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Loss of putative tumor suppressor EI24/PIG8 confers resistance to etoposide

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Abstract

Expression of p53-target gene EI24/PIG8 is lost in invasive breast cancers, suggesting that EI24/PIG8 is a tumor suppressor that prevents tumor spreading, and partially mediates p53-attributed tumor suppressor activity. EI24/PIG8 also has proapoptotic activity indicating that loss of EI24/PIG8 may modulate sensitivity to chemotherapy. Here it is demonstrated that suppression of EI24/PIG8 in fibroblasts and breast cancer cells significantly inhibits the apoptotic response to etoposide treatment. These findings suggest that loss of EI24/PIG8 contributes significantly to resistance of cells to chemotherapeutic agents that function through p53, and identify the EI24/PIG8 status as a potentially new prognostic marker of chemotherapy responsiveness.

Keywords

breast cancer; chemotherapy resistance; tumor development; tumor suppressor; apoptosis

1. Introduction

EI24 (etoposide-induced 2.4 kb transcript) was first isolated by differential display in 3T3 fibroblasts as an etoposide-induced gene associated with apoptosis by this drug [1]. Etoposide is a chemotherapeutic agent which inhibits topoisomerase II and is often used in combination regimens to treat breast cancer and other carcinomas [2]. The human *PIG8* gene (p53 induced gene 8) was discovered in screens of approximately 7200 mRNA transcripts as one of fourteen genes upregulated by restoring p53-dependent apoptotic activity in a p53-null cell line [3]. Murine *EI24* and human *PIG8* sequences are 98% homologous [4]. Murine *EI24* is also 52% similar to an open reading-frame found in *Caenorhabditis elegans* suggesting an important biological role for this highly-conserved gene [4].

Invasive cervical cancers and breast carcinomas frequently exhibit loss of heterozygosity at 11q23, the chromosomal location of the *PIG8* gene [5;6;7;8;9]. Among four genes examined at this location in 41 aggressive breast tumors, *EI24/PIG8* was the most often altered (by mutation, aberrant splicing, or deletion) [10]. Reduced EI24/PIG8 protein expression has also

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been documented in invasive ductal carcinomas, but not carcinoma *in situ* suggesting that EI24/PIG8 serves to prevent tumor invasion [11].

Studies to date have suggested a wide range of possible roles for EI24/PIG8 in apoptosis, some of which may possibly explain the correlation between *EI24/PIG8* status and tumor invasiveness. EI24/PIG8 is regulated by p53, a critical transcription factor that is a central mediator of the apoptotic response to DNA-damaging agents. EI24/PIG8 also causes growth arrest and apoptosis when ectopically over-expressed [4] and conversely depletion of EI24/PIG8 by antisense oligodeoxynucleotides results in suppression of apoptosis in response to the proapoptotic retinoid, CD437/AHPN [12]. p53 binds specifically to a p53-response element in the mouse gene promoter although the human EI24/PIG8 promoter has not yet been characterized. These data suggest that EI24/PIG8 functions downstream of p53, and that some of the apoptotic potential of p53 is transduced through EI24/PIG8. Importantly, loss of EI24/PIG8 in tumor cells may have additional adverse consequences for the host, because the efficacy of etoposide and other chemotherapeutic drugs is at least partially dependent on a p53-dependent apoptotic response [13;14;15].

We tested a potential role for EI24/PIG8 in modulating sensitivity to etoposide as a representative DNA damage-inducing agent used in chemotherapy, to assess the possibility of an important effector role for *EI24/PIG8* as a p53-regulated proapoptotic gene [1;16]. Our findings suggest a novel role for EI24/PIG8 in the clinically important phenomenon of chemotherapy resistance.

