Targeting the DNA damage response in cervical cancer

Wieringa, Hylke

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The role of ATM and 53BP1 as predictive markers in cervical cancer

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Abstract
Treatment of advanced stage cervical cancers with (chemo)radiation causes cytotoxicity through induction of high levels of DNA damage. Tumour cells respond to DNA damage by activation of the ‘DNA damage response’ (DDR), which induces DNA repair and may counteract chemoradiation efficacy. Here, we investigated DDR components as potential therapeutic targets and verified the predictive and prognostic value of DDR activation in cervical cancer patients treated with (chemo)radiation.

In a panel of cervical cancer cell lines, inactivation of ATM or its substrate 53BP1 clearly gave rise to cell cycle defects in response to irradiation. Concordantly, clonogenic survival analysis revealed that ATM inhibition, but not 53BP1 depletion, strongly radio-sensitised cervical cancer cells. In contrast, ATM inhibition did not radiosensitise non-transformed epithelial cells or non-transformed BJ fibroblasts. Interestingly, high levels of active ATM prior to irradiation were related with increased radioresistance.

To test whether active ATM in tumours prior to treatment also resulted in resistance to therapy, immunohistochemistry was performed on tumour material of advanced stage cervical cancer patients (n=375), treated with (chemo)radiation. High levels of phosphorylated (p-)ATM (P=0.006, HR=1.817) were related to poor loco-regional disease-free survival. Furthermore, high p-ATM levels predicted shorter disease-specific survival (HR=1.418, P=0.038). Presence of p-53BP1 was associated with p-ATM (HR=2.206, P=0.001), but was not related to any clinicopathological features or survival.

In conclusion, both our in vitro and patient-related findings indicate a protective role for ATM in response to (chemo)radiation in cervical cancer and point at ATM inhibition as a possible means to improve efficacy of (chemo)radiation.
Introduction
The standard of care for advanced stage cervical cancer patients has shifted over the last decade from radiotherapy alone to platinum-based chemoradiation [1]. Despite the shift in this curative treatment modality, 5-year survival is still around 66%, which leaves ample room for improvement [2]. Chemoradiation introduces high levels of DNA double-strand breaks (DSBs), with the aim to induce cell death [3-4]. At the molecular level, cells respond to DNA breaks with the activation of a distinct pathway called the ‘DNA damage response’ (DDR). The DDR recognizes DNA damage and subsequently coordinates a cell cycle arrest with the initiation of DNA repair [3-4]. Counteracting the effects of the DDR might thus be an attractive option to improve treatment results in advanced cervical cancer patients.

Central in the DDR is the ataxia telangiectasia mutated (ATM) kinase. ATM plays a key role in detecting DNA DSBs and in coordinating DNA repair, cell cycle arrest and the induction of apoptosis [4]. When DNA DSBs are induced, ATM is activated through autophosphorylation on serine 1981 (Ser1981), and subsequently phosphorylates numerous downstream substrates, including cell cycle regulators, DNA repair factors and proteins involved in apoptosis [3,5-6]. The importance of ATM is underscored by the observed increased radiosensitivity and cancer incidence in ataxia telangiectasia (AT) patients, bearing a mutation in the ATM gene [7]. One prototypical ATM substrate is the gene product of TP53-binding protein-1 (53BP1) [8-11], originally identified as a protein that binds p53 [12]. In response to DNA damage, 53BP1 is rapidly phosphorylated by ATM on multiple residues including serine 25 (Ser25) and serine 1778 (Ser1778) [6,13-14]. Phosphorylated 53BP1 localizes to irradiation-induced foci where it promotes the activation of p53 and Chk2 and mediates the recruitment of the repair factor BRCA1 [8-10]. 53BP1, like ATM, is also involved in repair of DNA breaks, by promoting non-homologous end-joining [15-16]. However, 53BP1 can also be detected on sites of homologous recombination and in addition influences this error-free type of repair [17]. Combined, these findings explain its important role in proper responses to DNA breaks and many of the cellular defects observed in AT, were recapitulated in 53BP1⁻/⁻ cells, including irradiation sensitivity, growth retardation and cancer predisposition [18-19].

The aim of this study was to investigate to which degree cervical cancer cells depend on the DDR after irradiation. For this purpose, we have analysed responses of a panel of cervical cancer cell lines to ionizing irradiation. We have subsequently investigated the role of ATM and 53BP1 as potential targets for radiosensitising approaches in vitro. Finally, we tested the predictive and prognostic properties of ATM pathway activity in tumours in a large, well-documented, consecutive series of cervical cancer patients, primarily treated with (chemo)radiation.

Results
ATM- and 53BP1-dependent cell cycle arrest in response to irradiation
We investigated the role of ATM and 53BP1 in the cellular response to irradiation in cervical cancer cells. In order to study cell cycle arrest in G1 and G2, a distinct early feature of the DDR, different doses of irradiation were tested for their ability to induce cell cycle arrest and foci formation of 53BP1 and γ-H2AX (Supplemental Fig. S1A-D). To subsequently study the role of ATM and 53BP1 in these
responses, we used chemical inhibition of ATM (KU55933) or interfered with the expression of 53BP1 using shRNA (Fig. 1A) in our panel of HPV-positive (SiHa, HeLa, CaSki) cell lines and the HPV-negative C33A cell line (harbouring a p53-mutant) [20]. Cell lines stably expressing 53BP1 shRNAs as well as cell lines in which ATM was inactivated showed normal proliferation, when compared with their control counterparts (Fig. 1B). Upon irradiation (5 Gy), clear cell cycle arrests in G1 and G2 were observed (Fig. 1B, D and E). Interestingly, ATM inhibition or 53BP1-depletion completely ablated the G1 arrest in response to irradiation in all examined HPV-positive cell lines (Fig. 1B, D). Similar results were obtained when we used a second shRNA against 53BP1 (Supplemental Fig. S2A-C). These results indicate that both ATM and 53BP1 are required for the irradiation-induced G1 arrest in HPV-positive cervical cancer cell lines. The p53-mutant cell line C33A did not show a significant irradiation-induced G1 arrest (Fig. 1D), which is in line with the p53-dependant G1 arrest after irradiation [21-22]. Indeed, neither ATM inhibition nor 53BP1-depletion further changed the proportion of G1 cells in C33A cells (Fig. 1D). We next investigated the ability of cervical cancer cells to arrest at the G2/M border. G2/M checkpoint activity was measured by the number of cells that enters mitosis after irradiation, judged by phospho-HistoneH3-positivity. Shortly after irradiation, control cells showed a clear decrease in the percentage of mitotic cells, as expected from cells with an intact G2/M checkpoint (Fig. 1B, E). Notably, ATM inhibition resulted in failure to properly arrest cells at the G2/M border after irradiation (Fig. 1B, E). In sharp contrast, cells depleted of 53BP1 still exhibited a clear decrease in mitotic cells upon irradiation, comparable to that of irradiated control cells. (Fig. 1B, E). These results indicate that ATM, but not 53BP1, is required for a proper G2/M arrest in response to irradiation and these results furthermore show that the G1 and the G2/M arrest have different molecular requirements in cervical cancer cells.

