Tumor cells can’t stand the heat

*Boosting the effectiveness of hyperthermia in cervical carcinoma*

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Chapter 4

Hyperthermia selectively targets human papillomavirus in cervical tumors via p53-dependent apoptosis

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Abstract

Human papilloma virus (HPV) is associated with cervical cancer, the third most common cancer in women. The high-risk HPV types 16 and 18 are found in over 70% of cervical cancers and produce the oncoprotein, early protein 6 (E6), which binds to p53 and mediates its ubiquitination and degradation. Targeting E6 has been shown to be a promising treatment option to eliminate HPV-positive tumor cells. In addition, combined hyperthermia with radiation is a very effective treatment strategy for cervical cancer. In this study, we examined the effect of hyperthermia on HPV-positive cells using cervical cancer cell lines infected with HPV 16 and 18, \textit{in vivo} tumor models, and \textit{ex vivo}-treated patient biopsies. Strikingly, we demonstrate that a clinically relevant hyperthermia temperature of 42°C for 1 hour resulted in E6 degradation, thereby preventing the formation of the E6-p53 complex and enabling p53-dependent apoptosis and G2-phase arrest. Moreover, hyperthermia combined with p53 depletion restored both the cell-cycle distribution and apoptosis to control levels. Collectively, our findings provide new insights for the treatment of HPV-positive cervical cancer and suggest that hyperthermia therapy could improve patient outcomes.
Introduction

Human papillomavirus (HPV)-associated cancers are responsible for approximately 5% of all tumors worldwide (Parkin, 2006). Over 100 different HPV types have been identified, although only a few are oncogenic. These high-risk HPVs, such as types 16 and 18, can develop precancerous lesions. If these lesions remain untreated, they can progress into cancer. HPV is associated with several types of cancers, such as carcinoma of the head and neck, anus, vagina, vulva and penis (Doorbar, 2006; Jayaprakash et al., 2011; Watson et al., 2008). HPV is particularly notorious for causing cervical cancer, which is the third most common cancer in women (www.cancer.gov) and the second most frequent cause of cancer-related death in women (Saslow et al., 2007a). The high-risk HPV types 16 and 18 are found in over 70% of cervical cancers (Munoz et al., 2004; Saslow et al., 2007b; Schiffman et al., 2007).

The standard treatment for cervical carcinoma is radical surgery and/or concurrent cisplatin chemoradiotherapy. Hyperthermia is clinically applied as concomitant therapy with either radiotherapy or chemotherapy, particularly as an alternative treatment if cisplatin-based chemotherapy is contraindicated or for recurrent cancers in a previously irradiated area (Franckena et al., 2009). Hyperthermia is given simultaneously with cisplatin-based chemotherapy and shortly before or after radiotherapy. Several phase III studies on cervical cancer have shown a significantly better overall survival after radiotherapy combined with hyperthermia compared to radiotherapy alone (Bergs et al., 2007; Franckena et al., 2008; Lutgens et al., 2010; van der Zee et al., 2000). Because a high percentage of cervical tumors are HPV infected we examined the response on HPV-positive cells to hyperthermia.

The high-risk HPV types 16 and 18 both produce early protein 6 (E6) which binds to the tumor suppressor protein p53 (Bernard et al., 2011; Chen et al., 1996; Scheffner et al., 1990; Werness et al., 1990; zur Hausen, 1987). E6 mediates ubiquitination of p53 and as a result targets p53 as well as itself for proteasomal degradation protein complex (Mani & Gelmann, 2005; Scheffner et al., 1990). As a consequence, both p53-induced cell-cycle arrest and apoptosis are abrogated (Lechner & Laimins, 1994). Therefore, E6 might be an interesting target in cancer therapies (Chung et al., 2014; Hellner & Munger, 2011). In this study, we describe that hyperthermia interferes in this degradation process and allows p53 to escape proteasomal degradation, reactivating its tumor-suppressive capacities.
Figure 1. HPV-positive cells are sensitive to hyperthermia (42°C for 60 min). (A) Western blot analysis of p53 in cervical cancer cells are shown. SiHa and HeLa cells were treated with or without hyperthermia prior to radiation (4 Gy). Accumulation of p53 is only observed after a 60-min treatment with a hyperthermia temperature of 42°C. (B) The induction of p53 starts immediately after hyperthermia and remains up to 4 h after treatment. (C) Immunocytochemistry staining of DAPI (blue) and p53 (green) are presented for different conditions. Accumulation of p53 is observed after HT and RT+HT, not after RT alone. (D and E) In SiHa cells grown in athymic mice (D) and in patient biopsies (E), p53 is accumulated after hyperthermia. (F and G) The upregulation of p53 after hyperthermia is also observed in western blots for three different xenografts (I, II and III) and four ex vivo treated patient biopsies (indicated with A, B, C and D). HT, hyperthermia; RT, radiation; RT+HT, radiation and hyperthermia.
Results

