Oncogenic human papillomaviruses block expression of the *B-cell translocation gene-2* tumor suppressor gene

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Human papillomavirus (HPV)-induced carcinogenesis is critically dependent on the activities of the viral *E6* and *E7* oncogenes. Here, we demonstrate that expression of the putative tumor suppressor gene *B-cell translocation gene-2* (*BTG2*) is reinduced in HPV16- and HPV18-positive cancer cells on silencing of viral oncogene expression, indicating that *BTG2* is repressed by oncogenic HPVs. Inhibition of *BTG2* expression was mediated by the HPV *E6* oncogene and occurred in a p53-dependent manner. Luciferase reporter gene analyses revealed that *BTG2* repression takes place at the transcriptional level and is dependent on the integrity of the major p53-response element within the *BTG2* promoter. Ectopic expression of *BTG2* acted antiproliferative in cervical cancer cells. Tissue specimens commonly exhibited reduced *BTG2* protein levels in HPV-positive high-grade lesions (CIN2/3) and cervical carcinomas, when compared with normal cervical epithelium. These findings identify the antiproliferative *BTG2* gene as a novel cellular target blocked by the HPV *E6* oncoprotein.

Key words: viral carcinogenesis; human papillomavirus; *E6*; cervical cancer; tumor suppressor genes; *BTG2*

Specific types of human papillomaviruses (HPVs) are closely associated with the development of cancers in humans, including cervical carcinoma.¹ The viral *E6* and *E7* genes are regularly expressed in HPV-positive cancer cells and are responsible for the maintenance of their malignant phenotype.¹ Both gene products target several cellular pathways linked to carcinogenesis. Among other activities, the *E7* protein can block the activity of the pRb tumor suppressor protein² and induce genomic instability,³ whereas the *E6* protein can inactivate the p53 tumor suppressor protein⁴ and stimulate telomerase expression.⁵ Notably, in cancers, which are not known to be related to viral infections, the same cellular pathways are often altered, e.g., by DNA mutation or epigenetic modification.⁶ Thus, the identification of novel downstream targets for the HPV oncogenes should not only increase our understanding of HPV-associated carcinogenesis but also could be informative for molecular carcinogenesis in general.

By whole genome microarray analysis, we have recently analyzed changes in the transcriptome of HeLa cervical carcinoma cells after specific inhibition of their endogenous HPV18 *E6/E7* expression by RNA interference.⁷ A total of 288 cellular genes were found to be upregulated on interference with HPV18 *E6/E7* expression, thereby representing potential targets to be downregulated by the viral oncogenes. These genes included the *B-cell translocation gene-2* (*BTG2*) gene.

BTG2 is a member of the antiproliferative gene family⁸ and has been recently linked to human carcinogenesis by exhibiting properties of a tumor suppressor gene. For example, *BTG2* reduces cell proliferation,^{9–13} blocks cell transformation by the *ras* oncogene,¹⁴ can induce cellular differentiation¹⁵ and modulates apoptosis.^{11,15,16} These pleiotropic effects may be linked to the ability of the *BTG2* protein to function as a broad activator of mRNA deadenylation.¹⁷ Notably, several cancers, including carcinomas of the breast,¹⁸ liver,¹⁹ kidney²⁰ and prostate,¹² exhibit decreased *BTG2* expression, when compared with corresponding normal

tissue. In light of these findings, it was interesting to investigate whether oncogenic HPVs may target *BTG2* expression in cervical cancer cells.

