Wee1 as therapeutic target in cervical cancer

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Abstract

Patients with advanced-stage cervical cancer are treated with chemoradiation, but 21-34% of these patients still die within 5 years. Novel treatment approaches for these patients are therefore eagerly awaited.

Cervical cancer is almost invariably caused by the human papillomavirus (HPV), which compromises G1/S cell cycle checkpoint function due to p53 inactivation by the HPV-E6 oncoprotein. Consequently, HPV-positive cancer cells rely on their G2/M checkpoint for cell cycle control. We tested whether G2/M checkpoint abrogation through inhibition of Wee1 has therapeutic potential in cervical cancer cells.

Immunohistochemical analysis of 204 advanced-stage cervical cancers showed Wee1 expression in 99.5% of cases. Reanalysis of a panel of 522 human cancer cell lines revealed that cervical cancer cell lines are very sensitive to Wee1 inhibition in vitro. Additionally, Wee1 inhibition in a subset of cervical cancer cell lines sensitized to cisplatin treatment. This effect was due to checkpoint abrogation, as judged by mitotic entry with DNA breaks. Few HPV-positive cervical cell lines, including SiHa, were not sensitive for Wee1 inhibition, and did not show sensitization to cisplatin after Wee1 inhibition. Insensitivity of these cells correlated with low HPV copy number and residual p53 levels. Surprisingly, siRNA-mediated removal of Rb1, but not p53, conferred sensitivity to Wee1 inhibition and resulted in cisplatin sensitization.

Combined, Wee1 is ubiquitously expressed in cervical cancers and Wee1 inhibition sensitized for cisplatin by abrogating G2/M checkpoint function in cervical cancer cell lines. Importantly, sensitivity to Wee1 inhibition not only depends on p53 loss-of-function, but also requires inactivation of Rb1.
5. Wee1 as therapeutic target in cervical cancer

Introduction

Worldwide, cervical cancer is the third leading cause of cancer-related deaths among women [1]. Currently, chemoradiation is the standard of care for locally advanced cervical cancer patients. However, still 21-34% of these patients will die within 5 years [2]. Regrettably no targeted therapies are as yet available for these patients, and novel approaches are therefore eagerly awaited.

The uniformity in cervical cancer etiology may provide common vulnerabilities of these cancers, which could serve as therapeutic targets. Cervical cancer is in virtually all cases caused by infection of the cervical epithelium with 'high risk' human papillomavirus (HPV) [3]. Expression of the viral HPV-E7 protein leads to inactivation of the retinoblastoma protein pRb, which initiates a cellular proliferation program [4, 5]. In parallel, the viral HPV-E6 protein promotes the degradation of the tumor-suppressor protein p53 and results in the inactivation of the p53 pathway in the far majority of cervical cancers [6-8].

P53 pathway inactivation leads to malfunctioning of cell cycle checkpoints. In p53-proficient cells, DNA damage can arrest the cell cycle at two phases. The G1/S checkpoint prevents entry into S-phase, whereas the G2/M checkpoint transition halts entry into mitosis when DNA damage is present. The maintenance of the G1/S arrest is largely dependent on the activation of the p53 tumor-suppressor protein. Specifically, p53 transcriptionally activates the Cdk inhibitor p21 (p21\textsuperscript{CIP1}/p21\textsuperscript{WAF1}), which directly inhibits G1/S-specific cyclin-Cdk complexes to prevent S phase entry [9]. The G2/M checkpoint depends less on the transcriptional activity of p53, and this checkpoint is usually intact in p53-deficient cancer cells [10]. As a result, p53- defective cancer cells rely heavily on their G2/M checkpoint for proper cell cycle control. It was therefore postulated that inhibition of G2/M checkpoint components might constitute a therapeutic approach to efficiently target p53-defective cancers.

