

Myeloid Cell Leukemia-1 Inversely Correlates with Glycogen Synthase Kinase-3 β Activity and Associates with Poor Prognosis in Human Breast Cancer

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Abstract

Myeloid cell leukemia-1 (Mcl-1), an antiapoptotic Bcl-2 family member, is overexpressed in many types of human cancer and associates with cell immortalization, malignant transformation, and chemoresistance. Glycogen synthase kinase-3 β (GSK-3 β), a key component of the Wnt signaling pathway, is involved in multiple physiologic processes such as protein synthesis, tumorigenesis, and apoptosis. Here, we report that expression of Mcl-1 was correlated with phosphorylated GSK-3 β (p-GSK-3 β) at Ser⁹ (an inactivated form of GSK-3 β) in multiple cancer cell lines and primary human cancer samples. In addition, Mcl-1 was strikingly linked with poor prognosis of human breast cancer, in which the high level of Mcl-1 was related to high tumor grade and poor survival of breast cancer patients. Furthermore, we found that activation of GSK-3 β could down-regulate Mcl-1 and was required for proteasome-mediated Mcl-1 degradation. Under some physiologic conditions, such as UV irradiation, anti-cancer drug treatment, and inhibition of growth factor pathways, Mcl-1 was down-regulated through activation of GSK-3 β . Our results indicate that Mcl-1 stabilization by GSK-3 β inactivation could be involved in tumorigenesis and serve as a useful prognostic marker for human breast cancer. [Cancer Res 2007;67(10):4564–71]

Introduction

Apoptosis is critical for normal embryonic development, tissue homeostasis, and cell functions. Deregulation of apoptosis contributes to diverse human diseases, including neurodegenerative disorder and cancer (1). The antiapoptotic Bcl-2-like proteins, including Bcl-2, Bcl-xL, and myeloid cell leukemia-1 (Mcl-1), which share three or four Bcl-2 homology (BH) regions (BH1–BH4), are required for cell survival and have been found to play key roles in the development of cancer due to their role in controlling mitochondria-mediated intrinsic pathways of apoptosis (2, 3). Mcl-1, originally characterized in differentiating

myeloid cells, contains three BH domains (BH1–BH3) but lacks a clearly defined BH4 domain at the NH₂ terminus. Mcl-1 also harbors a transmembrane domain at its COOH terminus, by which Mcl-1 localizes to various intracellular membranes, especially, the outer mitochondrial membrane (4). Similar to Bcl-2 and Bcl-xL, Mcl-1 can interact with Bax and/or Bak to inhibit mitochondria-mediated apoptosis (5, 6). Compared with Bcl-2 and Bcl-xL, Mcl-1 is unique in that its expression is quickly and readily induced after exposure to cytokines or growth factors (7). Increased Mcl-1 expression may enhance cell survival; that is, up-regulation of Mcl-1 not only promotes viability in a wide range of cell types in the short term but also contributes to immortalization and tumorigenesis in the long term (8, 9). As Mcl-1 is often overexpressed and plays a role in various human tumors; it may serve as a target for cancer therapy (10, 11).

Glycogen synthase kinase-3 β (GSK-3 β), a serine/threonine protein kinase involved in glycogen metabolism and the Wnt signaling pathway, plays important roles in embryonic development and tumorigenesis. GSK-3 β , through direct phosphorylation of a broad range of substrates (translation factor eIF2B, cyclin D1, c-Jun, c-myc, NFAT, cyclic AMP-responsive element binding protein, Tau, and Snail), is an integral part of multiple physiologic processes such as protein synthesis, microtubule dynamics, cell proliferation and differentiation, and cell motility (12, 13). GSK-3 β activity can be abrogated by direct phosphorylation on the Ser⁹ residue by phosphatidylinositol 3-kinase (PI3K)/Akt, mitogen-activated protein kinase (MAPK)/p90RSK, or mammalian target of rapamycin/S6K upon a number of extracellular stimuli, such as insulin, epidermal growth factor, and fibroblast growth factor (13). Recent studies have shown that overexpression of GSK-3 β can induce apoptosis in several cell types, whereas inactivation of GSK-3 β through phosphorylation of the Ser⁹ residue can reduce apoptosis (14, 15), indicating that GSK-3 β may play a critical role in linking multiple pathways to regulate cellular apoptosis. In the current study, we found that the activity of GSK-3 β was required for proteasome-mediated Mcl-1 degradation, and that Mcl-1 inversely correlated with GSK-3 β activity and associated with poor prognosis in human breast cancer.

