Targeted silencing of MLL5β inhibits tumor growth and promotes gamma-irradiation sensitization in HPV16/18-associated cervical cancers.

Dawn Sijin Nin1,#, Chow Wenn Yew1,#, Sun Kuie Tay2, Lih-Wen Deng1,*

1Department of Biochemistry, Yong Loo Lin School of Medicine, National University Health System, National University of Singapore and 2Department of Obstetrics & Gynaecology, Gynaecologic Oncology Section, Singapore General Hospital and National Cancer Center Singapore, Singapore.
#Nin DS and Yew CW contributed equally to this work.

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*Corresponding author: Lih-Wen Deng
Email: bchdlw@nus.edu.sg
Mailing Address: Department of Biochemistry, Yong Loo Lin School of Medicine, National University Health System, National University of Singapore
14 Medical Drive, Block MD6, #14-02S, Singapore 117599
Phone: 65-6516-1239 Fax: 65-6779-1453

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ABSTRACT

We previously identified a novel MLL5 isoform MLL5β, which was essential for E6 and E7 transcriptional activation in HPV16/18-associated cervical cancers. In this report, we investigated the potential of RNAi-mediated silencing of MLL5β through the use of MLL5β-siRNA as a novel therapeutic strategy for HPV16/18-positive cervical cancer. We observed concurrent down-regulation of E6 and E7 after MLL5β silencing, leading to growth inhibition via the activation of apoptosis and senescence in the HeLa cell model. This corresponded with the enhanced anti-tumor effects of MLL5β-siRNA compared to E6- or E7-siRNA single treatments. Significant reduction in tumor size after MLLβ-siRNA treatment in the HeLa xenograft tumor model further emphasized the importance of MLL5β in HPV16/18-associated tumor growth and the potential of RNAi therapeutics that target MLL5β. We also identified MLL5β as a modulator of cisplatin’s gamma-irradiation (IR) sensitization properties. We observed that while MLL5β silencing alone was enough to evoke cisplatin-like IR sensitization in tumor cells in vitro, overexpression of MLL5β inhibited cisplatin’s ability to sensitize HeLa cells to IR-induced cytotoxicity. MLL5β-siRNA-IR co-treatment was also observed to enhance tumor growth inhibition in vivo. Taken together, our findings highlight the potential of targeted silencing of MLL5β via the use of MLL5β-siRNA as a novel therapeutic strategy and propose that MLL5β-siRNA could be a viable alternative for cisplatin in the current cisplatin-based chemotherapeutics for HPV16/18-associated cervical cancers.
INTRODUCTION

Cervical cancer is the second most common and the fifth deadliest cancer in women worldwide (1, 2). The human papillomavirus (HPV) DNA is detectable in > 99% of all cervical cancer (3, 4), and of >120 subtypes of HPV identified, HPV16 and HPV18 account of almost 70% of all cases, making them the most prevalent high-risk HPV subtypes (5).

The viral oncogenes \textit{E6} and \textit{E7} have been widely recognized as the main mediators of HPV-induced cervical carcinogenesis (6-8). While the E6 oncoprotein is required for degradation of key tumor suppressor p53 (9-11), E7 competes with E2F for key cell cycle regulator phosphorylated retinoblastoma (pRb) binding, leading to cell cycle deregulation and uncontrolled cell proliferation (12, 13). Combining the effects of both E6 and E7 on p53 and pRb, cells undergo genomic instability and aberrant cell cycle progression (hallmarks of malignant transformation). Immortalization of keratinocytes when both E6 and E7 were over-expressed (7, 14, 15) further strengthens this theory.

Integration of the episomal HPV genome is a critical step for the expression of the \textit{E6} and \textit{E7} oncogenes. This integration results in the subsequent hijacking of the cell’s replication mechanism for the expression of various associated oncoproteins (16). As integration often occurs within the HPV viral \textit{E2} gene, E2 protein expression is lost after integration. Loss of E2, a known repressor of \textit{E6} and \textit{E7} transcription, results in the accumulation of E6 and E7 oncoproteins (17). Integration also allows for the continual expression of E6 and E7 as transcription of both \textit{E6} and \textit{E7} are controlled by a single promoter (18-20).

The current treatment strategy for cervical cancer is radical hysterectomy for early cancer and concurrent chemo-radiation for inoperable cases (21). One of the most widely used chemotherapeutic agents in current clinical practice is \textit{cis}-diamminecholoplatinum (II) (cisplatin) (22). Cisplatin has been found to repress \textit{E6} expression level in HeLa and SiHa, leading to the stabilization of p53 and up-regulation of p53 downstream genes (19, 23, 24). Besides that, cisplatin was reported to enhance radio-sensitivity of cervical cancer cells through the restoration of p53 functions (23, 25). However
the use of cisplatin is not without its disadvantages. The lack of specificity for cancer cells and the increasing incidence of cisplatin resistance have greatly limited its use (22). Therefore there is an urgent need for the identification of novel molecular targeted therapeutic strategies.

Due to the prominent roles of E6 and E7 in tumorigenesis and specificity to HPV-related cancer cells, there has been intense interest in targeting these two oncoproteins as a potential therapeutic strategy for HPV-positive cervical cancer (26, 27). In recent years, the development of RNA interference (RNAi) based strategies for disease treatment has gained prominence with many at various stages of clinical trials (28). With its precise targeting of specific oncogenes, RNAi stands out as one of the most favourable cancer therapeutics in the near future. In the development of RNAi-based therapeutic strategies for HPV-related cervical cancers, efforts have been focused on the potential of direct suppression of E6 and E7 expression through their siRNAs (26, 27, 29-32).