2. Material and Methods

Cell Culture and siRNA Transfections

NIH 3T3 murine fibroblasts were grown in DMEM with 10% (v/v) donor calf serum, and MCF-7 human breast cancer cells were grown in DMEM with10% (v/v) fetal bovine serum, at 37°C in humidified air with 5% CO_2 Cells were transfected with Lipofectamine Transfection Reagent and allowed to recover for 24 hrs before further treatment. Fluorescein-tagged siRNA oligo was obtained from Qiagen. Scrambled control and predesigned EI24/PIG8 siRNA oligos were purchased from Ambion with the sequence 5′ CCACGUAUUGUUAGUAGAAtt 3′ (murine EI24 siRNA) and 5′ GGAAUCAAAGACUCCAUCUtt 3′ (human PIG8 siRNA). Etoposide (Sigma) was prepared as a DMSO solution for each experiment.

Flow Cytometry

Cell cycle distribution and apoptosis were analyzed 24 hrs after treatment with etoposide (0.4 mM). Cells permeabilized in 70% ethanol and stained with propidium iodide (PI) (25 μg/ml) and RNase A (50 μg/ml) were analyzed by flow cytometry, collecting ≥ 20,000 cells/sample. Apoptotic cells were identified as a distinct sub-G_1 peak in logarithmic plots of PI fluorescence intensity generated using Cellquest Pro software.

Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling (TUNEL) Reaction

Cells were grown on Lab-Tek chamber slides, transfected with control siRNA or siEI24/PIG8 oligo, and treated with DMSO or etoposide (0.4 mM). Apoptosis was assessed using the TMR red *in situ* cell death detection kit (Roche). Briefly, cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate, and DNA strand breaks were labeled by terminal deoxynucleotidyl transferase. Slides were mounted with DAPI and DabCo anti-fade solution and observed using a Nikon Eclipse E800 microscope, digital camera, and Metasystems FISH software. The total number of cells in each field of view was recorded based on DAPI positive (blue) nuclei. The fraction of these that were TUNEL positive (red) was then

determined. Cells in at least five randomly selected fields of view were counted for each condition.

Real Time PCR

RNA was harvested from cells 24 hrs after exposure to etoposide (0.4 mM) and cDNA was synthesized using an Invitrogen Thermoscript Kit according to the manufacturer's instructions. SYBR Green master mix was used to quantitate product using Applied Biosystem's 7500 Fast Real-Time PCR system. The murine β-actin primers used as an internal control were: 5′ AGCCATGTACGTAGCCATCC 3′ and 5′ CTCTCAGCTGTGGTGGTGAA 3′. The murine EI24 primers spanning exons 9–10 were: 5′ TCTCTTCCCCATCCATCTT 3′ and 5′ TAACGTAACGACACTCCTTTC 3′. Human β-actin primers used as an internal control were: 5′ TCCCTGGAGAAGAGCTACGA 3′ and 5′ AGCACTGTGTTGGCGTACAG 3′. The human PIG8 primers were: 5′ CGGTAA CAGCCCGAATTATC 3′ and 5′ GGCTTCCTCCCTGATACCTC 3′.

3. Results

Knockdown of EI24/PIG8 suppresses etoposide-induced apoptosis in NIH 3T3 cells

To test the role of EI24/PIG8 in chemotherapy resistance, we used NIH 3T3 cells, the line in which EI24/PIG8 was originally discovered as a p53-regulated, apoptosis-associated gene. To determine whether loss of EI24/PIG8 affected the capacity of the cells to undergo apoptosis in response to etoposide, an siRNA targeting E124/PIG8, or control siRNA, was transfected into vehicle control (DMSO)- and etoposide-treated cells. The EI24/PIG8-specific siRNA (siEI24/PIG8) significantly and specifically suppressed EI24/PIG8 mRNA levels, compared to cells transfected with a control siRNA, as assayed by quantitative (q)RT PCR (Figure 1A). Flow cytometry was then used to measure the fraction of apoptotic cells by determining the percentage of cells with sub-G1 DNA content. As expected, treatment with etoposide induced apoptosis in cells transfected with control siRNA, compared with similarly transfected cells treated with vehicle (DMSO) alone (Figure 1B and 1C). In contrast, no induction of apoptosis was observed in those cells transfected with siEI24/PIG8 compared to treatment with DMSO. While a slight increase in baseline levels of apoptosis was observed between siRNA control- and siEI24/PIG8-transfected cells, likely due to nonspecific RNAi-associated toxicity, cells in which EI24/PIG8 mRNA levels had been knocked down were clearly protected from etoposide-induced apoptosis, with no significant differences in the percentage of cells exhibiting sub-G1 levels of DNA content between DMSO and etoposide-treated cells (35.9% and 36.6%).

