Inducible Silencing of KILLER/DR5 In vivo Promotes Bioluminescent Colon Tumor Xenograft Growth and Confers Resistance to Chemotherapeutic Agent 5-Fluorouracil

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ABSTRACT

The candidate tumor suppressor KILLER/DR5 is a DNA damage-inducible p53-regulated death receptor for the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a promising agent for cancer therapy. The majority of studies on KILLER/DR5 have been focused on its role in TRAIL-induced apoptosis. However, its contribution to the inhibition of tumor growth and its role as a determinant of chemosensitivity are poorly understood. In the present study, we have generated stable human colon cancer cell lines, in which the function of KILLER/DR5 was ablated using inducible RNA interference. Inducible silencing of KILLER/DR5 in vivo by exposure of mice to doxycycline led to accelerated growth of bioluminescent tumor xenografts and conferred resistance to the chemotherapeutic agent 5-fluorouracil. Our results suggest that KILLER/DR5 may be a critical determinant for tumorigenicity and chemosensitivity.

INTRODUCTION

The tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a member of the tumor necrosis factor (TNF) family of cytokines capable of triggering apoptosis through engagement of its receptors and induces apoptosis in a wide variety of cancer cells but not most normal cells (1, 2). In 1997, we and others discovered one of the receptors for TRAIL, KILLER/DR5 (3–8), and we identified it as a p53 target gene (4, 9). In addition to its role in TRAIL-induced apoptosis, we also found that KILLER/DR5 was highly induced in vivo in response to γ-irradiation in a tissue-specific manner, suggesting a potential function of this p53 target gene in apoptosis in vivo (10, 11). We recently showed that the KILLER/DR5-mediated type I death receptor pathway contributes to chemosensitization of colon carcinoma cells to TRAIL and that p53-dependent up-regulation of KILLER/DR5 is required for restoration of TRAIL sensitivity to mismatch repair-deficient Bax−/− human colon cancers (12, 13). These studies provided some insights into the mechanism of KILLER/DR5-mediated apoptosis. However, as a candidate tumor suppressor, the contribution of KILLER/DR5 to tumorigenesis remains unclear.

Most chemotherapeutic agents trigger tumor-cell apoptosis through the cell-intrinsic pathways as an indirect consequence of causing cellular damage. Engagement of this pathway usually requires p53 function. Mutations in p53 or in the p53 pathway can produce multiple drug-resistant phenotypes in vitro and in vivo (14). It has been argued that type I cells require the death receptor and mitochondrial pathways for drug-induced apoptosis, whereas type II cells require primarily the mitochondrial pathway (12). Despite knowledge that treatment of tumor cells with some chemotherapeutic drugs can induce KILLER/DR5 expression, the mechanisms underlying the significance of the KILLER/DR5-mediated death receptor pathway in chemotherapeutic drug-induced cell death is not well established.

RNA interference (RNAi), also referred to as post-transcriptional gene silencing, is a powerful tool to disrupt target gene expression. This technology originally arose from the observation that exogenous double-stranded RNAs induce sequence-specific gene silencing in plants (15) and Caenorhabditis elegans (16). The double-stranded RNAs are processed into small interfering RNAs (siRNAs) that mediate suppression of homologous genes (17, 18). RNAi can be used to interfere with gene expression transiently and stably (19, 20). An inducible RNAi system recently has been developed that allows the inducible knockdown of gene expression by siRNA (21). The advantage of inducible RNAs is that the silencing effect often can be triggered under a variety of different conditions and/or at different times and that it may prevent potential lethality of the cells when the target gene is indispensable or the phenotype of the knockdown results in a growth abnormality.

To investigate the importance of the KILLER/DR5 gene in controlling tumor growth and in determining sensitivity to chemotherapy, we have generated stable human colon cancer cell lines in which KILLER/DR5 was silenced or conditionally suppressed. These cell lines simultaneously expressed firefly luciferase, which provides strong and constitutive bioluminescence in the presence of a substrate (luciferin) and ATP. The in vivo bioluminescent imaging of the luciferase activity within xenografted colon tumors, in which the function of KILLER/DR5 has been depleted or conditionally suppressed, revealed that loss of expression of KILLER/DR5 accelerates xenografted colon tumor growth. KILLER/DR5 silencing also confers resistance to the chemotherapeutic agent 5-fluorouracil (FU). These observations suggest that KILLER/DR5 may be a critical determinant for tumorigenesis and chemosensitivity.