Our laboratory has recently discovered an HPV16/18-positive cell-specific novel protein isoform of Mixed Lineage Leukaemia 5 (MLL5β), essential for the activation of E6 and E7 gene expression in these cells (33). We reported that MLL5β was detected in HPV16/18-positive cell lines and primary human cervical carcinoma specimens. We also identified that MLL5β activates the transcription of both the E6 and E7 oncogenes through the formation of a complex with AP-1 at the distal AP-1 binding site of the HPV long control region (LCR). RNAi-mediated silencing of MLL5β via the use of a MLL5β-specific siRNA down-regulated both E6 and E7 gene and protein expression levels, leading to the restoration of p53 and pRb. Observing this combinatory effect of MLL5β silencing on both E6 and E7 expression in HPV16/18-positive cells, we are interested in the potential application of RNAi therapeutic targeting of MLL5β as a treatment strategy for HPV16/18-positive cervical cancer.

In this report, we assessed the effects of RNAi-targeted silencing of MLL5β on HPV16/18-positive tumor growth in vitro and in vivo. The effectiveness of MLL5β silencing on tumor growth suppression was also compared with the proposed use of RNAi therapeutics targeting E6 and E7.
Given reports that cisplatin works through the down-regulation of E6 and restoration of p53 levels (19, 23-25) (a phenomenon also observed after MLL5β silencing), the effectiveness of RNAi-mediated MLL5β silencing in combination with ionizing (gamma) radiation (IR) on inhibition of tumor growth was also investigated to elucidate the potential application of MLL5β-based RNAi therapeutics as a viable alternative to cisplatin in the current cisplatin-IR treatment regime.

MATERIALS AND METHODS

Cell lines, reagents and antibodies

Human cervical carcinoma SiHa, HeLa and C33A, embryonic kidney cells HEK 293T and diploid fibroblasts WI38 were cultured in Dulbecco’s Modified Eagles Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Hyclone), 2 mM glutamine (Gibco) and 100 units/ml penicillin/streptomycin (Gibco). All cell lines were purchased from American Type Culture Collection (ATCC) in 2006 and were last authenticated by short tandem repeat (STR) DNA typing (Genetica DNA Laboratories) in September 2013. Antibody against cleaved-PARP (Asp 214) was from Cell Signaling while antibodies against E6, E7 and β-actin were purchased from Santa Cruz Biotechnologies.

RNA interference, transfection and overexpression

All siRNA duplexes (summarized in Supplementary Table 1) were synthesized by 1st BASE (Singapore). For RNA interference using short hairpin RNA (shRNA), siRNA sequences were converted to shRNA through a web-based insert design tool by Invitrogen. Vectors containing shRNA sequences of MLL5β, HPV18 E6 and E7 were generated using pSilencer2.1-U6 Hygro (Invitrogen) as the backbone. siRNAs transfection was carried out using Lipofectamine RNAiMAX (Invitrogen). MLL5β-specific siRNA was designed to specifically target MLL5β (antisense sequence was BLASTed against the human and HPV16/18 genome and no sequence homology to either full length MLL5, HPV16/18 E6 or E7 mRNA was identified). HPV16 and HPV18 E7-siRNAs were designed to
specifically target each HPV subtype. Scrambled-siRNA was used as a control. MLL5β cDNA sequence was amplified from HeLa cDNA by polymerase chain reaction and the PCR amplicons were digested with BamHI and NotI and cloned into pEF6/V5-His vector (Invitrogen) to obtain the FLAG-tagged MLL5β-expressing vector (pEF6-FLAG-MLL5β). shRNA and plasmid transfections were carried out using the calcium phosphate-based transfection method.

**Clonogenic, attachment and soft agar assay**

shRNA was transfected into cells and subjected to 72 h of hygromycin (500 μg/ml) selection 24 h after transfection. Surviving cells were harvested for clonogenic, attachment and soft agar assays. For clonogenic assay, 400 cells were added into each well of a 6-well plate with hygromycin media (500 μg/ml). The plate was incubated for 20 days with changing of fresh hygromycin media every 3 days. The colonies formed were fixed with methanol and stained with 0.5 % crystal violet (Sigma-Aldrich). Colonies were observed under microscope and the percentage of surviving clone was calculated as the number of successful clones over total cells added. For attachment assay, 10000 cells were plated in to a 24-well and allowed to attach for 4 h. After 4 h, attached cells are trypsinized and counted. For each shRNA set, three wells were plated with 10000 each. Percentage of attached cells was counted for each well and the average was taken. Results are presented as mean of three experiments ± SD. $p < 0.05$ is taken to be statistically significant.

For soft agar assay, DMEM 2X and 0.6% as well as 0.8 % agarose solutions were prepared and kept warm at 40 °C. 500 μl of DMEM 2X was mixed with 500 μl of 0.8 % agarose and pipetted into a 6-well plate as the base layer for each well. This was allowed to cool and solidify for 30 min in an incubator. 800 cells were resuspended in 500 μl of DMEM 2X and mixed with 500 μl of 0.6 % agarose before being added on top of the settled base layer. 500 μl of hygromycin media was added to each well after the top layer has cooled and formed a semi-solid matrix. The plate was incubated for 30 days with the addition of 500 μl hygromycin media every 3 days. Colonies were fixed and stained as described above.
cDNA synthesis and quantitative real-time PCR (qPCR)
Total RNA was extracted with TRIzol (Invitrogen). RNA was treated with DNase I (Ambion) prior to cDNA synthesis using the First Strand cDNA Synthesis Kit (Invitrogen). Gene expression was measured with iQ5 qPCR (Biorad) using SYBR Green PCR Master Mix (BioRad) and in-house designed primers (Supplementary Table 2).