To confirm that NIH 3T3 cells lacking EI24/PIG8 were less sensitive to etoposide, the TUNEL reaction, which detects double-stranded DNA breaks and nicks, specifically identifying apoptotic cells, was employed. Significantly fewer tetramethylrhodamine (TMR) red-positive cells were present after etoposide treatment of cell cultures in which EI24/PIG8 levels had been suppressed by siRNA, compared with cells transfected with control siRNA. Approximately 12.6% of control siRNA-transfected cells treated with etoposide underwent apoptosis, compared to fewer than 1% of cells treated with DMSO (Figure 2). In contrast, only 2.7% of siEI24/PIG8-transfected cells treated with etoposide underwent apoptosis with similar basal levels of apoptosis (<1%) in DMSO-treated cells. Thus, etoposide-dependent apoptosis was reduced almost 5-fold in cells in which EI24/PIG8 mRNA levels were effectively suppressed.

MCF-7 breast cancer cells express EI24/PIG8 protein

Next, we wished to assess the relevance of EI24/PIG8 to chemotherapy resistance in p53-competent MCF-7 human breast cancer cells since loss of EI24/PIG8 has been established in this type of cancer. In addition, it needs to be established whether EI24/PIG8 serves the same

function in malignant cells. However, before initiating these experiments it was first necessary to ascertain that EI24/PIG8 is expressed in MCF-7 cells. For this purpose, MCF-7 cells were grown on chamber slides, fixed, blocked, and stained with the EI24/PIG8 antibody, as previously described [11]. Incubation with a fluorescein isothyocyanate (FITC)-conjugated secondary antibody allowed detection of EI24/PIG8 protein in cells by fluorescence microscopy. MCF-7 cells were found to express etoposide-inducible levels of cytoplasmic EI24/PIG8 (Figure 3A), consistent with its reported presence in the endoplasmic reticulum [11]. Additionally, sequence analysis showed no mutations in EI24/PIG8 cDNA cloned from these cells (data not shown).

Suppression of EI24/PIG8 mRNA levels inhibits etoposide-induced apoptosis in MCF-7 cells

Analysis of EI24/PIG8 transcript levels by qRT PCR showed that transfection of siEI24/PIG8 (optimally designed for human EI24/PIG8 transcripts) strongly suppressed etoposide-dependent induction of EI24/PIG8 mRNA by approximately 5-fold compared to levels in control siRNA-transfected cells (Figure 3B).

TUNEL assays were performed in control siRNA-transfected cells and in EI24/PIG8-depleted cells, in the absence or presence of etoposide. As shown in Figures 3C and 3D, 32% of control siRNA-transfected cells treated with etoposide underwent apoptosis, up from basal levels of 1.5% in the presence of DMSO. In contrast, only 10.5% of siEI24/PIG8-transfected cells treated with etoposide underwent apoptosis, a percentage essentially unchanged from the basal levels in cells treated with DMSO. Thus, similar to our findings in 3T3 fibroblasts, we observed a significant, 3-fold reduction in the percentage of apoptotic cells in etoposide-treated cell populations in which EI24/PIG8 levels had been suppressed.

4. Discussion

EI24/PIG8, a recently-discovered, p53-induced proapoptotic factor, is frequently lost in aggressive breast cancer and appears to play a role in prevention of tumor spread in invasive breast tumors. of EI24/PIG8 may also have additional adverse clinical consequences. We show in this report that suppression of EI24/PIG8 in murine fibroblasts and human breast cancer cells significantly inhibits etoposide-induced apoptosis. Therefore, EI24/PIG8 executes its p53-controlled apoptotic function in a species- and cell line-independent manner, in both benign and malignant cells. Although we have not yet fully elucidated the apoptotic molecular pathways controlled by EI24/PIG8, which appear to be initiated at the endoplasmic reticulum [11], the results of our studies clearly support an important role for EI24/PIG8 in susceptibility to genotoxic chemotherapy. Although many factors play important pro- and anti-apoptotic roles [17], and development of chemoresistance is a complex and multifactorial process [18], the EI24/PIG8 status of tumor cells deserves to be further analyzed as a possible prognostic factor for responsiveness to cancer treatments that utilize p53-dependent apoptosis pathways. Unpublished studies from our laboratory confirmed that the actions of EI24/PIG8 in transducing apoptotic signals is restricted to chemotherapeutic agents which act through p53, as apoptosis induced by staurosporine, which is independent of p53, was not ameliorated by suppression of EI24/PIG8 (data not shown). Screening patients for the presence of EI24/PIG8 may facilitate tailoring of unique chemotherapy drug regiments for individual patients.