Although previous studies have attributed an irradiation-induced G1 arrest to p53 function [23], we were surprised to observe a prominent G1 arrest after irradiation in all HPV-positive cervical cancer cell lines, in which p53 function is compromised by HPV E6. We therefore tested p53 function and observed expression of p53-target genes MDM2 and p21 in HeLa cells, albeit at low levels (Fig. 1C). Interestingly, depletion of 53BP1 resulted in a virtually complete loss of expression of MDM2 and p21, suggesting that the residual activity of p53 in cervical cancer cells depends on 53BP1 (Fig. 1C).

Regarding ATM inhibition as a therapeutic option, we compared the ability of ATM inhibition to induce apoptosis in HeLa cells versus human non-transformed RPE cells and human BJ foreskin fibroblasts. Whereas ATM inhibition clearly elevated the levels of apoptotic HeLa cells after irradiation, non-transformed RPE cells or BJ foreskin fibroblasts did not show any significant elevation of apoptosis levels in response to combined treatment with KU55933 and irradiation (Fig. 1F, Supplemental Fig. 1E).
Figure 1: ATM and 53BP1-dependent cell cycle arrest in irradiated cervical cancer cell lines. A, C33A, CaSki, SiHa and HeLa cells were infected with pRS control virus or pRS-53BP1 shRNA virus. Whole cell lysates of puromycin-resistant polyclonal cells were obtained and analysed with immunoblotting using indicated antibodies. B, HeLa-pRS, HeLa-pRS53BP1 and HeLa-pRS cells pretreated with KU55933. The role of ATM and 53BP1 as predictive markers in cervical cancer.
were irradiated (5 Gy) and harvested at indicated time points. Cells were fixed, stained for phospho-HistoneH3/Alexa-488 and propidium iodide/RNase. 1x10^4 events were measured by flow cytometry and representative DNA plots are shown. Inlays show phospho-HistoneH3 stainings and indicated percentages show phospho-HistoneH3-positive cells. C, HeLa cells, infected with pRS or pRS-53BP1 were irradiated (5 Gy) and harvested after indicated time periods. Immunoblotting was performed with indicated antibodies. D, C33A, CaSki, SiHa and HeLa cells were treated as for panel B and relative amounts of cells with 2N DNA content (G1-cells) are indicated at 24 hours after irradiation. Standard deviations of three independent experiments are shown. E, C33A, CaSki, SiHa and HeLa cells were treated as for panel B and relative amounts of cells with phospho-HistoneH3-positive cells at 3 hours after irradiation are indicated. Standard deviations of three independent experiments are shown. F, RPE, BJ foreskin fibroblasts and HeLa cells were treated with 10 μM KU55933 prior to irradiation. Twenty-four hours after irradiation, apoptosis was analysed by microscopic assessment of acridine orange staining. Representative images are indicated and averages of three experiments (with at least 100 cells per experiment) are shown. Arrowheads indicate apoptotic cells.

ATM, but not 53BP1 is required for clonogenic survival after irradiation
Our cell cycle analysis showed that ATM inhibition or loss of 53BP1 clearly lead to different defects in response to irradiation (Fig. 1). Reduced ability to initiate cell cycle arrest in response to irradiation, may also translate into altered survival kinetics of irradiated cells. To test this, we subsequently analysed clonogenic survival. To exclude that ATM inhibition resulted in altered growth rates and thereby influenced the results of the clonogenic survival assay upon irradiation, MTT proliferation assays were conducted and revealed that ATM inhibition on its own did not significantly alter growth rates of HeLa cells or SiHa cells (Suppl. Fig. 3A and data not shown). Importantly, inhibition of ATM resulted in a dramatic reduction in clonogenic survival after irradiation, observed in all cell lines tested (Fig. 2A, B). Notably, CaSki cells appeared much more sensitive to ATM inhibition when compared to other cell lines (Fig. 2B). In contrast, loss of 53BP1 only resulted in a very moderate loss of clonogenic survival after irradiation in all four cell lines (Fig. 2A, B). As expected, loss of 53BP1 did not further sensitize cells that were treated with ATM inhibitor (Fig. 2A, B).

Altogether, our results indicate a differential requirement for ATM or 53BP1 with respect to irradiation-induced cell cycle arrest and show that ATM inhibition dramatically radiosensitises cervical cancer cells as judged by survival assays. These results imply that the levels of ATM activity, perhaps even prior to irradiation, may determine the effect of (chemo)radiation.