Senescence assay
HeLa cells were plated in 6-well plate and the appropriate siRNA was transfected into the cells. Cells were cultured for 10 days with transfection of siRNA every 3 days. After 10 days, cells were washed and stained using the Senescence Cells Histochemical Staining Kit (Sigma-Aldrich). The number of senescent cells (stained blue) was calculated as a percentage of total cells in each sample.

Trypan blue exclusion assay
To assess the viability of cells after siRNA transfection, trypan blue exclusion assay was performed. Cells were washed with PBS and both attached and unattached cells collected and pelleted. Cells were resuspended in PBS and mixed with equal volume of trypan blue (Sigma-Aldrich). Both live and dead cells were counted. Cell viability was calculated as the percentage of the living cells divided by total number of cells. Experiment was performed in triplicates.

Cytotoxicity assay
To assess the toxicity caused by either cisplatin or siRNA, coupled with or without gamma-irradiation, cytotoxicity assay was performed with Cyto96 Non-Radioactive Cytotoxicity Assay (Promega). Each set was carried out in triplicates. For both siRNA and cisplatin-treated samples, the cells were harvested for assay 24 h after gamma-irradiation treatment.

In vivo tumor xenograft mouse study
Experiments were performed in accordance with NIH and IACUC guidelines for animal use using 6-week-old BALB/c immunoincompetent nude mice (Genscript). For tumor formation, a suspension
containing $5 \times 10^6$ HeLa cells in 100 µl of PBS plus matrigel (1:1) were injected subcutaneously in the right flank of nude mice. To investigate the effects of the various siRNAs on tumor growth, the tumor-bearing mice were randomly assigned into 4 groups (5 mice/group) and given intra-tumoral injections of specific siRNAs every other day for 20 days. In particular, each group received intra-tumoral injections of either scrambled-, E6-, E7- or MLL5β-siRNA at a dosage of 10 µg/mouse.

To investigate the IR sensitization effects of MLL5β-siRNA on tumor growth in vivo, the tumor-bearing mice were randomly assigned into 4 groups (6 mice/group). Groups 1 and 2 were injected (intra-tumoral) with 10 µg/mouse MLL5β-siRNA every other day for a 20-day period, and mice in Group 2 were irradiated with 1 Gy of IR on days 9 and 13 after siRNA treatment. Groups 3 and 4 were subjected to the same protocol where both groups were similarly injected with 10 µg/mouse scrambled-siRNA in place of MLL5β-siRNA over the same 20-day period and mice in Group 4 subjected to IR as per the protocol for Group 2.

Tumor growth was measured by caliper every other day. Volumes were calculated using $V = \frac{A \times B^2}{2}$, where $A$ and $B$ are the major and minor tumor axes, respectively. Tumor doubling time was calculated using the formula $(\ln 2 \times T_o)/\ln(V_o/V_i)$, where $T_o = \text{treatment duration}$, $V_i = \text{initial volume of tumor}$ and $V_o = \text{volume of tumor after the 20-day treatment}$.

**Statistical Analysis**

All data are presented as mean ± SD. Survival, senescence and tumor doubling time are analyzed with un-paired t-test. Average tumor growth and average-fold increase in tumor size for each group were analyzed with regression modeling following with ANOVA. $p \leq 0.05$ is considered statistically significant. Pixel quantitation was analyzed via ImageJ analysis software. E6 and E7 expression levels were normalized to actin loading control. Histograms are presented as relative pixel density compared to E6 or E7 protein levels at time point 0 h. Results were reported as the mean normalized pixel density of three independent experiments ± SD.
RESULTS

Targeted silencing of MLL5β reduces the colony-formation ability of HeLa cells in vitro.

To access the potential of MLL5β-targeted silencing as a therapeutic strategy for HPV16/18-positive cervical cancer, we first looked at the anti-tumor effects of the RNAi-mediated silencing of MLL5β in vitro using the HeLa model. HeLa cells have been chosen for our in vitro studies as they display consistently high transfection efficiencies (34). Clonogenic assay was first performed using shRNA-mediated RNAi, to test the effects of MLL5β silencing on cell survival and proliferation. As shown in Figure 1A, targeted silencing of MLL5β significantly reduced the number of colonies formed (more than 90% reduction compared to non-treatment/empty). This reduction in colony numbers was found to be greater than the targeted silencing of either E6 (~ 70% reduction) or E7 (~ 40% reduction) alone.

As HeLa cells were pre-transfected with the respective shRNAs, the ability of these cells to attach to the plate was also analysed. It was observed that after 4 h, the ability of cells pre-transfected with MLL5β-shRNA to attach to the plate was significantly reduced compared to scrambled-shRNA transfected cells. Similar to the observations in Figure 1A, the ability of E6- and E7-shRNA transfected cells to attach were also reduced but to a smaller extent compared to MLL5β-shRNA transfected cells (Supplementary Figure S1). These data suggest that the observed reduction in colony numbers after MLL5β-shRNA expression could also be a result of the inability of these cells to attach to the plate. Therefore to ascertain that MLL5β silencing affected the viability and proliferative ability of the cells, a soft agar assay was carried out. In this assay, the ability of the cells to attach is not a pre-requisite for colony formation. Upon silencing of MLL5β, HeLa cells failed to form any observable colonies compared to those treated with empty vector (Figure 1B). As expected, E6 and E7 silencing in HeLa cells also failed to form any observable colonies.