Materials and Methods

Constructs and reagents. pCGN-GSK-3 β (WT), pGEX-GSK-3 β , and constitutively active GSK-3 β (GSK-3 β -CA; S9A GSK-3 β) were kindly provided by Drs. A. Kikuchi (Hiroshima University, Hiroshima, Japan), M.J. Birnbaum (University of Pennsylvania School of Medicine, Philadelphia, PA),

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and J.R. Woodgett (Ontario Cancer Institute, Toronto, Canada). pHA-hMcl-1 was kindly provided by H-Y. Yan-Yen (Institute of Molecular Biology, Academia Sinica, Taipei, Taiwan). The full-length hMcl-1 cDNA was subcloned into vector pCMV5-MYC. Using the QuickChange multiple site-directed mutagenesis kit (Stratagene), kinase-dead GSK-3 β (GSK-3 β -KD; pCGN-GSK-3 β -KD) was generated according to the manufacturer's protocol and was further verified by automated sequencing. TDZD8, a GSK-3 β inhibitor, was purchased from Calbiochem. PD98059, an extracellular signal-regulated kinase (Erk)/MAPK inhibitor, was purchased from Cell Signaling. LY200294 (a PI3K/AKT inhibitor), MG132 (a proteasome inhibitor), insulin-like growth factor-1 (IGF-1), and staurosporine were purchased from Sigma.

Cell culture, UV and staurosporine treatment, and small interfering RNA transfection. Breast cancer cell lines MDA-MB-231, MDA-MB-361, MDA-MB-435, MDA-MB-453-MDA-MB-468, MCF-7, SKBR3, and T47D; ovarian cancer cell lines IP1, OVCAR3, and 2774; hepatoma cell lines HepG2 and Hep3B; and pancreatic cancer cell CFPAC1 were bought from the American Type Culture Collection. Cells were grown in DMEM/F12 medium supplemented with 10% fetal bovine serum. Transient or stable transfections with DNA were done with an optimal ratio of DNA to liposome based on prior experiment (16). HeLa cells were plated at a density of 3×10^6 in a 6-cm dish with DMEM supplemented with 10% fetal bovine serum for 1 day, then treated with 1.9 J/m²/s of UV irradiation for 2 min or with 0.1 μ M/L staurosporine at the indicated time points. For small interfering RNA (siRNA) experiments, cells were transfected with GSK-3 β siRNA expression plasmid pKD-GSK-3 β -v1 (Upstate Biotechnology) by electroporation (Solution T, program B-16) using Nucleofector 1(Amaxa), and then lysates were harvested 48 h after transfection.

Immunoblotting, immunoprecipitation, and *in vitro* kinase assay. Immunoblotting and immunoprecipitation were done as previously described (17) with the following antibodies: Mcl-1 and GSK-3 β (BD Transduction Labs), p-GSK-3 β (Ser⁹; Calbiochem and Cell Signaling Technology), phosphorylated Akt (Ser⁴⁷³), Akt, phosphorylated β -catenin (Ser^{33/37}/Thr⁴¹; Cell Signaling Technology), and phosphorylated Erk and Erk (Upstate Biotechnology). For *in vitro* kinase assays, purified GST- β -catenin protein was incubated with GSK-3 β immunocomplexes in the presence of 50 mmol/L ATP in kinase buffer for 30 min at 30°C. Reaction products were resolved by SDS-PAGE and then blotted with an anti-phosphorylated β -catenin antibody. For quantification of the Western analysis, the density of each band was quantified by Quantity One software; the background of each selected area (band) was automatically calculated and then deducted by the software.

Immunohistochemical staining. For immunohistochemical staining of human breast cancer tissue samples, each sample was stained with specific antibodies as indicated and scored by an H-score method that combines the values of immunoreaction intensity and the percentage of tumor cell staining. Briefly, the immunoreactivity of p-GSK-3 β and Mcl-1 were ranked into three groups according to the percentage of the positive tumor cells: high (++ , >25%), low (+ , 1–25%), and negative (– , 0%). One hundred twenty-four surgically resected human breast cancer specimens were collected from the Department of Pathology, Shanghai East Breast Disease Hospital, P.R. China. The clinical pathologic characteristics of specimens, including tumor size, lymph node status, tumor grade, and levels of estrogen receptor and progesterone receptor, were obtained from medical records. Tissue microarray slides (HistoArray IMH-302) were purchased from IMGENEX.

Statistical analysis. The linear regression analysis was used to analyze the correlation between p-GSK-3 β expression and level of Mcl-1, Bcl-2, and Bcl-xL, respectively, in multiple cell lines. The χ^2 test was employed to analyze the relation between Mcl-1 level with p-GSK-3 β expression and the stage of tumor grade in 125 primary breast cancer tissues. Kaplan-Meier curves for the overall survival of 125 primary breast cancer patients were plotted and compared. Log-rank test was used to evaluate the difference between three levels of Mcl-1 expression with regard to overall survival. Statistical analysis and graphs were done with SPSS software, and the level of significance was set at 0.05.