Material and methods

Cells and transfections

HPV18-positive HeLa and SW756 cervical carcinoma cells, HPV16-positive CaSki, SiHa and MRI-H-186 cervical carcinoma cells and HPV-negative U2OS osteosarcoma and MCF-7 breast cancer cells were all maintained in Dulbecco's minimal essential medium (pH 7.2), supplemented with 10% fetal calf serum, 50 U/ml penicillin and 50 µg/ml streptomycin sulfate. HeLa subclones stably expressing shRNAs blocking p53 expression have been described in detail previously.²¹ Primary human cervical and foreskin keratinocytes were grown in Keratinocyte Growth Medium 2 with supplements (Promocell, Heidelberg, Germany). Plasmids were transfected by calcium phosphate coprecipitation.²² Synthetic siRNAs were transfected with Oligofectamine (Invitrogen, Karlsruhe, Germany). Cells were plated on 6 cm dishes at 30–50% confluency. Oligofectamine (8 µl) and siRNAs at final concentrations between 50 and 200 nmol were both diluted in Opti-MEM I reduced serum medium (Invitrogen) and mixed in a final volume of 400 µl transfection solution. Before transfection, the complete medium was exchanged against 1.6 ml Opti-MEM I, which was supplemented 4 hr after transfection with 1 ml DMEM containing 30% fetal calf serum.

Plasmids and siRNAs

HPV16, HPV18 and HPV6 *E6* were expressed from the CMV promoter in expression vectors pRC/CMV and pBCH or from the Harvey murine sarcoma virus long terminal repeat in pLTR.^{21,23} HPV16 and HPV18 *E7* were expressed from the pCMX vector backbone.²⁴ Plasmid p53/248mut expresses a transdominant negative mutant p53 protein containing an arginine-to-tryptophan transversion at Codon 248.²⁵ Full length *BTG2* was expressed from vector pCI-BTG2, which is based on the pCI-neo backbone (Promega, Mannheim).⁹ The *p21* promoter and *BTG2*-promoter driven luciferase reporter plasmids have been described elsewhere.^{22,26} Synthetic siRNAs interfering with HPV18 and HPV16 *E6* or *E6/E7* gene expression have been characterized in detail before.^{22,27} Control siRNA siControl 5'-UAGCGACUAAACA CAUCAA-3' (Thermo Fisher Scientific, Lafayette, IN) contains at least 4 mismatches to all known human genes. The corresponding

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sequences were also expressed as shRNAs from vectors pSuper,²⁸ as described previously,^{22,27} or pCEPsh in which the BglIII-XhoI fragment of pCEP4 (Invitrogen) has been replaced by the BamHI-XhoI cassette of pSuper. pCEPshp53 expresses a shRNA blocking p53 expression.^{22,28}

Luciferase assays

For luciferase assays, cells were plated on 6 cm Petri dishes and transfected with 0.5 µg of the respective luciferase reporter plasmids, together with the indicated amounts of expression vectors or pCEPsh constructs, as indicated. Each transfection also contained 1.5 µg of the internal standard pACT-Gal, expressing the *E. coli* β -galactosidase gene under control of the human β -Actin promoter, to correct for possible variations in transfection efficiencies.²⁹ Cells were harvested 48–72 hr after transfection and processed as described.²⁹ Luciferase activities were quantitated using a Lucy 1 microplate luminometer (Anthos, Krefeld, Germany).

Protein and RNA analyses

Protein extracts were prepared 72 hr after transfection, essentially as previously described.²² For Western blot analyses, ~30 µg of protein were separated by 10% SDS-PAGE, transferred to an Immobilon-P membrane (Millipore, Eschborn, Germany) and analyzed by enhanced chemiluminescence (Amersham Biosciences, Freiburg, Germany) using anti-p53 antibody DO-1 (Pharmingen, San Diego, CA), anti-HPV18 E7 antibody 18E7C²² and anti-tubulin antibody Ab-1 (Oncogene, Boston, MA). For RT-PCR analysis, cellular RNA was isolated³⁰ and reverse transcription of 1 µg RNA was performed using the oligo-dT primer and SuperScriptIII First-Strand kit (Invitrogen, Karlsruhe, Germany). PCR analyses were performed using primers BTG2-for: 5'-CTC ACC TGC AAG AAC CAA GTG-3' and BTG2-rev: 5'-AGT TCC CCA GGT TGA GGT ATG T-3',²⁰ GAPDH-for: 5'-GAA GGT GAA GGT CGG AGT C-3' and GAPDH-rev: 5'-GAA GAT GGT GAT GGG ATT TC-3'.³¹ Cycle number for each primer set was established to be in the linear range of amplification. After separation on a 2% nondenaturing agarose gel, the amplification products were visualized with ethidium bromide. Individual amplification products were quantitated in the linear range of the PCR by densitometric scanning (ImageJ 1.41, NIH, Bethesda, MD) and determined relative to the GAPDH amplification product. For quantitative real time PCR, BTG2 and GAPDH expression were determined with a 7300 Real-Time PCR System detector (Applied Biosystems, Forster City, CA), using SYBR green PCR Master Mix (Applied Biosystems), supplemented with 250 nM (for BTG2) or 500 nM (for GAPDH) of each forward and reverse primer (see earlier). The integrity of the PCR products was initially analyzed by agarose gel electrophoresis and subsequently checked by melting point analysis after each reaction. Relative quantification was performed using the comparative Ct ($2^{-\Delta\Delta C_t}$) method.³² Data are presented as the fold difference in gene expression normalized to the expression levels of GAPDH, as an internal standard, and relative to a calibrator sample (corresponding to the respective control transfectants).