Enhanced anti-tumor effect of MLL5β-targeted silencing is a consequence of the induction of both apoptosis and senescence as a result of concurrent E6 and E7 down-regulation.

It has been established that E6 knockdown induces apoptosis in HPV16/18-positive cell lines while E7 knockdown leads to senescence (12, 30, 35). We have previously reported that MLL5β is essential in the activation of both the E6 and E7 transcription in HPV16/18-positive cells (33). With these
observations, we hypothesized that the more pronounced anti-tumor effects of MLL5β-targeted silencing in HeLa cells was due to the combined effects of both apoptosis and senescence as a consequence of the down-regulation of both E6 and E7 transcript levels. To validate this, we first determined the effects of MLL5β knockdown on the expression of the E6 and E7 transcripts levels. As expected, knockdown of MLL5β resulted in the concurrent reduction of both E6 and E7 transcript levels in HeLa cells (Figure 1C, left panel). This was accompanied by the concurrent down-regulation of both E6 (~50% decrease) and E7 (~80% decrease) protein levels (Figure 1C, right panel, Supplementary Figure S2). We next sought to investigate if the observed down-regulation of both E6 and E7 levels after MLL5β knockdown resulted in both apoptosis and senescence. First, the degree of apoptosis after MLL5β, E6 or E7 knockdown in HeLa cells was determined via the analysis of apoptotic marker cleaved-PARP in western blotting analysis. A marked accumulation of cleaved-PARP was observed after MLL5β and E6 knockdown but not after E7 knockdown (Figure 1D).

Next, senescent levels after MLL5β, E6 or E7 knockdown were assessed. We observed a high percentage of cells in senescence only after MLL5β and E7 knockdown while E6 knockdown induced little effect after 1 week (Figure 1E). These observations strongly suggest that the enhanced anti-tumor effects of MLL5β-targeted silencing compared to E6 or E7 silencing alone is due to the concurrent induction of apoptosis and senescence as a result of both E6 and E7 down-regulation.

**RNAi-targeted silencing of MLL5β mediated by MLL5β-siRNA selectively reduces the viability of HPV16/18-positive cells.**

RNAi-mediated gene silencing can be achieved via the use of either shRNA or siRNAs. However, due to the major challenges facing the use of shRNAs such as the safety issues concerning the need for a virus-based delivery system (36), we decided to focus on potential of MLL5β-siRNA as a possible RNAi-based therapeutic agent for HPV16/18-positive cervical cancers. With our previous discovery of the specificity of MLL5β expression in HPV16/18-positive cells (33), we first sought to assess the cytotoxic specificity of MLL5β-siRNA for these cells. Effects of MLL5β-, E6- or E7-siRNAs on the growth of HPV16/18-positive cervical cancer cell lines SiHa (HPV16) and HeLa
(HPV18), HPV16/18-negative cervical cancer cell line C33A and the normal diploid lung fibroblast cell line WI38 were determined using trypan blue exclusion after 72 and 96 hours of siRNA treatment. HPV16/18-positive SiHa and HeLa cells displayed much lower viability when treated with MLL5β-siRNA compared to the HPV16/18-negative C33A and normal diploid WI38 cells (Figure 2A and B). While all the siRNA treatments have minimal effects on C33A and WI38 indicating their specificity for HPV16/18-positive cells, the reduction of cell viability for HPV16/18-positive cell lines was more pronounced in MLL5β-siRNA-treated cells when compared to either E6- or E7-siRNA treatment alone. This observation further suggests the enhanced efficacy of MLL5β-targeted silencing compared to the silencing of E6 or E7 alone. It was also of interest to note the HPV-subtype specificity of E6- and E7-siRNA treatment, where HPV16-positive SiHa cells were more sensitive to the growth inhibitory effects of the HPV16 E6- and E7-siRNAs and HPV18-positive HeLa was more sensitive to the HPV18 E6- and E7-siRNAs.

**MLL5β-siRNA exhibits anti-tumor effects in vivo.**

Next, the anti-tumor properties of MLL5β-siRNA were determined in vivo. Using a nude mice HeLa xenograft model, the effects of scrambled-, E6-, E7- or MLL5β-siRNA injected intra-tumorally on tumor growth rate were monitored over 20 days. Consistent with the results observed in vitro, MLL5β-siRNA showed the highest tumor-suppressive ability compared to E6- or E7-siRNA treatment alone when a graph of average tumor volume increase at each reading was calculated and plotted (Figure 2C). The average-fold increase in tumor volume was also calculated and MLL5β-siRNA-treated tumors registered an approximately 0.5-fold increase in tumor volume after the 20-day treatment period. This is significantly ($p<0.001$) smaller than the 1.79-fold increase observed in the scrambled-siRNA-treated group as well as tumors treated with either E6- or E7-siRNA alone, which averaged a 0.94 and 1.31-fold increase in tumor volume respectively (Figure 2D). Average tumor doubling time (DT), a widely used measure of tumor growth after therapy in the clinics, was also calculated for each group and presented in Table 1. Consistent with the above results, the average DT after MLL5β-siRNA treatment was slowed to approximately 38 days compared to the 14.6 days of the scrambled-siRNA-treated group. This growth was also significantly slower than that of either E6-
siRNA (28.6 days) or E7-siRNA (21.2 days) treatment alone. Taken together, these data strongly suggest the potential of MLL5β-siRNA as a potential RNAi-based therapeutic agent for HPV16/18-positive cervical cancer.