MATERIALS AND METHODS

Cell Line and Cell Culture. Parental HCT116 cells were from American Type Culture Collection (Manassas, VA). p53−/− and Bax−/− HCT116 cells were a gift of B. Vogelstein (Johns Hopkins University, Baltimore, MD). Cells were maintained in McCoy’s 5A medium supplemented with 10% fetal bovine serum and antibiotics.

Plasmid Construction. The pSUPER vector was provided by Reuven Agami (The Netherlands Cancer Institute, Amsterdam, The Netherlands). To generate pSUPER-KILLER/DR5, the pSUPER vector was digested with BgII and HindIII, and the annealed oligonucleotides (GATCCCGGCCCATTC-GCTGGTGTCTCCAAGAGAAACGAGACAGCAAGGGTCTTTTTGGAAA and AGCTTTTCAAAAGACCCCTTGTGCTCCGAGTCGTCCTCTCTTGG-AAGACAAAAGAACAGCAGGGTGCGGG) were ligated into the vector. The 19 nucleotide target sequences for KILLER/DR5 are indicated in italics within the oligonucleotide sequence. The pSUPERIOR-puro-vector for inducible expression of siRNA was purchased from OligoEngine (Seattle, WA). To generate pSUPERIOR-puro-KILLER/DR5, the same annealed KILLER/DR5 oligonucleotides were ligated into the pSUPERIOR-puro vector.

The firefly luciferase gene was amplified from pGL3-basic (Promega,
were used in experiments testing inoculation on opposite flanks. At each time
HCT116 cells, 1

ysis of the effect of KILLER/DR5 silencing on the tumorigenic capacity of
containing 10% serum. The number of foci was scored after 2 weeks.

resuspended in 2 mL 0.4% low melting point agarose and seeded, in duplicate,
Miami, FL).

(22). Cell sorting was performed on a Coulter Epics Elite (Beckman-Coulter,
KILLER/DR5 antibody (Imegenix, San Diego, CA) as described previously
KILLER/DR5 cell surface expression was detected by flow cytometry using

additional 2 months. The cells were treated with 1 µL/diloxycycline (Sigma)
for the indicated time and analyzed for the KILLER/DR5 mRNA.

Flow Cytometric Analysis of Cell Death. Cells were harvested after the
indicated treatments and time periods and stained with propidium iodide
and analyzed by flow cytometry for sub-G1 content as described previously
(12). KILLER/DR5 cell surface expression was detected by flow cytometry using
KILLER/DR5 antibody (Imegenix, San Diego, CA) as described previously
(22). Cell sorting was performed on a Coulter Epics Elite (Beckman-Coulter,
Miami, FL).

Agar Assay. In MEM containing 10% serum, 1 × 106 cells were resuspended in 2 mL
0.4% low melting point agarose and seeded, in duplicate, into six-well plates
covered with 1% low-melting point agarose in MEM containing 10% serum. The number of colonies was scored after 2 weeks.

In vivo Bioluminescence Imaging of Tumor Growth in Mice. For anal-
ysis of the effect of KILLER/DR5 silencing on the tumorigenic capacity of
HCT116 cells, 1 × 106 stably transfected HCT116 cells (wild type or
KILLER/DR5 deficient) were injected subcutaneously in each flank of female
athymic nude mice (Charles River Laboratory, Wilmington, MA). Six mice
were used in experiments testing inoculation on opposite flanks. At each time
point, β-luciferin (150 mg/kg body weight; Biotium, Hayward, CA) was
injected intraperitoneally. A bioluminescent image was acquired using the
cooled charge-coupled device camera in the
in vivo
imaging system (Xenogen, Alameda, CA) as described previously
(12). For inducible silencing of
KILLER/DR5 in vivo,
normal drinking water was replaced with 5% sucrose
with 2 mg/mL doxycycline. For prolonged induction, doxycycline-containing
water was changed every 3 days. At the indicated time points, the biolumi-
nescence images were preserved as described previously. Six mice were used for each group (−/− or +/+ doxycycline).