Cell count analyses and colony formation assays

For cell count analyses, cell suspensions were diluted in CASY-ton (Innovatis AG, Reutlingen, Germany) 48 and 72 hr after transfection with either pCI-BTG2 or empty control vector. Total cells per milliliter were measured using a CASY TTC cell counter (Innovatis AG). For colony formation assays, cells were grown on 6 cm dishes and transfected with 3 µg of pCI-BTG2 or pCI-neo. After 12 days selection for G418 (Invitrogen) resistance, colonies were fixed with formaldehyde and stained with crystal violet.

Immunohistochemical analyses

Serial cryosections (5 µm) of normal ectocervical tissue, high grade CIN (CIN2/3) and cervical squamous cell carcinomas were

mounted on 3-aminopropyl-triethoxysilane-coated slides and fixed in 4% paraformaldehyde in TBS for 4 min at 4°C. Endogenous peroxidase activity was blocked by 0.6% hydrogen peroxidase in TBS for 30 min at room temperature. Anti-BTG2 (1.0 µg/ml, ARP33561_P050, AVIVA, San Diego, CA), anti-p16 (1.2 µg/ml, E6H4, MTM Laboratories, Heidelberg, Germany) or anti Ki-67 (0.8 µg/ml, MIB-1, Dako, Hamburg, Germany) antibodies were incubated over night at 4°C. Subsequent thorough washing in TBS-1%Tween20 was performed. Specifically, bound primary antibodies were detected by the use of the EnVision technology (DAKO, Denmark) in accordance with the manufacturer instructions. Tissue specimens were examined at random order and grouped into negative (–), positive (+) and strongly positive (++) by 2 independent examiners.

Results

BTG2 expression is blocked by the HPV E6 oncogene

To investigate whether BTG2 expression is modulated by oncogenic HPVs, HPV16- and HPV18-positive cervical cancer cells were treated with siRNAs, which block viral E6/E7 expression concomitantly (si18E6/E7 and si16E6/E7).^{22,27} BTG2 is a very labile protein with a short half-life and is usually undetectable by immunoblotting at endogenous levels. Therefore, we assessed possible alterations of BTG2 expression at the mRNA level. As shown in Figure 1a, treatment of a series of HPV16- and HPV18-positive cells with E6/E7-targeting siRNAs led to a clear increase of BTG2 transcripts. In contrast, BTG2 expression was not upregulated by both E6/E7-targeting siRNAs in HPV-negative cells, such as MCF-7 or U2OS, corroborating that the effect is HPV dependent. Treatment with si18E6 and si16E6, which specifically block E6 expression,^{22,27} also led to the induction of BTG2 expression in HPV-positive cancer cells (Fig. 1a), indicating that E6 alone is sufficient to decrease BTG2 transcript levels.

To further differentiate whether BTG2 repression is E6- and/or E7 dependent, we ectopically expressed the HPV16 and HPV18 E6 and E7 genes in the HPV-negative cell line MCF-7 and investigated their influence on endogenous BTG2 expression. Both HPV16 and HPV18 E6 strongly reduced endogenous BTG2 transcript levels (Fig. 1b). In contrast, BTG2 expression was not affected by expression of either HPV16 or HPV18 E7. These findings indicate that BTG2 expression is mainly suppressed by the viral E6 oncogene.