Figure 2: ATM and 53BP1-dependent clonogenic survival in irradiated cervical cancer cell lines. A, C33A, CaSki, SiHa and HeLa cells were infected with pRS control virus or pRS-53BP1 shRNA virus. Cells were plated in 6-well plates and subsequently irradiated with indicated amounts of ionizing irradiation, and allowed to grow colonies. If indicated, cells were treated with KU55933 prior to irradiation. Surviving colonies were stained. B, Quantification of colony numbers. If no colonies survived, a dashed line is shown. Shown data are from 3 independent experiments.
Predictive value of ATM and 53BP1 for response to treatment

To investigate the predictive value of ATM activity for response to irradiation in cervical cancer cells, we analysed the levels of phospho-Ser1981-ATM before and after irradiation (Fig. 3A). HeLa and SiHa cells have very low baseline levels of phospho-ATM, in contrast to CaSki cells that have activated ATM even prior to irradiation (Fig. 3A). Interestingly, baseline amounts of phospho-ATM seem to correlate with radioresistance, since CaSki cells were significantly more resistant to irradiation than HeLa and SiHa cells as observed in clonogenic survival assays (Fig.
These findings suggest that ATM activity prior to treatment can be used as a predictor of response to irradiation. To next test the specificity of antibodies in paraffin-embedded material, we analysed paraffin-embedded cervical cancer cell lines, irradiated in the presence or absence of ATM inhibitors (Suppl. Fig. S4A). Our results showed that irradiation clearly increases phospho-S1981-ATM levels as well as phospho-S25-53BP1 levels, a process that is completely reverted after pretreatment with ATM inhibitor KU55933 (Suppl. Fig. S4A). These findings indicate that phospho-S1981-ATM and phospho-S25-53BP1 can be detected specifically in paraffin-embedded cervical cancer cells and that both stainings reflect ATM activity.

Figure 3: Relation of ATM and 53BP1 expression to loco-regional disease-free survival

A. HeLa, SiHa and CaSki cells were left untreated or irradiated (5Gy). Thirty minutes after irradiation, cells were lysed and immunoblotted for phospho-Ser1981-ATM and β-Actin. B. HeLa, SiHa and CaSki cells were plated in 6-well plates and subsequently irradiated with indicated amounts of ionizing irradiation, and allowed to grow colonies. Surviving colonies were stained and average colony numbers of three experiments are indicated.

C. Representative immunostaining for p-ATM and p-53BP1 in advanced stage cervical cancer are shown. D. Kaplan-Meier plots of loco-regional disease-free survival, related to expression of p-ATM and p-53BP1.
To investigate whether indeed ATM activity has predictive value for response to (chemo)radiation, we examined phospho-Ser1981-ATM (p-ATM) levels in pre-treatment cervical cancer tissues. Representative p-ATM stainings in cervical cancer tissue are shown in Figure 3C. Any positive nuclear staining (≥10% of intensity ≥1) for p-ATM was observed in 344 out of 349 patients (98.6%), indicating that ATM is activated at least to some degree in virtually all patients. High levels of p-ATM expression, however, were only observed in 183 patients (52.4%).

We analysed expression levels of the ATM substrate 53BP1. Positive nuclear expression for 53BP1 was observed in 100% of the tumours with similar intensity and thus no statistical analysis for 53BP1 expression could be performed. Positive nuclear phospho-S25-53BP1 (p-53BP1) expression, representing ATM activity, was observed in 180/311 tumours (57.9%). As expected for a direct substrate of ATM [6,10,13], a positive signal for p-53BP1 was more frequently found in tumours with high p-ATM in comparison to negative/low p-ATM (OR=2.206; 95%CI=1.383-3.519; \( P=0.001 \)).

We evaluated the relation between clinicopathological data versus p-ATM and p-53BP1 expression (Table 1). Logistic regression analysis showed higher expression levels of p-ATM in advanced stage (≥IIb) tumours (OR=1.851; \( P=0.007 \)). In addition, tumour diameter (≥4 cm; OR=1.848; \( P=0.014 \)) and age (OR=1.005; \( P=0.006 \)) were also related to p-ATM expression. None of the clinicopathological features showed a statistical relationship with positivity for p-53BP1 (Table 1).

### Table 1: Relation between tumour staining for p-ATM and p-53BP1 versus clinicopathological data

<table>
<thead>
<tr>
<th>High p-ATM positive</th>
<th>OR (95% C.I.)</th>
<th>( P )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.981 (0.968-0.994)</td>
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<tr>
<td>Stage ≥ IIb</td>
<td>1.851 (1.179-2.905)</td>
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<tr>
<td>Adenocarcinoma</td>
<td>1.778 (0.954-3.311)</td>
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</tr>
<tr>
<td>Poor differentiation</td>
<td>1.259 (0.799-1.983)</td>
<td>0.321</td>
</tr>
<tr>
<td>Lymphangio invasion</td>
<td>0.715 (0.389-1.314)</td>
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<tr>
<td>Tumour diameter ≥ 4 cm</td>
<td>1.848 (1.135-3.010)</td>
<td>0.014</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>p-53BP1 positive</th>
<th>OR (95% C.I.)</th>
<th>( P )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.989 (0.975-1.004)</td>
<td>0.148</td>
</tr>
<tr>
<td>Stage ≥ IIb</td>
<td>1.253 (0.786-1.996)</td>
<td>0.343</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>0.764 (0.407-1.434)</td>
<td>0.401</td>
</tr>
<tr>
<td>Poor differentiation</td>
<td>1.170 (0.724-1.891)</td>
<td>0.521</td>
</tr>
<tr>
<td>Lymphangio invasion</td>
<td>0.724 (0.385-1.362)</td>
<td>0.317</td>
</tr>
<tr>
<td>Tumour diameter ≥ 4 cm</td>
<td>1.118 (0.669-1.869)</td>
<td>0.670</td>
</tr>
</tbody>
</table>

