Advances in Brief

Modulation of Signal Transducer and Activator of Transcription 3 Activities by p53 Tumor Suppressor in Breast Cancer Cells

Jiayuh Lin, Xiaohong Jin, Kori Rothman, Huey-Jen Lin, Huiqin Tang, and William Burke

Division of Gynecologic Oncology, Department of Obstetrics and Gynecology, University of Michigan Comprehensive Cancer Center, Ann Arbor, Michigan 48109-0916

Abstract

The constitutive activation of the Stat3 oncogene product and mutation of the p53 tumor suppressor are both frequently detected in human breast cancer. We sought to determine whether there is functional regulation of Stat3 by wild-type (wt) p53. We demonstrate that expression of wt p53, but not mutant p53, significantly diminished phosphorylation of Stat3, reduced Stat3 DNA binding activity, and inhibited Stat3-dependent transcriptional activity in breast cancer cells expressing constitutively active Stat3. Expression of wt p53 did not cause a reduction in the phosphorylation of three unrelated protein kinases in other signal transduction pathways, AKT, extracellular signal-regulated kinase (ERK)1, and ERK2 or a reduction of phosphorylation of epidermal growth factor receptor. Furthermore, the expression of the p53 downstream target, p21 WAF1, did not have an inhibitory effect on Stat3 phosphorylation. Wt p53 also induced significant apoptosis in breast cancer cell lines that express constitutively active Stat3. Interestingly, the p53-dependent apoptosis occurred in the presence of high levels of phosphorylated AKT and ERK1/2. Therefore, these findings demonstrate a novel p53-dependent cellular process that regulates Stat3 phosphorylation and activity.

Introduction

Stat3 signaling pathways are activated in response to cytokines and growth factors (1). A growing number of tumor-derived cell lines, as well as tumor specimens from human cancers, is reported to express constitutively activated Stat proteins, very frequently Stat3 (2). The constitutive activation of Stat3 is frequently detected in breast carcinoma cell lines but not in normal breast epithelial cells (3, 4). Constitutive activation of Stat3 has also been detected in prostate, ovarian, head and neck, and other cancers (2, 5). Stat3 has been classified as an oncogene because the constitutively active Stat3 can mediate oncogenic transformation in cultured cells and tumor formation in nude mice (6). Stat3 activation may not only provide a growth advantage, allowing accumulation of tumor cells, but may also confer resistance to conventional therapies that rely on apoptotic machinery to eliminate tumor cells. The events downstream from constitutively active Stat3 that promote tumorigenesis are unclear but could include deregulation of cell cycle progression and/or providing protection against apoptosis. Constitutive activation of Stat3 signaling confers resistance to apoptosis in human U266 myeloma cells (7) and in fibroblasts (8).

In addition to the constitutive activation of Stat3 detected in breast cancer, alterations in the p53 tumor suppressor gene are also frequently detected in human breast carcinoma (9). Two of the major activities of p53 include its ability to transcriptionally activate downstream genes that induce both GI cell cycle growth arrest and apoptosis, particularly after DNA damage (10, 11). At present, it is unknown whether constitutive activation of Stat3 selectively occurs in human breast cancers that harbor inactivating mutations of the p53 gene. It is also unknown whether wt p53 modulates Stat3 activities. In this study, we sought to explore the functional relationship between p53 and Stat3 proteins in human breast cancer cells.

Materials and Methods

Human Cancer Cell Lines. Human breast cancer cell lines, MDA-MB-468 and SK-BR-3, and human ovarian cancer cell line, MDAH2774, were maintained in DMEM containing 10% FBS and antibiotics (5000 units/ml penicillin G and 5000 µg/ml streptomycin; Life Technologies, Inc., Grand Island, NY). All three cancer cell lines express both constitutively active Stat3 and mutant p53 protein (4, 5, 12–14).