Next, we compared BTG2 expression levels in HPV-positive cervical cancer cells with the levels in human primary cervical and foreskin keratinocytes, which are the natural target cells for HPVs. Because BTG2 mRNA expression can correlate with cell density,²⁰ care was taken to study BTG2 mRNA levels in the exponential growth phase of the cells. We found that HPV-positive cervical cancer cells exhibited substantially lower BTG2 transcript levels than primary cervical keratinocytes (Fig. 1c), which would be consistent with the notion that BTG2 expression is blocked by oncogenic HPVs.

Repression of BTG2 expression by HPV E6 is p53 dependent

Several factors have been reported to stimulate BTG2 expression, which include retinoic acid receptors,³³ NF-κB,¹⁸ ARF³⁴ and p53.⁹ Because E6 can functionally antagonize p53,⁴ we assessed whether the observed BTG2 stimulation on E6 inhibition was p53 dependent. In this experimental setting, we used HeLa cell subclones, which stably express shRNAs repressing the p53 gene.²¹ Parental HeLa cells endogenously expressed low levels of p53 protein, which were strongly increased on interference with viral oncogene expression (Fig. 2a), most likely due to the loss of the p53-destabilizing E6 activity. Subclone HeLa C2 tightly blocks p53 expression in that basal p53 protein levels were undetectable, and siRNA-mediated silencing of HPV18 expression resulted in only barely detectable p53 amounts. Subclone HeLa A1 exhibited an intermediate level of p53 reconstitution after HPV inhibition. Notably, the levels of reconstituted p53 protein correlated well

FIGURE 1 – Analysis of *BTG2* expression in HPV-positive cervical cancer cells. (a) RT-PCR analysis of HPV18-positive HeLa and SW756 and HPV16-positive SiHa and CaSki cells on treatment with siRNAs targeting viral *E6/E7* or *E6* expression. MCF-7 and U2OS, HPV-negative control cells. *GAPDH*, internal standard. Numbers indicate relative *BTG2* mRNA levels above control-transfected cells, as quantified by densitometry. (b) Modulation of *BTG2* mRNA expression by the HPV oncogenes. MCF-7 cells were transfected with expression vectors for the *E6* and *E7* proteins of HPV16 and HPV18. *BTG2* mRNA levels were determined by real-time PCR analysis and are indicated relative to control transfected cells. (c) Comparison of *BTG2* expression levels among primary cervical keratinocytes (CxK2), primary foreskin keratinocytes (FK107) and HPV-positive cancer cell lines (HeLa, SW756, CaSki, MRI-H-186, SiHa) by RT-PCR analysis. Numbers indicate relative *BTG2* mRNA levels in percent, when compared with the value for primary cervical keratinocytes (arbitrarily set at 100%), as quantified by densitometry. *GAPDH*, internal standard.

FIGURE 2 – Reinduction of *BTG2* expression on *E6/E7* inhibition is p53 dependent. (a) Differential reinduction of *BTG2* expression in HeLa cell subclones differing in their p53 response. Wild-type HeLa cells and HeLa subclones HeLa A1 and HeLa C2 were transfected with si18E6/E7 or siControl. Upper panel: Reconstituted p53 levels were measured by immunoblotting. *E7* protein levels were assessed as positive control for the functionality of si18E6/E7. Tubulin, loading control. Lower panel: Concomitant determination of *BTG2* mRNA levels by real-time PCR analysis. *BTG2* mRNA levels of siControl-transfected cells were set at 1.0. (b) A transdominant negative p53 mutant blocks reinduction of *BTG2*. HeLa cells were transfected with pSuper18E6/E7, together with either an expression vector for the p53 mutant protein p53/248mut (+) or vector control (-). Real time PCR analysis, *BTG2* mRNA levels of control-transfected cells were set at 1.0. Standard deviations are indicated.

with the amounts of *BTG2* stimulation (Fig. 2a), in line with the interpretation that *BTG2* induction on *E6/E7* inhibition is mediated by p53.

