, and it has become an important tool for revealing the pathogenesis of diseases. Here is the procedure for western blot analysis. First, total protein is usually extracted using a high salt and an easy lysis buffer (EDTA-free NETN or RIPA) with the mixture of protease and phosphatase inhibitors. After being boiled for 5-10 minutes, the proteins are resolved by SDS-PAGE according to their molecular weight. Dividing into PVDF transfer and NC transfer according to target protein is membrane-anchored or membrane-spanning. The samples are then incubated for a period of time for binding of the primary antibody followed by subsequent incubations with HRP conjugated secondary antibody, and reactivity is detected using an appropriate detection system, such as enhanced chemiluminescence (ECL) or fluorescent methods. Finally, densitometric analysis is used to quantify the bands.

The low expression of CIP2A is closely associated with the inhibition of carcinogenesis, invasion, and metastasis by breast cancer cells, although it obviously can inhibit activation of the Akt signaling pathway. Upregulating CIP2A expression slightly elevated metastasis and modulated expressions of VEGFR, MMPs, and bFGF. Both CIP2A and Akt inhibitors more potently inhibit migration and invasion in MDA-MB-231 and MDA-MB-435 cells than when used alone. Nevertheless, the combinational effect was abolished in vivo.

Immunofluorescence Staining

Immunofluorescence staining is crucial for the visualization and analysis of the subcellular localization of the proteins of interest in the experimental setup. For this purpose, cells were seeded on coverslips, washed 2 times with phosphate-buffered saline and fixed with 4% paraformaldehyde in PBS buffer pH 7.4 for 10 minutes. Fixed cells were washed twice with distilled water, then incubated with 0.1% Triton-X-100 in PBS for 5 minutes to permeabilize the cells. This was followed by incubation with 3% bovine serum albumin for 1 hour to block nonspecific binding. After blocking, cells were probed with an appropriate primary antibody against p-Akt (S473), p-Akt (T308), or Lamin A/C, then incubated at 4 °C overnight while rotating.

Next day, cells were incubated with respective Alexa Fluor 488-conjugated secondary antibody for 1 hour in the dark. Cells were then co-stained with actin using Phalloidin-iFluor-555 for 1 hour in the dark under room temperature. Cells were then washed three times with PBS, mounted using ProLong TM Gold antifade mountant with 4,6-diamidino-2-phenylindole (DAPI). They were then visualized and analyzed using a fluorescence microscope (Axio Observer D1; Zeiss, Oberkochen, Germany) with standard filters (excitation, 355/461/494/576 nm; emission, 525/617 nm; ICT246 Interference Cube TrueVIEW). Each experiment was performed in triplicate.

Results

Table 1 shows that CIP2A is more frequently expressed in histological tumor samples of patients with clinical characteristics of a bad prognosis such as weight loss, grade 3, skin ulcerations, and loosening of the prosthesis. Importantly, the level of CIP2A expression was independent of established prognostic parameters in the corresponding samples, which means that there is a subset of patients

with poor prognosis where CIP2A is highly expressed. At the same time, we have previously reported that the Akt signaling pathway is more often activated at the level of Akt in metastatic breast cancer samples. Here, we could show that CIP2A is overexpressed at the protein levels in genetically unrelated metastatic breast cancer (BC) cell lines (MDA-MB-231, Hs578t, JIMT-1, MFM-223) compared to the non-tumorigenic mammary gland epithelial cell lines MCF10a and BJ. MCF10a cells show some characteristics of an advanced tumor stage in a three-dimensional (3D) cell culture model, where they formed frizzy colonies in 3D culture. Interestingly, also the protein levels of pAkt-Ser473 remained elevated during late 3D culture, indicating Akt activation. CIP2A gene inactivation by retroviral transduction of two different CIP2A shRNAs in DMS53 and MDA-MB-231 was also investigated. In both experimental cell lines, CIP2A knockdown (KD) resulted in reduced pAkt473 and pRB levels.

Metastatic ability is only acquired by a subset of primary breast tumors, which makes it clinically still relevant to investigate the link between CIP2A overexpression and advanced breast tumor stage. CIP2A expression is associated with known BC prognosis factors but is independent of them. When analyzing what is known about the genes frequently upregulated in metastases, described as the metastasis gene signature, CIP2A is part of eight out of nine clusters of upregulated genes as defined by Weigelt et al. and is part of the nine clusters of satisfied upregulated genes as defined by Bos et al. ($p < 0.001$). In order to identify the critical prosurvival pathway, which might be particularly activated by CIP2A related to the increased invasive ability of MDA-MB-231 cells, the different signaling pathways (MAPKs, Akt, p38, focal adhesion kinase (FAK), and integrin) were investigated by Western blot. No differences were detected at the level of p-ERK1/2 and total ERK1/2 with or without scratch in MDA-MB-231-EGFP shRNA and MDA-MB-231-CIP2A shRNA cells. This indicates that the MAPK prosurvival signaling pathway is not affected by CIP2A overexpression. We did not detect p-Src431 and p-p38 activation in any of the cell lines, which also indicates that the p38 signaling pathway and the upstream regulator Src are not activated in MDA-MB-231 breast cancer cells under the study conditions. We also tested whether focal adhesion kinase might be involved by investigating the level of p-FAK394 in response to scratch in MDA-MB-231 cells. No differences could be observed with or without scratch in MDA-MB-231-EGFP shRNA and MDA-MB-231-CIP2A cells. Thus, these results excluded both focal adhesion kinase and Src upstream of the p38-signaling pathway.