Future studies are needed to examine the specific molecular mechanisms by which EI24/PIG8 mediates p53-dependent apoptosis. It will also be important to determine if suppression of EI24/PIG8 mediates sensitivity to other chemotherapeutic agents, particularly stimuli that act at discrete steps in the apoptotic pathway. Such studies would generalize the importance of EI24/PIG8 beyond the actions of etoposide alone, and could serve to pinpoint the location of EI24/PIG8 activity in the apoptosis pathway.

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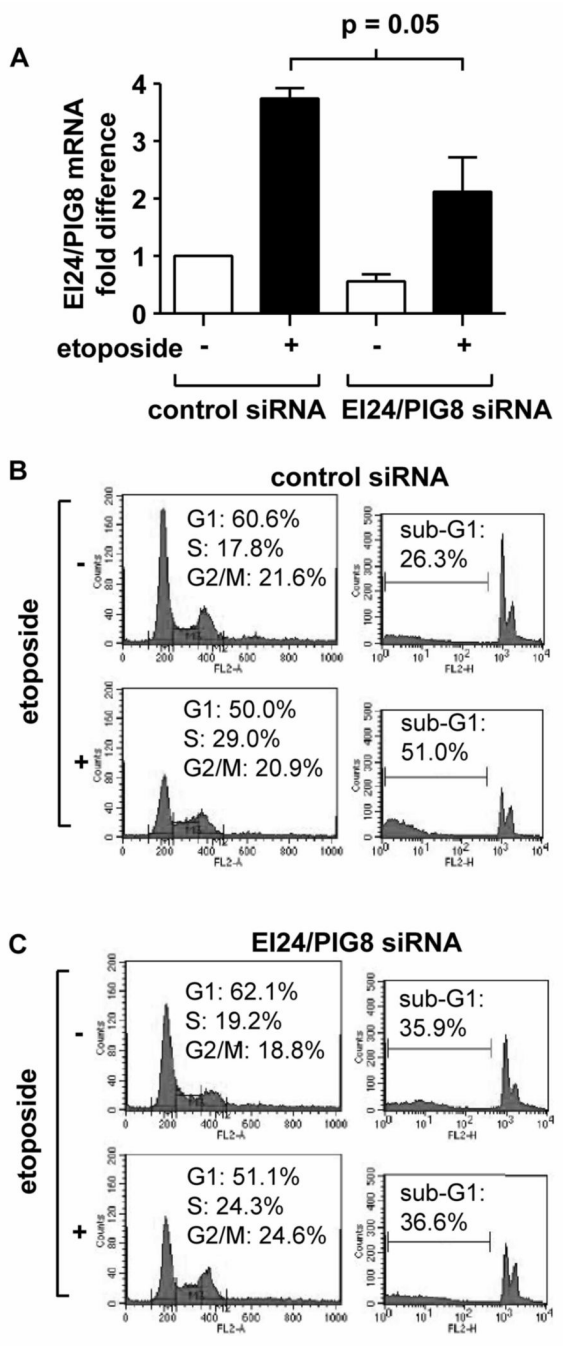