Results

Level of Mcl-1 expression is related to phosphorylation of GSK-3 β at Ser⁹ in multiple cancer cell lines and primary tumor samples. To investigate the mechanism of GSK-3 β -induced apoptosis, we searched for correlations between GSK-3 β activity and expression of the apoptosis-associated molecules Bcl-2, Bcl-xL, and Mcl-1. Because phosphorylation of Ser⁹ is known to inactivate GSK-3 β kinase activity and can be used as a measurement of GSK-3 β inactivation (18), we compared expression of Bcl-2, Bcl-xL, and Mcl-1 with GSK-3 β phosphorylation level at Ser⁹. We found that Ser⁹ phosphorylation of GSK-3 β was significantly correlated with Mcl-1 expression in a panel of 11 cancer cell lines of breast, ovary, and pancreas; however, there is no statistically significant correlation between the level of GSK-3 β phosphorylation at Ser⁹ and the expression of Bcl-2 or Bcl-xL (Fig. 1A). To further test whether the correlation between p-GSK-3 β (Ser⁹) and Mcl-1 expression in cell culture is a general phenomenon in multiple tumor types, we examined 21 human tumor specimens from tumor tissue microarray using immunohistochemical analysis. GSK-3 β phosphorylation at Ser⁹ was correlated with Mcl-1 expression in 18 of the 21 tumor specimens (Fig. 1B), indicating that the relationship between Ser⁹ phosphorylation of GSK-3 β and Mcl-1 expression is a general phenomenon in multiple types of human cancer.

Mcl-1 expression associates with poor survival in human breast cancer. To justify pathologic relevance of the relationship between p-GSK-3 β (Ser⁹) and Mcl-1 expression observed in cancer cell lines, we analyzed these two proteins in 125 human breast cancer tissue samples. Consistently, immunohistochemical stainings showed that there is a positive correlation between the levels of Mcl-1 and p-GSK-3 β in human breast cancer samples ($P = 0.025$; Fig. 2A and B). Next, we analyzed expression level of Mcl-1 and clinical-pathologic characteristics of 125 breast cancer samples: age, tumor size, lymph node metastasis, tumor grade, and expression of estrogen receptor. We found that a high level of Mcl-1 was strikingly correlated with high tumor grade, whereas negative/low expression of Mcl-1 was correlated with low tumor grade (Fig. 2B). We then compared the Mcl-1 expression in tumor tissues with the patients' survival follow-up. The results suggested that the patients' survival is decreased significantly with the increasing expression of Mcl-1 (Fig. 2C), indicating that the expression level of Mcl-1 could be a potential candidate as a predictive marker of poor prognosis in breast cancer patients.

GSK-3 β kinase activity is required for proteasome-mediated Mcl-1 degradation. The positive correlation between Ser⁹-phosphorylated GSK-3 β and Mcl-1 up-regulation suggested that there is an inverse correlation between GSK-3 β activity and Mcl-1 expression, which prompted us to test whether activation of GSK-3 β could inhibit the expression of Mcl-1. A wild-type GSK-3 β (GSK-3 β -WT), GSK-3 β -CA, or GSK-3 β -KD was cotransfected with Mcl-1 to 293 T cells for 48 h. The expression level of Mcl-1 was much lower in the presence of GSK-3 β -CA or GSK-3 β -WT than in the presence of GSK-3 β -KD (Fig. 3A, lanes 1 and 2 versus lane 3). In addition, the proteasome inhibitor MG132 blocked GSK-3 β -WT- or GSK-3 β -CA-induced down-regulation of Mcl-1 (Fig. 3A, lanes 4 and 5 versus lanes 1 and 2), suggesting that proteasome-mediated degradation is involved in active GSK-3 β -induced Mcl-1 down-regulation. We then tested whether

