HPV-16 E6 and E7 oncogene expression is downregulated as a result of Mdm2 knockdown

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Abstract. The carcinogenic potential of HPV infections is based on the integration and constitutive expression of the E6 and E7 genes which inhibit the p53 and Rb tumor suppressor proteins. In normal cells, Mdm2 regulates p53 in a negative feedback loop, and although Mdm2 is apparently functional in HPV-infected cells, E6 is the protein responsible for repressing p53 replacing Mdm2 function. The role of Mdm2 in HPV-positive cells is still elusive. In this study, Mdm2 was knocked down in an HPV-positive cervical cancer cell line; as a result we found downregulation of the expression of E6 and E7 and p53 upregulation.

Introduction

The infection with human papilloma virus (HPV) has been associated with cervical cancer in 90% of cases (1); over 200 serotypes of HPV have been identified but only 23 are known to infect the human cervical tract and of these, only 13 are considered ‘high risk’ (2-4). HPV-16 and -18 are found in the latter category and between them are responsible for over 70% of cervical carcinomas worldwide (5,6).

The carcinogenic potential of HPV is attributed to the partial or total integration of the viral DNA into the host cell genome, resulting in the constitutive expression of E6 and E7 which inhibit p53 and pRb tumor suppressor activity (7), although it is known that E6 has other carcinogenic activities independent of its interaction with p53 (8,9).

Basal p53wt expression can be found in HPV-positive cancer cells, and although the p53Pro72 allele is more susceptible to E6 mediated degradation than its p53Pro72 counterpart (12), cervical carcinomas in which both HPV infection and mutant alleles of p53 coincide are rarely seen (10,11).

It has been suggested that E6 expression levels are closely related to p53 activity (13) and that the continuous expression of the E6 and E7 genes in transformed HPV-positive cells is required to maintain their malignant state (14); therefore, p53 stabilization in these cells by inhibiting E6 or E7 expression has been attempted in the past (15).

On the other hand, Mdm2 has been identified as an E3 ligase capable of binding and exporting p53 outside the nucleus (16) where it is degraded by ubiquitin thereby inhibiting p53 tumor suppressor activities such as growth arrest or apoptosis (17-19), hence it is known as the natural repressor of p53. In transformed HPV-infected cells, however, p53-Mdm2 interaction is impaired and Mdm2 is completely replaced by E6 in the process of nuclear export and ubiquitin mediated degradation of p53 (20). The role of Mdm2 in these cells has not been entirely determined yet. It is suggested, however, that Mdm2 may associate with the HPV E2 protein enhancing the viral protein's transcriptional activity (21).

In the last decade, the use of RNA interference (RNAi) has been widely applied in diverse studies. In HPV-infected cancer cells, RNAi silencing of E6 and E7 has been done with different results (7,15,22); for instance, the co-suppression by RNAi of both E6 and E7 in HPV-infected cells has been found to enhance the effect of growth arrest in these cells than otherwise obtained by silencing only one of them (23), on the other hand, the gene knockdown of E6, Mdm2 and other p53 antagonists shows a higher apoptosis rate than silencing only E6 (24).

The side effect of inhibiting E6 in HPV-transformed cells is the accumulation and stabilization of p53 and the subsequent changes in the expression of some of the p53-dependent genes, such as Bax, which is necessary for apoptosis in HPV-infected cervical cancer cell lines (25). However, by knocking down E6 in HPV-infected lung cancer cell lines, the expression level of Mdm2, which is downregulated in these cells, was restored to similar levels found in HPV-negative cells (26).

Regardless, the effect that silencing Mdm2 could have over the expression of p53, E6 and E7 and why the co-suppression of Mdm2 and the E6 oncogene enhances apoptosis in HPV-infected cervical cancer cells has not been fully addressed. In this study...
we found that the RNAi knockdown of Mdm2 in a cervical cancer cell line not only results in the upregulation of p53 but also in downregulation of E6 and E7 gene expression.

