SIRT6 modulates paclitaxel and epirubicin resistance and survival in breast cancer

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In this study, we report the identification of a novel role of SIRT6 in both epirubicin and paclitaxel resistance in breast cancer. We found that SIRT6 protein levels are elevated in paclitaxel- and epirubicin-resistant MCF-7 cells compared with the parental sensitive cells. SIRT6 knockout and depletion sensitized cells to both paclitaxel and epirubicin treatment, whereas SIRT6 ectopic overexpression led to increased resistance to paclitaxel and epirubicin. Moreover, our data suggest that SIRT6 could be mediating epirubicin resistance through enhancing the DNA repair response to epirubicin-induced DNA damage. Clonogenic assays also revealed that mouse embryonic fibroblasts (MEFs) lacking SIRT6 have decreased long-term viability in response to epirubicin. The tumour suppressor FOXO3a increases its levels of acetylation in MEFs depleted of SIRT6, whereas its induction by epirubicin is attenuated in breast cancer cells overexpressing SIRT6. Further cell viability studies demonstrate that deletion of FOXO1/3 in MEFs can confer sensitivity to both paclitaxel and epirubicin, suggesting that SIRT6 reduces paclitaxel and epirubicin sensitivity, at least in part, through modulating FOXO acetylation and expression. Consistently, immunohistochemical analysis of 118 breast cancer patient samples revealed that high SIRT6 nuclear staining is significantly associated with poorer overall survival (P = 0.018; Kaplan–Meier analysis). Multivariate Cox analysis demonstrated that nuclear SIRT6 staining remained associated with death after correcting for tumour stage and lymph-node involvement (P = 0.033). Collectively, our data suggest that SIRT6 has a role in paclitaxel and epirubicin sensitivity via targeting FOXO proteins and that SIRT6 could be a useful biomarker and therapeutic target for paclitaxel- and epirubicin-resistant cancer.

Introduction

Aside from non-melanoma skin cancer, breast cancer is the most common women’s cancer in developed countries and one of the leading causes of cancer death among women of all races. Anthracyclines, including epirubicin and doxorubicin, and taxanes, including paclitaxel (Taxol) and docetaxel (Taxotere), are the most active and widely used chemotherapeutic agents for treating breast cancer in hormone receptor-negative patients and those who do not respond to hormone therapy (1,2). These agents are particularly important in the treatment of advanced or metastatic breast cancer, as they sometimes represent the sole treatment or management option. Treatment of metastatic breast cancer with anthracyclines and taxanes typically results in response rates of 30–70%, but responses often are not durable due to the development of resistance to one or both drugs (3,4). Drug resistance, whether intrinsic or acquired during treatment, is believed to cause treatment failure in over 90% of patients with metastatic cancer (5). Once resistance to anthracyclines and taxanes occur, few treatment options exist. These patients are usually treated with agents like capecitabine, gemcitabine and vinorelbine, showing low response rates and limited survival (6,7). Understanding of the molecular mechanisms underlying taxane and anthracycline resistance is thus crucial for the development of effective chemotherapeutic strategies for treatment of breast cancer and other cancers.

Taxanes and anthracyclines act through very different mechanisms. Paclitaxel binds to β-tubulin preventing the depolymerization, causing the dynamic instability of microtubules (8). The main cellular consequence of its action is the inhibition of the mitotic spindle dynamics, leading to a transient block at the transition between G2 and M phase and consequent cell death (9). Experimental evidence from in vitro models has suggested different mechanisms by which tumour cells can develop resistance to taxanes, including taxane exclusion from the cell by adenosine triphosphate-binding cassette transporters, the expression of certain tubulin isoforms and microtubule-associated proteins, tubulin gene mutations, and alterations in the survival or mitotic checkpoint signalling (10). The principal mechanism of action of anthracyclines is its ability to intercalate into DNA, binding to DNA topoisomerase II and inducing DNA cleavage in an adenosine triphosphate-dependent manner (11). Anthracyclines also induce the formation of reactive oxygen species, which contributes to the cytotoxic activity of these drugs (12). Thus, the cellular DNA damage response that includes initiation of DNA damage repair, cell cycle checkpoint activation and induction of apoptosis or senescence is a key determinant of the efficacy of genotoxic therapy. It has also been proposed that drug transport proteins, antioxidant defences and topoisomerase modulation may mediate anthracycline resistance (10).