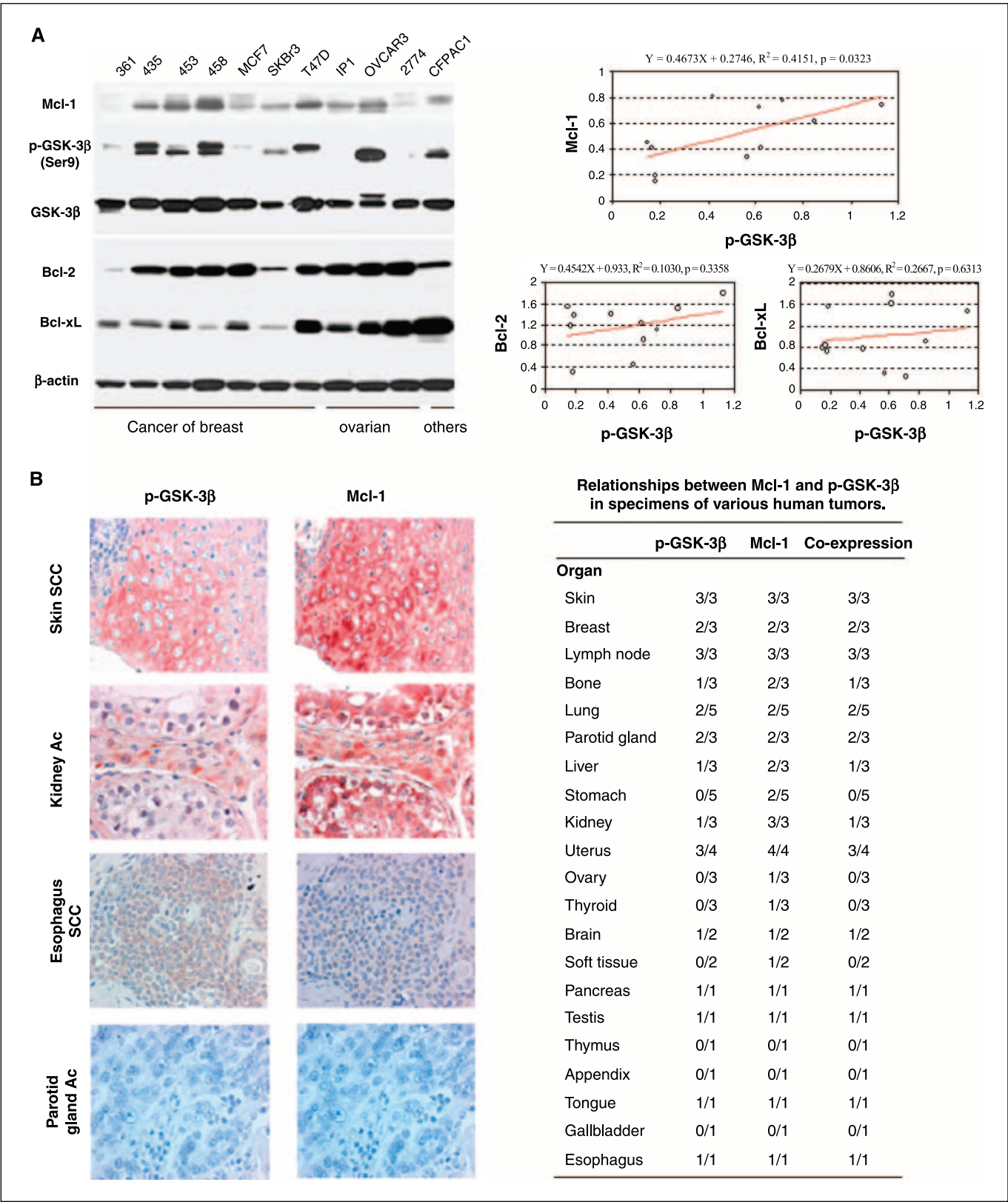


Figure 1. Level of Mcl-1 expression is correlated with phosphorylation of GSK-3β at Ser⁹ in multiple cancer cell lines and primary tumor samples. **A**, the expression of Mcl-1, Bcl-2, and Bcl-xL and Ser⁹-phosphorylated status of GSK-3β were studied in the indicated cancer cell lines (left). The density of Mcl-1, Bcl-2, Bcl-xL, and p-GSK-3β (Ser⁹) was quantitated using Quantity One software and then analyzed by linear regression (right). p-GSK-3β was normalized by GSK-3β. Mcl-1, Bcl-2, and Bcl-xL were normalized by β-actin. For the four multiple bands, all bands were quantified. **B**, level of Mcl-1 and p-Ser⁹ of GSK-3β were detected in human primary tumor specimens by immunohistochemistry staining. Expression of Mcl-1 and p-GSK-3β (Ser⁹) in representative human tumor samples (left). AC, adenocarcinoma; SCC, squamous cell carcinoma. Summary of the expression of Mcl-1, p-GSK-3β (Ser⁹), and the coexpression number in total examined human tumor samples (right).

GSK-3 β inhibitors or proteasome inhibitors might up-regulate endogenous Mcl-1 in human cancer cells. The protein level of endogenous Mcl-1 was increased in human cancer cell lines treated with GSK-3 β inhibitors lithium or TDZD8 or the

proteasome inhibitor MG132 (Fig. 3B). Taken together, the results indicate that activation of GSK-3 β results in Mcl-1 down-regulation. To investigate the molecular mechanisms for GSK-3 β -mediated down-regulation of Mcl-1, we investigated whether