Materials and methods

Plasmids. Short hairpin RNA (shRNA) were expressed from vector pSilencer 1.0 (Ambion, Austin, TX). The zeocin drug resistance cassette was amplified by PCR from the pcDNA4/TO plasmid (Invitrogen, Carlsbad, CA) using the primers 5'-AACCGGATCCGAGGAGCGACCC-3' and 5'-GCGCGGTTTCGACGAGCGACCC-3' and cloned in the KpnI site of pSilencer 1.0. The shRNA target sequences were designed using the siRNA Design Tool from Ambion and further screened for thermodynamic stability (27). The designed shRNA DNA oligonucleotides consisted of the Apal cloning sequence, 19 nucleotides (nt) of the target sequence followed by a 9-nt loop sequence (TTCAA GAGA), the reverse complement to the 19-nt target sequence, the stop codon for the U6 promoter (TTTTT), and the EcoRV restriction sequence. The sequence used for Mdm2 was 5'-CG CCACAAAATCTGTATGTA-3'; and as an RNAi target sequence for EGFP which was also used as a control RNAi sequence we used 5'-GAAGCAGACGACTTCTTC-3'. The complementary strands were mixed in an equimolar ratio (10 µM) in annealing buffer (1X PBS pH 6.8, 2 mM MgCl2), heated at 95°C/5 min and cooled at room temperature. The shRNA constructs were cloned in the pSilencer_ApoI/EcoRV restriction sites.

Cell lines and transfections. SiHa epithelial cell line is derived from a human cervical carcinoma and contains 1-2 copies of HPV-16 genome integrated per cell. It was grown and maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B (Invitrogen). For transfection, the cells were trypsinized 48 h later and bedded in 12-well plates; stable selection was done adding zeocin (100 µg/ml) for 15 days. The cells were genotyped by PCR using the primers: U6fwd (5'-GGTACCCGCTCTAGAACTAGTGG-3') and 5'-AACGGGGTACCGGAACAACACTCAACCCTATCT-3'; and as an RNAi target sequence the stop codon for the U6 promoter (TTTTT), and the restriction sequence. The sequence used for Mdm2 was 5'-CG CCACAAAATCTGTATGTA-3'; and as an RNAi target sequence for EGFP which was also used as a control RNAi sequence we used 5'-GAAGCAGACGACTTCTTC-3'. The complementary strands were mixed in an equimolar ratio (10 µM) in annealing buffer (1X PBS pH 6.8, 2 mM MgCl2), heated at 95°C/5 min and cooled at room temperature. The shRNA constructs were cloned in the pSilencer_ApoI/EcoRV restriction sites.

GFP assay. Cells were cultured in a 24-well plate and transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The cells were trypsinized 48 h later and bedded in 12-well plates; stable selection was done adding zeocin (100 µg/ml) for 5 days. The cells were genotyped by PCR using the primers: U6fwd (5'-GGTACCCGCTCTAGAACTAGTGG-3'), U6rsv (5'-AAACAAGGCTTTTCTCAAGGG-3') and pSrvs (5'-TTACGCCAACGGCGCAATTAC-3') to verify construct integrity. Thermal cycle was 94°C/30 sec; 59°C/30 sec; 72°C/1 min (35 cycles).

RT-PCR. Total RNA was treated with RQ1 RNase-Free DNase (Promega, Madison, WI) according to the manufacturer's instruction. Single strand cDNA was obtained from 4 µg of total RNA using AMV Reverse Transcriptase (New England Biolabs, Ipswich, MA) following the manufacturer's instructions.

Semi-quantitative PCR. For semi-quantitation, 200 ng of cDNA was amplified in multiplex reaction using Taq polymerase (GenScript, Piscataway, NJ); the following primers were used: for GAPDH, 5'-AGGTCCGAGATCAACGGATTGTG-3' and 5'-CATGTGGGCCATGAGGTCCACCAC-3'; for Mdm2, 5'-GGCCGAAAACCCCGGATGGTGAGG-3' and 5'-CCTG TGTCCTTCTCACAGAGAAGC-3'; for p53, 5'-GGGTTCACTG AAGACCCAGGTCC-3' and 5'-ACCATCGCTATCTGAGCA GGCC-3'; for E6 5'-CAGGACCCCACGGAGCGACC-3' and 5'-GGACACAGTGCCGGGTCTTTTACAG-3'; and for E7, 5'-CCCAGCTGTAATCATGCATG-3' and 5'-CCCACTGTAATCATGCATG-3'. The thermal cycle used was: 94°C/30 sec, 59°C/30 sec, 72°C/1 min. Twenty-eight cycles were used for Mdm2; 29 cycles for p53; and 25 cycles for E6 and E7. Mdm2, p53, E6, and E7 DNA bands were quantified and normalized against the corresponding GAPDH bands to obtain relative expression levels.