Restoration of p53 after hyperthermia

To investigate changes in p53 levels after irradiation and after hyperthermia, Western blots and immunocytochemistry stainings were performed. Highly elevated levels of p53 were found in SiHa and HeLa cells after 1 hour of hyperthermia at 42°C and after a combinational treatment of hyperthermia and radiation, but not after a treatment of radiation alone. A temperature of 42°C is required to achieve a significant increase of p53 and either 40°C or 41°C was not sufficient to cause induction of p53 (Fig. 1A). The effectiveness of radiation, hyperthermia, and their combination on p53 levels is tested on HPV-positive (Supplementary Fig. S1A) and HPV-negative cells (Supplementary Fig. S1B). In HPV-positive cells, a treatment including hyperthermia was required to induce p53, whereas radiation alone was sufficient in upregulating p53 levels in HPV-negative p53wt cells. The HPV-negative p53mut or p53null cells did not show any upregulation of p53. Accumulation of p53 was observed immediately after hyperthermia (at 42°C for 1 hour) and lasted until 4 hours after treatment (Fig. 1B).

In immunocytochemistry staining, p53 was only seen after hyperthermia or after the combinational treatment of radiation and hyperthermia (Fig. 1C), p53 was neither observed in controls nor after radiation treatment only. Moreover, in vivo-treated tumors of SiHa cells grown in athymic mice and ex vivo-treated patient biopsies show a p53 induction after a 60-minute in vivo treatment with 42°C (Fig. 1D-G). Results of more patients are shown in Supplementary Fig. S1C and S1D. Quantification of immunofluorescence staining is presented in Supplementary Fig. S1E.

Hyperthermia causes E6 downregulation

Protein E6, produced by human papillomavirus 16 and 18, is present in the untreated SiHa and HeLa, as shown with immunocytochemistry (Fig. 2 A and B), whereas E6 has disappeared immediately after hyperthermia (42°C for 1 hour). In order to test the role of E6 in HPV-infected cells after hyperthermia, an E6-siRNA transfection was used. After transfection with E6-siRNA, neither E6 nor p53 is observed in immunocytochemistry staining. Next, cells were transfected with E6-siRNA and irradiated (4 Gy). Again, there was no E6 measurable, but now an accumulation of p53 was observed, which suggests that hyperthermia also causes stress to the cells that induce p53. Similar results were obtained with western blots examination as shown in Supplementary Fig S2A.

In SiHa cells grown in athymic mice, E6 was present in the cells of untreated tumors, but no E6 was detected after treatment with hyperthermia (Fig. 2C). Furthermore, after ex vivo treatment of patient biopsies, E6 disappeared after
hyperthermia treatment, which was similar to the outcomes of the cell and xenograft experiments (Fig. 2D and Supplementary Fig S2B and S2C).

Cells were also treated for 30 minutes at 42°C to understand the time needed before hyperthermia is fully effective on both E6 and p53 (Supplementary Fig. S2D). In the untreated situation, all cells were E6 positive, whereas none of the cells express p53. After a 30-minute treatment only a few cells
were completely positive for p53, and E6 expression is lower compared with the control. This shows that a 30-minute treatment is not sufficient and 60-minute treatment at 42°C is needed to induce p53 in all cells and to degrade E6 completely. Quantification of immunofluorescence staining is presented in Supplementary Fig. S2E.

**Besides functional p53, degradation of p53 still occurs mainly via the proteasomal pathway**

To examine whether p53 accumulation is newly produced or due to interference with the degradation, cells were treated with 1 μmol/L cycloheximide for 1 hour. This did not result in a p53 accumulation, whereas treatment with 10 μmol/L MG132 for 1 hour did show p53 accumulation (Fig. 3A). It was concluded that p53 is produced *de novo* after a hyperthermia treatment at 42°C for 60 minutes.