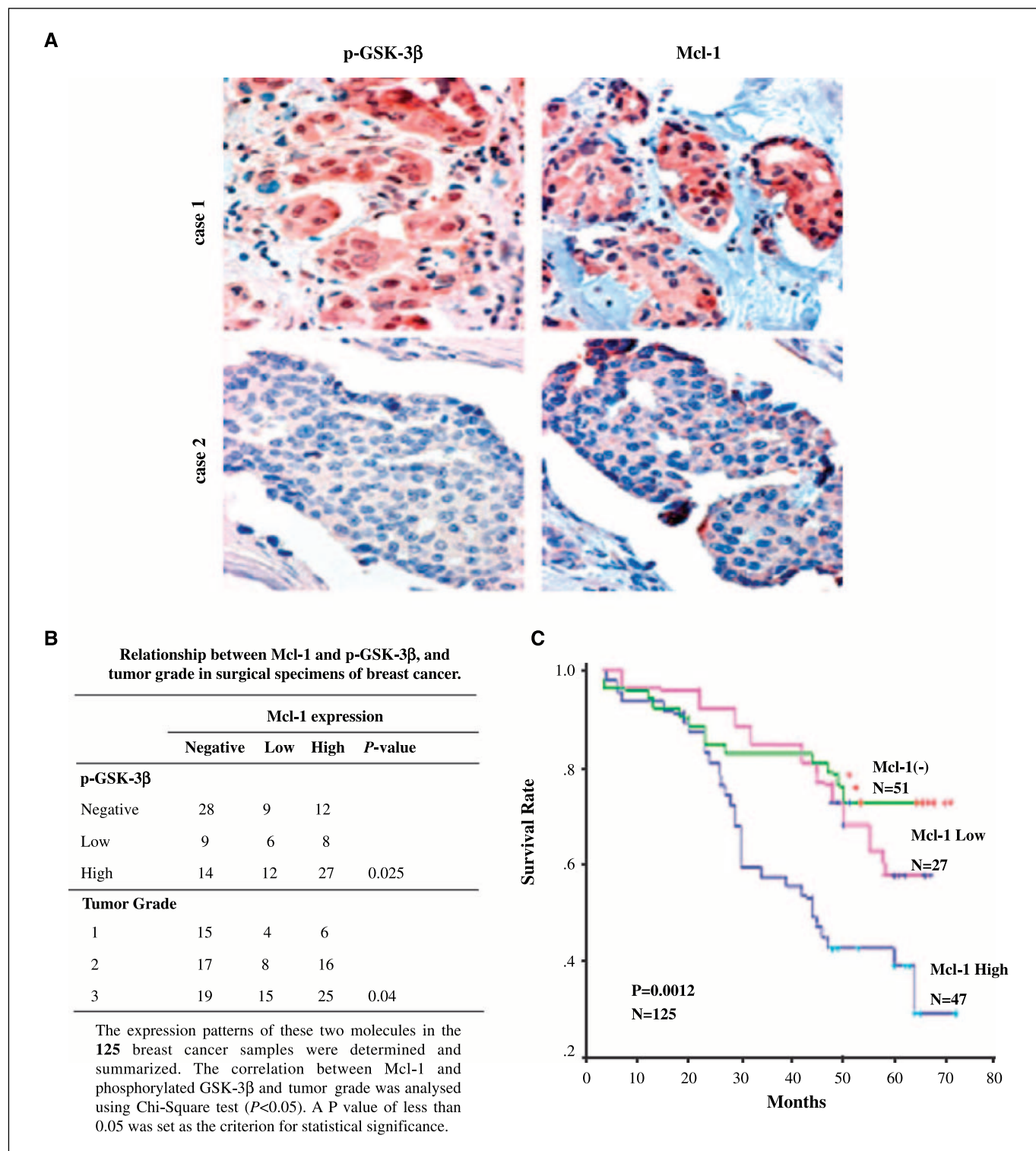


Figure 2. Expression of Mcl-1 correlates with Ser⁹-phosphorylated GSK-3 β and is associated with poor survival in 125 human breast cancers. **A**, human primary breast tumor specimens were immunostained with antibodies specific to p-Ser⁹ of GSK-3 β and Mcl-1, respectively. Case 1 is a representative specimen with high expression of p-GSK-3 β and Mcl-1. Case 2 is a specimen with low or no expression of p-GSK-3 β and Mcl-1. **B**, relationship between expression of Mcl-1 and p-GSK-3 β (Ser⁹) and tumor grade in human breast cancer specimens. **C**, Kaplan-Meier overall survival curves for breast cancer patients with high, low, or no expression of Mcl-1.