Adenoviral p53 and p21 WAF1 Vectors. The adenovirus wt p53 that encodes wt p53 gene is as described previously (15). NCV contains the empty vector as the same backbone as the adenovirus p53. The adenovirus p53-175 vector (provided by Bert Vogelstein) encodes p53 gene containing single amino acid substitution at residue 175. The adenovirus p21 WAF1 vector (provided by Tsong Hsieh) encodes full-length wt p21 WAF1 (CDNA).

Western Blot Analysis. To analyze the protein levels of phosphorylated Stat3, AKT, EGFR, ERK1/2, and p53-targets, cells were plated with 1.2 × 10^6 cells/10-cm dish. Later (20 h), the serum concentration was reduced to 2%; then cells were infected with adenovirus wt p53, p53-175, wt p21 WAF1, or NCV at MOI of 100–200 pfu/cell. After infection (4 h), the adenovirus was removed, and medium was replaced with DMEM plus 10% FBS. After infection (26–30 h), 100 µg of total proteins from cell lysates were electrophoresed through SDS polyacrylamide gels and immunoblotted with antibodies against phospo-specific Stat3 (Tyr705), phospo-dependent Stat3, phospo-specific AKT (Ser473), and phospo-specific EGFR (Tyr1068; Cell Signaling Technology, Beverly, MA), respectively. The same blots were also analyzed with antibodies against p21 WAF1 and p53, respectively. The same blots were also analyzed with antibodies against p21 WAF1 and p53, respectively. The same blots were also analyzed with antibodies against p21 WAF1 and p53, respectively.

Apoptotic Assays. To quantify the induction of apoptosis by wt p53, cells were plated with 3 × 10^5 cells/6-cm dish. Later (20 h), the serum concentration was reduced to 2%; then cells were infected with adenovirus wt p53, p53-175, wt p21 WAF1, or NCV at MOI of 100–200 pfu. After infection (4 h), the adenovirus was removed, and medium was replaced with DMEM plus 10% FBS. After infection (26–30 h), cells were harvested and fixed with 70% alcohol. The cells were then stained with PI and analyzed for the Sub-G1 profile using software on a FACScan flow cytometer (Becton Dickinson, San...
breast cancer cells that express constitutively active Stat3 (4). Cells were infected with NCV, adeno-virus wt p53, or adeno-virus p53-175. A dramatic reduction of phosphorylation of Stat3 protein at Tyr residue 705 was observed in cells infected with adeno-virus wt p53 (Fig. 1A). In contrast, in uninfected cells or cells infected with NCV or adeno-virus p53-175, the levels of phosphorylated Stat3 protein remained unchanged (Fig. 1A). Because Stat3 is activated by phosphorylation on Tyr705, which leads to dimer formation and recognition of Stat3-specific DNA binding elements and activation of target gene transcription, we next examined whether wt p53 also reduces Stat3 DNA binding activity. Our results demonstrated that wt p53 inhibited constitutive Stat3 DNA binding activity in MDA-MB-468 cells (Fig. 1C). Expression of wt p53 did not affect the expression of phosphorylation-independent Stat3 protein. This cell line expresses high levels of endogenous mutant p53 (12). However, only cells infected with adeno-virus wt p53 demonstrated induction of two p53 targets, p21\textsuperscript{WAF1} and mdm2 (Fig. 1A). To exclude the possibility that infection of this cell line with adeno-virus wt p53 may cause global reduction of phosphorylated proteins, we further examined distinct protein kinases in other signal transduction pathways, AKT, ERK1, and ERK2. Expression of wt p53 did not cause a reduction of phosphorylated AKT and ERK1/2 proteins (Fig. 1A). Therefore, the expression of wt p53 specifically reduces phosphorylated Stat3 and inhibits Stat3 DNA binding activity without affecting the phosphorylation of AKT and ERK1/2.