The molecular wiring of the G2/M checkpoint is centered on the phosphorylation of the G2/M-specific cyclin B-Cdk1 complex. The activity of this complex is in large part determined by phosphorylation of residues Thr-14 and Tyr-15 within Cdk1 [11, 12]. Phosphorylation at these residues has an inhibitory effect on Cdk1 activity and is accounted for by the Myt1 and Wee1 kinases [13-15]. The Wee1 kinase is expressed in a cell cycle-dependent fashion and is mainly localized in the cytoplasm during interphase [16]. Prior to cells entering mitosis, Wee1 accumulates in the nucleus, where it prevents the premature activation of cyclin B-Cdk1 [16, 17]. In turn, dephosphorylation and consequent activation of cyclin B-Cdk1 is mediated by one of the three Cdc25 phosphatase isoforms [18]. In case of DNA damage, Cdc25 phosphatases are rapidly inhibited [19-22], whereas the activity of the Wee1 kinase is up regulated [23-25]. Combined, this results in Cdk1 inactivation upon DNA damage and installs a G2 arrest [26].

One approach to sensitize p53-deficient cancer cells, such as HPV-infected cells, for DNA damaging agents through G2/M checkpoint abrogation is to interfere with Wee1 [27]. Indeed, genetic inactivation of Wee1 could enhance the anti-tumor efficacy of DNA damaging agents by forcing premature mitotic entry [28]. Since cervical cancer is almost always characterized by HPV-mediated p53 inactivation, treatment of these cancers may benefit from Wee1 inhibition [29, 30]. Here we therefore immunohistochemically analyzed the expression of Wee1 in tumors of cervical cancer patients. Subsequently, we reanalyzed a large panel of human cancer cell lines, and assessed the Wee1 inhibitor sensitivity with emphasis on the cervical cancer cell lines. Finally,
we studied the effects of Wee1 inhibition on cisplatin sensitivity and investigated the underlying mechanism in a subset of cervical cancer cell lines.

Results
Wee1 is expressed in cervical cancer specimen
We first determined the specificity of immunohistochemical Wee1 analysis using paraffin-embedded HeLa cervical cancer cells. Cytoplasmic and nuclear Wee1 staining was present and this staining pattern matched previously reported Wee1 localization [16]. Next, we depleted Wee1 expression using siRNA (Supplemental Figure S1A). Importantly, the phosphorylation status of the Wee1 downstream target Cdk1 at Tyrosine-15 was greatly diminished after siRNA-mediated Wee1 down regulation, indicative of a functional depletion of Wee1 (Supplemental Figure S1B). Importantly, Wee1 expression also was strongly reduced, underscoring the specificity of our immunohistochemical Wee1 analysis (Supplemental Figure S1A, S1B). Subsequently, we analyzed Wee1 expression in tumor tissues of cervical cancer patients. We analyzed tumor tissue of 204 advanced-stage cervical cancer patients, as this subgroup of cervical cancer patients is treated with chemoradiation and could benefit from novel treatment approaches. Clinico-pathological data of the 204 patients is described in Supplemental Table 1. Representative Wee1 stainings in these tumors are illustrated in Figure 1A. Based on staining intensity, we classified Wee1 expression as negative (n=1), low (n=112), moderate (n=81) or strong (n=10) (Figure 1B). Positive nuclear Wee1 staining was present in 203 of 204 patients (99.5%), whereas in only 1 patient no Wee1 expression was observed (0.5%). Furthermore, in 198 out of 204 evaluable patients (97.1%), Wee1 expression was present in over 75% of the tumor cells.

Figure 1: Wee1 protein expression in cervical cancer patients (A) Representative immunohistochemical Wee1 stainings of the tumor, of patient with advanced-stage cervical cancer. (B) Distribution of Wee1 staining intensity in advanced-stage cervical cancer tissues.
Figure 2: Cytotoxicity of MK-1775 in cancer cell lines (A) Analysis of MK-1775 cytotoxicity in 522 human cancer cell lines. Each circle represents a cell line, where green circles represent non-cervical cancer cell lines and red circles represent cervical cancer cell lines. Cytotoxicity data of these cell lines were adapted from data published in [31] and plotted as the log of the IC50 concentration in μM. The mean IC50 concentration is indicated with a dashed line. (B) Left panel: Analysis of MK-1775 sensitivity of non-cervical cancer cell lines versus cervical cancer cell lines based on their IC50 values. Right panel: Analysis of MK-1775 cytotoxicity of HPV-negative versus HPV-positive cancer cell lines based on their IC50 values. Statistical testing was done using a two-sided Students T-test, where p=0.05 is considered statistically significant. (C) MK-1775 cytotoxicity of MK-1775 (left panel) or PD-166285 (right panel) was tested in C33A, HeLa and SiHa cervical cancer cell lines using MTT assays. To this end, cells were treated for 4 days with indicated concentration of MK-1775 or PD-166285, and metabolic MTT conversion was related to DMSO-treated cultures and indicated as relative viability.