Sirtuins constitute a family of NAD+-dependent deacetylases that are highly conserved from lower organisms to humans. In mammals, there are seven different sirtuins, SIRT1–7, that are involved in the regulation of critical biological processes, including metabolism, cell division, differentiation, survival and senescence (13). Sirtuin family members have been proposed to play a role in the development of cancer and drug resistance. SIRT1, the most extensively studied sirtuin, is upregulated in a wide spectrum of cancers, including non-melanoma skin cancers (14), lung (15), prostate (16) and pancreatic cancer (17), hepatocarcinoma (18) and leukaemia (19). Increased SIRT1 expression has also been reported in chemoresistant neuroblastoma, osteosarcoma, prostate, ovarian and breast cancer cells when compared with their drug-sensitive counterparts (20,21). Interestingly, small interfering RNA (siRNA)-mediated downregulation of SIRT1 has been shown to partially reverse the resistance phenotype of cancer cells and reduce the expression of the multidrug resistance molecule P-glycoprotein. Conversely, ectopic overexpression of SIRT1 induces the expression of P-glycoprotein and renders cells resistant to doxorubicin (20).

SIRT6 is a chromatin-associated nuclear protein that shows both deacetylase and mono-ADP-ribosylase activities (22,23). Sirt6 deficiency in mice leads to genomic instability, metabolic defects and degenerative pathologies associated with ageing (24). Accumulating
evidence suggests that SIRT6 is involved in modulating DNA repair. Recent studies have revealed that SIRT6 is involved in base excision repair (24) and in enabling efficient repair of DNA double-strand breaks (DSBs) through both non-homologous end joining and homologous recombination (25–27). SIRT6 is recruited to DSB sites where it interacts and stabilizes the DSB repair factor DNA-PK to promote DNA repair (27). SIRT6 also promotes DNA end resection, a crucial step in DSB repair by homologous recombination, through CtIP deacetylation (26) and mono-ADP-ribosylates PARP1 enhancing DSB repair under oxidative stress (25). In the present study, we show that SIRT6 is overexpressed in paclitaxel- and epirubicin-resistant breast cancer cell lines and provide evidence to demonstrate that SIRT6 contributes to the development of chemotherapy resistance in breast cancer.

Materials and methods

Cell culture

The human breast cancer cell line MCF-7 originated from the American Type Culture Collection and was acquired from the Cell Culture Service, Cancer Research UK, where it was tested and authenticated. Mouse embryonic fibroblasts (MEFs) isolated from wild-type (WT) and Sirt6−/− mice were transformed by SV40 large T antigen expression (28). p53−/− MEFs have been described previously (29). FosO1/Hic4−/− MEFs were kind gifts from Professor Boudewijn Burgering. UMC, Utrecht, the Netherlands and have been described (30). All cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% foetal calf serum, 2 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin in a humidified incubator at 37°C with 5% CO2.

Transfection

The FLAG-tagged SIRT6 expression vector has been described previously (31). See also Supplementary Materials and methods, available at Carcinogenesis Online.

Western blotting and co-immunoprecipitation

Whole-cell lysates were obtained as described (32). See also Supplementary Materials and methods, available at Carcinogenesis Online.

RNA extraction and quantitative reverse transcription–polymerase chain reaction

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Crawley, UK) according to the manufacturer’s protocol. Complementary DNA generated by Superscript III reverse transcriptase (Invitrogen) was analysed by quantitative reverse transcription–polymerase chain reaction (qRT–PCR) using Power SYBR Green PCR Mix from Applied Biosystems, Warrington, UK. All data were normalized to ribosomal protein L19 mRNA expression.

Salforhodamine B assay

For the sulforhodamine B (SRB) assay, 3000 cells were seeded in each well of a 96-well plate. After culture, cells were fixed with 100 μl of trichloroacetic acid and incubated for 1 h at 4°C. Plates were then washed five times with slow running tap water and 100μl of SRB solution (0.4% SRB in 0.1% acetic acid) was added. After incubation for 1 h at room temperature, plates were rinsed three times with 1% acetic acid and air-dried. One hundred microlitres of 10mM Tris base solution was then added to the wells to solubilize the protein-bound dye, and optical density at 492 nm was measured in a microplate reader (Sunrise, Tecan, Dorset, UK).