We next examined the inhibition of Stat3 phosphorylation by wt p53 in another breast cancer cell line, SK-BR-3, which also expresses constitutively active Stat3 (14). Similarly, a reduction of phosphorylated Stat3 was observed in cells infected with adeno-virus wt p53 but not with adeno-virus p53-175 or NCV (Fig. 2A). Only cells infected with adeno-virus wt p53 were p53 downstream targets, p21\textsuperscript{WAF1}, Bax, and mdm2 proteins, significantly induced (Fig. 2A). Expression of wt p53 did not decrease phosphorylation-independent Stat3 protein in MDA-MB-468 or SK-BR-3 cell lines (Figs. 1A and 2A), suggesting that the decreased levels of phosphorylated Stat3 is not attributable to the degradation or the reduction of total Stat3 protein. Expression of wt p53 slightly up-regulated the levels of phosphorylated AKT and ERK1/2.

**Results**

Wt p53 but Not Mutant p53 Reduces Stat3 Phosphorylation. Constitutive activation of Stat3 has been reported in breast cancer specimens and cancer cell lines (3, 4). We first examined the potential effect of wt p53 on the phosphorylation of Stat3 in MDA-MB-468
ERK1/2, p53, mdm2, Bax, and p21WAF-1 protein were analyzed as described in the legend.

We next evaluated whether wt p53 could induce apoptosis in SK-BR-3 breast cancer cells. SK-BR-3 cancer cells were infected with NCV, adenovirus wt p53, or adenovirus p53-175 at MOI of 100–200 pfu. In A, 26–30 h after infection, phosphorylated Stat3, phospho-independent Stat3, phosphorylated AKT, phosphorylated ERK1/2, p53, mdm2, Bax, and p21WAF-1 protein were analyzed as described in the legend to Fig. 1. In B, cells were stained with PI and examined for apoptosis with sub-G1 profile analysis using a FACSScan flow cytometer. The results are given as the average and SDs of Log PI from three independent experiments.

These results demonstrate that wt p53 specifically inhibits Stat3 phosphorylation in both breast cancer cell lines expressing constitutively active Stat3.

Wt p53 Induces Apoptosis in Cancer Cell Lines Expressing Constitutively Active Stat3. Overexpression of wt p53 has been shown to induce apoptosis in some human breast cancer cell lines (18). We next evaluated whether wt p53 could induce apoptosis in MDA-MB-468 and SK-BR-3 cancer cell lines that express constitutively active Stat3. Because the overexpression of constitutively active Stat3 may inhibit apoptosis induced by Fas, IFN-γ, and TNF-α (Fas, Interferon-γ, and Tumor Necrosis Factor-α) (7, 8), we predicted that these cancer cell lines may become resistant to apoptosis induced by wt p53. Surprisingly, both breast cancer cell lines were still sensitive to apoptosis induced by adenovirus wt p53 (Figs. 1B and 2B). As a control, infection of these cancer cells with NCV or adenovirus p53-175 demonstrated a minimal induction of apoptosis when compared with uninfected cells (Figs. 1B and 2B). Reduction of phosphorylated Stat3 was concurrent with p53-dependent apoptosis in these cancer cells (Figs. 1A and 2A). Interestingly, the p53-dependent apoptosis occurred regardless of high levels of phosphorylated AKT and phosphorylated ERK1/2 expression in these cancer cell lines (Figs. 1A and 2A). We also observed a similar reduction in the phosphorylation of Stat3 and the induction of apoptosis in MDAH2774 ovarian cancer cells (data not shown).