Sensitivity of cancer cell lines to chemical Wee1 inhibition
To analyze the sensitivity to chemical Wee1 inhibition of cervical cancer cell lines in comparison to cell lines of other cancer types, we re-analyzed data on a panel of 522 human cancer cell lines, which were treated with the small molecule Wee1 inhibitor MK-1775 [31]. In this data set, MK-1775 sensitivity was judged on 3-day cytotoxicity assays and plotted as IC50 values (Figure 2A). On average, cervical cancers cell lines (n=21) showed lower IC50 values when compared to non-cervical cancer cell lines (n=501) (IC50 of 0.56 nM versus 0.71 nM respectively, p=0.482, Figure 2B, left panel). Also, when cell lines were categorized based on HPV-positivity regardless of tissue of origin, we found that HPV-positive cell lines (n=17) were on average more sensitive to MK-1775 when compared to HPV-negative cell lines (n=505) (IC50 of 0.44 nM versus 0.72 nM respectively,
\( p = 0.24 \), Figure 2B, right panel). However, no statistically significant differences between cervical cancer cell lines or HPV-positive cell lines and their control groups were found, possibly due to the fact that 2 out of 21 HPV-positive cervical cell lines showed remarkable insensitivity to Wee1 inhibition, including the SiHa cell line. In order to verify these findings, we assessed the cytotoxicity of MK-1775 in HeLa (HPV-positive), C33A (HPV-negative, TP53 mutant) and SiHa (HPV-positive) cervical cancer cell lines in short-term survival assay (Figure 2C). In line with results from the cell line panel data, we observed that both HeLa and C33A cells were markedly more sensitive to Wee1 inhibition (IC50's of 2.66 nM and 2.34 nM, respectively) when compared to the SiHa cells (IC50 of 8.63 nM) (Figure 2C, left panel). Additionally, we assessed the cytotoxicity of an independent chemical second Wee1 inhibitor, PD-166285 (Figure 2C, right panel) [32]. Again, we observed that SiHa cells (IC50 of 3.49 nM) were less sensitive to Wee1 inhibition compared to HeLa and C33A cells (IC50s of 2.71 nM and 2.24 nM, respectively), suggesting that these differences are related to Wee1 inhibition, rather than off-target effects (Figure 2C).

**Sensitization to cisplatin by Wee1-inhibition**

We next tested whether Wee-1 inhibition could sensitize cervical cancer cell line to cisplatin in short-term cell survival assays. HeLa, C33A and SiHa cells were treated with increasing concentrations of cisplatin in combination with MK-1775 (250 nM). We observed that MK-1775 enhanced the cytotoxicity of cisplatin in both HeLa and C33A cells (Figure 3A, B, left panel). In contrast, MK-1775 treatment did not potentiate the cytotoxic effects of cisplatin in SiHa cells (Figure 3C), in analogy with this cell line being insensitive to Wee1 inhibition as a single agent treatment (Figure 2C). Similar effects were observed with the Wee1 inhibitor PD-166285 (Figure 3A, B, C, right panel). Thus, in the cervical cell lines that are sensitive to Wee1 inhibition, also cisplatin sensitization can be achieved using Wee1 inhibition.
Figure 3: Sensitization to cisplatin using Wee1 inhibition in cervical cancer cell lines (A) Left panel: HeLa cells were treated with indicated concentrations of cisplatin in combination with DMSO or MK-1775 (250 nM). After 4 days of treatment, cells were incubated with MTT and the viability of cells was determined by colorimetric measurement. Right panel: HeLa cells were treated with indicated concentrations of cisplatin in combination with DMSO or PD-166285 (250 nM) and harvested after 4 days and analyzed as for left panel. (B) Left panel: C33A cells were treated and analyzed as for A (left panel). Right panel: C33A cells were treated and analyzed as for A (right panel). (C) Left panel: SiHa cells were treated and analyzed as for A (left panel). Right panel: SiHa cells were treated and analyzed as for A (right panel).
Wee1 inhibition leads to G₂/M checkpoint abrogation and elevated levels of DNA damage in mitosis.