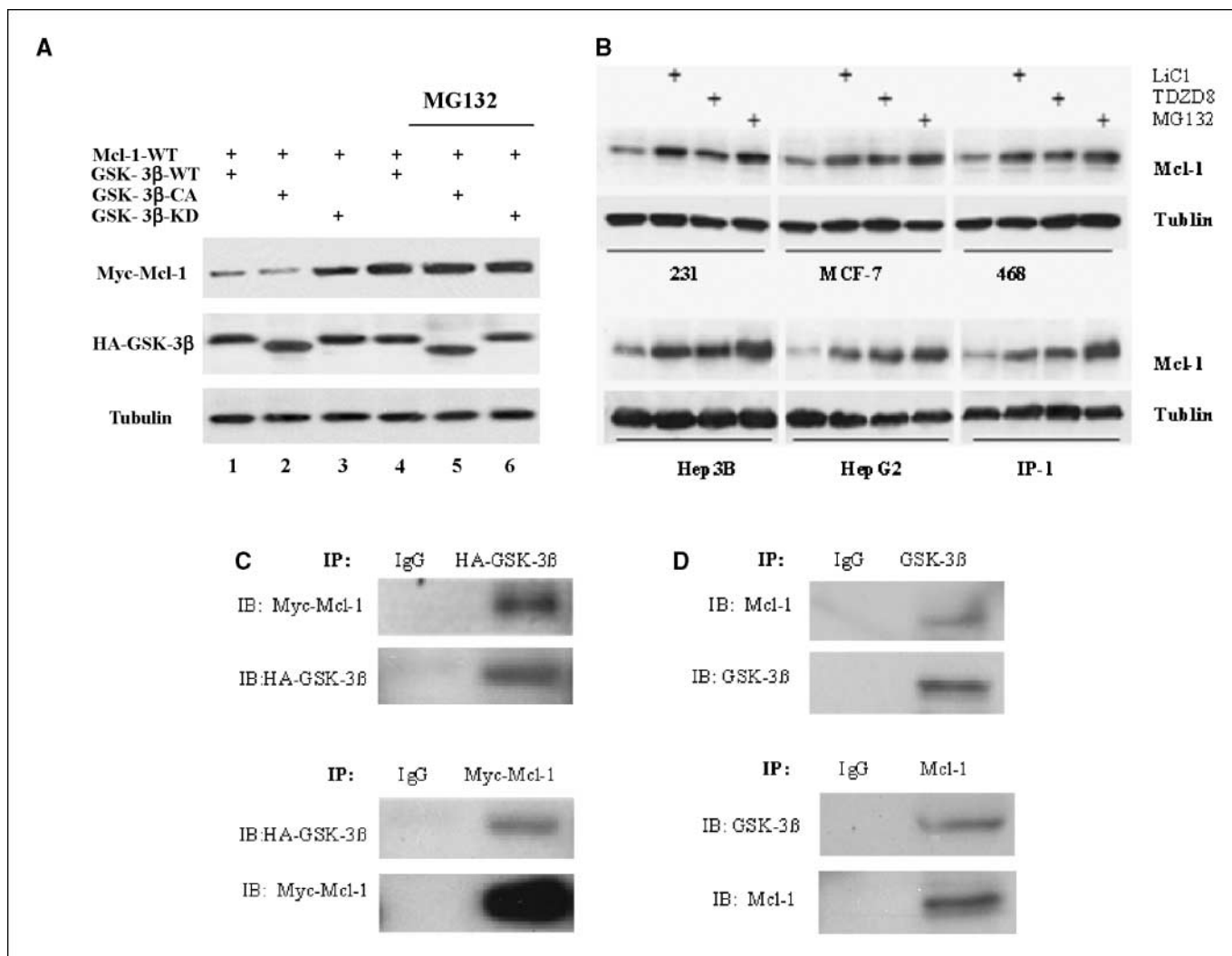


Figure 3. GSK-3 β associates with Mcl-1, and its kinase activity is required for proteasome-mediated Mcl-1 degradation. **A**, 293T cells transfected with Myc-Mcl-1 and HA-GSK-3 β -WT, GSK-3 β -CA, or GSK-3 β -KD were treated with or without the proteasome inhibitor MG132 (10 μ M) for 10 h and then analyzed by Western blotting with antibodies against Myc-Mcl-1 and HA-GSK-3 β , respectively. The data were consistent with Fig. 1E in our recent publication in *Molecular and Cellular Biology* (24). **B**, human cancer cell lines were treated with GSK-3 β inhibitors LiCl (20 mmol/L), TDZD (4 μ M) or the proteasome inhibitor MG132 (10 μ M) for 10 h, respectively, and then cell lysates were subjected to Western blotting to detect endogenous Mcl-1. **C**, HA-tagged GSK-3 β and Myc-tagged Mcl-1 were cotransfected into 293T cells, and GSK-3 β and Mcl-1 were immunoprecipitated from cotransfected 293T cell lysates, respectively, and then subjected to Western blotting. **D**, endogenous GSK-3 β and Mcl-1 were immunoprecipitated from the breast cancer cell line MDA-MB-453, respectively, and then subjected to Western blotting.

GSK-3 β could interact with Mcl-1. The hemagglutinin-tagged GSK-3 β was transfected into 293T cells, and then GSK-3 β and Mcl-1 were immunoprecipitated from cell lysates. The association between GSK-3 β and Mcl-1 was clearly detected by Western blotting (Fig. 3C). Furthermore, we also detected *in vivo* association of endogenous GSK-3 β and Mcl-1 in MDA-MB-453, a breast cancer cell line (Fig. 3D), whereas under the same conditions, we did not detect any association between GSK-3 β and two other Bcl-2 family proteins, Bcl-2 and Bcl-xL (data not shown).

UV irradiation, staurosporine, or inhibition of growth factor pathways down-regulates Mcl-1 through activating GSK-3 β . To investigate whether GSK-3 β -mediated down-regulation of Mcl-1 occurs under other physiologic and pathologic conditions, two cancer cell lines (Hep3B, a hepatoma cell line and MDA-MB-231, a breast cancer cell line) were treated with

IGF-1, which is known to inhibit GSK-3 β activity through activation of the PI3K/Akt and Erk/MAPK pathways (18, 19). After IGF-1 treatment in both cell lines, we observed that Mcl-1 was stabilized, and GSK-3 β was inactivated via phosphorylation of Ser⁹ (Fig. 4A). Furthermore, once GSK-3 β activity was recovered after cells were treated with the PI3K/AKT inhibitor LY200294 and the MAPK inhibitor PD98059, which indicated by reduced Ser⁹ phosphorylation of GSK-3 β , the expression level of Mcl-1 was down-regulated (Fig. 4A). These findings provided further evidence that Mcl-1 expression depends on the activity of GSK-3 β . It is known that UV irradiation and certain anticancer drugs can down-regulate Mcl-1, but the underlying mechanism is yet to be clear. We investigated whether GSK-3 β plays a role in down-regulating Mcl-1 under these conditions; HeLa cells were treated with UV irradiation or the anticancer drug, staurosporine, which can induce apoptosis (20). Following

these treatments, the level of p-GSK-3 β was reduced (Fig. 4B); concurrently, the Mcl-1 level was decreased, indicating that UV and staurosporine could stimulate GSK-3 β activity and down-regulate Mcl-1. We then investigated whether the activity of