**MLL5β is a modulator of cisplatin’s gamma-irradiation sensitization property.**

The current standard therapy for cervical cancer includes chemotherapy using platinum-based derivatives such as cisplatin in combination with radiotherapy. Cisplatin is widely believed to exert some of its anti-tumor actions through the activation of the p53-dependent apoptotic pathway by down-regulating E6, and stabilizing p53 expression (19, 23-25) in HPV16/18-positive cervical cancer cells. This phenomenon is thought to result in the sensitization of cisplatin-treated cells to IR due to the amplification of the p53-dependent apoptotic cascade. As E6 down-regulation and p53 restoration was also observed when MLL5β was silenced, we hypothesize that cisplatin may exert its IR sensitization property through MLL5β. To test this hypothesis, we first determined if cisplatin had any effect on the expression level of MLL5β in HPV16/18-positive cells. Treatment of both SiHa and HeLa cells with cisplatin reduced the expression of MLL5β in a dose-dependent manner (Figure 3A), with the greatest reduction observed after treatment with 40 μM cisplatin. This was also accompanied by an observed reduction in both E6 and E7 transcript levels.

To verify that MLL5β plays a role in cisplatin’s IR sensitization property, a rescue experiment was conducted. In this experiment, when HeLa cells were transfected with the pEF6-empty vector, cisplatin and IR combinatory treatment on HeLa cells increased cytotoxicity levels by almost 2-fold (from about 35% to almost 90% cytotoxicity) compared to just treatment with cisplatin alone. However when MLL5β was overexpressed in HeLa cells through the exogenous introduction of the pEF6-Flag MLL5β plasmid, the sensitivity of these MLL5β-overexpressing cells to IR after cisplatin treatment was abrogated (Figure 3B). Taken together, these observations strongly suggest that cisplatin-induced sensitization of HPV16/18-positive tumor cells to IR is modulated by MLL5β.

Although cisplatin has been widely applied in the clinics as a chemotherapeutic strategy in cervical cancer treatment, the general cytotoxicity of cisplatin on non-tumor remains a primary concern. With
the high specificity of MLL5β-siRNA for HPV16/18-positive tumor cells observed in our earlier experiments, we thought that MLL5β-siRNA could be a good alternative to cisplatin with reduced cytotoxicity on non-tumor cells. To validate this hypothesis, cytotoxicity levels caused by different cisplatin concentrations on HPV16-positive SiHa and HPV18-positive HeLa, HPV16/18-negative C33A and normal diploid WI38 cells were first assessed. In this experiment, IR was also included to show the sensitization effects of cisplatin. Cisplatin treatment alone induced cytotoxicity in all four cell lines tested regardless of their tumorigenicity, especially at 40 µM treatment where >40% cytotoxicity was observed in all cell lines (Figure 4A). At 40 µM, cisplatin significantly sensitized HPV16/18-positive SiHa and HeLa to IR-induced cytotoxicity. In contrast, HPV16/18-negative C33A and normal diploid WI38 cells neither of which expresses MLL5β, showed less pronounced cisplatin-induced IR sensitization compared to cisplatin treatment alone. This suggests that cisplatin-IR sensitization effects could be modulated in part through MLL5β expression in HPV16/18-positive cells.

Next, a similar cytotoxicity assay was conducted using siRNA treatment in place of cisplatin. In this assay, while IR induced significant increases in cytotoxicity across all cell lines treated with the various siRNAs, only MLL5β-siRNA showed enhanced cisplatin-like IR sensitization effects in both SiHa and HeLa cells. This was in contrast to E6-siRNA induced IR sensitization which was subtype specific, while E7-siRNA-treated cells showed minimal IR sensitization effects (Figure 4B). Compared to Figure 4A, MLL5β-siRNA induced < 20% cytotoxicity in HPV16/18-negative cells with or without IR, suggesting the selectivity and specificity of MLL5β-siRNA on cytotoxicity and IR sensitization for HPV16/18-positive cells.

**MLL5β-siRNA-IR co-treatment showed enhanced tumor growth inhibition in vivo.**

Finally, to investigate the potential application of MLL5β-siRNA-IR combinational therapy, we next validated the ability of MLL5β-siRNA to work with IR to inhibit tumor growth in vivo using the previously established HeLa xenograft tumor model (Figure 2C and D). The effects on tumor growth of groups of mice treated with either MLL5β-siRNA or IR (1-Gy) single treatment were compared
with groups of mice treated with MLL5β-siRNA-IR (1-Gy) combinatory treatment. Consistent with our *in vitro* findings, MLL5β-siRNA-IR combination therapy significantly reduced tumor growth (*p* ≤ 0.05) compared to scrambled-siRNA, MLL5β-siRNA or IR single treatment (Figure 4C). Similarly, MLL5β-siRNA-IR combination-treated tumors registered lower-fold increase in tumor volumes compared to either MLL5β-siRNA or IR single therapy. A significant retardation of tumor growth was observed in the group subjected to combinatory treatment (Figure 4D), which registered only a 1.19-fold increase in tumor volume over the 20-day treatment period compared to the 2.97-fold increase in the non-treated group. This reduction was also significant compared to the 1.82 and 1.94-fold increase in the MLL5β-siRNA or IR single treatment groups. Average tumor doubling time (DT) was also calculated for each group and presented in Table 2. Again, consistent with the above results, it was revealed that the average DT of MLL5β-siRNA-IR combinatorial therapy was significantly longer than that of either MLL5β-siRNA or IR single treatment.