In order to test how Wee1 inhibition affects cellular behavior in the absence or presence of cisplatin, we assessed cell cycle progression and levels of DNA breaks. When cervical cell lines were treated with cisplatin, the level DNA breaks clearly increased, as judged by gamma-H2AX positivity (Figure 4B and data not shown). Importantly, none of the 3 cell lines showed mitotic cells (MPM2-positive) that were gamma-H2AX-positive, indicating that all three cell lines are proficient for a DNA damage-induced G₂/M arrest (Figure 4B). When cisplatin was combined with MK-1775 treatment, again high levels of gamma-H2AX-positivity were observed (Figure 4B). However, the combination treatment resulted in both interphase and mitotic cells that display DNA damage, illustrative for G₂/M checkpoint abrogation. Strikingly, the increase in mitotic cells with DNA damage was only found in HeLa and C33A cells, and was not observed in SiHa cells (Figure 4C).

Since checkpoint abrogation by Wee1 inhibition only occurs in cell lines that are sensitive for MK-1775, these results suggest that this mechanism may be underlying the sensitivity to Wee1 inhibition in cervical cancer cells.

Figure 4: G₂-M checkpoint abrogation in MK-1775-sensitive cervical cancer cell lines. (A) HeLa cells were left untreated and were fixed in 70% ice-cold ethanol and stained with anti-gamma-H2AX/Alexa-488 and anti-MPM2/Alexa-647. Subsequently, cells were labeled with propidium iodide/RNase and analyzed by flow cytometry. Representative FACS profiles of cell cycle distribution are presented. Numbers indicate the percentages of cells in each phase of the cell cycle. Mitotic cells were gated based on MPM2-positivity and subsequently analyzed for gamma-H2AX positivity. (B) HeLa cells were left untreated or treated with cisplatin (0.5 µM), MK-1775 (200 nM) or the combined treatment with cisplatin and MK-1775. After 24 hours of treatment, cells were fixed in 70% ice-cold ethanol and stained as described in panel A. Representative examples of FACS plots for gamma-H2AX-Alexa488 and MPM2-Alexa-647 are presented. Numbers indicate the percentage of gamma-H2AX/MPM2 double positive cells. (C) HeLa, C33A and SiHa cells were treated and analyzed as for B. The average percentage of gamma-H2AX/MPM2 double positive cells from two independent experiments and standard deviations are represented (* represents p<0.05).
Combined inactivation of p53 and Rb1 determines the response to Wee1 inhibition.

Wee1 inhibition enhances the cytotoxic effects of chemotherapeutic agents mainly in p53-deficient cell lines [33-35]. Although SiHa and HeLa cell lines are both HPV-positive, clear differences in their sensitivity to Wee1 inhibition were observed. One explanation that could underpin this difference is the higher expression levels of Wee1 in sensitive cell lines HeLa and C33A compared to the insensitive SiHa cell line. Importantly, the level of Wee1 corresponded with the phosphorylation status of its downstream target Cdc2, indicating that higher Wee1 expression goes along with increased Wee1 activity. Another reason that could explain the difference in sensitivity to Wee1 inhibition could be a differential degree of p53 inactivation. Indeed, when levels of p53 were examined, SiHa cells showed residual p53 levels, whereas HeLa showed a virtual absence of p53 (Figure 5A). Notably, C33A cells show extremely high levels of
p53, due to TP53 mutation. To test whether the residual p53 levels in SiHa cells indeed conferred insensitivity to Wee1 inhibition, SiHa cells were stably depleted from p53 using retroviral shRNA (Figure 5B). Surprisingly, p53-depleted SiHa cells did not show increased sensitivity to MK-1775, when compared to SiHa cells, stably transfected with a control shRNA virus (Figure 5C). Besides targeting p53, HPV also inactivates the Retinoblastoma (Rb1) tumor-suppressor [36]. Notably, we observed that Rb1 resides mostly in its hypophosphorylated active form in SiHa cell lines whereas in HeLa and C33A only the hyperphosphorylated inactive form of Rb1 was detected (Figure 5A). Therefore, to investigate whether this residual Rb1 function in SiHa cells might explain the insensitivity to Wee1 inhibition, we inactivated Rb1 using siRNA (Figure 5D). Rb1 depletion caused a clear sensitization to MK-1775 treatment both in SiHa cells harboring control shRNA as well as in p53-depleted SiHa cells (Figure 5E). These results suggest that Rb1 inactivation is a key determinant of the sensitivity of cervical cancer cells to Wee1 inhibition. To test whether a similar requirement for Rb1 inactivation determines cisplatin sensitization by Wee1 inhibition, we investigated the combined treatment of MK-1775 and cisplatin in Rb1-depleted SiHa cells. In line with the results of single agent treatment with MK-1775, depletion of Rb1, but not p53, resulted in sensitizing effects of Wee1 inhibition to cisplatin (Figure 5F). Taken together, these data indicate that cervical cancer cells may be sensitized to Wee1 inhibition through combined inactivation of Rb1 and p53.