To gain insight into degradation of E6 and p53 after hyperthermia, cells were treated after incubation with a proteasomal inhibitor (MG132) or a lysosomal inhibitor (chloroquine). In untreated conditions, no p53 is detected in HPV-positive cells. After blocking the lysosome using chloroquine, no difference was observed using Western blot, but after blocking the proteasome using MG132, a substantial increase of p53 was observed. Compared to blocking of the proteasome alone, combined blocking of both lysosome and proteasome showed a similar increase in p53. After a 60-minute treatment at 42°C, the

**Figure 2. E6 downregulation after hyperthermia (42°C for 60 min).** (A and B) Immunocytochemistry staining of DAPI (blue), E6 (red) and p53 (green) are shown for different conditions on SiHa (A) and HeLa (B) cells. Untreated cells are positive for E6, but negative for p53. After hyperthermia accumulation of p53 is observed, but there is no E6 anymore. After transfection with E6-siRNA, neither E6 nor p53 is observed. Any treatment after an E6-siRNA transfection causes induction of p53. (C and D) After HT, there is no E6 present in Xenograft (C) and in a Patient biopsy (D). HT, hyperthermia; RT, radiation; RT+HT, radiation and hyperthermia.
lysosomal inhibition with chloroquine resulted in a slight increase in the levels of p53. However, this effect was much more pronounced when the activity of the proteasomal pathway was blocked, indicating that the degradation of p53 is to a large extent dependent on the proteasome (Fig. 3B).

In order to study the localization of p53 within the cells, the proteasome was blocked using an inhibitor (MG132) to prevent degradation. After hyperthermia p53 is only present in the nucleus, whereas after radiotherapy p53 is also present in the cytoplasm (Fig. 3C and D; top).

After studying localization of p53, the localization of E6 has been investigated as well. As we cannot detect E6 after hyperthermia, a proteasomal inhibitor (MG132) was used before treating the cells with hyperthermia or radiation. After both radiation and hyperthermia treatment, in the presence of MG132, E6 is localized in the cytoplasm and nucleus (Fig. 3C and D; bottom).

To understand the interaction of E6 and p53 after hyperthermia, immunocytochemistry stainings were performed using both lysosomal and proteasomal inhibitors. In untreated SiHa cells, E6 was present in control, and also after blocking the lysosome (bafilomycin) and after blocking the proteasome (MG132). However, p53 was absent both in control and
after blocking the lysosome, whereas p53 was detected after blocking the proteasome, showing p53 is only degraded via the proteasome after complex formation with E6. Both E6 and p53 were present after treating cells with hyperthermia before blocking either the lysosome or proteasome (Fig. 3E and Supplementary Fig. S3A), suggesting that after hyperthermia E6 is degraded via both the lysosomal and proteasomal pathway. Besides functional p53, it is observed that some p53 is still degraded via the proteasomal pathway.
Moreover, conditions containing components of the standard treatment (radiation with platin-based chemotherapy) were tested. Cisplatin (cDDP) or standard chemoradiation (cDDP+RT) did not result in an accumulation of p53 compared to untreated controls (Supplementary Fig. S3B). A treatment with hyperthermia was necessary to induce p53. Quantification of immunocytochemistry staining is presented in Supplementary Fig. S3C.

**Accumulation of p53 results in p53-dependent apoptosis and G\textsubscript{2}-arrest**

To test whether the accumulated p53 is functional, apoptosis was tested by the Nicoletti assay. About 6% of the untreated SiHa and HeLa cells were apoptotic (Fig. 4A). No difference was observed after radiation or after transfection of p53-siRNA prior to hyperthermia. Both after hyperthermia and after hyperthermia combined with radiation, a significant increase in apoptosis was observed. The effects of more treatments and treatment combinations on apoptosis in SiHa and HeLa cells are summarized in Supplementary Fig. S4A. Additional HPV-positive cell lines (Supplementary Fig. S4B) show similar results as were found in SiHa and HeLa cells: induction of apoptosis occurs only if hyperthermia is part of the treatment. In the p53\textsubscript{wt} HPV-negative cell lines, radiation alone already caused a significant increase in apoptosis (Supplementary Fig. S4C), whereas there was no apoptosis observed in the p53\textsubscript{mut} or p53\textsubscript{null} cell lines. Cleaved caspase-3 immunohistochemistry staining has been performed on paraffin-embedded coupes to test the apoptotic induction in xenograft and patient biopsies. After hyperthermia, there is a clear upregulation of the cleaved caspase-3, indicating that apoptosis is induced (Fig. 4B).