To obtain more direct evidence for this notion, we analyzed whether a transdominant negative p53 mutant protein can block the stimulation of *BTG2*. As shown in Figure 2b, the increase of *BTG2* mRNA levels on *E6/E7* inhibition could be completely reversed by concomitant expression of the p53 mutant p53/248mut, which blocks wild-type p53 activity.²⁵ These results further corroborate the notion that the reinduction of *BTG2* expression after HPV *E6/E7* repression is p53 dependent.

BTG2 induction after HPV16 or HPV18 inhibition occurs at the transcriptional level

Next, we investigated whether *E6*-mediated regulation of *BTG2* expression occurred at the transcriptional level. Luciferase reporter assays revealed that the full length *BTG2* promoter (*BTG2* nucleotides -2658/-1)²⁶ was stimulated in HeLa cells on interference with endogenous *E6* or *E6/E7* expression (Fig. 3a). Similarly, the p53-inducible p21 promoter,³⁵ which served as a positive control, was activated on repression of *E6* and *E6/E7* expression (Fig. 3a). Stimulation on interference with HPV oncogene expression was also observed for a truncated *BTG2* promoter fragment (*BTG2* nucleotides -266/-1), which contains the major p53-response element of the *BTG2* promoter.²⁶ Mutational deletion of this p53 response element (p266mutA) completely abolished activation by both sh18E6 and sh18E6E7. These data indicate that *BTG2* stimulation occurred, at least in part, at the transcriptional level and was mediated by the major p53-response element contained within the *BTG2* promoter.

FIGURE 3 – HPV16 and HPV18 E6 modulate the activity of the *BTG2* promoter. (a) Activation of the *BTG2* promoter on inhibition of HPV E6 expression. Luciferase reporter plasmids under the control of the complete *BTG2* promoter (p2658) or a truncated *BTG2* promoter, either containing the major p53 response element (p266) or not (p266mutA), were cotransfected in HeLa cells with 4 µg of vectors encoding shRNAs which block HPV18E6 or HPV18E6/E7 expression. pGL3, negative control (promoterless luciferase reporter plasmid), p21, positive control (luciferase under transcriptional control of the p53-responsive *p21* promoter). Indicated are relative luciferase activities (RLA) above the individual reporter plasmids cotransfected with pCEPshControl (arbitrarily set at 1.0). Standard deviations are indicated. (b) Repression of the *BTG2* promoter (p2658) by HPV16 or HPV18 E6 in MCF-7 cells. Left panel: Cotransfection of 0.5 µg of HPV16 or HPV18 E6 expression vectors. Central panel: Repression by a shRNA blocking p53 expression. Right panel: Cotransfection of 1 µg of HPV16E6 or HPV6E6 expression vectors. Relative luciferase activities (RLA) are indicated in percent, relative to the luciferase activities of cells cotransfected with p2658 and the respective vector controls (arbitrarily set at 100%). Standard deviations are indicated.

Vice versa, ectopic expression of the HPV16 or HPV18 E6 proteins in HPV-negative MCF-7 cells led to a strong reduction of *BTG2* promoter activities (Fig. 3b). Activity of the *BTG2* promoter was also strongly reduced on intracellular expression of a shRNA blocking p53 expression,²² further underlining the p53 dependence of its activity (Fig. 3b). To investigate whether the ability to block *BTG2* expression is shared by the E6 protein of an HPV type, which is usually not associated with cancers, we tested the effect of HPV6 E6 on *BTG2* expression. As shown in Figure 3b, HPV6 E6, which does not induce p53 destabilization, did not affect *BTG2* promoter activities.