**P-ATM and p-53BP1 in relation to response to (chemo)radiation and survival**

To analyse the relationship of p-ATM and p-53BP1 protein expression with response to (chemo)radiotherapy, two models were used as described previously [22]. In Model I, where treatment response is based on loco-regional disease-free survival, 364 patients (97.1%) could be analysed. Evaluation of loco-regional disease-free survival was employed since it is a relevant measurement of local effects induced by (chemo)radiation, which induces ATM pathway activation. In this model, high p-ATM was related to poor loco-regional disease-free survival in univariate Cox regression analysis (HR=1.82; \( P=0.006 \)) as well as in multivariate analysis (HR=1.65; \( P=0.022 \)), while p-53BP1
expression was not. Figure 3D depicts locoregional disease-free survival in relation to high p-ATM and p-53BP1 expression. In this analyses, the log-rank P-value for high p-ATM expression was $P=0.003$. To further strengthen our hypothesis that high levels of p-ATM are associated to the response to (chemo)radiotherapy, we evaluated response-to-treatment in a second model. In Model II, we separated our data in two subsets of patients with the highest contrast in treatment response (see suppl. document SD1 patients and methods section) [22]. In this model, a statistical significant association between high p-ATM and poor response to treatment was found in univariate logistic regression analysis (OR=2.57; $P=0.011$) as well as in multivariate analysis (OR=2.34; $P=0.039$). P-53BP1 was not related to response to (chemo)radiation. After selection of these extreme groups, the relation of p-ATM with poor response-to-treatment was even stronger when compared to Model I. These data indicate that pre-treatment ATM activity levels, but not phosphorylation status of 53BP1, is relevant for the response to (chemo)radiation in cervical cancer patients.

Finally, we analysed the expression of p-ATM and p-53BP1 in relation to disease-specific survival. During follow-up, 201 of 375 (54.6%) of patients died. In 155 (78%) of these patients, death was related to cervical cancer (disease-specific survival). In line with our results described above, no relations were observed between p-53BP1 expression and disease-specific survival. However, we found that high p-ATM expression was related to worse disease-specific survival (HR=1.42; $P=0.038$) in univariate analysis (Table 3), again underscoring a role for ATM activity in cervical cancer behaviour.

Table 2: Immunostaining in relation to poor response to therapy

<table>
<thead>
<tr>
<th>Model II</th>
<th>Univariate</th>
<th>Multivariate*</th>
</tr>
</thead>
<tbody>
<tr>
<td>OR</td>
<td>(95% C.I.)</td>
<td>P-value</td>
</tr>
<tr>
<td>Age</td>
<td>1.014</td>
<td>(0.991-1.037)</td>
</tr>
<tr>
<td>Stage ≥IIb</td>
<td>2.966</td>
<td>(1.292-6.810)</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>4.621</td>
<td>(1.888-11.311)</td>
</tr>
<tr>
<td>Poor differentiation</td>
<td>0.852</td>
<td>(0.413-1.758)</td>
</tr>
<tr>
<td>Lymphangio invasion</td>
<td>1.630</td>
<td>(0.694-3.827)</td>
</tr>
<tr>
<td>Tumour diameter ≥4 cm</td>
<td>3.046</td>
<td>(1.196-7.760)</td>
</tr>
<tr>
<td>High p-ATM positive</td>
<td>2.567</td>
<td>(1.243-5.299)</td>
</tr>
<tr>
<td>p-53BP1 positive</td>
<td>0.659</td>
<td>(0.305-1.423)</td>
</tr>
</tbody>
</table>

* Multivariate analysis adjusted for treatment modality.
‡ Not in final step of multivariate analysis.
Table 3: Immunostaining of p-ATM and p53BP1 in relation to disease-specific survival

<table>
<thead>
<tr>
<th>Disease-specific survival</th>
<th>Univariate</th>
<th>Multivariate*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR (95% C.I.)</td>
<td>P-value</td>
</tr>
<tr>
<td>Age</td>
<td>1.003 (0.993-1.013)</td>
<td>0.520</td>
</tr>
<tr>
<td>Stage ≥IIb</td>
<td>2.170 (1.463-3.217)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>1.507 (0.999-2.273)</td>
<td>0.050</td>
</tr>
<tr>
<td>Poor differentiation</td>
<td>1.211 (0.873-1.681)</td>
<td>0.251</td>
</tr>
<tr>
<td>Lymphangio invasion</td>
<td>1.125 (0.726-1.744)</td>
<td>0.597</td>
</tr>
<tr>
<td>Tumour diameter ≥4 cm</td>
<td>1.967 (1.308-2.959)</td>
<td>0.001</td>
</tr>
<tr>
<td>High p-ATM positive</td>
<td>1.418 (1.019-1.972)</td>
<td>0.038</td>
</tr>
<tr>
<td>p-53BP1 positive</td>
<td>0.819 (0.576-1.165)</td>
<td>0.267</td>
</tr>
</tbody>
</table>

* Multivariate analysis adjusted for treatment modality.
‡ Not in final step of multivariate analysis.

Discussion

This study shows a critical role for active ATM in the response of cervical cancer to irradiation, both in cervical cancer cell lines as well as in cervical cancer patients treated with (chemo) radiation. Our in vitro studies indicate that cervical cancer cells, even in the presence of HPV E6 expression, which blocks p53 function, still undergo a robust G1 cell cycle arrest in response to irradiation. HPV-positive cervical cancer cells require both ATM activity and the presence of 53BP1 for induction of this irradiation-induced G1 arrest. In addition, ATM but not 53BP1 is required for induction of a G2/M cell cycle arrest.

The requirements for ATM and 53BP1 for induction of an irradiation-induced G1 arrest are likely explained by residual p53 function in HPV-positive cervical cancer cells, which most often carry wild type alleles of p53 [24]. Both ATM and 53BP1 have been described to regulate p53 function. Whereas ATM has been extensively described to directly regulate p53 [4], a role for 53BP1 in the regulation of p53, is controversial. 53BP1 was initially demonstrated to bind p53 and promote p53 activity [12, 25], but T-cells from 53BP1−/− mice still showed robust irradiation-induced p53 responses [26]. Our results indicate that 53BP1 is required for p53 function, albeit in a background with compromised p53 levels. However, in HPV-positive cervical cancer cells, this does not appear to have functional consequences, since 53BP1 depletion did not dramatically alter clonogenic survival of cell lines after irradiation, nor was the activation status of 53BP1 associated with loco-regional disease-free survival of cervical cancer patients.