Untreated SiHa cells showed a cell percentile distribution of 62.6±3.1 in G\textsubscript{0}-G\textsubscript{1}, 15.8±1.1 in S and 26.0±0.5 in G\textsubscript{2}-M. After hyperthermia a clear G\textsubscript{2}-phase arrest is observed as the percentages change to 44.2±1.2 in G\textsubscript{0}-G\textsubscript{1}, 11.0±0.6 in S and 49.1±0.7 in G\textsubscript{2}-M. Using p53-siRNA prior to hyperthermia, the G\textsubscript{2} arrest is abrogated again as the distribution of cell phases almost returned to control level, showing 65.6±1.0 in G\textsubscript{0}-G\textsubscript{1}, 13.5±0.6 in S and 25.9±1.2 in G\textsubscript{2}-M. Similar results were found in HeLa cells: In untreated conditions 65.2±1.6 in G\textsubscript{0}-G\textsubscript{1}, 12.1±1.2 in S and 22.7±0.4 in G\textsubscript{2}-M; after hyperthermia 44.0±1.2 in G\textsubscript{0}-G\textsubscript{1}, 14.6±0.2 in S and 41.4±1.3 in G\textsubscript{2}-M. After transfection with p53-siRNA and hyperthermia, cell distribution almost returned to control levels 66.4±0.5 in G\textsubscript{0}-G\textsubscript{1}, 11.5±0.6 in S and 22.2±1.2 in G\textsubscript{2}-M. These data in combination with more HPV-positive cell lines are shown in Supplementary Table SI. The effectiveness of hyperthermia or hyperthermia and radiation on HPV-negative cell lines is presented in Supplementary Table SII. Histograms of cell cycle show a clear G\textsubscript{2} arrest and induction of apoptosis after hyperthermia (Fig 4C). Additional apoptotic assays performed on SiHa and HeLa (cleaved caspase-3 and Annexin-V staining) confirm results found by the Nicoletti assay, only apoptosis induction after a treatment including hyperthermia (Supplementary Fig S4D and S4E. In these figures, the functionality of p53-siRNA was demonstrated as...
Figure 4. Activity of p53 after hyperthermia results into G2-arrest and apoptosis.

(A) Nicoletti assay on SiHa and HeLa cells. A low percentages of apoptosis is found in control samples. Hardly any difference is found after radiation or after p53-siRNA prior to hyperthermia. After hyperthermia or hyperthermia combined with radiation a 3-5 fold increase in apoptosis is observed. (B) Cleaved caspase-3 is increased after hyperthermia in Xenograft and ex vivo treated Patient biopsy in immunohistochemistry stainings. (C) Nicoletti assay presenting cell cycle distribution and apoptosis in SiHa and HeLa cells. In untreated samples a high G1 population and a very low G2 population is observed. After hyperthermia, an substantial increase in G2 population is noticed and a 3-4 fold increase of apoptosis is measured. Transfecting cells with p53-siRNA prior to hyperthermia resulted into more or less normal cell distributions. (D) Survival fractions of all HPV-positive cell lines are lower after radiation combined with hyperthermia compared to radiation alone. Values are shown ± SEM. HT, hyperthermia; RT, radiation; RT+HT, radiation and hyperthermia. *** p<0.001
well. Transfection with scrambled p53-siRNA prior to hyperthermia caused apoptosis, whereas the normal p53-siRNA prior to hyperthermia was able to prevent the induction of p53. This has been confirmed using Western blot (Supplementary Fig. S4F).

Cell survival of HPV-positive cells were tested using a clonogenic assay (Franken et al, 2006). The survival curves are shown in Fig. 4D. All cell lines show a consistent significantly lower survival after radiotherapy combined with hyperthermia compared to radiation alone. This hyperthermic sensitization is shown for clinically relevant radiation doses. The corresponding α- and β-values of the linear-quadratic analysis can be found in Supplementary Table SIII, showing an impressive enhancement of the linear parameter, α.
Discussion