Ectopic expression of *BTG2* acts antiproliferative in HeLa cells

Next, we tested whether the growth of HPV-positive cervical cancer cells is affected by reincreasing intracellular *BTG2* levels. For this set of experiments, we used HeLa cells in which we obtain ~90% transfection efficiencies, thereby enabling direct assessment of the growth inhibitory potential of *BTG2* by transient transfections. Cell count analyses showed that ectopic expression of *BTG2* clearly reduced HeLa cell numbers (Fig. 4a). To assess

FIGURE 4 – Ectopic expression of *BTG2* acts antiproliferative in HeLa cells. (a) Determination of cell numbers of HeLa cells, 48 or 72 hr after transfection with pCI-*BTG2* or the empty control vector, as indicated. Control transfectants were set at 100%. Standard deviations are indicated. (b) Colony formation assays of HeLa and U2OS cells on stable transfection with a *BTG2* expression vector (pCI-*BTG2*) compared with pCI-neo control transfectants.

long-term effects of ectopic *BTG2* expression on cell growth, we performed colony formation assays of HeLa cells stably transfected with a *BTG2* expression vector. Both the size and the number of HeLa cell colonies were reduced after 12 days selection for cells transfected with pCI-*BTG2*, when compared with control transfectants (Fig. 4b). Reduction of colony formation capacity was also observed after ectopic *BTG2* expression in HPV-negative U2OS osteosarcoma cells (Fig. 4b), in line with reports that *BTG2* can act antiproliferative in different cell types.^{9–12,18}

HPV-positive tissues exhibit loss of *BTG2* expression

The experiments described earlier define *BTG2* as a novel target gene being blocked by the HPV E6 oncogene in HPV-positive cancer cell lines *in vitro*. To investigate whether these data correlate with the *in vivo* situation, we analyzed biopsies derived from normal ectocervical tissues ($n = 10$), cervical intraepithelial neoplasias grade 2/3 (CIN2/3) ($n = 10$) and invasive cervical squamous cell carcinomas ($n = 10$) for the relation between HPV and *BTG2* expression. If E6 also blocks *BTG2* expression *in vivo*, reduced *BTG2* protein levels would be expected in HPV-positive tissues.

Immunohistochemical analyses revealed homogeneous *BTG2* expression in all biopsies of normal cervical epithelial tissue. In stark contrast, 30% of CIN 2/3 lesions and 40% of the cervical carcinomas did not exhibit any detectable *BTG2* expression (Fig. 5 and Table I). Moreover, if signal intensities were taken into account, only 1 (5%) of the 20 HPV-positive lesions exhibited high levels of *BTG2* protein expression, in contrast to 6/10 (60%)

FIGURE 5 – BTG2 protein expression in cervical epithelia. Immunohistochemical staining of a normal ectocervical tissue (upper panel) and of a cervical carcinoma (lower panel) for expression of BTG2, p16 (a surrogate marker for HPV oncogene expression) and the proliferative marker Ki-67. The signal intensity observed for BTG2 in the normal tissue is representative of a case scored as strongly positive. Overall, BTG2 staining was homogeneous, also at lower expression levels. Magnification: 40-fold.

normal cervical tissues (Table I). Areas in CIN and cervical cancer lesions, which stained positive for p16, a well-established surrogate marker for the activity of oncogenic HPVs,³⁶ typically showed reduced BTG2 protein amounts (Fig. 5). Taken together, these findings indicate a negative correlation between HPV-positivity and BTG2 protein levels *in vivo*, consistent with the *in vitro* data demonstrating the potential of oncogenic HPVs to inhibit BTG2 expression.

Discussion

This study identified the putative tumor suppressor gene *BTG2* as a novel target for inhibition by oncogenic HPVs. Mechanistically, *BTG2* repression was caused by E6, which blocks p53-mediated transcriptional activation of the *BTG2* promoter. This conclusion is supported by the findings that (i) the reinduction of *BTG2* expression on interference with viral oncogene expression in HPV-positive cancer cells was p53 dependent, (ii) the E6 proteins of the oncogenic HPV types 16 and 18—which induce the proteolytic degradation of p53—can block *BTG2* gene expression and repress the *BTG2* promoter in p53 wild-type cells, (iii) the levels of *BTG2* expression in HeLa cell subclones correlated well with the amounts of p53 protein reconstituted on interference with viral oncogene expression and (iv) the stimulation of the *BTG2* transcriptional promoter on E6 inhibition was dependent on its major p53-response element.