Figure 1. Suppression of EI24/PIG8 induction in NIH 3T3 cells by siRNA reduces their sensitivity to etoposide

siRNA targeting EI24/PIG8 mRNA (siEI24/PIG8), or negative control siRNA, and fluorescein-tagged siRNA were transfected into NIH 3T3 cells (10:1 ratio). The fluorescein signal was clearly visible inside the cells after transfection. After 24 hrs transfected cells were treated for an additional 24 hrs with etoposide (0.4 mM) or DMSO (vehicle). Cells were analyzed by two methods, qRT PCR (**A**) and flow cytometry (**B** and **C**). (**A**) RNA was harvested, cDNA was synthesized and qRT PCR, using EI24/PIG8-specific primers, was used to quantify the amount of EI24/PIG8 mRNA. mRNA expression was measured relative to an internal control (endogenous β-actin) and reported as relative values normalized to control

siRNA/DMSO-treated sample. Data are expressed as the mean +/− SEM ($n \geq 3$). p values were calculated using Student's t test. (**B** and **C**) PI-staining of nuclear DNA was used to examine cell cycle profiles. To facilitate quantification of relative fractions of cells in G1 (M1), S (M3), or G2/M (M2) phases, cells were assayed on a linear fluorescence scale, while sub-G1 (apoptotic) populations were assayed on a logarithmic scale. The results from one of two independent experiments yielding equivalent outcomes are shown.

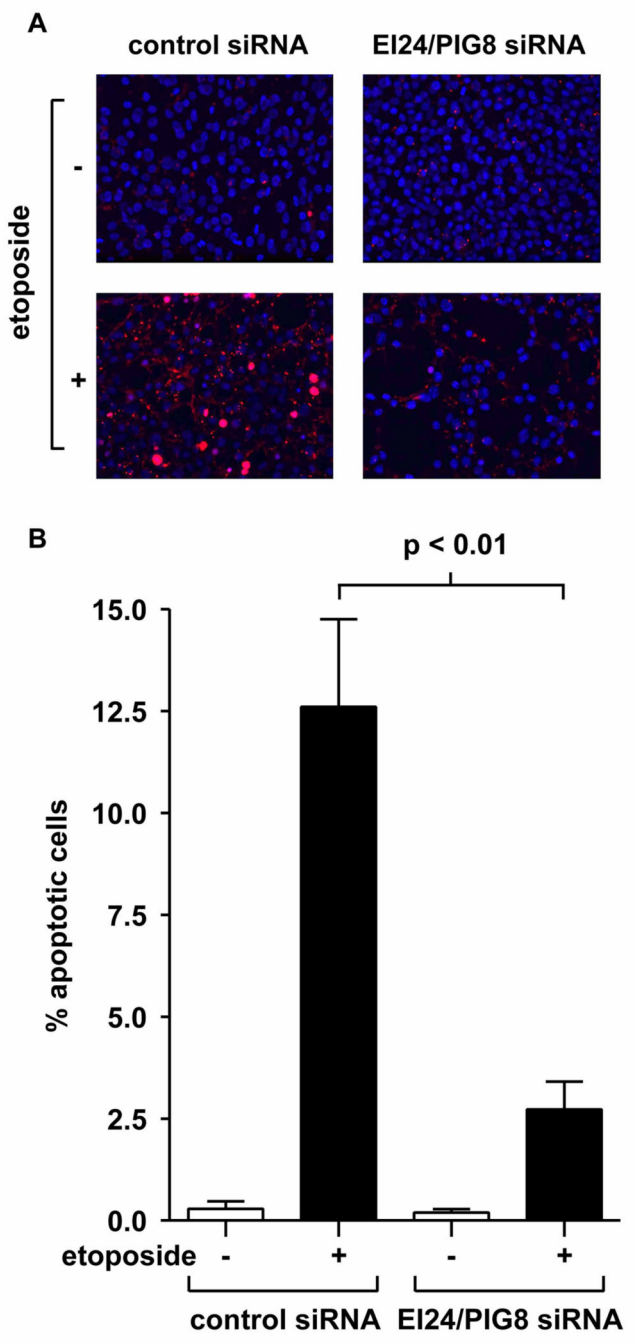

Figure 2. Suppression of EI24/PIG8 induction in NIH 3T3 cells reduces their sensitivity to etoposide as assayed by TUNEL reactivity

Cells transfected with EI24/PIG8 siRNA or a negative-control siRNA were incubated for 24 hrs, with or without etoposide, and stained for DNA breaks. TMR-red (TUNEL)-positive cells were observed only in the populations transfected with control siRNA and treated with etoposide (0.4 mM). (**A**) Representative experiment showing apoptotic effects of etoposide on normal and EI24/PIG8-depleted cells. (**B**) Quantification of multiple fields of view and demonstrated statistical significance. On average, over 1,000 DAPI-positive cells were counted for each reaction condition. Data are expressed as the mean +/− calculated SEM (average

number of cells counted for each condition = 1338). p values were calculated using Student's t test.