In this study we aimed to elucidate the cellular mechanisms that may explain why hyperthermia is particularly clinically effective in HPV-positive cancers. In HPV-positive carcinoma cells, E6 binds and ubiquitinates p53, after which this complex is degraded via the proteasomal pathway (Bernard et al., 2011; Chen et al., 1996; Scheffner et al., 1990; zur Hausen, 1987). As a consequence, neither p53-dependent G2-arrest nor apoptosis can be induced. We found that hyperthermia at 42°C for a duration of 60 minutes abrogates the interaction of p53 with E6, resulting in a major accumulation of p53. This was demonstrated in multiple HPV-positive cell lines, both in vitro and in xenografts and in patient cervical carcinoma biopsies. We found that hyperthermia restored p53 function inducing p53-dependent apoptosis and G2-arrest in HPV-positive cells, which could be inhibited by p53-siRNA (Fig. 4A and C). Our data highlight the difference between HPV-negative and positive cells, because the HPV-negative cells induce p53 and causes apoptosis after radiation alone, whereas HPV-positive cells require hyperthermia to accomplish these effects. G2-phase arrest and apoptosis induction after hyperthermia at 42°C for 1 hour in HPV-E6-transfected cells were observed earlier (van Bree et al., 1999). This important finding may explain why patients with HPV-positive cervical cancers respond particularly well to the combination of standard radiotherapy or chemotherapy with mild hyperthermia and may boost further clinical studies with hyperthermia in other HPV-associated cancers. Activation of the p53-dependent apoptotic pathway by hyperthermic degradation of E6 is specific for HPV-positive tumor cells, which may provide a therapeutic benefit to cancer patients by minimizing normal tissue damage compared to classical cisplatin-based treatments.

Hyperthermia induces de novo p53 (Fig. 3A). This induction of p53 is observed after 1-hour treatment at 42°C; treatment at lower temperatures did not show any accumulation of p53 (Fig. 1A). Furthermore, this p53 accumulation occurs immediately after hyperthermia and remains elevated up to 4 hours later (Fig. 1B). Also in immunofluorescence staining, p53 upregulation is observed directly after hyperthermia, in both HPV16 and 18-positive tumor models (Fig. 1C). Previous studies support the hypothesis that blocking E6 might lead to reactivation of p53 (Mantovani & Banks, 1999). In these studies blocking of E6 alone was not effective, and it was postulated that an additional stimulus would be necessary to transport and activate p53. Our results confirm these earlier findings by showing that p53 is not elevated in E6-siRNA-transfected cells, but that p53 levels in these cells are highly increased after adding radiation. This p53 accumulation appears to be about similar in quantity to the p53 induction found after hyperthermia (Fig. 2).

By blocking the proteasome or lysosome it can be observed that E6 is degraded via the proteasomal and lysosomal pathways, and p53 is degraded exclusively via the proteasomal pathway (Fig. 3). After hyperthermia no significant
differences in p53 levels were detected, suggesting that p53 degradation remains via the proteasomal pathway. Figure 5 presents a schematic overview summarizing these findings.

Reactivating or restoration of p53 function in tumor cells has long been studied as a rational cancer treatment (Issaeva et al, 2004; Ventura et al, 2007). Also targeting E6 resulted into effective killing of HPV-positive cancer cells (Butz et al, 2003). Targeting of E6, resulting in reactivation of p53, has also been studied in HPV-positive head and neck tumors (Xie et al, 2014). The main difference between the present study and previous investigations is our use of hyperthermia; although this clinical treatment has been used for decades, the sensitization mechanism in tumor cells is only now starting to unfold (Krawczyk et al, 2011).

Our study presents a possible biological explanation for the tumor-specific radiosensitization by hyperthermia for HPV-positive tumor cells. These findings suggest that patients with HPV-positive tumors benefit from mild hyperthermia due to abrogation of the p53-E6 complex causing activation of the p53-dependent apoptotic pathway.

**Competing interests**
Authors declare that they have no competing interests.

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**Materials and methods**

**Cell culture.** The cervical carcinoma cell lines: SiHa, HeLa, C33A, Caski, C4I, and HT3; the human prostate carcinoma cells lines: Du145, LNCaP, and PC3 were all obtained from American Type Culture Collection. Colon carcinoma cell lines: RKO, RC10.1, and RC10.2 were kindly provided by Dr. Kathleen Cho, University of Michigan (Ann Arbor, MI), and HCT116 p53wt and p53null were obtained from Horizon Discovery, Cambridge, UK. All cell lines were passed for fewer than 6 months after receipt, except for RKO, RC10.1, and RC10.2. Their p53-status have been tested regularly using western blots. Human cervical carcinoma cells, SiHa (HPV 16-infected), HeLa (HPV 18-infected) and C33A cells (HPV-negative) were grown in Media Modified Eagle’s Medium (BioWhittaker/Lonza). HPV 16-infected cervical carcinoma Caski cells were grown in RPMI-1640 (Gibco-Brl Life Technologies). HPV-negative human cervical carcinoma cell line C4I was grown in Waymouth’s medium (Gibco-Brl Life Technologies). HPV-negative human cervical carcinoma cell line Caski cells were grown in RPMI-1640 (Gibco-Brl Life Technologies). HPV-negative human cervical carcinoma cell line HT3, colon carcinoma cell lines HCT116 p53wt and p53null, RKO, RC10.1, and RC10.2 were grown in McCoy’s 5a (Gibco-Brl Life Technologies) supplemented with 10% heat-inactivated FBS and 2 mmol/L glutamine. Cells were maintained at 37°C in an incubator with humidified air supplemented with 5% CO₂. The doubling time of these cells during exponential growth is approximately 24 to 60 hours.