Comet assay

The alkaline single-cell gel electrophoresis assay was performed by a modified method of Singh et al. (33). See also Supplementary Materials and methods, available at Carcinogenesis Online.

Phospho-hH2AX immunofluorescent staining and foci quantification, tissue microarray, immunoistochemistry, statistical analysis, clonogenic assays and cell cycle analysis

See Supplementary Materials and methods, available at Carcinogenesis Online.

Results

SIRT6 is upregulated in both paclitaxel-resistant and epirubicin-resistant MCF-7 cells

Accumulating evidence has suggested that sirtuins could play a key role in the development of chemotherapeutic drug resistance (34). To test this hypothesis, we first compared the expression levels of the seven mammalian sirtuins in both sensitive and drug-resistant breast cancer cells. To this end, we generated paclitaxel-resistant (MCF-7-TaxR) and epirubicin-resistant (MCF-7-EpiR) breast cancer cell lines by continuous exposure of the parental drug-sensitive MCF-7 cells to increasing concentrations of the drug until resistance to 1 and 5 μM was reached, respectively, as shown by SRB proliferation assay (Figure 1A). Both sensitive and drug-resistant MCF-7 cells were then treated with either paclitaxel or epirubicin (0–48 h) and the expression levels of the seven mammalian sirtuins (SIRT1–7) were analysed by western blot (Figure 1B) and qRT–PCR (Supplementary Figure S1, available at Carcinogenesis Online). As shown in Figure 1B (left panel), no significant changes in the levels of SIRT1, SIRT4 and SIRT7 proteins were found for the MCF-7-TaxR cells when compared with its drug-sensitive counterparts. We observed that SIRT2 protein levels were decreased, whereas SIRT3, SIRT5 and SIRT6 protein levels were increased in the MCF-7-TaxR cells. Sirtuin levels remained relatively constant after paclitaxel treatment in both sensitive MCF-7 and MCF-7-TaxR cell lines. MCF-7-EpiR cells displayed higher levels of SIRT3, SIRT4, SIRT5, SIRT6 and SIRT7 proteins when compared with the parental MCF-7 cells (Figure 1B, right panel). Like the MCF-7-TaxR cells, the MCF-7-EpiR cells showed lower levels of SIRT2 than the sensitive cells, whereas no differences were observed for SIRT1 between the two cell lines. No significant changes in sirtuin levels were observed following epirubicin treatment neither in sensitive nor in drug-resistant MCF-7. Notably, SIRT6 protein expression is consistently and significantly upregulated in the drug-resistant cell lines, suggesting a role for SIRT6 in chemoresistance (Figure 1B and Supplementary Figure S2, available at Carcinogenesis Online). Consistent with this notion, the expression of the previously described SIRT6 markers H3K9ac and H3K56ac (23,28,35) was also downregulated in the drug-resistant MCF-7 cells compared with the parental cells (Supplementary Figure S2, available at Carcinogenesis Online). This increase in SIRT6 protein expression in the drug-resistant cells does not appear to be due to an increase in SIRT6 mRNA levels, as revealed by qRT–PCR analysis (Supplementary Figure S1, available at Carcinogenesis Online).

SIRT6 mediates paclitaxel and epirubicin resistance in MCF-7 cells

To investigate if SIRT6 confers paclitaxel and epirubicin resistance in breast cancer cells, we analysed the effect of SIRT6 knockdown on the proliferation of MCF-7-TaxR and MCF-7-EpiR cells following paclitaxel or epirubicin treatment, respectively. SRB proliferation assays showed that the siRNA-mediated knockdown of SIRT6 (Figure 2A and B, upper panels; Supplementary Figure S3A, available at Carcinogenesis Online) resulted in a decrease in the rate of cell proliferation of both MCF-7-TaxR and MCF-7-EpiR (Figure 2A and B, lower panels). This result suggests that both paclitaxel and epirubicin resistance are mediated, at least in part, through SIRT6 in MCF-7 cells. We next investigated if SIRT6 overexpression confers chemotherapeutic drug resistance to MCF-7 cells. Sensitive MCF-7 cells were transiently transfected with an empty or a SIRT6-overexpressing vector (Figure 2C and D, upper panels) and cell proliferation was analysed by SRB assay after treatment with increasing concentrations of either paclitaxel or epirubicin. As shown in Figure 2C and D (lower panels), the decrease in cell proliferation observed upon paclitaxel and epirubicin treatment was significantly lower (P < 0.05; t-test) over a range of drug concentrations in SIRT6-overexpressing cells compared with the control cells, thus supporting a role of SIRT6 in mediating both paclitaxel and epirubicin resistance in MCF-7 cells.