GSK-3 β is required for UV- or staurosporine-induced Mcl-1 down-regulation. We showed that both Ser⁹-phosphorylated GSK-3 β and expression of Mcl-1 were increased in LiCl-pretreated cells upon treatment with UV or staurosporine (Fig. 4C).

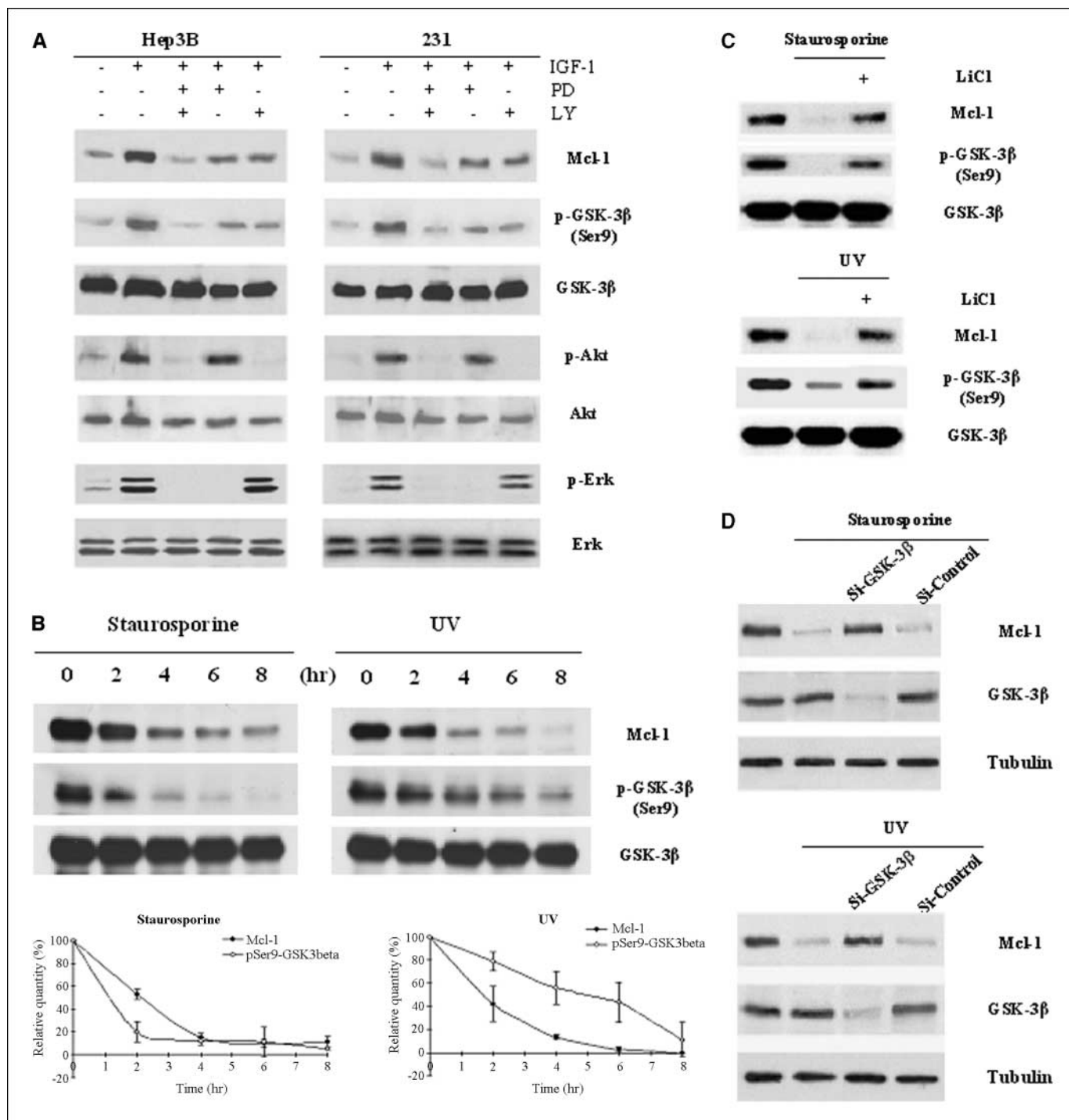


Figure 4. UV irradiation, staurosporine, or inhibition of growth factor pathway down-regulates Mcl-1 through activating GSK-3 β . **A**, Hep3B and MDA-MB-231 (231) cells were pretreated with the Erk/MAPK inhibitor PD98059 (PD; 20 μ mol/L) and/or the PI3K/Akt inhibitor LY200294 (LY; 2 μ mol/L) before treatment with IGF-1 (40 ng/mL). The expression of Mcl-1 and the phosphorylation levels of GSK-3 β , Erk (p-Erk), and Akt (p-Akt) were examined by Western blotting. **B**, HeLa cells were treated with UV or staurosporine at several time points (2, 4, 6, and 8 h). The Ser⁹-phosphorylated status of GSK-3 β and Mcl-1 expression levels were determined in cell lysates by Western blotting, and then the densities of Mcl-1 and p-GSK-3 β (Ser⁹) were quantified by software Quantity One. p-GSK-3 β and Mcl-1 were normalized by GSK-3 β , respectively, then the result of each time point will be compared with the time point without treatment in each group (the result of without treatment was treated as 100%). The experiments were triplicated. Results were included with error bars. **C**, HeLa cells were pretreated with the GSK-3 β inhibitor LiCl (40 mmol/L) before stimulation with UV or staurosporine for 6 h, then the Ser⁹-phosphorylated status of GSK-3 β and Mcl-1 expression levels were determined in cell lysates by Western blotting. **D**, cells were transfected with GSK-3 β siRNA or nontargeting control siRNA (Dharmacon) for 48 h followed by treatment of UV irradiation or staurosporine for 6 h, and cell lysates were subjected to Western blotting to detect Ser⁹-phosphorylated status of GSK-3 β and Mcl-1 expression level.

Furthermore, GSK-3 β siRNA also blocked UV- or staurosporine-induced Mcl-1 turnover (Fig. 4D). Taken together, these findings indicate that inactivation of growth factor pathways, UV irradiation, and staurosporine can down-regulate Mcl-1 through activation of GSK-3 β .

Discussion

It is well documented that antiapoptotic Bcl-2 family members, such as Bcl-2, Bcl-xL, and Mcl-1, play critical roles in the development of human cancers. Transformation of T lymphocytes, myeloid cells, and mammary epithelial cells were observed in Bcl-2 or Bcl-xL transgenic mice (21, 22). Mcl-1 also plays an important role in embryonic development and in apoptotic control, resulting in long-term immortalization and tumorigenesis. However, following a wide range of environmental stimuli, the expression patterns of Mcl-1 and other members of the Bcl-2 family are significantly different (7). The underlying mechanism contributing to the different expression patterns among Bcl-2 family members remains