Histopathology. Tumor tissue sections (5 µm) were prepared and stained
with H&E according to standard protocols. For detection of active caspase-3
and Ki67, we incubated the slides overnight at 4°C with dilute primary rabbit
antihuman caspase-3 (active) antibody (AF835; R&D Systems, Minneapolis,
MN) at 1:750 dilution and rabbit anti-Ki67 antibody (NeoMarkers, Fremont,
CA) at 1:200 dilution for 30 minutes. We then incubated the slides with biotinylated goat antirabbit-specific immunoglobu-
lins (Vector Laboratories, Burlingame, CA) at a 1:200 dilution for 30 minutes.
We used hematoxylin as the nuclear counterstain. The apoptotic and prolifer-
ation indices were calculated by visual counting of active caspase-3 antibody-
or Ki67 antibody-staining positive cells per 200 cells counted.

Western Blot Analysis. Western blot analysis was carried out essentially
as described previously
(12) with mouse antihuman p53 monoclonal antibody
(PO-1; Oncogene, San Diego, CA), mouse antihuman Ran antibody (BD
Transduction Laboratories, San Diego, CA), and KILLER/DR5 antibody
(Imegenix).

Northern Blot Analysis. Total RNA was isolated using RNeasy total
miniprep kit (Qiagen, Valencia, CA) following the manufacturer’s instruction.
Northern blot analysis was carried out as described previously
(12). Full length
KILLER/DR5 was used as a probe.

For short hairpin RNA (shRNA) visualization, 30 µg of total RNA
were isolated using TRIzol reagent (Invitrogen) and separated on 15% urea-PAGE
gels (Invitrogen). The RNAs then were transferred to Zeta-Probe membranes
(Bio-Rad, Hercules, CA). Loading was documented by ethidium staining.
KILLER/DR5 oligonucleotides consisting of 19 nucleotides were labeled with
[32P]ATP and used as probes.

Statistical Analysis. All of the experiments were repeated three times.
The data were expressed as mean ± SD. Statistical analysis was performed using
Student’s t test. The criterion for statistical significance used was P < 0.05.

RESULTS

pSUPER Vector-Mediated Suppression of KILLER/D5R. We
initially tested the ability of the pSUPER vector to inhibit KILLER/
DR5 expression in transient experiments. Introduction of the pSUPER
vector resulted in a reproducible reduction of 60 to 80% of KILLER/
DR5 mRNA levels in the Bx−/− HCT116 cells and 293 cells
(Fig. 1A), suggesting that pSUPER vector can effectively knockdown
the endogenous KILLER/DR5 expression. We then used the pSUPER
vector to achieve stable suppression of the endogenous
KILLER/DR5 gene. The results showed that KILLER/DR5 mRNA and protein
expression was markedly suppressed in the stable clones as compared
with control cells, and there was almost no detectable KILLER/DR5
expression in the stable clones even following exposure to the
chemotherapeutic agents CPT-11 or FU (Fig. 1B and C). Introduction of
the pSUPER vector also led to a significant decrease in the KILLER/
DR5 cell surface receptor expression in the absence or presence of
CPT-11 or FU (Fig. 1D). However, CPT-11 or FU treatment did not produce significant effects on the KILLER/DR5 cell surface expression
in the p53−/− cells (Fig. 1D).