In our study, the inhibition of ATM in cervical cancer cells interfered with the irradiation-induced G1 and G2/M cell cycle arrest, in line with loss of checkpoint function in cells from AT patients [4,7,27]. ATM inhibition furthermore severely decreased clonogenic survival for all tested cervical cancer cell lines, indicating that cervical cancer cells heavily depend on the ATM signalling axis for survival after irradiation. Interestingly, when ATM was inhibited in irradiated non-transformed RPE cells or BJ foreskin fibroblasts, no induction of apoptosis was observed in contrast to the apoptotic effects of ATM inhibition on HeLa cells, implying that a therapeutic
window for ATM inhibition may be present. Further research, however, is required to investigate the long-term toxicity profile of ATM inhibition in normal cells. In addition, investigation of the combined effects of radiotherapy and ATM inhibition in normal tissues are needed to further establish ATM as a therapeutic target for radiosensitisation.

These observations, as well as our findings that high baseline levels of active ATM correlate to increased clonogenic survival after irradiation, suggest that high levels of activated ATM levels are beneficial for cervical tumour cell survival. Previously, it has been acknowledged that the ability to repair therapy-induced DNA damage counteracts the efficacy of therapy in a number of tumour types. For instance, high expression levels of the repair enzyme MGMT counteracts the effects of alkylating agents and predicts poor prognosis in gliomas [28]. Conversely, low expression level of the DNA damage repair gene ERCC1 correlates with prolonged survival of non-small cell lung cancer patients [29].

To analyse whether activation status of ATM was also related to therapy outcome, we assessed the expression of p-ATM and the expression of p-53BP1. To our knowledge this is the first time that ATM and 53BP1 have been assessed by immunohistochemistry in their activated phosphorylated state in a large, well-documented, series of cervical cancer patients primarily treated with (chemo) radiation. Our data suggest that high expression levels of p-ATM are related to poor loco-regional disease-free survival. Currently, only very limited data concerning ATM and 53BP1 expression in relation to response to (chemo)radiation and survival in other malignancies is present. The few studies that investigated ATM expression show an ambiguous picture. In pancreatic cancer patients ATM expression in their tumours was not a prognostic factor [30]. In contrast, expression of ATM in colorectal cancer was related to good survival in a large series of patients, although only a subset was treated with genotoxic therapy [31]. Both in oesophageal cancer and early stage breast cancer, ATM expression did not predict response to therapy [32-33].

Our results show that expression of p-ATM was related to response-to-treatment, while expression of p-53BP1 was not. Importantly, our clinical data were in line with our cell line data, in which we show that 53BP1 inhibition did not affect clonogenic survival upon irradiation. Although predictive roles for 53BP1 were unknown in cervical cancer, high expression levels of 53BP1 were associated with poor responses to cisplatin-based therapy in lung cancer [34]. In analogy, 53BP1 was overexpressed specifically in those ovarian tumours that showed resistance to paclitaxel/carboplatin-based therapy [35]. In addition, 53BP1 was frequently lost in hereditary breast cancers, where it was suggested to relieve the genomic instability caused by BRCA1 loss. [17]. The diversity of these results could be explained by differences in treatment modality between and even within these studies. Moreover, our study focussed predominantly on the activation status of both proteins, rather than expression levels only. Finally, carcinogenesis of cervical cancer is fundamentally different from that of the tumour types under study in the latter reports. It may very well be that early inactivation of p53 through HPV infection account for differences in prognostic factors, especially those functioning within the DDR.

ATM is activated in response to chemoradiation. The relatively high levels of p-ATM that we observed in our therapy-naive cervical tumour specimens could represent continuous activation of the ATM-regulated DDR as a result of deregulated proliferation, as also reported for other tumour types [36-37]. Alternatively, elevated levels of p-ATM prior to (chemo)radiation may point
at DNA damage-independent functions of ATM, such as the recently reported role for ATM in sensing oxidative stress levels [38].

In summary, based on our results, targeting ATM kinase activity is an interesting potential therapeutic option to sensitize tumor cells for (chemo)radiation in cervical cancer patients who have high tumor levels of active ATM before start of (chemo)radiotherapy. Our data, in line with other reports, show that inhibition of ATM by targeted drug application results in enhanced sensitivity to radiotherapy [20,39-40], recapitulating the radiosensitivity phenotypes of AT patients' cells [4,7,41]. Moreover, a therapeutic window appears to be present when the toxicity to non-cancer cells is taken into account. This differential sensitivity to ATM inhibition may be related to HPV-dependent rewiring of the cell cycle machinery. Other investigations have reported enhanced radiosensitivity by inhibition of ATM in several malignancies [42-46]. Although ATM inhibitors are still in preclinical development, our study suggests relevance of ATM-targeted agents and warrants a further assessment of ATM inhibition as a (chemo)radiosensitising treatment in advanced stage cervical cancer patients. However, besides increased radiosensitivity AT patients also show increased cancer development [4,7,41], so prolonged ATM inhibition should be avoided. In clinical settings, only a scenario in which short-term ATM inhibition can be combined with the local induction of DNA damage seems feasible. In this respect, it is very relevant that reversible ATM inhibitors have recently been described and that transient ATM inhibition was shown to reach radiosensitising effects in cancer cells [44].

Materials and methods

Cell line studies
The human HPV-positive cervical cancer cell lines HeLa, CaSki and SiHa (all p53 wt) as well as the HPV-negative C33A cell line (mutant p53) were cultured in DMEM:Ham's F12 (1:1), supplemented with 10% fetal calf serum, 100 units/mL penicillin, and 100 μg/mL streptomycin. Human embryonic 293T kidney cells, non-transformed human retinal pigment epithelial (RPE) cells and human BJ foreskin fibroblasts were cultured in DMEM, supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/mL streptomycin. Authenticity of cell lines was verified by DNA short tandem repeat analysis (Baseclear, the Netherlands). If indicated, cells were irradiated using a CIS international/IBL 637 equipped with a cesium137 source (0.01083 Gy/s). If indicated, cells were incubated with 10 μM of ATM inhibitor KU55399 (Tocris Bioscience).