**Xenograft tumors.** Human SiHa cells were injected into the hind leg of athymic mice. When tumors had grown to a volume of about 100 mm³, mice were treated with or without hyperthermia. The animals were cooled, which prevented an increase of the body core temperature. During all treatments, the animals were anesthetized with a mixture of 2.5% isoflurane in oxygen. Directly after treatment mice were sacrificed and the tumors were taken out. both untreated and treated tumors were prepared for either western blotting or immunofluorescence. Animal experiments were approved by the animal welfare committee of the Academic Medical Center as required by Dutch law LEX102767.

**Patient biopsies.** Cervical carcinoma (AMC/MEC 03/137) biopsies of patients were divided into two parts, one half was left untreated, whereas the other half was treated ex vivo with hyperthermia. Four biopsies were sliced and sonificated into cell suspensions to perform western blotting. Three biopsies were submerged in paraformaldehyde, to be used for paraffin coupes.

**Hyperthermia.** Hyperthermia of the cells was performed by partially submerging the culture dishes in a thermostatically controlled water bath (Lauda aqualine AL12, Beun de Ronde) for 1 hour at 40, 41 or 42°C and 30 minutes at 42°C. Temperature was checked in parallel dishes, and the desired temperature (±0.1°C) was reached in approximately 5 minutes. The atmosphere was adjustable by a connection with air and CO₂ supplies. All cell cultures were heated in a 5% CO₂/95% air atmosphere and air inflow of 2 L/min.

Hyperthermia of mouse tumors was performed by submerging the tumor-bearing hind leg in a thermostatically controlled water bath for 1 hours at 42.7°C, resulting in a tumor temperature of 42.0°C, as confirmed by thermocouple measurements at the tumor surface. Hyperthermia (1 hour at 42°C) of patient biopsies was performed in culture dishes in the
same thermostatically controlled water bath as described for the in vitro cultures.

**Radiation treatment.** Cells were irradiated with or without prior hyperthermia treatment. In order to allow observation of an additional effect of hyperthermia all irradiation treatments were performed with single dose (4 Gy) of gamma-rays from a $^{137}$Cs source at a dose rate of about 0.5 Gy/min. For survival curves cells were irradiated with single doses up to 8 Gy.

**Inhibitors.** Proteasomal degradation was tested using an inhibitor (MG132, 10 μmol/L for 1 hour; Sigma). For lysosomal degradation, a lysosomal inhibitor, chloroquine (100 μmol/L for 16 hours; Sigma), was used and a second compound inhibiting fusion between autophagosomes and lysosomes, so called bafilomycin A1 (200 nmol/L for 4 hours; Sigma). To check the effect of hyperthermia on the p53-E6 interaction, an E6-siRNA (Santa Cruz Biotechnology) transfection was performed. Furthermore cells were transfected with p53-siRNA and scrambled p53-siRNA (Cell Signaling Technology) prior to hyperthermia in order to study the p53-dependent apoptosis. Cells were also incubated with MG132 in order to investigate the localization of E6 after radiation. To test whether the accumulated p53 was produced de novo, cycloheximide (1 μmol/L for 1 hour; Sigma-Aldrich) was used, which prevents translation from taking place.

**Survival assays.** Clonogenic assays were conducted to investigate the radiosensitization of hyperthermia. Experiments were performed in HPV 16 and 18-positive cervical cancer cell lines. Cells were plated before treatment into six-well culture plates (Costar). Dishes were placed in an incubator with 5% CO$_2$ at 37°C until sufficiently large colonies were formed. Afterwards the medium was removed and cells were washed with PBS. A mixture of 6.0% glutaraldehyde and 0.5% crystal violet were added for at least 30 minutes at room temperature (20°C). After removing the mixture of glutaraldehyde and crystal violet, plates were washed with water and eventually dishes were dried in normal air at 20°C. Colonies were counted under a light microscope. Survival fractions were calculated by dividing the plating efficiency of treated cells by that of control cells ± SEM (Franken et al., 2006; van Bree et al., 2001). Surviving fractions after dose D, \( S(D)/S(0) \), were corrected for the cytotoxicity of hyperthermia alone, and survival curves were analyzed to calculate values of the linear and quadratic parameters α and β, using SPSS 14.0 statistical software by means of a fit of the data by weighted linear regression, according to the linear-quadratic formula: Ln(S(D)/S(0))=-(αD+βD²)(Barendsen, 1982; Barendsen et al, 2001; Franken et al, 1997; Franken NAP, 1997). Data on clonogenic assays and apoptosis were analyzed using a t test.