Figure 5: Rb1 inactivation determines cytotoxicity after Wee1 inhibition
(A) HeLa, C33A and SiHa cell were irradiated (5 Gy) and harvested at 5 hours after irradiation. Immunoblotting was performed with anti-p53 and anti-actin antibodies. For HeLa and SiHa p53 levels from the same long exposure are shown. For C33A, a short exposure is shown. (B) SiHa cells were stably infected with pRS-Scr or pRS-p53 and selected using puromycin. Subsequently, SiHa cells were left untreated or irradiated (5Gy) and harvested and analyzed as for panel A. (C) Stably infected SiHa cell lines harboring indicated shRNAs were treated with indicated concentrations of MK-1775. After 4 days of treatment, viability of cells was analyzed by colorimetric analysis of MTT conversion. Viability of cells was related to DMSO-treated cultures. Averages and standard deviations of three independent experiments are indicated. (D) SiHa cells were transiently transfected with two independent siRNAs targeting Rb1 or a scrambled siRNA. Cells were harvested at 24 hours after transfection and lysates were immunoblotted using anti-Rb1 and anti-Actin antibodies. (E) SiHa cells, stably infected with pRS-Scr (solid lines) or pRS-p53 (dashed lines) were transiently transfected with Rb1 siRNA (#1, red lines) or control siRNA (green lines). After 24 hours post-transfection, cell were trypsinized, replated in 96-well plates and treated with indicated concentrations of MK-1775. After 4 days of treatment, cells were incubated with MTT and viability of cells was determined by colorimetric measurement. DMSO-treated cultured were used as a reference. (F) SiHa cells, stably infected with pRS-Scr (green lines) or pRS-p53 (red lines) were transiently transfected with Rb1 siRNA (#1, dashed lines) or control siRNA (solid lines). Cells were related in 96-well plates at 24 hours after treatment and treated with indicated concentrations of cisplatin in combination with MK-1775 (250 nM) or DMSO. After 3 days of treatment, cells were incubated with MTT and colorimetric MTT conversion was used to calculate cellular viability.
Discussion
In this study we investigated the expression of Wee1 in advanced-stage cervical cancer and showed that Wee1 is ubiquitously expressed in the far majority (99.5%) of these tumors. Subsequently, we analyzed the sensitivity of a large panel of human cancer cell lines to the Wee1 inhibitor MK-1775 and found that cervical cancer cell lines on average were more sensitive to Wee1 inhibition than non-cervical cancer cell lines. Finally, we found that some HPV-positive cervical cancer cell lines showed unusual resistance to Wee1 inhibitor treatment, including SiHa, which was related to the level of Rb1 inactivation rather than p53 inactivation.

5. Wee1 as therapeutic target in cervical cancer
A strikingly high percentage of cervical cancers showed protein expression in our study. We checked the data of The Cancer Genome Atlas consortium (http://www.cbioportal.org/public-portal/) on cervical cancers and observed that no homozygous deletions of the WEE1 gene were reported. This finding is in line with our results that Wee1 expression is observed in 99.5% of cases. Taken together, it appears that Wee1 is ubiquitously expressed in cervical cancers, which makes it an attractive potential therapeutic target for most of these cancers. In this respect this tumor type with it ubiquitous Wee1 expression differs from other solid tumors. Wee1 expression was found in 90% of vulvar squamous carcinomas [37] and 70% of metastatic melanomas [38]. In addition, substantial Wee1 overexpression compared to normal tissues was reported in 80% of osteosarcomas [39] and 35% of the luminal and HER2-positive breast cancers [40].