Sirt6−/− MEFs are more sensitive to paclitaxel and epirubicin treatment than WT MEFs

To investigate further the role of SIRT6 on chemotherapeutic drug response, we used SV40 large T antigen-transformed WT or Sirt6−/− MEFs. The Sirt6−/− MEFs provide a more definitive SIRT6-depletion cell system to study the function of SIRT6 without the need for siRNA.
transfection. SIRT6 deletion and the subsequent increase in the acetylation of the SIRT6 markers H3K9ac and H3K56ac were confirmed by western blotting (Figure 3A and Supplementary Figure S3, available at Carcinogenesis Online). WT and Sirt6−/− MEFs were treated with increasing concentrations of either paclitaxel or epirubicin and cell viability was measured by SRB assay. As indicated in Figure 3B, the decrease in cell viability following paclitaxel and epirubicin treatment was significantly higher in Sirt6−/− MEFs compared with WT MEFs over a range of concentrations tested, thus suggesting that Sirt6-deficient cells are more sensitive to both drugs and supporting the notion that SIRT6 confers resistance to paclitaxel and epirubicin.

**SIRT6 modulates the effects of paclitaxel on cell cycle arrest and cell death in MCF-7 cells**

Paclitaxel and its derivatives act mainly through the binding to β-tubulin, preventing the depolymerization of microtubules and consequently inhibiting the mitotic spindle dynamics (8). However, how SIRT6 mediates paclitaxel resistance remains enigmatic. Treatment of MCF-7 cells with paclitaxel has been shown to induce a G2/M cell cycle arrest followed by cell death (36,37). Taking into account these findings, we next investigated if SIRT6 overexpression or knockdown has an effect on the cell cycle distribution of sensitive and paclitaxel-resistant MCF-7 cells, respectively (Supplementary Figures S3 and S4, available at Carcinogenesis Online). Cell cycle analysis was performed on propidium iodide-stained cells after treatment with paclitaxel for different times using flow cytometry. As expected, siRNA-mediated knockdown of SIRT6 did not cause any drastic changes in cell cycle distribution, probably due to the fact that the parental MCF-7 cells have already low expression levels of SIRT6 (Supplementary Figure S5, available at Carcinogenesis Online). However, it is also notable that the SIRT6-silenced MCF-7 cells consistently have marginally higher levels of sub-G1 dying cells and lower amounts of G2/M arrested cells. In contrast, as shown in Supplementary Figure S6, available at Carcinogenesis Online, paclitaxel treatment did not induce a transient G2/M arrest in MCF-7-TaxR cells transfected with control siRNA. Interestingly, SIRT6 knockdown in paclitaxel-resistant cells caused an increase in cell death (sub-G1 fraction) for all the time points analysed, thus suggesting an increased sensitivity to paclitaxel and supporting the notion that SIRT6 can act as a modulator of paclitaxel resistance in MCF-7-TaxR cells (Supplementary Figure S6, available at Carcinogenesis Online). We next examined the effect of SIRT6 overexpression on the cell cycle phase distribution of sensitive MCF-7 cells treated with paclitaxel. As shown in Supplementary Figure S6, available at Carcinogenesis Online, SIRT6 overexpression resulted in a small decrease in the number of cells in the sub-G1 fraction, thus confirming that SIRT6-overexpressing cells are less sensitive to paclitaxel than control cells. In addition, the proportion of cells arrested in the G2/M phase of the cell cycle after paclitaxel treatment was lower in the SIRT6-overexpressing cells than in the control cells (Supplementary Figure S6, available at Carcinogenesis Online). This result suggests that SIRT6 expression could deregulate the G2/M checkpoint and the subsequent cell death induced by paclitaxel.