**Western blotting.** To understand the additional effect of hyperthermia to radiation on HPV-positive tumors, western blots were carried out to study p53 levels. Controls and treated cells were harvested 4 hours after treatment. Pellets were lysed in ice-cold RIPA buffer (20 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mmol/L Na$_2$EDTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L 8-glycerophosphate, 1 mmol/L Na$_3$VO$_4$, 1 μg/mL leupeptin) for 30 minutes on ice with protein inhibitors (van Bree et al, 2004). Laemmli buffer with 2-mercaptoethanol (355 mmol/L) was added to the supernatant (1:1) and heated in boiling water for 2 to 5 minutes. Finally, samples were sonificated (Sonic & Materials Inc.). One microgram of protein was resolved by 10% SDS-PAGE precast gels.
(BioRad) and transferred to PVDF membranes. Equal protein loading was checked by Ponceau S staining (van Bree et al., 2004). Immunodetection was performed for p53 mAb (Dako) in combination with a horseradish peroxidase-conjugated secondary anti-mouse IgG (Southern Biotech). Housekeeping protein ERK2 was detected using mAb (Bethyl Laboratories) and a secondary anti-rabbit mAb (Invitrogen Life Technologies). All samples were enhanced using chemoluminescence (Amersham Pharmacia Biotech). Finally, blots were analyzed using LAS4000 (GE, Healthcare life sciences).

**Cell cycle and apoptosis analyses.** The Nicoletti assay (Riccardi & Nicoletti, 2006) is another way to study p53 functionality. Apoptosis was studied in all cell lines using this assay. At 48 hours after treatment (p53-siRNA with or without hyperthermia), cells were collected and pellets were resuspended in Nicoletti buffer (0.1% w/v Sodium citrate, 0.1% v/v Triton X-100 in demi water, pH 7.4). We have also performed additional apoptotic assays (cleaved caspase-3 and Annexin V) on SiHa and HeLa cells. Analyses were made using flow cytometry (FACS Canto; BD Biosciences). Statistical analysis was performed using a t-test.

**Immunocytochemistry and immunohistochemistry.** SiHa and HeLa cells were grown on coverslips. Inhibitors chloroquine, bafilomycin A1 and MG132 were added to study the breakdown of p53 and E6, in normal conditions and after hyperthermia. Also, a transfection with HPV 16/18-E6-siRNA was performed to investigate whether the same results were found after hyperthermia and a specific E6-siRNA transfection. Afterwards, cells were fixed with 4% paraformaldehyde for 20 minutes on ice. Before blocking cells (PBS containing 0.1% Triton X-100 and 5% normal goat serum), cells were washed with PBS. Cells were incubated overnight at 4°C in HPV 16/18-E6 protein (Santa Cruz Biotechnology) or p53 mAb (Transduction Labs). The next day, samples were washed and incubated for 1 hour at room temperature with a secondary antibody Alexa Fluor 488 (Invitrogen Life Technologies), washed and incubated for 10 minutes in PBS containing 1 μg/ml DAPI (Sigma-Aldrich). After washing, coverslips were stuck to slides using ProLong Gold anti-fade reagent (Invitrogen Life Technologies). Paraffin-embedded coups were made from xenograft tumors and patient biopsies. They were deparaffinized and rehydrated. Afterwards a heat-induced antigen retrieval at pH 9.0 for 20 minutes was performed, followed by a 30-minute cooling period. Next, a 15-minute PO block including H2O2 was performed. Then coups were incubated overnight at 4°C with HPV 16/18-E6 protein or p53 mAb (Transduction Labs). Next, tissue was embedded in Alexa Fluor 488 (Invitrogen Life Technologies), after washing in PBS. DAPI was used to stain the nuclei blue before covering tissue with ProLong Gold antifade reagent (Invitrogen Life Technologies) and a coverslip. In order to study the cleaved caspase-3 in immunohistochemistry stainings, the heat-induced antigen retrieval was performed at pH 6.0. Primary antibody cleaved caspase-3 antibody (Cell Signaling Technology) was diluted in Primary antibody dilution (KliniPath) and incubated overnight at 4°C. Afterwards, tissue was embedded in Powervision Poly-HRP-GAM/R/R IgG (Immunologic, Immunovision Technologies). For counterstaining Eosinhematoxyline (Fluka) was used, before covering tissue in mounting solution (Pertex) and a coverslip.
Supplementary Figures