Wt p53 Inhibits Stat3-dependent Transcription Activity. Because wt p53 reduces Stat3 phosphorylation and DNA binding activity, which may affect its biological activities such as transcription, we further examined whether wt p53 inhibits Stat3-dependent transcription activity in a luciferase assay. Transfection of the Stat3 luciferase reporter, pLuc TKS3, into MDA-MB-468 cells that express constitutively active Stat3 resulted in high levels of luciferase activity (Fig. 3A). Significantly, cotransfection of wt p53 expression vector with pLuc TKS3 inhibited Stat3-dependent luciferase activity (Fig. 3A). In contrast, cotransfection of the expression vector p53-175 or p53-273 that harbors a single amino acid substitution at residues 175 or 273 had no inhibitory effect on Stat3-dependent luciferase activity (Fig. 3A). An inactivating mutation at residue 175 or 273 of p53 frequently occurs in breast cancer (9). To determine the specificity of this inhibition of Stat3-dependent luciferase activity, we examined the effect of wt p53 on two other distinct promoters, SV40 and CMV. The expression of wt p53 or mutant p53 did not have any inhibitory effect on the transcription activity from the SV40 and CMV promoters (Fig. 3, B and C). Together, these results demonstrate that wt p53 reduces phosphorylated Stat3, diminishes Stat3 DNA binding, inhibits Stat3-dependent transcriptional activities, and induces apoptosis in cancer cells expressing constitutively active Stat3.

p53 but Not p21WAF-1 Reduces Stat3 Phosphorylation. We further examined whether the p53 downstream target, p21WAF-1, is involved in the inhibition of Stat3 phosphorylation. MDA-MB-468 cells were infected with adenovirus wt p53 or adenovirus wt p21WAF-1. Only cells infected with adenovirus wt p53 demonstrated a significant induction of mdm2 protein (Fig. 4A). Slightly higher levels of p21WAF-1 protein were expressed in cells infected with adenovirus wt p21WAF-1 as compared with cells infected with adenovirus wt p53 (Fig. 4A). Although wt p53 significantly reduced Stat3 phosphorylation, expression of high levels of p21WAF-1 in cells infected with adenovirus wt p21WAF-1 did not have an effect on Stat3 phosphorylation (Fig. 4A). Expression of wt p53 but not wt p21WAF-1 also induced dramatic apoptotic (Fig. 4B). Therefore, these results suggest that p21WAF-1 is very unlikely to mediate either the inhibition of Stat3 phosphorylation or the induction of apoptosis in these cancer cells. MDA-MB-468 cells have been shown to overexpress EGFR (19), which is an upstream activator of Stat3 (19). We further examined whether p53 inhibits EGFR phosphorylation that may subsequently cause a decrease in Stat3 phosphorylation. Our results demonstrated that wt p53 had little effect on the EGFR phosphorylation in MDA-MB-468 cells (Fig. 4C). Therefore, the reduction of Stat3 phosphorylation is unlikely attributable to the inhibition of EGFR phosphorylation by wt p53 in these cancer cells.

Discussion

Constitutive activation of Stat3 has frequently been detected in breast cancer cells but is absent in normal breast epithelial cells (3, 4). These studies suggest that activated Stat3 may play a role in breast cancer carcinogenesis. Mutation of the p53 tumor suppressor gene is one of the most commonly detected genetic alterations in human cancers, including breast cancer. p53 functions to transcriptionally activate downstream genes to induce G1 cell cycle growth arrest, as well as induction of apoptosis, particularly after DNA damage (10, 11). Our results showed that wt p53 inhibits Stat3 phosphorylation, Stat3 DNA binding, and Stat3-dependent transcriptional activities in breast cancer cells expressing constitutively active Stat3. The molec-
ular mechanism through which wt p53 inhibits Stat3 is currently unclear. Our results demonstrated that wt p53 does not complex with Stat3 in MDA-MB-468 cells (data not shown). Therefore, it is unlikely that direct physical interaction between wt p53 and Stat3 inhibit Stat3 phosphorylation. One p53 downstream target, p21WAF-1, did not affect Stat3 phosphorylation (Fig. 4A). Therefore, other p53-downstream target(s) may be involved in the inhibition of Stat3 phosphorylation and activity. One potential mechanism is that a Tyr phosphatase dephosphorylating Stat3 at residue Tyr705 may be induced directly or indirectly by wt p53, e.g., a protein phosphatase, Wip1, is induced in response to DNA damage in a p53-dependent manner (20). We are currently examining whether a Tyr phosphatase that dephosphorylates Stat3 is induced by wt p53.