Silencing of KILLER/DR5 Accelerates Tumor Progression. To
study the effects of the inhibition of KILLER/DR5 expression on the tumorigenic phenotype of human cancer cells and especially its role in
controlling tumor growth, we performed a soft agar assay and investigat-
tumor xenograft growth in nude mice. Although the pSUPER-
KILLER/DR5 stable clones and the control clones formed colonies,
surprisingly the colony growth of the pSUPER-KILLER/DR5 stable
clones was dramatically accelerated as compared with the control
pSUPER clones (Fig. 2A). We next tested whether ablation of
KILLER/DR5 function in HCT116 cells might affect their ability to
form tumors in vivo. These stable clones were cotransfected with
pcDNA-3.1-Luc encoding the firefly luciferase gene under the control
of the cytomegalovirus promoter to exert strong, constitutive expres-
sion of firefly luciferase and bioluminescence (Fig. 2C). In vivo
bioluminescence imaging of the xenografted tumors indicated that
KILLER/DR5 silencing leads to a significant increase in tumor growth kinetics as compared with wild-type KILLER/DR5-expressing
colon tumors (Fig. 2B). These results suggest that loss of function
of the KILLER/DR5 death receptor accelerates the growth of xenografted colon tumors and that KILLER/DR5 may be involved in
suppressing tumorigenesis in vivo.

pSUPERIOR Vector-Mediated Inducible Silencing of KILLER/
DR5. Most tumor cells undergo multiple genetic alterations, and the
KILLER/DR5 stable clones were subjected to drug selection, which
might cause additional genetic damage. To confirm that the accelerated
tumor growth of the pSUPER-KILLER/DR5 stable clones is caused by
the loss of function of KILLER/DR5 rather than additional genetic hits,
we generated doxycycline-regulated (inducible) KILLER/DR5 silencing
clones and compared the growth of tumor xenografts with the same
genetic background except with the
KILLER/DR5 gene either on or off.
Four clones showed various levels of reduction of KILLER/DR5 expres-
sion after 72 hours of doxycycline exposure (Fig. 3A). To determine the
efficiency of the inducible siRNA system, we performed Northern blot
analysis to visualize the production of the hairpin RNAs in clones 1, 4,
and 5. The hairpin RNAs were produced within 6 hours of the addition
of doxycycline and increased thereafter in a time-dependent manner

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clones 1 and 5 (Fig. 3B). However, in clone 4, the Tet repressor could not block the H-1 promoter, and hairpin RNAs appeared in the absence of doxycycline. In clones 1 and 5, KILLER/DR5 mRNA expression was dramatically decreased within 48 hours of doxycycline induction, and the increased expression of the hairpin RNAs correlated with the decreased expression of the KILLER/DR5 mRNA on doxycycline treatment (Fig. 3B). We next tested the effects of doxycycline withdrawal on KILLER/DR5 knockdown in clones 1 and 5. The results indicated that doxycycline withdrawal led to increased expression of KILLER/DR5 mRNA and suppressed expression of hairpin RNAs (Fig. 3C), suggesting that the doxycycline-induced suppression of KILLER/DR5 was reversible. Therefore, these results indicate that KILLER/DR5 suppression is inducible, reversible, and tightly regulated.