Supplementary Figure 1. (A) All HPV-positive cell lines show an induction of p53 after a treatment including hyperthermia (42°C for 60 min). (B) In the p53wt HPV-negative cell lines radiation alone can already cause an induction of p53. In the p53mut and p53null HPV-negative cells, no increase of p53 is observed. (C and D) Additional patient biopsies, demonstrating the p53 induction after hyperthermia. (E) Quantification of immunofluorescence staining of ten fields per condition. From left to right: SiHa (Fig. 1C, upper panel), HeLa (Fig. 1C, lower panel), Xenograft I (Fig. 1D), Patient E (Fig. 1E), Patient F (Fig. S1C), Patient G (Fig. S1D). Staining was significantly higher for all samples treated with HT compared to untreated samples (*, p<0.001). HT, hyperthermia; RT, radiation.
Supplementary Figure 2. (A) E6-siRNA in combination with RT induces p53 in SiHa and HeLa cells. (B and C) Additional patient biopsies, demonstrating downregulation of E6 after HT. (D) In untreated SiHa cells, the level of p53 is too low to be detected. After a 30-min treatment at 42°C, a small accumulation of p53 is noticed, while expression of E6 is less intense compared to control. After a 60-min treatment at 42°C, all cells are p53 positive, and cells no longer express E6. (E) Quantification of immunofluorescence staining of ten fields per condition. From left to right: SiHa (Fig. 2A), HeLa (Fig. 2B), Xenograft I (Fig. 2C), Patient E (Fig. 2D), Patient F (Fig. S2B), Patient G (Fig. S2C), SiHa cells treated with 30 or 60 min HT (Fig. S2D). Green bars represent p53, red bars E6. Graph bars indicated with * are significantly different from untreated samples (*, p<0.001). HT, hyperthermia; RT, radiation.
Supplementary Figure 3. (A) Immunofluorescence staining of DAPI (blue), E6 (red) and p53 (green) are shown for different conditions. High levels of p53 are observed after incubation with a proteasomal inhibitor (MG132), while there is no p53 seen after incubation with the lysosomal inhibitor (Bafilomycin) in HeLa cells. (B) Western blot analysis of p53 in SiHa cells only show p53 detection after inhibition of the proteasomal pathway in untreated, radiation (RT) and cisplatin (cDDP) conditions. Any hyperthermia (HT) treatment, combined with RT or cDDP or HT alone, is seen to yield a p53 accumulation without the presence of any lysosomal or proteasomal inhibitor. (C) Quantification of immunocytochemistry staining of ten fields per condition. From left to right: SiHa (Fig. 3C), HeLa (Fig. 3D), SiHa (Fig. 3E), HeLa (Fig. S3A). Green bars represent p53, red bars E6. All graph bars were significantly different from the untreated samples (*, p<0.001).
Supplementary Figure 4. (A) Nicoletti assay showing apoptosis in SiHa and HeLa cells. Only after a treatment including hyperthermia, significantly higher percentages of apoptosis are observed. (B) Nicoletti assay showing apoptosis in HPV-positive cells. Only after a hyperthermia or hyperthermia and radiation, significantly higher percentages of apoptosis are detected compared to the untreated situation. After transfecting cells with p53-siRNA prior to hyperthermia apoptotic levels almost return to the untreated levels. (C) Nicoletti assay showing apoptosis in HPV-negative cells. In the p53wt cells, radiation alone already causes apoptosis. Induction of apoptosis is not observed in p53mut or p53null cells. (D and E) Annexin V (D) and Cleaved caspase-3 (E) analysis on SiHa (left) and HeLa (right) cells. Positive cells are detected after hyperthermia, or hyperthermia and radiation. Scrambled p53-siRNA prior to hyperthermia also induces apoptosis. (F) Western blot analysis demonstrating only p53 induction of scrambled p53-siRNA prior hyperthermia. HT, hyperthermia; RT, radiation; RT+HT, radiation and hyperthermia. *, p<0.05; **, p<0.01; ***, p<0.001
References


Chapter 4 | Hyperthermia targets HPV-positive cervical carcinoma