**Sirt6−/− MEFs sustain higher degrees of epirubicin-induced DNA damage**

It is known that anthracyclines cause DSB DNA damage through different mechanisms, including topoisomerase II inhibition and generation of free radicals (38–40). After identifying SIRT6 as a mediator of epirubicin resistance, we next sought to evaluate the DNA damage response to epirubicin in SV40 large T antigen-transformed WT and Sirt6−/− MEFs. First, we analysed by immunostaining the
nuclear foci formation of phosphorylated histone H2AX, a marker for cellular DSBs that facilitates the assembly of checkpoint and DNA repair factors to the damaged sites (41) (Figure 3C). Quantification of γH2AX foci at an early time point of 2 h following treatment with a range of epirubicin concentrations showed that there are no significant differences in the epirubicin-induced foci formation between the WT and Sirt6−/− MEFs (Supplementary Figure S7, available at Carcinogenesis Online and Figure 3C), indicating that epirubicin can cause DSBs equally well in WT and Sirt6−/− MEFs. However, at the later time point of 24 h, a greater number of γH2AX foci was observed in the Sirt6−/− MEFs, suggesting that these cells are less effective in repairing DSBs compared with WT MEFs (42). To further analyse the influence of SIRT6 in the efficiency of epirubicin-induced DNA DSB repair, we used alkaline comet assay to compare levels of DNA damage in single cells. Treatment with 0.5 or 1 μM epirubicin for 6 and 24 h led to an increase in the number of visible comet tails in both WT and Sirt6−/− MEFs (Figure 3D). Measurement of the percentage of DNA in tail, tail moment and olive moment (Figure 3D, lower panel) showed that the epirubicin-induced increase in DNA damage was significantly higher for Sirt6−/− MEFs compared with control WT MEFs. Together such data suggest that SIRT6 ablation leads to impaired repair of epirubicin-induced DNA DSBs and increased accumulation of broken DNA in Sirt6−/− MEFs.
Fig. 3. SIRT6 modulates drug sensitivity and DNA damage in MEFs. (A) Western blot analysis of SIRT6, H3K9ac, H3K56ac, total H3 and β-tubulin protein levels in SV40 large T antigen-transformed WT and Sirt6−/− MEFs. (B) WT and Sirt6−/− MEFs were treated with increasing doses of paclitaxel or epirubicin for 72 h and proliferation rates were measured by SRB assay. Data are represented as average ± SD. Statistical significance was determined by Student’s t-test (*P ≤ 0.05 versus control). (C) Representative images showing γH2AX foci formation induced by 0, 2 and 24 h treatment with 1 μM epirubicin. The treated cells were stained for γH2AX (green) and nuclei counterstained with 4′,6-diamidino-2-phenylindole (blue). Images were acquired with Leica TCS SP5. Quantification of the average number of γH2AX foci per cell induced by 1 μM epirubicin at times indicated following treatment (right panel). Bars represent average ± SD. Statistical significance was determined by Student’s t-test (*P ≤ 0.05 versus control). (D) Representative images of comet assay showing DNA migration pattern of SV40 large T antigen-transformed Sirt6−/− or control WT MEFs following treatment with 1 μM epirubicin for 0, 6 and 24 h. DNA damage represented by various comet assay parameters: average percentage of DNA in tail (lower left panel), average tail moment (lower middle panel) and average olive tail moment (lower right panel) after treatment with 0.5 or 1 μM epirubicin for 6 and 24 h. These parameters were measured with Comet Assay IV image analysis software for at least 100 cells per dose. Bars represent average ± SD. Statistical significance was determined by Student’s t-test (*P ≤ 0.05 versus control).
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Fig. 4. Effects of DNA damage on WT and Sirt6\textsuperscript{−/−} MEFs. (A) WT and Sirt6\textsuperscript{−/−} MEFs were exposed to 0, 1, 2.5, 5 and 10 Gy of \(\gamma\)-irradiation and their cell viability measured by SRB assay. Representative data from three independent experiments are shown. Statistical analyses were performed using Student’s \(t\)-tests and compared with the WT MEFs for the correspondent dosage of irradiation. *\(P \leq 0.05\); Significant for 1, 2.5, 5 and 10 Gy. (B) WT and Sirt6\textsuperscript{−/−} MEFs seeded on culture chamber slides were exposed to 5 Gy of \(\gamma\)-irradiation. At 0, 4 and 48 h after irradiation, cells were fixed and immunostained for anti-\(\gamma\)H2AX with Alexa Fluor 488 anti-rabbit sera (green). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (blue). Images were acquired with Leica TCS SP5 (\(\times63\) magnification). The average number of foci per cell was quantified using the CellProfiler software. Results represent average of three independent experiments \(\pm\) SD. Statistical analyses were conducted using Student’s \(t\)-tests against the correspondent time point. *\(P \leq 0.05\); ns, non-significant. (C) Clonogenic assays. Soft agar colony formation efficiency of Sirt6\textsuperscript{−/−} and WT MEFs treated with 0, 50 and 75 nM of epirubicin. Representative images of colonies after crystal violet staining (left panel). The data point is the mean of nine replicate dishes from three independent experiments. Bars represent average \(\pm\) SD. Statistical significance was determined by Student’s \(t\)-test (*\(P \leq 0.05\)).
Fig. 5. SIRT6 modulates the acetylation status and expression of FOXO3a and p53.  
(A) Sensitive MCF-7 cells transfected with control FLAG or a SIRT6-overexpressing vector were treated with 1 μM epirubicin for the times indicated and harvested for western blot analysis. Whole-cell extracts from WT and Sirt6−/− MEFs (B) or from MCF-7 cells transfected with siControl/siSIRT6 siRNAs (C) were subjected to immunoprecipitation with specific antibodies against acetylated lysine (Ac-Lys), FOXO3a or normal rabbit immunoglobulin G as a control. The immunoprecipitates were then assessed for the presence of the indicated proteins by western blot. (D) WT and Foxo1/3/4−/− MEFs were treated with increasing doses of paclitaxel or epirubicin for 72h and proliferation rates were measured by SRB assay. Western blot analysis of FOXO1, FOXO3a, FOXO4 and tubulin expression (left panel) and cell viability analysis (right panel) are shown. Data are represented as average ± SD. Statistical significance was determined by Student’s t-test (*P ≤ 0.05 versus control).
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DNA in response to the drug. Characterization of the drug-resistant MCF-7 cell lines revealed that the multidrug-resistant protein MDR1/Pg-P is overexpressed in both epirubicin and paclitaxel-resistant cell lines (Supplementary Figure S2, available at Carcinogenesis Online), suggesting that the overexpression of MDR1/Pg-P could, at least partially, account for the drug resistance of these cell lines. In order to eliminate the influence of MDR1/Pg-P and to further confirm that SIRT6 is essential for genotoxic drug resistance and DNA damage repair, we compared the sensitivity of Sirt6−/− and WT MEFs with γ-irradiation, a direct DNA damaging agent that cannot be expelled by multidrug-resistant proteins. The results indicated that Sirt6−/− MEFs were more sensitive to γ-irradiation compared with WT MEFs (Figure 4A). γH2AX staining studies also showed that Sirt6−/− MEFs had similar kinetics for γH2AX foci accumulation as WT cells at the earlier time of 4 h following γ-irradiation but had higher number of foci at the longer time point (24 h). These data suggest that the Sirt6−/− deficient MEFs are less effective in repair rather than more susceptible to DNA damage. To study the role of SIRT6 in long-term survival of cells upon epirubicin treatment, we performed clonogenic assays comparing the ability of WT and Sirt6−/− MEFs to survive and form colonies in response to epirubicin treatment. As shown in Figure 4C, Sirt6−/− MEFs demonstrated considerable greater inhibition of cell colony formation in a soft agar clonogenic assay at epirubicin concentrations of 50 and 75 nM compared with WT MEFs.