RNA interference
Short-hairpin RNA sequences against the human TP53BP1 gene were previously described and validated [47]. To produce VSV-G pseudotyped retrovirus particles, 293T cells were transfected with either pRetrosuper (pRS), pRetrosuper-53BP1#1 (targeting sequence 5'-GAACGAGGAGACGGTAATA-3') or pRetrosuper-53BP1#2 (5'-GATACTGCTCATCACAGT-3') and the packaging plasmids pMDG/P and pMDG in a 3:2:1 ratio using a calcium phosphate protocol. Virus-containing supernatant culture medium was filtered (0.22 μm, Millipore), mixed with Polybrene (4 μg/mL) and used for infection for three consecutive 12-hour periods. Twenty-four hours after the third infection, puromycin was added (1 μg/mL) for selection.
Western blotting and immunofluorescence

For Western blotting, cell lysates were obtained using MPER (Thermo Scientific), supplemented with protease inhibitor and phosphatases inhibitor cocktail (Roche). Thirty μg of protein was used for SDS-PAGE. Separated proteins were transferred to PVDF membranes and blocked in 5% milk in TBS-0.01% Tween20. Immunodetection was done with antibodies directed against 53BP1 (rabbit, H-300, Santa Cruz Biotechnology), MDM-2 (mouse, AB1, Calbiochem), phospho-Thr68-Chk2 (rabbit, C13C1, Cell Signaling), p21 (mouse, EA10, Calbiochem), β-Actin (mouse, A5441, Sigma) and phospho-Ser1981-ATM (rabbit, EP1890Y, Epitomics). HRP-conjugated antibodies (Dako) were used as secondary antibodies. Visualisation was performed using Enhanced Chemiluminiscence (Lumilight, Amersham) and a Biorad Bioluminescence device, equipped with Quantity One/Chemidoc XR software.

For immunofluorescence, cells were grown on glass cover slips. One hour after treatment cells were fixed in 3.7% formaldehyde, blocked in 5% bovine serum albumin (BSA) and stained overnight using anti-53BP1 (rabbit, H-300, Santa Cruz Biotechnology) and anti-γ-H2AX (mouse, phospho-Ser139, #05-636, Millipore). Cells were counterstained with Alexa-488 and Alexa-568-conjugated secondary antibodies (Molecular Probes) and DAPI (Sigma).

Clonogenic survival assays

Depending on the amount of irradiation, cells were seeded at 100 (0Gy), 500 (2Gy), 2000 (4Gy) or 5000 cells/well (6Gy) in 6-well plates, and allowed to adhere for four hours. Cells were subsequently irradiated at indicated doses. If indicated, cells were pretreated with ATM inhibitor (KU55933, 10 μM) for 30 minutes. ATM inhibitor (KU55933)-treated CaSki cells were seeded up to 80,000 cells/well due to extreme irradiation sensitivity observed in initial experiments. When colony size reached an approximate minimum size of 50 cells per colony after 10-14 days, cells were fixed and stained using methanol/acetic acid/water mixture (50%, 20% and 30% respectively), containing 0.01% Coomassie brilliant blue. Surviving fraction was calculated using the plating efficiencies, using the non-irradiated controls as a reference. Results shown are averages of three independent experiments performed in triplicate.

Apoptosis assays and proliferation measurements

Twenty-four hours after plating in 6-well plates, cells were irradiated (10Gy). If indicated, cells were pretreated with ATM inhibitor (KU55933, 10 μM) for 30 minutes. Twenty-four hours after irradiation, apoptosis was assessed visually by fluorescence microscopy after staining nuclear chromatin with acridine orange. Apoptosis assays were independently performed in triplicate. In order to measure cell proliferation, 7000 HeLa cells or SiHa cells were plated in 96-well plates in the presence or absence of KU55933 (10 μM). Directly after plating, or at 24, 48 or 72 hours after plating, 20 μl of 5 mg/ml (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg/ml) was added for 2 hours. Subsequently, culture medium was removed and cells were incubated in DM50 for 30 minutes. Absorbance was measured at 520 nm using a Biorad microplate reader. Cell growth was calculated by calculating relative increases of MTT conversion. MTT conversion at day 1 of plating was used as a reference.
Flow cytometry
Cells were harvested at indicated time points after irradiation and fixed in ice-cold 70% ethanol. Cells were stained with rabbit anti-phospho-Ser10-Histone H3 antibody (rabbit, Upstate, #06570, 1:200), and subsequently stained with Alexa-488-conjugated anti-rabbit antibody (Molecular Probes) and counterstained with propidium iodide/RNase (Sigma). Cell cycle distribution and phospho-Histone H3 positivity were analysed on a FACSCalibur (Becton Dickinson) equipped with CellQuest software. Per sample, at least 1x10^4 events were analysed and indicated results show averages and standard deviations of three independent experiments.

Cervical cancer patients
Immunohistochemical analysis was performed on pretreatment tissue specimens of 375 advanced stage cervical cancer patients primarily treated with chemoradiation, collected between January 1980 and December 2006. Tissues specimens were used to generate a tissue microarray (TMA) as described previously [48-49]. Clinicopathological data of patients, analysed in this study, are summarised in Supplemental Table 1. The mean follow-up time was 3.99 years (range: 0.1–18.3) for all patients. For patients who were still alive at time of their final follow-up, median follow-up time was 6.3 years. 189 (50.4%) patients only received radiotherapy, whereas 186 (49.6%) patients received chemoradiation. Patients who received chemoradiation were younger compared to patients who received RT alone (median 46.8 vs. 64.8, P<0.001). All other baseline characteristics were comparable in both groups (data not shown). Immunohistochemistry was performed with antibodies against phospho-Ser1981-ATM (rabbit, EP1890Y, S1981, Epitomics), 53BP1 (rabbit, H-300, SC-22760, Santa Cruz), phospho-Ser25-53BP1 (rabbit, AB82559, Abcam), and anti-γ-H2AX (mouse, #05-636, Millipore). As chemoradiation is associated with a better survival and is a time-dependent factor, we adjusted for treatment modality in the multivariate analyses. Additional detailed information about staining protocols, patient information, evaluation of stainings and statistical analysis can be found in the supplemental document SD1.