The small molecule Wee1 inhibitor MK-1775 has single agent activity in various p53-deficient cancer cell lines, both in vitro and in vivo [33, 35, 41]. Also, treatment with MK-1775 sensitized p53-deficient cancer cells for several chemotherapeutic agents [33, 35, 41]. In addition, Wee1 inhibition efficiently sensitized p53-deficient tumor cells for radiotherapy [34, 42]. Since p53 inactivation is a prerequisite for effective Wee1 inhibitor treatment, this approach appeared promising for cervical cancer, which has frequent HPV-mediated p53 inactivation. Surprisingly, the HPV-positive cervical cancer cell line SiHa did not follow this pattern. Mechanistically, SiHa cells appeared to have residual cell cycle checkpoint activity, as Wee1 inhibition did not lead to G2/M checkpoint abrogation. As a consequence, combined treatment with cisplatin and MK-1775 did not result in mitotic entry in the presence of DNA damage. This residual checkpoint activity may be caused by low copy numbers of HPV in SiHa. Specifically, SiHa cells only contain 2 copies of HPV-16, whereas HeLa cells have ~40 copies of HPV-18 [43]. Indeed, we observed that the reported low copy number of HPV in SiHa cells corresponded to residual levels of p53 as assessed by Western blotting (Figure 5A). Unexpectedly, down-regulation of p53 in SiHa cells did not confer to Wee1 inhibition. Rather, our results indicated that down-regulation of Rb1, in addition to p53 inactivation, determines the sensitivity to Wee1 inhibition in cervical cancer cells. The notion that additional factors to p53 determine Wee1 inhibitor sensitivity is in line with the observation that not all p53-mutant pancreatic cancer xenografts were sensitive to Wee1 inhibition in vivo [35]. Whether differences in Rb1 levels, or other genes involved in G1/S cell cycle control, can explain the variable responses to Wee1 inhibitor treatment in other cancer types remains to be experimentally addressed.

Combined, our study indicates that Wee1 is a potential therapeutic target in cervical cancer as a single agent and as an agent that potentiates the effects of cisplatin. Of note, it seems that susceptibility for Wee1 inhibitor treatment requires sufficient levels of HPV integration, to reach a threshold level of checkpoint abrogation. The ongoing clinical trials, in which MK-1775 is tested in cervical cancer patients (NCT01958658) and head-and-neck cancer patients (NCT01935037), allow addressing these questions.

Materials and Methods
Immunohistochemical analysis of patient material
Immunohistochemical analysis of Wee1 expression was performed on pre-treatment tumor tissue specimens of 255 patients with locoregionally advanced cervical cancer (stage IIIB-IVA),
primarily treated with radiotherapy or chemoradiation between March 1981 and December 2006 in the University Medical Center Groningen or affiliated hospitals [44]. International Federation of Gynecologists and Obstetricians (FIGO) guidelines were used for staging. Clinico-pathological data of patients analyzed in this study are summarized in Supplemental Table 1. A tissue microarray was generated with tumor specimens as described previously [45]. Sections of 3 μm were prepared for immunohistochemical staining by deparaffinization in xylene and rehydration in ethanol. Antigen retrieval was done using microwave treatment in Tris/HCl buffer (pH 9) for 15 minutes. Endogenous peroxidase activity was blocked by incubation with 0.3% hydrogen peroxidase for 30 minutes. Sections were incubated with a monoclonal antibody against Wee1 (Clone B-11, SC-5285, 1:50, Santa Cruz Biotechnology), phospho-Cdk1 (phospho-Tyr15, clone 10A11, #4539, 1:100, Cell Signaling Technology) or control antibodies: IgG₃ (X0931, Dako, Supplemental Figure S1C) and IgG (X0903, Dako) overnight at 4°C. Subsequently, sections were incubated for 30 minutes with horse-radish-peroxidase (HRP)-conjugated secondary rabbit anti-mouse antibody or goat anti-rabbit antibody and a tertiary HRP-conjugated goat anti-rabbit antibody or rabbit anti-goat (both at 1:100, Dako). For antibody detection, sections were incubated with 3’3-diaminobenzidine tetrahydrochloride (DAB) (Dako) and counterstained with hematoxylin.