SIRT6 regulates the acetylation status and expression levels of FOXO3a and p53
We next compared the expression patterns of these tumour suppressors in MCF-7 cells transfected with a SIRT6 expression vector with those transfected with a control (FLAG) expression vector upon epirubicin treatment. The results showed that although p53 and FOXO3a accumulated upon epirubicin treatment in the control MCF-7 cells over the time course, the induction of p53 and FOXO3a was curtailed in the cells overexpressing SIRT6 (Figure 5A). Notably, the levels of the SIRT6-specific markers H3K9ac and H3K56ac, but not acetylated α-tubulin, were also reduced in the MCF-7 cells overexpressing SIRT6. Similar results were also observed for the parental and paclitaxel-resistant MCF-7 cells (Supplementary Figure S4, available at Carcinogenesis Online). To explore further the mechanism of action of SIRT6, we performed co-immunoprecipitation experiments on WT and Sirt6−/− MEFs using an acetylated lysine antibody in order to identify cellular proteins whose acetylation status is modulated by the loss of SIRT6. The results showed that the levels of acetylation of the cell proliferation regulators FOXO3a and p53 increased in the Sirt6 knockout MEFs compared with the WT MEFs (Figure 5B). To confirm this, we next compared the acetylation status of FOXO3a in MCF-7 cells and those with SIRT6 silenced. Consistently, the results again showed that the degrees of acetylation were higher in the MCF-7 cells with SIRT6 knockdown compared with the controls (Figure 5C). These results suggest that the cell cycle and cell death regulators FOXO3a and p53 may be in vivo targets of SIRT6. Given that we have shown previously that FOXO3a and p53 are important mediators of paclitaxel and epirubicin resistance in breast cancer cells (29,36,37), collectively these results suggest that SIRT6 modulates paclitaxel and epirubicin sensitivity, at least partially, through targeting FOXO3a and p53 in breast cancer cells. Consistently, the expression of FOXO3a and its downstream antiproliferative targets, p27kip1 and BIM, was induced in response to epirubicin and paclitaxel treatment after SIRT6 depletion in drug-resistant MCF-7 cells and in Sirt6−/− MEFs (Supplementary Figure S2, available at Carcinogenesis Online). To examine this idea further, we compared the sensitivity of WT MEFs and MEFs deficient of Foxo1/3/4 with a range of paclitaxel and epirubicin concentrations. The cell viability results showed that Foxo1/3/4-deficient MEFs were significantly less sensitive to both paclitaxel and epirubicin over a wide range of concentrations (0.05–μM) (Figure 5D), whereas both WT and p53+/− MEFs have similar sensitivity to paclitaxel and epirubicin, except at low drug concentrations (e.g. 0.005 and 0.01 μM) where p53+/− MEFs appeared to be more resistant (Supplementary Figure S8, available at Carcinogenesis Online).