Acknowledgements
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References


3. The role of ATM and 53BP1 as predictive markers in cervical cancer

Supplemental document S1

Immunofluorescence
Cells were grown on glass cover slips and at the indicated time points after radiation, coverslips were removed from the culture dish and washed twice with PBS. Cells were then fixed for 10 minutes in 3.7% paraformaldehyde at room temperature and subsequently washed with PBS. Cells were permeabilised using PBS, containing 0.1% Triton-X100 for 5 minutes and subsequently washed extensively. Cells were stained with primary mouse antibodies against γ-H2AX (Upstate, pS139, #05-636, 1:200) or rabbit antibodies against 53BP1 (Santa Cruz Biotechnology, B-300, 1:200) in PBS-0.05% Tween20, supplemented with 5% BSA for 3 hours at room temperature. After extensive washing, cells were incubated with anti-mouse or anti-rabbit secondary antibodies, conjugated with Alexa-488 and Alexa-568 respectively and were counterstained using DAPI. Images were obtained on a Leica DMRXA fluorescence microscope and a minimum of 20 nuclei were scored for the presence of γ-H2AX or 53BP1 foci per condition.

Patients
Our group has established a large database of paraffin-embedded formalin-fixed tumour material and follow-up data from cervical cancer patients treated at the University Medical Center Groningen (UMCG). Routine patient follow-up time was at least 5 years or until September 2010. Staging of patients was according to FIGO guidelines. This patient cohort and corresponding treatments, review board approval and tissue microarray (TMA) construction has been previously described [1].

Evaluation of response to (chemo)radiation
Approximately 8-10 weeks after completion of (chemo)radiation either hysterectomy or biopsy was performed if a patient was (technically) classified as operable. Therefore, not all patients in our database underwent a post-treatment biopsy and/or hysterectomy. As a consequence, the response to (chemo)radiation could not be evaluated based on this parameter in all patients. Therefore, we evaluated response to (chemo)radiation retrospectively in two ways. First (Model I), the response to (chemo)radiation was determined based on loco-regional disease-free survival. Loco-regional disease-free survival is defined as the period from diagnosis until loco-regional progression of disease during treatment or loco-regional recurrence. Patients were excluded from the analysis, if the location of the recurrence was unknown. Second (Model II), response to (chemo) radiation was determined in two subsets of patients, with supposedly the largest difference in treatment response. In the first subset, patients with no residual tumour-material in their post treatment specimen and without loco-regional recurrence during follow-up with a minimum of 2 years were selected. The second subset consists of patients with clinical evidence of disease progression during treatment or clinical evidence of disease persistence at examination after completion of primary treatment. Both models also have been described and used previous [1].

Immunohistochemistry
Tissue microarray (TMA) construction has been previously described [1]. TMAs were immunohistochemically stained with monoclonal antibodies against p-ATM (1:50; Rabbit IgG,
Epitomics, Clone EP1890Y, S1981) exclusively detects the phosphorylated (Ser1981) ATM product [2]. Total levels of 53BP1 were stained using a rabbit polyclonal antibody against 53BP1 (53BP1, 1:1000; 16h; Rabbit IgG, Santa Cruz Biotechnology, clone H-300; SC-22760). Phosphorylated 53BP1 was detected using polyclonal anti-phospho-Ser25-53BP1 (1:25, 16h; Rabbit IgG, Abcam, Clone A882559) and polyclonal anti-phospho-Ser1778-53BP1 (1:50, 16h; Rabbit IgG, Cell Signaling, Clone 2675). Ser139 phosphorylated H2AX was detected using monoclonal anti-γ-H2AX (1:200, Mouse IgG, Millipore, #05-636).

From our TMAs, 3 µm sections were cut and placed on amino-propyl-etoxy-silan (APES) coated glass slides. Slides were subsequently deparaffinised using xylene and rehydrated using a multistep process from ethanol to phosphate buffered saline (PBS). Antigen retrieval for the p-ATM antibody was done using microwave treatment in EDTA buffer (pH=8). For 53BP1 and p53BP1 stainings, antigen retrieval was performed microwave treatment in citrate buffer (pH=6). Endogenous peroxidase activity was blocked by incubating with 0.3% hydrogen peroxidase for 30 minutes. All antibodies were detected using rabbit anti-goat/biotin labelled secondary antibodies and horse-radish-peroxidase (HRP) conjugated streptavidin, except for γ-H2AX, where rabbit anti-mouse/HRP and goat anti-rabbit/HRP were used. Counterstaining was achieved using hematoxylin. All incubations with primary antibodies were performed at room temperature for 1 hour. Anti-γ-H2AX, anti-53BP1 and anti-phospho-53BP1 stainings were detected using 3′3-diaminobenzidinetetrahydrochloride (DAB). Anti-p-ATM staining was visualised using EnVision (DAKO). Counterstaining was achieved using hematoxylin.

Antibody validation for immunohistochemistry

In order to validate our antibodies for immunohistochemistry, we used paraffin-embedded HeLa or MCF-7 cell pellets that were left untreated or were radiated with 5 Gy irradiation. 53BP1 was present in untreated and irradiated cells (data not shown). To analyze the phosphorylation-status of 53BP1, we used antibodies that recognize two previously validated ATM phosphorylation sites on 53BP1, Ser25 and Ser1778 [3-4]. Phospho-Ser25-53BP1 and phospho-Ser1778-53BP1 hardly showed reactivity in non-irradiated cells, but showed massive reactivity in irradiated paraffin-embedded cells, in line with 53BP1 being phosphorylated on both these sites by ATM in response to irradiation (Supplemental Figure S2A and data not shown). Surprisingly, the anti-phospho-Ser1778-53BP1 showed extensive cytoplasmic staining (data not shown) and these results indicated that the phospho-Ser1778 antibody may also stain a non-specific protein in paraffin-embedded material and our further analyses of 53BP1 phosphorylation were therefore restricted to phospho-Ser25-53BP1 (referred to as p-53BP1).