These data furthermore indicate that the endogenous p53 levels which are reconstituted on silencing of HPV oncogene expression suffice to transactivate the *BTG2* promoter in HPV-positive cancer cells. This is in contrast to other p53-responsive promoters (e.g., *BAX*), which are not markedly transactivated under identical experimental conditions,³⁷ possibly because they contain relatively low affinity p53 binding sites.^{37–39} Similarly, transcriptome analyses indicated that only a subset of potential p53 target genes are reinduced by the p53 amounts which are reconstituted on inhibition of E6 or E6/E7 expression in HPV-positive cell lines.^{7,40–42} In contrast, the induction of *BTG2* promoter activities and the concomitant increase of BTG2 transcript levels on silencing of E6 expression indicate that E6 is a critical determinant in continuously maintaining the downregulation of *BTG2* expression in HPV-positive cancer cells.

TABLE I – *BTG2* STAINING IN CERVICAL BIOPSIES

	Staining intensity		
	Negative (–)	Positive (+)	Strongly positive (++)
Normal ectocervical tissue (n = 10)	0 (0%)	4 (40%)	6 (60%)
CIN 2/3 lesions (n = 10)	3 (30%)	7 (70%)	0 (0%)
Cervical squamous cell carcinomas (n = 10)	4 (40%)	5 (50%)	1 (10%)

In line with our experimental data showing that oncogenic HPVs can repress the *BTG2* gene, we observed that HPV-positive cancer cell lines exhibited lower *BTG2* expression levels than primary human keratinocytes, the natural target cells for infection by HPVs. Moreover, the observed patterns of BTG2 expression *in vivo* are largely consistent with this interpretation. BTG2 expression was clearly detectable in all 10 HPV-negative, normal cervical tissues. In contrast, 3/10 CIN lesions and 4/10 cervical cancers did not exhibit any detectable BTG2 expression at all. Furthermore, high signal intensities for BTG2 expression were observed in 6/10 normal tissues but only in 1/20 HPV-positive lesions. It will be interesting to study in the future whether BTG2 expression levels may have an impact on the progression risk of CIN lesions or on the clinical course of cervical cancer patients, an issue which will require the investigation of larger patient numbers.

Reduced or absent expression of the antiproliferative *BTG2* gene has been associated with other tumor entities as well, such as cancers of the breast,¹⁸ liver,¹⁹ kidney²⁰ and prostate.¹² Thus, the identification of *BTG2* as a novel target for HPV E6 supports the idea that the HPV oncogenes represent valuable tools to identify molecular pathways, which are more generally affected in human carcinogenesis, i.e., in cancers which are not known to be related to HPV infections. Conceivably, *BTG2* repression in these latter cancers could be due to the mutational inactivation of p53 and, consequently, the loss of p53-mediated transcriptional activation of the *BTG2* promoter. However, reduced BTG2 expression is also often observed in renal cell carcinoma, a cancer which relatively infrequently harbors p53 mutations, suggesting that additional genetic events may lead to *BTG2* downregulation in human tumors.^{14,20}

Previous reports showed that BTG2 exerted antiproliferative effects in different cell types.^{9–13} Consistent with these observations, we found that ectopic BTG2 expression inhibited HeLa cell proliferation, indicating that their growth rate can be modulated in dependence on their intracellular BTG2 expression levels. No obvious signs for apoptosis induction after ectopic BTG2 expression were observed in these experiments. However, there is also experimental evidence linking BTG2 to the regulation of apoptosis. Both proapoptotic^{9,15} and antiapoptotic¹⁶ effects have been observed for BTG2, depending on the cell type. Notably, BTG2 has been recently found to enhance the apoptotic sensitivity of HeLa cells toward the chemotherapeutic agent doxorubicine.¹⁶

Therefore, it is tempting to speculate that the inhibition of *BTG* expression by the viral E6 oncoprotein may also contribute to the apoptotic resistance of HPV-positive cancer cells, an issue which is currently under investigation.

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