**DISCUSSION**

We have previously identified a novel MLL5 isoform, MLL5β which was found to activate E6/E7 expression in HPV16/18-positive cells through the distal AP-1 site in the long control region (LCR) (33). Knocking down MLL5β via a siRNA-duplex designed specifically to target only the isoform, down-regulated E6 and E7, at both transcript and protein levels. In this study, we report the selective pro-apoptotic and growth-suppressing properties of this MLL5β-specific siRNA (MLL5β-siRNA) on HPV16/18-positive cells. MLL5β-siRNA promoted cell death and suppressed the growth of cancer cells, *in vitro* and *in vivo*. These MLL5β-siRNA anti-cancer properties were related to the induction of both apoptosis and senescence as a result of its ability to down-regulate both E6 and E7 levels. We also assessed the effectiveness of MLL5β-siRNA as an alternative to cisplatin in the sensitization of HPV16/18-positive cervical cancer cells to IR. MLL5β-siRNA sensitized HPV16/18-positive cancer cells towards IR as effectively as cisplatin but with better selectivity. Overexpression of MLL5β in HeLa cells negated cisplatin’s IR sensitization effects, suggesting its role in modulating cisplatin’s IR sensitization property. This study is the first to assess the potential of using MLL5β-siRNA as a new...
therapeutic agent and as a more tumor-specific alternative for cisplatin in the treatment of HPV16/18-positive human cervical cancers.

The current gold standard treatment for cervical cancer includes chemotherapy using cisplatin, in combination with IR-radiotherapy. This chemoradiotherapy targets rapidly dividing cancer cells by inducing DNA damage, triggering the up-regulation of p53 activating p53-dependent apoptotic cascades. However, the major drawback of this current strategy is the lack of tumor cell specificity resulting in a multitude of unwanted side effects due to their cytotoxic effects on non-tumor cells. Besides, some patients eventually develop resistance to cisplatin, greatly limiting its effectiveness (22, 37, 38). Therefore, there is an urgent need for the development of more novel and better molecular-based therapeutics in HPV16/18-associated cervical cancer therapy.

Recently, targeted-therapy via RNA interference (RNAi) has emerged as a promising treatment strategy for many types of cancer, with a number already in clinical trials (39, 40). The precise targeting of specific genes by RNAi mediated by siRNAs is a major advantage of such therapy, and it presents as one of the most favourable cancer treatment options in the near future. HPV-induced tumors have emerged as an ideal model system for the therapeutic use of RNAi due to the exclusive expression of the $E6$ and $E7$ oncogenes in cancer cells. As such, many efforts have been made to explore the therapeutic potentials of siRNAs targeting the $E6$ and $E7$ oncogenes in HPV-induced cancers. However, due to the highly specific nature of the siRNAs, subtyping of the HPV involved has to be accurately performed before the corresponding E6- or E7-siRNAs can be administered. Moreover, a number of HPV infections are reported to be heterogeneous in nature with the infection of multiple subtypes in one individual (41). This makes the selection of a suitable siRNA a challenge.

In view of the limitations in both cisplatin-based chemoradiotherapy and E6- or E7-siRNA based therapy, targeting MLL5β in HPV16/18-positive cancers (which makes up almost 70% of all HPV-related cervical cancers) with MLL5β-siRNA shows great potential as an alternative therapeutic approach. First of all, due to the ability of MLL5β-siRNA to concurrently down-regulate both E6 and
E7 expression, it has an enhanced anti-tumor effect compared to the use of E6- or E7-siRNA alone. As shown in both in vitro and in vivo assays, the combined effects on E6 and E7 expression of MLL5β-siRNA on tumor cells resulted in the activation of both the apoptotic and senescence pathways in these cells, resulting in an enhanced tumor growth inhibition (Figure 1 and 2). Moreover, since MLL5β activates E6/E7 expression in both HPV16 and HPV18 subtypes, it can be employed as a therapeutic strategy in both HPV16- and HPV18-positive tumors, circumventing the subtype specificity issue of E6- or E7-siRNA-based therapy. Secondly, in comparison to cisplatin, cytotoxic effects of MLL5β-siRNA are highly specific to HPV16/18-positive cancer cells because its target, MLL5β, is expressed exclusively in these cancer cells (Figure 2A and B). This high specificity of the MLL5β-siRNA significantly lowers the adverse side effects brought about by cisplatin’s cytotoxic effects on non-tumor cells. Besides, we also established that cisplatin exerts its anti-cancer effect through a mechanism that involves MLL5β (Figure 3A and B). Therefore, by targeting MLL5β directly using the MLL5β-siRNA, we could achieve a comparable anti-cancer effect compared to cisplatin but with minimal cytotoxicity on non-tumor cells.

In this study, we also explored the effectiveness of MLL5β-siRNA-IR combinational therapy in vitro and in vivo. MLL5β-siRNA was found to sensitize HPV16/18-positive cells to the cytotoxic effects of IR in vitro (Figure 4B). We also observed a significant reduction in tumor growth in vivo when tumors were co-treated with MLL5β-siRNA and IR, suggesting the potential application of MLL5β-siRNA in place of cisplatin. It would also be interesting to compare if MLL5β-siRNA-treated tumors are more sensitive to IR compared to cisplatin-treated tumors in vivo. This could help us determine if a reduced dose of IR can be used if tumors are treated with MLL5β-siRNA instead of cisplatin to further minimize any side effects brought about by IR-based radiotherapy. Together these experiments could provide us with valuable insights to the potential translation of MLL5β-siRNA-IR combinatory therapy from bench to bedside. In light of the findings reported in this study, we are also interested in gaining more insights into the molecular mechanisms behind the regulation of the HPV LCR by MLL5β to aid in the identification of small molecular inhibitors that could be clinically relevant for cervical cancer therapy.
In summary, we have successfully demonstrated the potential of RNAi-targeted silencing of MLL5β via the use of MLL5β-specific siRNA as a novel therapeutic strategy for HPV16/18-positive human cervical cancer. The use of MLL5β-siRNA offers higher tumor specificity compared to the conventional treatment of using cisplatin and better efficacy compared to the proposed method of employing E6- and E7-specific siRNAs. Our study highlighted that MLL5β-siRNA-mediated targeted silencing of MLL5β induces both apoptosis and senescence in cancer cells via its combined action on both E6 and E7 expression. We also offered a possible mechanism for cisplatin-induced IR sensitization effects through its action on MLL5β and suggest the potential of MLL5β-siRNA as an alternative to cisplatin in the treatment of HPV16/18-related cervical cancers.