elusive. In the current study, we found that Ser⁹-phosphorylated GSK-3 β , as an inactive form of GSK-3 β , was correlated with up-regulation of Mcl-1, but not with Bcl-2 or Bcl-xL in multiple tumor cell lines and primary human cancer samples. Furthermore, GSK-3 β physically interacted with Mcl-1, but not with Bcl-2 and Bcl-xL. Our results may explain the differential expression of Mcl-1 and other members of Bcl-2 family following a wide range of environmental stimuli (7). In addition, we found that high level expression of Mcl-1 was linked to high tumor grade and poor survival in breast cancer patients, and that no or low level expression of Mcl-1 was correlated with low tumor grade and good survival. These results suggest that up-regulation of Mcl-1 plays an important role in development of breast cancer; and high-level expression of Mcl-1 could be used as a predictive marker for poor prognosis of breast cancer patients.

GSK-3 β is a key regulator of numerous signaling pathways such as the Wnt/ β -catenin, growth factor, and receptor tyrosine kinase pathways. Recently, several studies indicate that GSK-3 β -induced apoptosis is related to the mitochondrial-dependent

intrinsic caspase pathway. In the current study, we found that GSK-3 β down-regulates Mcl-1, and that its kinase activity is required for proteasome-mediated Mcl-1 degradation, indicating that activation of GSK-3 β may facilitate cell apoptosis through inhibiting Mcl-1. Our observation is consistent with a newly published report stating that GSK-3 β phosphorylates Mcl-1, resulting in destabilization of Mcl-1 (23). It is well known that Mcl-1 protein is tightly regulated and subjected to rapid degradation when cells undergo apoptosis in response to various stimuli, such as withdrawal of growth factor, ionizing radiation, and DNA-damaging reagents. Here, we show that these pathophysiologic conditions, such as UV and anticancer drug treatment, activate GSK-3 β , causing degradation of Mcl-1. Importantly, it is well documented that Mcl-1 is overexpressed and significantly contributes to chemoresistance in various human malignancies such as leukemia, melanoma, pancreatic cancer, hepatocellular carcinoma, and others. Chemoresistance, which may be caused by highly expressed receptor tyrosine kinase Her2 and other growth factors in breast cancer, is one of the major obstacles in treating breast cancer patients. The current study provides a plausible mechanism that Her2 and growth factors may inactivate GSK-3 β through the PI3K/AKT and MAPK pathways and then stabilize Mcl-1 (11), resulting in chemoresistance in breast cancer. Further studies are required for elucidating whether activation of GSK-3 β may lead to chemosensitization of breast cancer cells through down-regulation of Mcl-1, which may provide a novel approach for breast cancer treatment.

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