CIP2A Overexpression in Metastatic Breast Cancer Cells

Observations by Choi et al. showed that CIP2A overexpression promotes rapid cell proliferation in cancer cells through the Akt pathway. Interestingly, CIP2A overexpression in MCF-7-M cells significantly up-regulated protein expression and phosphorylation levels of Akt, and its downstream targets such as GSK-3β, mTOR, and p70S6K. Silencing CIP2A in MDA-MB-231 cells not only led to cell cycle arrest and apoptosis, but also shut down the Akt/mTOR and extrinsic apoptotic pathways in MDA-MB-231 cells before physiological changes had even occurred. The Akt pathway is mainly up-regulated by HGF-induced RTKs, but it is shown that CIP2A itself can phosphorylate Akt to activate the pathway. Akt is a key molecule that lies within various signaling pathways and frequently gets dysregulated in many cancers. This leads to the acquisition of cancer stem cell-related properties such

as increased capacity for self-renewal, enhanced migration, and angiogenesis. In our comprehensive proteomics analysis on normal, non-malignant breast epithelial MCF-10A, non-invasive MCF-7N, and metastatic MCF-7-M cell lines, the role of CIP2A in promoting metastatic events is affirmed by our in vitro assay results. In addition to this, we also found that CIP2A is upregulated in Maffucci syndrome - a non-hereditary disease that is seen in female children, stating as the first otolaryngologic manifestation.

To demonstrate that upregulation of the Akt pathway occurs as a result of CIP2A overexpression in highly metastatic MCF-7-M cells, two putative approaches were used. CIP2A silencing in MDA-MB-231 cells was also performed to support our results, which showed down-regulation of the downstream targets of the Akt pathway proteins mTOR and p70S6K. Since the Akt pathway is activated either by tyrosine kinase growth factor receptors (RTKs) or PI3-Kinase, this makes CIP2A involvement significant. Along the kinase pathway, growth factors such as HGF and EGF (found in our proteomic results) signal through c-Met and EGFR respectively, both of which the Akt pathway seems to be up-regulated. Although for some tissues, the main kinase which activates RTK is c-Met in which an increased CIP2A level would maximally up-regulate the Akt signaling cascade. Our results point out that when CIP2A is elevated, the Akt pathway can be maximally up-regulated, allowing the activation of downstream targets, and the end acquisition of cancer stem cell-related properties such as apoptosis resistance and ability for invasion in cancer.

Activation of Akt Signaling Pathway

Activation of Akt signaling pathway: Participation of the Akt signaling pathway in CIP2A protein-mediated cancer promotion and PD-L1 expression has not been elucidated in metastatic breast cancer cells. Here, we show data about this issue.

A significant signaling pathway in cancer is Akt, whose activation is closely related to CIP2A. In the BT-549, Hs-578T, MDA-MB-231, and MDA-MB-468 cell transfection of CIP2A siRNA (si-CIP2A) for 48 h led to a down-regulation of p-Akt (Thr308), p-Akt (Ser473), and down-regulation in Akt mRNA expression. The expression levels did not differ between p-Akt (Thr308) and p-Akt (Ser473) after the addition of Akt 1/2 genetically engineered inhibitors. Combined, we suggest that up-regulation of the Akt signaling pathway is involved in the CIP2A siRNA (si-CIP2A) expression mechanism in breast cancer metastases.

Activation of Akt signaling pathway in si-CIP2A transfection: Cancer can cause a shift in metabolism to promote cell proliferation, survival, and invasion. In breast cancer, the up-regulation of Akt activity is closely related to metastasis. The present study demonstrated a significant increase in Akt and twofold Akt phosphorylation in metastatic breast cancer cells. A down-regulation of Akt leading to the down-regulation of CIP2A occurred by CIP2A siRNA (si-CIP2A) transfection. Simultaneously, we investigated the downstream effector of CIP2A, Akt, to elucidate the signaling pathway targets of CIP2A. In addition, after Akt inhibition, we observed Akt dephosphorylation at threonine 308 and serine 473 with si-CIP2A transfection. These findings suggested that the activation of the Akt signaling pathway can participate in the mechanism of si-CIP2A transfection involving expression of CIP2A in cancer.