Nuclear SIRT6 expression in breast cancer is associated with poorer survival
The expression pattern of SIRT6 was studied using immunohistochemical staining on a previously described panel of breast cancer samples (118 cases) with high proportion of invasive ductal carcinoma (93 cases) (Figure 6A) (43). SIRT6 staining was found in both nuclear and cytoplasmic compartments with representative expression patterns shown in Figure 6B. Immunohistochemical analysis results revealed that high SIRT6 nuclear staining was significantly associated with poorer survival (P = 0.018 for overall survival, log-rank test, Kaplan–Meier estimate analysis) (Figure 6C), whereas cytoplasmic staining was significantly related to better survival (P = 0.014 for overall survival, Kaplan–Meier estimate analysis) (Supplementary Figure S9, available at Carcinogenesis Online). On multivariate analysis, SIRT6 staining remained associated with death after correcting for tumour stage and lymph-node involvement (P = 0.033) (Figure 6D). Despite not statistically significant, there was a strong trend of SIRT6 nuclear staining associated with disease-specific survival in both Kaplan–Meier estimate analysis (P = 0.057) (Supplementary Figure S10, available at Carcinogenesis Online) and Cox multivariate regression analysis (P = 0.063) (Supplementary Figure S10, available at Carcinogenesis Online). We, therefore, conclude that SIRT6 nuclear expression is associated with poor prognosis in breast cancer patients, further suggesting that SIRT6 expression is associated with cytotoxic chemotherapeutic drug resistance, as at least half of these patients received treatments and monitored after diagnosis (Supplementary Figure S11, available at Carcinogenesis Online).