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REFERENCES


Table 1: Average tumor volume doubling time (DT) after MLL5β-, E6- and E7-siRNA treatment in HeLa xenograft tumor model.

<table>
<thead>
<tr>
<th>siRNA Treatment</th>
<th>Average tumor volume DT (days)</th>
<th>t-test (vs scrambled)</th>
</tr>
</thead>
<tbody>
<tr>
<td>scrambled</td>
<td>14.60 ± 13.16</td>
<td></td>
</tr>
<tr>
<td>HPV18 E6</td>
<td>28.60 ± 13.16</td>
<td>N.S.#</td>
</tr>
<tr>
<td>HPV18 E7</td>
<td>21.60 ± 13.16</td>
<td>N.S.#</td>
</tr>
<tr>
<td>MLL5β</td>
<td>38.00 ± 12.14</td>
<td>(p &lt; 0.05)</td>
</tr>
</tbody>
</table>

N.S.= not significant  \(^#\ p > 0.05\)
Table 2: Average tumor volume doubling time (DT) after MLL5β-siRNA and gamma-irradiation (IR) combinatory treatment in HeLa xenograft tumor model.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Average tumor volume DT (Days)</th>
<th>t-test</th>
</tr>
</thead>
<tbody>
<tr>
<td>scrambled-siRNA (no IR)</td>
<td>10.12 ± 0.62</td>
<td></td>
</tr>
<tr>
<td>MLL5β-siRNA (no IR)</td>
<td>13.67 ± 1.53</td>
<td><em>p &lt; 0.001</em> [vs scrambled-siRNA (no IR)]</td>
</tr>
<tr>
<td>scrambled-siRNA + 1-Gy</td>
<td>13.44 ± 2.26</td>
<td><em>p &lt; 0.01</em> [vs scrambled-siRNA (no IR)]</td>
</tr>
<tr>
<td>MLL5β-siRNA + 1-Gy</td>
<td>18.77 ± 4.66</td>
<td><em>p &lt; 0.01</em> [vs scrambled-siRNA (no IR)] <em>p &lt; 0.05</em> [vs scrambled-siRNA + 1-Gy] <em>p &lt; 0.05</em> [vs MLL5β-siRNA (no IR)]</td>
</tr>
</tbody>
</table>
FIGURE LEGEND

Figure 1. Targeted silencing of MLL5β displays anti-tumor properties through the induction of apoptosis and senescence.

(A) Knockdown of MLL5β reduced colony-formation ability *in vitro*. HeLa cells were treated with either the empty pSilencer vector or the pSilencer vector containing the MLL5β-, HPV18 E6- or E7-shRNA. MLL5β-shRNA-treated cells showed significant reduction in colony numbers compared to other shRNAs (* p<0.05, *** p<0.001). (B) Loss of ability for anchorage-independent growth in HeLa cells after MLL5β knockdown. HeLa cells were treated with the respective shRNA as in (A). No visible colonies can be observed after treatment of MLL5β-, E6- or E7-shRNA. Results are representative of three independent experiments. (C) Targeted silencing of MLL5β induces both apoptosis and senescence in HPV18-positive HeLa cells. Knockdown of MLL5β with MLL5β-siRNA down-regulates both E6 and E7 levels in HPV18-positive HeLa cells. HPV18-positive HeLa cells were treated with either scrambled-siRNA or MLL5β-siRNA. Cells were harvested for RNA at 24, 48 and 72 h, and E6 and E7 mRNA levels were determined by qRT-PCR. Average-fold decrease in transcript expression of three independent experiments is plotted (left panel). A representative blot of E6 and E7 protein levels after scrambled-siRNA or MLL5β-siRNA knockdown (right panel). (D) Knockdown of MLL5β in HPV18-positive HeLa cells activates the apoptotic pathway. HeLa cells were treated with either scrambled-, MLL5β-, HPV18 E6- or E7-siRNA before cell lysates were collected at 24, 48 and 72 h intervals for western blotting. Cleaved-PARP was used as a marker for apoptosis. An accumulation of cleaved-PARP was observed in both MLL5β and E6-siRNA-treated cells. (E) Knockdown of MLL5β in HPV18-positive HeLa cells induces senescence. HeLa cells were treated with either scrambled-, MLL5β- or HPV18 E6- or E7-siRNA for up to 1 week and cells were stained for β-galactosidase to detect senescence. Cells stained blue (marked by arrows) indicate a cell in senescence. Total cell number and the total number of senescent cells were counted (* p < 0.05, *** p < 0.001). A higher percentage of senescent cells can be observed in both MLL5β- and E7-siRNA-treated cells.
Figure 2. MLL5β-siRNA selectively suppresses the growth of HPV16/18-positive cells in vitro and inhibits in vivo tumor growth.