Overexpression of oncogene products, such as ras and c-myc, has been shown to stabilize p53 protein and activate p53 activities (21, 22). It may be possible that the constitutively activated Stat3 oncogene product may function similarly to ras or c-myc that it causes the activation of p53 and subsequent induction of growth arrest/apoptosis. p53 may then serve as a safeguard against oncogenic deregulation of
Stat3. Only cancer cells that further mutate p53 or inactivate the p53 pathway may be able to escape p53-dependent apoptosis/growth arrest and be able to continue tumor progression. Therefore, constitutive activation of Stat3 may selectively occur in cancer cells that harbor inactivating mutations or deletions of the p53 gene. This may enable cancer cells to escape inhibition by the wt p53 pathway, particularly after DNA damage. This hypothesis is partially supported by the published reports from others, as well as our laboratories on the status of Stat3 and p53 in breast (MDA-MB-468 and SK-BR-3) and ovarian (MDAH2774, SKOV-3, and Caov-3) cancer cell lines. These reports demonstrated that all of these cancer cell lines express both constitutively active Stat3 and mutant or null p53 (4, 5, 12–14, 23). In contrast, MCF-7 breast cancer cells and A2780 ovarian cancer cells that do not express constitutively active Stat3 still retain endogenous wt p53 (4, 5, 12, 23). Furthermore, p53 mutants that contain hot spot mutations at residues 175 and 273 are unable to inhibit Stat3 phosphorylation or Stat3-dependent transcription activity, which is consistent with this hypothesis (Fig. 3A). Alternatively, it is possible that constitutive activation of Stat3 and inactivation of p53 in breast cancer may be two independent events leading to tumor progression, e.g., certain mutations of p53 could gain new functions that are not shared by wt p53 (24). Gain of functions of p53 mutations may confer growth advantages to tumor cells and be an independent step from the constitutive activation of Stat3. However, additional experiments will be needed to verify these two hypotheses.

Wt p53 induced dramatic apoptosis in both breast cancer cell lines (Figs. 1B and 2B). Interestingly, apoptosis occurred in the presence of phosphorylated AKT and ERK1/2, whereas only phosphorylated Stat3 is down-regulated (Figs. 1 and 2). A similar reduction of phosphorylated Stat3 and induction of apoptosis by wt p53 were also observed in MDAH2774 ovarian cancer cells (data not shown). AKT and possibly ERK1/2 and Stat3 are cell survival pathways that inhibit apoptosis (7, 8, 25–27). These results suggest that inhibition of the AKT survival pathway by wt p53 is unlikely to be involved in p53-dependent apoptosis, at least in MDA-MB-468 and SK-BR-3 cancer cells. It is possible that cancer cells that express constitutively active Stat3 cells may be dependent on Stat3 for survival. These cells undergo dramatic apoptosis when Stat3 is inhibited by a dominant-negative Stat3 (7, 27, 29). However, more experiments will be necessary to test this hypothesis. In summary, our results identified a novel p53-dependent cellular process that plays an important role in the regulation of Stat3 phosphorylation and activity in breast and ovarian cancer cells.

Acknowledgments

We thank Bert Vogelstein at the Johns Hopkins Oncology Center and Tsong Hsieh at the University of Texas Southwestern Medical Center for generously providing the adenovirus p53–175 and p21WAF1–1, respectively. We also thank Richard Jove at the H. Lee Moffitt Cancer Center and Xinhin Chen at the Medical College of Georgia for generously providing the pLuc TKS3 luciferase reporter and p53-HE epitope-tagged expressing vectors, respectively. We also thank Stephen P. Ethier and Rebecca Liu at the University of Michigan for helpful comments of this work and carefully reading of this manuscript, respectively.