Wee1 stainings were scored semi-quantitatively by two independent persons in line with a previously reported immunohistochemical Wee1 analysis [37]. In short, Wee1 stainings were categorized according to intensity in four categories: 0-negative, 1-low, 2-moderate, 3-strong. In addition the percentage of positive cells was determined and categorized as: 1 (<5%), 2 (5-25%), 3 (25-50%), 4 (50-75%) and 5 (>75%). Only patients with at least two evaluable tumour cores were included for analysis in Figure 1B and are listed in Supplemental Table 1. 51 out of 255 patients (20%) were not evaluable due to core loss. A concordance of more than 90% was found between both observers, for all immunostainings. Subsequent evaluation of discordant cases was performed to reach a consensus score.

Cell lines
The human cervical cancer cell lines HeLa, SiHa and C33A were obtained from the American Type Culture Collection (ATCC). The identity of all cell lines was validated using STR profiling (BaseClear, the Netherlands). All cell lines were cultured in D-MEM:HAM’s F12 media in a 1:1 ratio. Media were supplemented with 10% fetal bovine serum (Thermo Scientific), 100 units/ml penicillin and 100 μg/ml streptomycin. All cell lines were cultured at 37°C in a humid atmosphere with 5% CO2.

Flow cytometry
For cell cycle analysis, cell lines were treated with indicated doses of MK-1775 (Axon Medchem) and/or cisplatin for 24 hours. Cells were harvested using trypsin and fixed in ice-cold 70% ethanol. After washing, cells were incubated in PBS-0.5% Tween-20 with anti-gamma-H2AX (#9718, Cell Signaling Technology) and anti- MPM-2 (#5368, Millipore) overnight at 4°C. Cells were subsequently washed and incubated with Alexa-488-conjugated or Alexa-647-conjugated secondary antibodies for 45 minutes and then treated with RNAse (100 μg/ml) in combination with propidium iodide (50 μg/ml). Cells were analyzed on a FACS-Calibur (Becton Dickinson) using Cell Quest software (Becton Dickinson). A minimum of 30,000 events was counted per sample and used for further analysis.
Cytotoxicity assays
HeLa, C33A and SiHa cells were plated at a density of 2,000 cells (HeLa and C33A) or 3,000 cells (SiHa) per well in 96-well plates. One day after plating, cells were treated with indicated concentrations of the Wee1 inhibitor (MK-1775) or the Wee1 inhibitor PD-166285 (kindly provided by Pfizer), in combination with increasing concentration of cisplatin. After 3 days, MTT solution [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was added to a final concentration of 0.5 mg/ml and plates were incubated for 3 hours at 37°C. The MTT-containing medium was then discarded and 200 μl DMSO was added to cells. Absorbance was measured at 520 nm using a microplate spectrophotometer (iMark, Bio-Rad). Background absorbance was subtracted, and untreated cultures were used as a reference to obtain relative levels of MTT conversion. We made use of a previously published data set in which the sensitivity of 522 human cancer cell lines to Wee1 inhibition was tested [31]. In this data set, IC50 values of the Wee1 inhibitor MK-1775 were obtained using 3-day MTT cytotoxicity assays. Manual annotation of the cancer tissue origin and HPV status of these cell lines was performed to identify a subgroup of 21 cervical cancer cell lines, and a subgroup of 17 cancer cell lines which were confirmed HPV positive.

RNA interference
Retroviral shRNA interference was performed as described previously [46]. In summary, to produce retroviral particles, HEK293T cells were transfected using pMDG and pMDg/p along with either pRetrosuper-scrambled (5’- TTCTCGAAGGCTGGCTCT-3’) or pRetrosuper TP53 (5’- GACTCCAGTGGAATCTAC-3’, kindly provided by Dr. R. Agami, Netherlands Cancer Institute, Amsterdam, the Netherlands) in a 1:3:4 ratio. SiHa cells were infected using three consecutive 12 hours periods and infected cells were subsequently selected using puromycin selection (Sigma, final concentration 1 μg/ml).