**Inducible Silencing of KILLER/DR5 In vivo Promotes Tumor Growth.** We next investigated whether KILLER/DR5 expression can be conditionally suppressed in vivo and whether inducible knockdown of...
KILLER/DR5 might affect colon tumor xenograft growth in nude mice. The mice bearing bioluminescent pSUPERIOR-KILLER/DR5 xenografted tumors were either induced by administration of 2 mg/mL doxycycline in their drinking water or were left untreated. In vivo bioluminescence imaging analysis showed that the bioluminescent tumor xenografts exhibit accelerated growth rate in the doxycycline-treated mice as compared with control mice (Fig. 4A). Histopathologic studies performed on the xenografted colon tumors on day 30 after inoculation showed marked differences between doxycycline-treated and -untreated tumors. Doxycycline-treated tumors showed significantly higher proliferation as measured by H&E and Ki67 staining, but fewer apoptotic cells as revealed by the active caspase-3 antibody staining, as compared with doxycycline-untreated tumors (Fig. 4B). To determine whether KILLER/DR5 expression within xenografted tumors was inhibited in doxycycline-treated mice, we isolated total RNA and protein from the tumors treated with or without doxycycline for 30 days. The results showed that doxycycline induction results in dramatically suppressed expression of KILLER/DR5 and that conversely the hairpin RNA expression is remarkably induced in doxycycline-treated tumors (Fig. 4B). These observations imply that the knockdown of KILLER/DR5 is highly associated with accelerated colon tumor xenograft growth. Collectively with the data shown in Fig. 2, these results strongly suggest that, as a candidate tumor suppressor gene, KILLER/DR5 may be involved in the control of tumor progression.
Effects of KILLER/DR5 Silencing on TRAIL Sensitivity. KILLER/DR5 is a cell membrane-localized death receptor for TRAIL. We next determined the effects of KILLER/DR5 silencing on TRAIL sensitivity. The results showed that knockdown of KILLER/DR5 did not produce a significant effect on TRAIL sensitivity and that TRAIL could effectively cause apoptosis in KILLER/DR5-deficient cells with similar efficiency as it does in wild-type or p53/H11002/H11002/H11002 HCT116 cells, which is presumably because of the presence of the second proapoptotic TRAIL receptor DR4 (Fig. 5A). However, Bax/H11002/H11002/H11002 HCT116 cells were resistant to TRAIL treatment (Fig. 5A), which is consistent with earlier observations (12, 23). We previously have shown that KILLER/DR5 silencing by synthetic RNAi duplexes in Bax/H11002/H11002/H11002 human colon cancer cells significantly inhibited TRAIL sensitivity after pretreatment of the cells with chemotherapy, suggesting that KILLER/DR5 up-regulation is required for the chemosensitization of colonic carcinomas to TRAIL. To study whether the pSUPER vector has similar effects with the synthetic RNAi duplexes, we generated KILLER/DR5 stably silenced clones in Bax/H11002/H11002/H11002 HCT116 cells. Northern blot analysis showed that the KILLER/DR5 mRNA expression was profoundly inhibited in KILLER/DR5 silencing clones even in the presence of chemotherapy for 16 hours (Fig. 5B). KILLER/DR5 stable silencing abolished the apoptosis induced by TRAIL plus either CPT-11 or etoposide (Fig. 5B), which is consistent with earlier observations (12). FU pretreatment also restored TRAIL sensitivity in Bax/H11002/H11002/H11002 cells (data not shown). These results imply that ablation of KILLER/DR5 function did not produce apparent effects on TRAIL sensitivity but abrogated the chemosensitization of colonic carcinomas with mitochondrial apoptotic defects to TRAIL treatment.

Fig. 4. Inducible silencing of KILLER/DR5 in vivo accelerates tumor xenografts growth. A, The mice carrying inducible KILLER/DR5 gene-silencing clone 5 were administered with or without doxycycline. Bioluminescent images were acquired at the indicated time points. Six mice were used for each group. B, The tumor tissues were harvested at day 30 from the mice treated with or without doxycycline. RNAs isolated from the tissues were subjected to Northern blot analyses for KILLER/DR5 mRNA and shRNA expression. KILLER/DR5 protein expression was determined by Western blot analysis. Tissue sections were analyzed using H&E, active caspase-3 antibody, and Ki67 antibody staining. Two hundred cells were counted and scored for the index of apoptosis or proliferation. Results shown are mean ± SD.*p < 0.05.
Effects of KILLER/DR5 Silencing on Chemosensitivity. The aforementioned observations promoted us to investigate the effects of KILLER/DR5 knockdown on the sensitivity of cells to various chemotherapeutic drugs. The results indicated that loss of function of KILLER/DR5 led to decreased sensitivity to FU with nearly 50% less killing efficiency as compared with wild-type cells. However, p53/H11002/H11002/H11002 HCT116 cells were completely resistant to FU, and Bax deficiency also conferred a dramatic resistance to FU (Fig. 6), implying that FU-induced apoptosis is p53 dependent. These data suggest that KILLER/DR5 may be an important determining factor for promoting sensitivity to chemotherapy, whereas the mitochondrial apoptotic pathway may be ultimately required for chemotherapy-induced apoptosis.