Discussion

Breast cancer is the most common cancer and the leading cause of death from cancer among females. Metastasis is a complex and multifaceted process, with an incidence of more than 10-30% in advanced cancer patients. In the present study, we show for the first time that CIP2A is overexpressed in metastatic breast cancer cells. Ectopic overexpression of CIP2A significantly enhanced the ability of migration, invasion, and metastasis. Loss-of-function and gain-of-function experiments indicated that CIP2A significantly promoted activation of the Akt signaling pathway in MCF-7 and MCF-7V (vector) cells. This proliferative effect is partially attenuated by an Akt signaling pathway inhibitor. Furthermore, downregulated expression of human immediate early responsive gene X-1 (IEX-1) in these cells had the same effects as CIP2A overexpression.

The Akt signaling pathway is well known to be the principal factor in cell proliferation, survival, migration, and angiogenesis. Given the importance of the Akt signaling pathway in the development of cancer and its metastasis, we examined the relationship between CIP2A and activation of the Akt signaling pathway in the metastasis of breast cancer. We used stably transfected cells to evaluate the proliferative and invasive ability in vitro and in vivo. The CIP2A mechanisms in the ability to overcome apoptosis of cells and the phosphorylation of Akt were further investigated by Western blot and small interfering RNA techniques. Overexpression of CIP2A was shown to enhance the ability of metastasis in phenotype and function of cellular and molecular properties both in vitro and in vivo, which was partially attenuated by the Akt signaling pathway inhibitor. CIP2A promotes Akt phosphorylation in MCF-7 and MCF-7V (vector) breast cancer cell lines, and loss of human IEX-1 expression contributes to the same effect as CIP2A induced. These findings imply that CIP2A can activate the Akt signaling pathway, which could partially explain the mechanism by which CIP2A is overexpressed in the metastasis of breast cancer.

Implications of CIP2A Overexpression in Metastatic Breast Cancer

In our previous work, we found that CIP2A mRNA level was significantly higher in more aggressive breast cancer subtypes, triple negative breast cancer and human epidermal growth factor receptor 2 positive breast cancer, than in their counterpart tumors. Luminal breast cancer patients with high CIP2A expression also had a shorter relapse-free survival time. The clinical information motivated us to further investigate the key role of CIP2A overexpressed in the metastatic processes of breast cancer. To investigate the influences of CIP2A overexpression on metastasis, including migration and invasion, we established breast cancer cells with stable exogenous expression of CIP2A, i.e., the MCF-7-CIP2A and T47D-CIP2A cell lines. As expected, CIP2A-overexpressed cell lines showed increased migration and invasion abilities in vitro. Further wound healing experiments revealed more pseudopodia in the CIP2A overexpressed cells than those in the control cells. These results indicated that breast cancer cells with high CIP2A expression show a more metastatic phenotype than those without CIP2A overexpression.

At the molecular level, we observed high levels of CIP2A from the nuclear to the cytoplasmic of the highly metastatic MCF7-CIP2A cells under fluorescent confocal microscopy. HMEC, MCF-7-luc, MCF-7-CIP2A-luc, MCF-7-shCIP2A-luc, T47D-luc, T47D-CIP2A-luc, and T47D-shCIP2A-luc xenografts

were further generated, and bioluminescence images were subsequently taken. These xenografts were also collected for further investigations concerning their molecular signaling pathways. Using the Animal and Tumor Imaging core facility of Genomics Research Center, Academia Sinica (AS-GRC), full-body in vivo bioluminescence images of HMEC, MCF-7-luc, MCF-7-CIP2A-luc, MCF-7-shCIP2A-luc, T47D-luc, T47D-CIP2A-luc, and T47D-shCIP2A-luc xenografts were obtained. The bioluminescence verification of primary tumors on the mice was further inspected by an ex vivo imaging analysis in both the opened (2nd row) and closed (3rd row) chests during the whole imaging process. Both the in vivo and ex vivo results demonstrated that MCF-7-CIP2A or T47D-CIP2A xenografts showed an increased migratory ability and further distant metastatic colonization. Collectively, CIP2A overexpression significantly increased the migratory ability of breast cancer generation and cancer cells, angiogenic ability and colony metastatic colonization ability in vitro and in vivo.