For transient down-regulation of Rb1 and Wee1, SiHa cells were transfected with 133 nanomolar siRNAs targeting Rb1 (#1: HSS109090, #2: HSS109091; Invitrogen) and HeLa cells were transfected with siRNA targeting Wee1 (#1: HSS111337; #2: HSS111338, Invitrogen) using oligofectamine (Invitrogen). Transfections with scrambled siRNAs were used as controls in both cell lines (#12935-300, Invitrogen). Cells were harvested at 24 hours after transfection, and Rb1 and Wee1 depletion was confirmed by Western blotting.

Western blotting
Cells were lysed at 4°C in mammalian protein extraction reagent (M-PER, Thermo Scientific), supplemented with protease and phosphatase inhibitors (Roche). Protein concentrations were determined by Bradford assay. For SDS-PAGE, cell lysates were mixed with sample buffer (125 mM Tris-HCl, pH 6.8, 10% β-mercaptoethanol, 4.6% SDS, 20% glycerol, and 0.003% bromophenol blue) and were boiled for 10 minutes prior to loading. Samples were subsequently separated by electrophoresis on 8% SDS-polyacrylamide gels and transferred to polyvinyl difluoride membranes for 1 hour (PVDF, Immobilon-P, Millipore). After blocking nonspecific binding sites for 1 hour using 5% skim milk (Sigma) in Tris-buffered saline (TBS), supplemented with 0.05% Tween-20 (TBS-T), membranes were incubated overnight with primary antibody anti-Wee1 (Clone B-11, SC-5285, 1:1000, Santa Cruz Biotechnology), anti- Rb1 (IF8, sc-102, Santa Cruz, 1:100), anti-p53
(DO-1, SC-126, 1:1000, Santa Cruz Biotechnology) and anti-actin (#MA1-744, MD Biosciences) at 4°C. Membranes were then washed extensively in PBS-T and incubated with HRP-conjugated secondary antibodies for 30 minutes. After extensive washing, enhanced chemiluminescence (Lumi-Light PLUS, Roche) was used to visualize proteins using X-ray film exposure or a Bio-Rad chemiluminescence imager (Bio-Rad, Gel Doc EZ Imager), equipped with Bio-Rad Quantity One software.

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Supplemental data

5. Wee1 as therapeutic target in cervical cancer
References


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Supplementals

Supplemental Table S1: Patient and tumor characteristics in the 204 patients with sufficient tumor material available for Wee1 expression measurement

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<td>(12.7)</td>
</tr>
<tr>
<td>Other</td>
<td>11</td>
<td>(3.4)</td>
</tr>
<tr>
<td><strong>Tumor differentiation grade</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Good/moderate</td>
<td>124</td>
<td>(60.8)</td>
</tr>
<tr>
<td>Poor</td>
<td>68</td>
<td>(33.3)</td>
</tr>
<tr>
<td>Unknown</td>
<td>12</td>
<td>(5.9)</td>
</tr>
<tr>
<td><strong>Lymphangioinvasion</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>130</td>
<td>(63.7)</td>
</tr>
<tr>
<td>Yes</td>
<td>29</td>
<td>(14.2)</td>
</tr>
<tr>
<td>Unknown</td>
<td>45</td>
<td>(22.1)</td>
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<tr>
<td><strong>Tumor diameter</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-4cm</td>
<td>34</td>
<td>(16.7)</td>
</tr>
<tr>
<td>≥4cm</td>
<td>142</td>
<td>(69.6)</td>
</tr>
<tr>
<td>Unknown</td>
<td>28</td>
<td>(13.7)</td>
</tr>
</tbody>
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FIGO=International Federation of Gynecologists and Obstetricians
Supplemental figure S1 (A) HeLa cells were transiently transfected with two independent siRNAs targeting Wee1 or a control siRNA. Cells were harvested at 24 hours after transfection for Western blot analysis with anti-Wee1 and anti-actin antibodies. (B) Representative immunostaining for Wee1 and phospho-Tyr15-Cdk1 in paraffin embedded HeLa cell line pellets are indicated. (C-D) Representative immunostainings using control IgG antibodies.