MLL5β-siRNA suppresses the growth of HPV16/18-positive cancer cells but not HPV16/18-negative cells. HPV16/18-positive HeLa and SiHa (black bars) and HPV16/18-negative C33A and WI38 cells (white bars) were each treated with either scrambled-, MLL5β-, HPV16/18 E6- or E7-siRNA and trypan blue exclusion assay was used to determine the number of surviving cells after (A) 72 h and (B) 96 h. Graphs were plotted by normalizing the number of surviving cells after siRNA treatments against the number of surviving cells in the scrambled-siRNA-treated samples. Results are representative of three independent experiments. Data were analyzed with un-paired t-test and significance was calculated with respect to percentage of surviving cells in the scrambled-siRNA-treated group for each cell line (* p ≤ 0.05, ** p < 0.01, *** p < 0.001). (C) MLL5β-siRNA significantly reduces average tumor volume in the HeLa xenograft tumor model. Effects of either scrambled-, MLL5β-, E6- or E7-siRNAs on the average volume increase of the tumor over the 20-day treatment period were recorded. MLL5β-siRNA-treated group showed a significant reduction in tumor volume (* p ≤ 0.05) compared to scrambled-treated group. (D) Fold of increase in tumor volume after the 20-day treatment was recorded and the MLL5β-siRNA-treated group showed a significantly smaller-fold increase in average tumor volume after 20 days (*** p < 0.001) compared the E6-siRNA treatment group (* p ≤ 0.05). Each treatment group consists of five mice (n=5).

Figure 3. Cisplatin-induced gamma-irradiation sensitization properties are modulated in part through MLL5β. (A) Cisplatin treatment reduces MLL5β transcript levels in a dose-dependent manner. HeLa and SiHa cells were treated with cisplatin at the indicated doses for 6 h, after which RNA was harvested. MLL5β, E6 and E7 transcript levels were determined by qRT-PCR. All reduction in expression after cisplatin treatment were statistically significant with p ≤ 0.05 when analyzed using un-paired t-test versus vehicle treated. (B) Overexpression of MLL5β in HeLa cells negates cisplatin’s ability to sensitize these cells to gamma-irradiation (IR). MLL5β was overexpressed in HeLa cells for 24 h before treatment with cisplatin for 6 h, after which the cells were subjected to IR of 5-Gy, and cytotoxicity assay was carried out 24 h post-irradiation (*** p < 0.001).
Figure 4. MLL5β-siRNA sensitized HPV16/18-positive cells to gamma-irradiation (IR) in vitro and MLL5β-siRNA-IR combinatory treatment enhanced tumor growth inhibition in vivo. (A) Cisplatin sensitized HPV16/18-positive cell lines to IR. Cytotoxicity assay was carried out to assess the effects of cisplatin on HeLa, SiHa, C33A and WI38 cells, with or without IR. Cisplatin sensitization of SiHa and HeLa to IR-induced cytotoxicity was observed at all doses of cisplatin used while its sensitization effects of C33A and WI38 were minimal. General cytotoxicity was observed in all cells after cisplatin treatment (* p ≤ 0.05, ** p < 0.01, *** p < 0.001). (B) MLL5β-siRNA sensitized HPV16/18-positive cell lines to IR. Cytotoxicity assay was carried out to assess the effects of scrambled, MLL5β-, E6- and E7-siRNA on HeLa, SiHa, C33A and WI38 cells, with or without IR. MLL5β-siRNA selectively sensitized both HPV16/18 positive SiHa and HeLa cells to IR while its effects on HPV16/18-negative C33A and normal diploid WI38 were less pronounced. MLL5β-siRNA was also less cytotoxic to normal cells compared to cisplatin treatment as shown in (A). IR alone induced significant increases in cell cytotoxicity for all treatments (* p ≤ 0.05). Asterisks on individual bars represent significance with respect to the scrambled-treated cells in the respective with or without IR treatment groups (* p ≤ 0.05, ** p < 0.01, *** p < 0.001). (C) MLL5β-siRNA in combination with gamma-irradiation (IR) effectively reduced the average tumor size in the HeLa-induced xenograft mouse model. MLL5β-siRNA and IR combinatory treatment significantly reduces average increase in tumor volume over the 20-day treatment period compared to either scrambled-siRNA treatment (*** p < 0.001) or MLL5β-siRNA (* p ≤ 0.05) and IR single treatments (* p ≤ 0.05). (D) Fold of increase in tumor volume at the end of 20 days for each treatment was recorded. MLL5β-siRNA-IR co-treatment significantly reduced tumor volume increase compared to scrambled-(*** p< 0.001) or MLL5β-siRNA (* p ≤ 0.05) or IR single treatment (* p ≤ 0.05). Each treatment group consists of 6 mice (n=6).
Figure 1
Figure 2

A) Cell survival after 72 h of siRNA knockdown

B) Cell survival after 96 h of siRNA knockdown

C) The effect of MLL5β-, E6- and E7-siRNA on tumor growth in the HeLa xenograft tumor model

D) Average-fold increase in tumor volume after MLL5β-, E6- and E7-siRNA treatment in HeLa xenograft tumor model
Figure 3
**A** Effect of cisplatin treatment coupled with IR in cells

- Without IR
- With IR

**B** Effect of siRNA knockdown coupled with IR in cells

- HeLa
- SiHa
- C33A
- WI38

**C** Combinatory effects of MLL5β-siRNA and 1-Gy IR on tumor growth in the HeLa xenograft tumor model

**D** Average-fold increase in tumor volume after MLL5β-siRNA and IR combinatory treatment in the HeLa xenograft tumor model

- Without IR
- With IR

[Graphs and data from mct.aacrjournals.org on June 8, 2017. © 2014 American Association for Cancer Research.]
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