Discussion
Taxanes and anthracyclines are widely used in the treatment of breast cancer. However, resistance to one or both drugs often develops in a high proportion of patients with metastatic disease (4,5). Understanding the molecular mechanisms underlying chemotherapeutic drug resistance is thus essential in order to develop effective treatments for these patients. In the present study, we have identified a novel role of SIRT6 in the development of both paclitaxel and epirubicin resistance in breast cancer cells. We observed that the basal levels of SIRT6 protein are higher in the MCF-7-Tax and MCF-7-Epi cells compared with the parental drug-sensitive MCF-7 cells. This upregulation of SIRT6 seems to be mediated at the post-transcriptional level, as qRT–PCR analysis shows that basal SIRT6 mRNA levels are in fact decreased in the drug-resistant cells when compared with its drug-sensitive counterparts. Interestingly, the levels of other sirtuins, like SIRT3 and SIRT5, are also upregulated in the drug-resistant cell lines, whereas SIRT2 levels are downregulated, thus suggesting that other sirtuins apart from SIRT6 may also be associated with the development of resistance to these chemotherapeutic agents in breast cancer. Surprisingly, we did not find a significant change in the protein levels of SIRT1, which had been reported previously to be upregulated in different drug-resistance cell lines (20,21) and had been proposed to induce doxorubicin resistance through the regulation of the multidrug resistance molecule P-glycoprotein in osteosarcoma SaOS2 cells (20). This discrepancy suggests that the role of SIRT1 in drug resistance may vary depending on the type of tumour and the chemotherapeutic treatment used. It is also possible that different cell lines contain distinct genetic backgrounds that may influence the cellular sensitivity to these drugs.

SIRT2 is predominantly cytoplasmic (44) and SIRT3, SIRT4 and SIRT5 are mainly mitochondrial proteins (45,46) and SIRT1, SIRT6 and SIRT7 are located in the nucleus (44), consistent with a role in the regulation of chromatin structure and gene expression. Initially described as a mono-ADP-ribosylase, SIRT6 has been shown to catalyse the NAD+-dependent deacetylation of histone H3 lysines 9 and 56 (23,35). This histone deacetylase activity of SIRT6 has been linked.
to the regulation of the telomeric chromatin, gene expression and the dynamic chromatin association of DNA repair factors (47). The significant and consistent upregulation of SIRT6 protein expression in drug-resistant MCF-7 cells led us to investigate a possible role of SIRT6 in mediating chemoresistance.

Knockdown of SIRT6 expression in MCF-7-TaxR and MCF-7-EpiR cells causes an increase in the sensitivity of the resistant cells to both paclitaxel and epirubicin, thus indicating that chemotherapeutic drug resistance is in part mediated by SIRT6 in MCF-7 cells. This is supported by the results obtained in MEFs, showing that Sirt6−/− cells are more resistant.

**Fig. 6.** High nuclear SIRT6 expression is associated with poor outcome in breast cancer patients. One hundred and thirty-three cases of breast cancer diagnosed between the years 1992 and 2001 with clinical follow-up data were retrieved from the records of the Department of Pathology, Queen Mary Hospital, Hong Kong. The patients’ ages at diagnosis ranged from 30 to 90 years old, with a mean of 53 years. Histological sections of all cases were reviewed by the pathologist, the representative paraffin tumour blocks chosen as donor block for each case and the selected areas marked for construction of tissue microarray blocks. The tissue arrays were stained with the polyclonal SIRT6-specific antibody (Cell Signaling #2590) diluted at 1:50. Allowing of loss of tissue cores from previous sectioning for other studies, a total of 118 could be assessed and scored for SIRT6 expression. The expression pattern and subcellular localization were correlated with histological type, histological grade, clinical stage, oestrogen and progesterone receptor status, HER2 oncoprotein overexpression, lymph-node metastasis and survival time. (**A**) The clinical and pathological features of patients used in the study. (**B**) Four representative tumour cases showing high–high, high–low, low–high and low–low corresponding cytoplasmic-nuclear staining patterns (magnification: ×100; insets ×400). (**C**) Kaplan–Meier analysis of overall survival for nuclear SIRT6 staining with log-rank test P-values indicated. High expression of nuclear SIRT6 significantly inversely correlated with poor overall and disease-specific survival in breast cancer patients. (**D**) Cox univariate and multivariate regression analysis of SIRT6 staining and clinical parameters.
Role of SIRT6 in paclitaxel and epirubicin sensitivity

In summary, in this study, we identified a novel role of SIRT6 in the development of resistance to both epirubicin and paclitaxel in breast cancer. Our findings also suggest that SIRT6 could be a potential marker for identification of tumours that are likely to be resistant to these drugs and indicate a novel therapeutic strategy to overcome acquired paclitaxel and epirubicin resistance through inactivation of SIRT6 expression in breast cancer.

Supplementary material

Supplementary Materials and methods and Figures S1–S11 can be found at http://carcin.oxfordjournals.org/

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References


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