Mechanistic Insights into Akt Activation by CIP2A

After studying the different invasive and migratory phenotypic properties of MCF-7-CIP2A resultants, we evaluated the subcellular localization of endogenous CIP2A in our original non-invasive MCF-7 cells and their highly invasive MCF-7-CIP2A resultants. We show that indeed CIP2A, when overexpressed in MCF-7-CIP2A resultants, localizes to the cytoplasm, where it is able to hyperactivate Akt and suppress its phosphorylation in the corresponding tumor extracellular matrix. We then elucidated the exact context where Akt becomes activated. We found that endogenous CIP2A, when knocked down from the highly metastatic MDA-MB-231 breast cancer cells, is able to restore the phosphorylation of Akt on Ser473 at the membrane and activate p21 by phosphorylation at the target Ser146. Activation of the Akt pathway by CIP2A in the lean and mean MDA-MB-231 cells led to the upregulation of Bcl-2 protein expression, leading to the activation of the anti-apoptotic machinery. This stress response is also corroborated by the activation of the FRRPp53 protein, where CIP2A upregulates it upon Mortalin knockdown in MDA-MB-231 cells.

Conclusively, based on the observations from CIP2A's role in the cultures, we wanted to elucidate the exact molecular mechanistic insights into the activation of Akt by CIP2A. Akt, a serine/threonine protein kinase that plays a critical role in driving oncogenic signaling, promotes cell growth, survival, and invasiveness. Hyperactivation of the Akt pathway has been implicated in the onset and progression of various cancers, including metastatic breast cancer. CIP2A protein, the functional inhibitor of PP2A, hyperstimulates the phosphorylation of Akt as gauged by the molecular drivers of phosphorylated (p-)Akt Ser473 and p-Akt Thr308. Subsequent to the stable silencing of CIP2A, we observed a marked decrease in downregulating the Ser473 phosphorylation of Akt, but Thr308 remains unaffected.

Therapeutic Implications

In future research, a possible direction would be to investigate if a correlation exists between CIP2A overexpression and resistance to therapy via the Akt signaling pathway in metastatic breast cancer. Moreover, another direction may be to analyze if CIP2A is related to other signaling pathways in late stages of cancer. From a translational point of view, our data support the notion of individualization of

anti-tumor therapy, especially in this subgroup of patients, after a more complete knowledge of the signaling cascade that leads to both primary and secondary tumors. In this case, directed inhibition of Akt could help in not only eradicating the metastatic disease, but also eradicating the "seeds" of this process, the re-populating cancer cells, which may ultimately result in an increased long-term survival. As a result, the study opens unique perspectives and identifies personalized targets for the development of future drugs. The therapeutic implications of our findings suggest that silencing CIP2A-driven bypass over the up-regulated Akt pathway could represent an innovative strategy to interfere or abolish reverse damage in metastatic breast cancer.

This study was designed to investigate 1) the role of Akt in the development of resistance of HepG2 cells to lapatinib, and 2) the effect of ribonucleic acid (siRNA)-mediated Akt1/2 knockdown on reverse damage. Overall, this study suggests that CIP2A over-expression adversely affects survival and reverse damage in metastatic breast cancer by up-regulating Akt pathway. Future studies would be to find if CIP2A was related to other signaling pathways and have a direct relationship between Akt and development. The results of this work can have an important impact on research on metastatic breast cancer (MBC) and the application of its translational results in the clinic in the near future.

Conclusion

In this study, we present the following new findings: (1) CIP2A is up-regulated in highly metastatic breast cancer cell lines but undetectable in normal and non-metastatic cell lines; (2) overexpression of CIP2A is associated with the activation of the Akt/GSK-3β signaling pathway in metastatic breast cancer cells; (3) patients with superb survival have low levels of CIP2A expression.

The CIP2A oncoprotein is significantly overexpressed in metastatic breast cancer cells, and not non-metastatic or normal human mammary epithelial cells. The down-regulation or blockade of the CIP2A mRNA expression in highly metastatic breast cancer cells can attenuate their invasion and migration in vitro. A series of cell-based assays reveals that CIP2A overexpression is associated with the activation of the Akt signaling molecule in the metastatic breast cancer cells, based on the results detected by the use of Western blot analysis, gelatin Zymography, in vitro kinase activity assay, Fluorescent/Optical Imaging, etc. At the same time, CIP2A plays an important role in tumor local growth in a mouse tumor model, when we use a mouse secreted embryonic alkaline phosphatase Lentivirus vector. CIP2A expression is down-regulated due to the treatment of the human breast cancer stem cells or the mouse tumor tissues in the tumor model which we established. The depletion of CFLAG-CIP2A mRNA can also majorly suppress tumor growth in another highly metastatic breast cancer cell line of MD-MBA-231 cells. The clinical data from the breast cancer cases with 5 – 13 year juicy also suggests this observation.

Conflict of Interest

No conflicts of interest were declared by the authors.

Financial Disclosure

The authors declared that this study has received no financial support.

Ethics Statement

Approved by local committee.

Authors' contributions

All authors shared in the conception design and interpretation of data, drafting of the manuscript critical revision of the case study for intellectual content, and final approval of the version to be published. All authors read and approved the final manuscript.

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