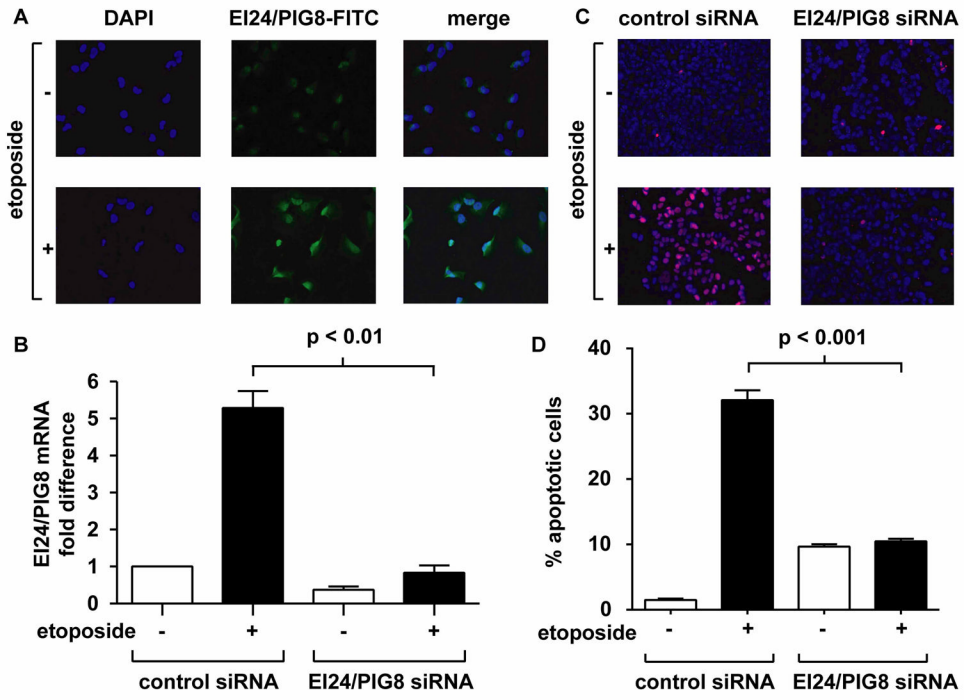

Figure 3. Suppression of EI24/PIG8 in MCF-7 cells results in reduced sensitivity to etoposide

(**A**) MCF-7 cells express etoposide-inducible EI24/PIG8. Cells were grown on chamber slides and treated with DMSO (vehicle control) or etoposide (0.4 mM) for 24 hrs. Cells were fixed, blocked with 5% BSA in PBS, and stained with anti-EI24/PIG8 antibody overnight prior to application of a FITC-conjugated antibody for visualization. Etoposide, but not DMSO vehicle control, induces EI24/PIG8, as detected by immunofluoresence. (**B**) siRNA targeting EI24/PIG8 mRNA or negative control siRNA, again with fluorescein-tagged siRNA of irrelevant sequence (10:1 ratio), were cotransfected into MCF-7 cells and incubated for 24 hrs with or without etoposide (0.4 mM). As before, qRT PCR analysis shows that EI24/PIG8 siRNA specifically and effectively suppressed EI24/PIG8 induction. (**C**) Increased numbers of TMR-red (TUNEL)-positive cells were only observed only in the populations transfected with negative control siRNA that were treated with etoposide. (**D**) Quantification of multiple frames confirms the visual images. Data are expressed as the mean +/− calculated SEM (average number of cells counted for each condition = 2228). p values were calculated using Student's t test.