In order to confirm that ATM is responsible for phosphorylation of Ser25 on 53BP1, in response to irradiation, we analysed paraffin-embedded HeLa cell pellets, and included cells that were incubated with ATM inhibitor KU55933 prior to irradiation (Suppl. Fig. S2A). Reassuringly, ATM inhibition completely ablated the phosphorylation of 53BP1 (Suppl. Fig. S2A) as well as phosphorylation of the established ATM substrate H2AX on Ser139 under the same conditions (Suppl. Fig. S2B). We next examined the phosphorylation status of ATM itself. To this end, we used the extensively characterised ATM autophosphorylation site Ser1981[5] (Suppl. Fig. S2B). Already in the absence of irradiation-induced DNA damage, HeLa cells showed high levels of Ser1981-
phosphorylated ATM (Suppl. Fig. S2B), which were further induced after irradiation (Fig. S2B). As expected, reactivity of phospho-Ser1981-ATM (referred to as p-ATM) was virtually absent when cells were pretreated with the ATM inhibitor KU-55933 (Suppl. Fig. S2B).

**Evaluation of immunohistochemistry**

Staining, intensities were semi-quantitatively scored as negative (0), weak positive (1), positive (2), and strong positive (3). In addition, the percentage of positive tumour cells per staining intensity was documented for each core, since not only the level of expression of either p-ATM or (p-)53BP1, but also the proportion of cells that are positive could play an important role in the response to chemoradiation.

We have classified p-ATM immunostaining data into ‘high’ expression, which is defined as patients with positive nuclear immunostaining with an intensity of at least 2 present in at least 75% of tumour cells. For p-53BP1 staining, we have classified into the following three groups: ‘Negative’, which is defined as all patients with negative (0) staining or patients with weak staining intensity (1) in 0-25% of tumour cells, ‘Positive’, which is defined as patients with a staining intensity of 1 in more than 25% of tumour cells, or patients with a staining intensity of 2 or 3. TMA evaluation was performed independently by two observers without prior knowledge of the clinical data. 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. Only patients with at least two evaluable tumour cores were included for statistical analysis.

**Statistical analysis**

Statistical analysis was performed using SPSS 16.0 for Windows (SPSS Inc., Chicago, IL). The Student’s t-test was used to analyse differences in age. Other baseline characteristics were compared with the Pearson’s χ² test. In addition, logistic regression models were used to evaluate positive staining and clinicopathological characteristics, with immunostaining being the dependent factors and the clinicopathological characteristics being the independent factors. Logistic regression was also used to evaluate associations between immunostainings of p-53BP1 and p-ATM. To identify factors involved in response to (chemo)radiation, response to (chemo)radiation (dependent) was evaluated in relation to clinicopathological factors and immunostaining (independent) using Cox-regression analysis for Model I and logistic regression analysis in Model II. Disease-specific survival was defined as the time from diagnosis until the last follow-up alive, death due to other causes than cervical cancer or death due to cervical cancer. Survival was visualised using the Kaplan-Meier method and Mantel-Cox log rank test was used to evaluate the differences between these curves. Disease-specific survival was analysed using the Cox regression analysis. Since (chemo) radiation is a time-dependent factor and associated with a better survival, multivariate analyses were adjusted for treatment modality. Variables with a P-value of <0.10 in univariate analysis were excluded stepwise in multivariate analysis; in the final step, only factors with a P-value of <0.05 were included. P-values <0.05 were considered statistically significant.
References
## Supplemental data

### Supplemental Table 1: Patient and tumour characteristics

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<tr>
<td>IIIa</td>
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*Abbreviation: FIGO, International Federation of Gynaecologists and Obstetricians.*
Supplemental Figure 1: DNA damage responses in cervical cancer cell lines

A-C, HeLa cells were left untreated or were irradiated with indicated doses of IR. Twenty-four hours after irradiation, cells were harvested, fixed and stained using propidium iodide/RNAse. In panel A, the average amount of G1 cells (containing 2N DNA) is indicated. In panel B, the average amounts of S-phase cells are indicated. Data represent averages and standard deviations of three independent experiments. C, HeLa cells were fixed at one hour after irradiation (5 Gy). Cells were permeabilised and stained for γ-H2AX-Alexa-568 and 53BP1-Alexa-488. Representative cells are indicated and inlays represent magnified images of nuclear areas. D, C33A, CaSki, SiHa and HeLa cells were treated as for panel C. Amounts of γ-H2AX and 53BP1 foci per nucleus were counted and averages and standard deviations of at least 20 cells per condition are indicated. E, BJ foreskin fibroblasts were left untreated or treated with KU55933 for 30 minutes prior to irradiation (10 Gy). Twenty-four hours after irradiation. Representative images are indicated.
Supplemental Figure 2: DNA damage responses after 53BP1-depletion. 

A. HeLa cells were infected with pRS control virus, pRS-53BP1#1 or pRS-53BP1#2 shRNA virus. Whole cell lysates of puromycin-resistant polyclonal cells were obtained and analysed with immunoblotting using indicated antibodies. 

B. HeLa-pRS, HeLa-pRS53BP1#1 and HeLa-pRS53BP1#2 were left untreated or were irradiated (5 Gy) and harvested after 24 hours. Cells were fixed, stained with propidium iodide/RNAse. 1x10^4 events were measured by flow cytometry and representative DNA plots are shown. 

C. Quantification of the results obtained for panel B. Amounts of G1 cells from three independent experiments were quantified and averages and standard deviations are indicated.
Supplemental Figure 3: A. HeLa cells were plated at 7000 cells per well in 96-wells plates in the presence or absence of KU55933. After 24, 48 or 72 hours, cell growth was measured using MTT conversion. Shown averages and standard deviations are from six replicates and growth was related to MTT conversion levels at t=0h.
Supplemental Figure 4: Validation of antibodies for immunohistochemistry. HeLa cells were left untreated or were irradiated (5 Gy). Alternatively HeLa cells treated with KU55933 for 30 minutes prior to irradiation. Thirty minutes after irradiation, cells were trypsinised and incorporated in solidifying agarose to yield blocks containing HeLa cells. Agarose blocks were further processed for fixation in formalin and embedded in paraffin blocks, using identical conduction as are used in diagnostic pathology. Paraffin-embedded cells were sliced, de-paraffinised, stained for γ-H2AX, phospho-Ser1981-ATM or phospho-Ser25-53BP1, and counter-stained with hematoxylin.