References


6. ase reporter and p53-HA epitope-tagged expressing vectors, respectively. We thank Bert Vogelstein at the Johns Hopkins Oncology Center and Tsong Hsieh at the University of Texas Southwestern Medical Center for generously providing the pLuc TKS3 luciferase reporter and p53-HA epitope-tagged expressing vectors, respectively. We thank Stephen P. Ethier and Rebecca Liu at the University of Michigan for helpful comments of this work and carefully reading of this manuscript, respectively.

References


6. ase reporter and p53-HA epitope-tagged expressing vectors, respectively. We thank Bert Vogelstein at the Johns Hopkins Oncology Center and Tsong Hsieh at the University of Texas Southwestern Medical Center for generously providing the pLuc TKS3 luciferase reporter and p53-HA epitope-tagged expressing vectors, respectively. We thank Stephen P. Ethier and Rebecca Liu at the University of Michigan for helpful comments of this work and carefully reading of this manuscript, respectively.

References


6. ase reporter and p53-HA epitope-tagged expressing vectors, respectively. We thank Bert Vogelstein at the Johns Hopkins Oncology Center and Tsong Hsieh at the University of Texas Southwestern Medical Center for generously providing the pLuc TKS3 luciferase reporter and p53-HA epitope-tagged expressing vectors, respectively. We thank Stephen P. Ethier and Rebecca Liu at the University of Michigan for helpful comments of this work and carefully reading of this manuscript, respectively.

References


Announcements

MEETING OF THE RADIATION RESEARCH SOCIETY

The annual meeting of the Radiation Research Society will be held at the State University of Iowa, Iowa City, on June 22–24, 1953. The Society will be the guest of the University, and all meetings will be held on the campus. The program will consist of: (1) Two symposia, one on “The Effects of Radiation on Aqueous Solutions,” which includes the following speakers: E. S. G. Barron, Edwin J. Hart, Warren Garrison, J. L. Magee, and A. O. Allen. The second is “Physical Measurements for Radiobiology” and companion talks by Ugo Fano, Burton J. Moyer, G. Failla, L. D. Marinelli, and Payne S. Harris. (2) On Monday night, June 22, a lecture by Dr. L. W. Alvarez on meson physics has been tentatively scheduled. On Tuesday night, June 23, Dr. L. H. Gray of the Hammersmith Hospital, London, will speak on a topic to be announced. Dr. Gray’s lecture is sponsored by the Iowa Branch of the American Cancer Society. Those desiring to report original research in radiation effects, or interested in attending or desiring additional information, please contact the Secretary of the Society, Dr. A. Edelmann, Biology Department, Brookhaven National Laboratory, Upton, L.I., New York.

ERRATUM

The following correction should be made in the article by Beck and Valentine, “The Aerobic Carbohydrate Metabolism of Leukocytes in Health and Leukemia. I. Glycolysis and Respiration,” November, 1952, page 891; substitute for the last paragraph:

The data in Table 8 permit several interesting calculations. If one compares the amount of glucose actually disappearing with the sum of the amount equivalent to lactic acid produced plus that equivalent to O2 consumption, it is seen that the amount of glucose “cleavage products” exceeds the amount of glucose utilized by 12 per cent in N and 27 per cent in CML and is exceeded by the glucose utilized by 16 per cent in CLL. If the assumption is made that, in this respect, the myeloid and lymphoid cells of leukemia are similar to those of normal blood, it may be that the computed normal figure represents a summation of the myeloid (M) and lymphoid (L) cells that make up the normal leukocyte population. Thus, if M = +0.27 and L = −0.16 and the normal differential is 65 per cent M and 35 per cent L, then

\[ 0.65 (+0.27) + 0.35 (-0.16) = +0.12 \]

a figure identical to the observed +0.12 for normal leukocytes.
Modulation of Signal Transducer and Activator of Transcription 3 Activities by p53 Tumor Suppressor in Breast Cancer Cells

Jiayuh Lin, Xiaohong Jin, Kori Rothman, et al.