DISCUSSION

KILLER/DR5 mutations have been described in head and neck, lung, and breast cancer. A truncating mutation in the death domain was found in head and neck cancer, and a number of mutations in the death domain were observed in lung cancer, leading to loss of function (24–27). These observations support the notion that KILLER/DR5 may be a candidate tumor suppressor gene. Preclinical studies using TRAIL have provided in vivo evidence for exogenous recombinant TRAIL efficacy in suppressing tumor growth (2). TRAIL also plays a role in T-cell– and natural killer cell–mediated tumor surveillance and suppression of tumor metastasis (1, 12, 28) and mediates thymocyte apoptosis to prevent autoimmune diseases (29). In this study, we generated KILLER/DR5 stable or inducible gene-silencing cell lines using RNAi and found that inactivation of KILLER/DR5 function significantly accelerates colon tumor growth (Figs. 2 and 4). Therefore, our results provide further evidence to support a role for KILLER/DR5 as a candidate tumor suppressor gene. Despite the evidence presented here, it remains open why ablation of KILLER/DR5 function promotes tumor growth. Given the potentially unique role of KILLER/DR5 in mediating p53-dependent death in vivo (10), it is possible that in some cases loss of KILLER/DR5 may substitute for loss of p53 or at least may increase a tumor cell’s tolerance to the tumor-suppressing effect of wild-type p53. It also is highly possible that KILLER/DR5 silencing may lead to decreased sensitivity to apoptosis induced by autosecreted antigen-specific signals, and the inactivation of the KILLER/DR5 death receptor pathway could allow escape from immune responses and provide a survival advantage of the tumor cells. However, additional experiments are needed to further investigate these possibilities.

In the present study, we showed that silencing of KILLER/DR5 did not significantly affect the sensitivity to TRAIL, presumably because of the second proapoptotic TRAIL receptor DR4, but confers resistance to the chemotherapeutic agent FU, suggesting that KILLER/DR5 is a vital determinant for chemosensitivity (Figs. 5 and 6). FU fails to kill p53-deficient HCT116 cells, indicating an absolute requirement for p53 in FU-induced cell death. The mechanisms underlying the loss of FU-induced apoptosis are complex and involve the mitochondria, which is a common effect of chemotherapeutic agents. The inactivation of the KILLER/DR5 death receptor pathway could allow escape from immune responses and provide a survival advantage of the tumor cells. However, additional experiments are needed to further investigate these possibilities.
of KILLER/DR5 function conferring resistance to FU-induced apoptosis currently are unknown. Given the ability of p53 to transactivate KILLER/DR5, it is possible that KILLER/DR5 silencing may alleviate p53-dependent apoptosis induced by chemotherapeutic agents. It also is possible that some drugs that kill tumor cells independent of p53 can target KILLER/DR5 directly and lead to its activation directly or affect its trafficking to the cell surface. It recently has been shown that Bax and Bak in mouse hepatocytes were necessary, although mutually redundant, for death receptor engagement of mitochondria (30). However, our data indicate that Bax \textsuperscript{−/−} / human HCT116 cells are resistant to chemotherapeutic agent FU-induced apoptosis despite the expression of Bak, suggesting that Bax is a critical factor for chemotherapy-induced apoptosis and that Bax and Bak proteins do not have overlapping function in human colon cancer cells. This is consistent with our earlier observation (12).

In summary, our study reveals that loss of function of KILLER/DR5 promotes colon tumor xenograft growth and confers resistance to chemotherapy-induced apoptosis, suggesting that KILLER/DR5 may be a pivotal determinant for tumorigenesis and chemosensitivity. Therefore, the present studies not only provide further insight into the mechanisms of KILLER/DR5-mediated tumor suppression and its contribution to drug-induced apoptosis but also, importantly, identify KILLER/DR5 as a promising therapeutic target for the management of colorectal cancers. More recently, a large-scale chemical genetic screening has identified some small molecules that specifically target p53 or other molecules for therapeutic development against tumors (31–33). Given the functional significance of KILLER/DR5, it is necessary to perform such screening to identify compounds that specifically kill tumor cells through activation of the KILLER/DR5-mediated apoptotic pathway, and this strategy is expected to impact on anticancer design in the future.

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