Statistical methods. Densitometry data from DNA bands was obtained using Gel Pro Analyzer 4.0 software (Media Cybernetics), these data were analyzed using a Student's two-tailed t-test. Mean average and standard deviation of at least four independent experiments is given; significance was set at ≤0.05.
**Results**

**shRNA constructs and GFP assay.** The Mdm2 shRNA target sequence was selected based on the thermodynamic stability of its 3’-end, which would facilitate the entry of the antisense strand into the RISC complex (27), this construct also meets 5 out of 7 (28) and 4 out of 4 (29) criteria for siRNA design. This siRNA targets position 858 of the \textit{hmdm2} mRNA (PubMed access: M92424). The target sequence for EGFP was designed in a similar fashion. These shRNA constructs were cloned in the pSilencer 1.0 vector, which has the murine RNA pol III promoter, U6 (Fig. 1).

To verify the functionality of the murine U6 promoter in SiHa cells as well as the general design of our shRNA constructs, we transfected a GFP reporter plasmid alone in SiHa cells (Fig. 2D), and co-transfected the GFP reporter plasmid with either the shRNAmdm2 or shRNAGFP constructs.

After 72 h of transfection, GFP expression was efficiently suppressed when co-transfected with pS-shRNAGFP (Fig. 2F) but not when co-transfected with the pS-shRNAmdm2 vector (Fig. 2E); showing that the RNAi constructs were expressed in SiHa cells using a U6 murine promoter and that the design of the siRNA sequences was adequate.

**Stably transfected cell lines and genotyping.** It was previously noted that, in our assays, transfection efficiency was very low in SiHa cells so, in order to obtain more uniform data, the zeocin resistance gene was amplified from the pcDNA4/TO plasmid and cloned in the shRNA expression vectors. After transfecting the RNAi plasmids, the SiHa cells were selected using the drug zeocin for 15 days.

The cell lines were later genotyped amplifying the murine U6 promoter by PCR (Fig. 3A). The primers U6fwd and U6rvs were annealed downstream of the RNAi construct (C).
Semi-quantitative PCR analysis. After obtaining the stable SiHa cells, two clones of each transfected construct were chosen at random. Total RNA was obtained from these clones and from untransfected SiHa cells which were used as an untreated control. The cDNA was obtained in a reverse transcription assay and a multiplex amplification curve was done to establish the cycle number in which each gene was to be analyzed (data not shown).

The shRNA_{mdm2}, control shRNA_{GFP} cell clones and SiHa cells were cultured in 60-mm plates, and harvested after 72 h, total RNA was extracted, and cDNA synthesis was performed; 200 ng of this cDNA was later used to amplify mdm2, p53, E6 and E7 making a semi-quantitative analysis by testing band intensity against GAPDH (Fig. 4).

The analysis of Mdm2 showed that mRNA level had diminished 65% when compared with control shRNA_{GFP} clones (p≤0.05) and against untreated SiHa cells (p≤0.05) (Fig. 5), showing effective Mdm2 knockdown.

It has been previously reported that Mdm2 silencing does not, or only in a negligible manner, result in the activation of p53 (24,30). In agreement with previous reports, our stably transfected shRNA_{mdm2} cell lines did not present any of the characteristics of p53 activity, such as growth arrest or apoptosis.

However, the effect that mdm2 knockdown alone could have on p53 expression in a cervical cancer cell line has not yet been reported to the best of our knowledge. So, with this in mind we analyzed the expression level of p53 in these cells; we found that, although p53 activation is not apparent in the cells that have Mdm2 knockdown, the mRNA level of p53 is upregulated, showing a 56% increase when compared with the untreated SiHa cells or with the control shRNA_{GFP} cells (p≤0.05) (Fig. 5). Although p53 expression was increased, it is
likely that its transcription activity could not be achieved since E6 expression levels are in close relation to those of p53 activity (13). 

Trying to determine how E6 and E7 mRNA levels behaved in response to p53 upregulation by Mdm2 knockdown, we analyzed the treated and untreated cells. Unexpectedly, we found that E6 mRNA level had decreased 30% when compared against the untreated and control shRNA GFP cells (p≤0.05); moreover, the level of E7 mRNA expression was also decreased by 42% when compared with the untreated and control cells (p≤0.05) (Fig. 5).

These results show that by silencing Mdm2 using RNAi in an HPV-infected cervical cancer cell line, the expression level of p53, E6 and E7 are modified. The obtained results were somewhat unexpected and though p53 mRNA expression is upregulated and E6 and E7 mRNA levels are decreased, no growth arrest or apoptotic effect was apparent.

Discussion

The data obtained in this study showed that the inhibition of Mdm2 using RNAi, results in expression changes of p53, E6 and E7 in an HPV-infected cervical cancer cell line. The tumor suppressor activity of p53 in an HPV-infected cell is impaired by E6; moreover, E6 completely replaces Mdm2 in the nuclear export and ubiquitin mediated degradation of p53 in these cells (20); on the other hand, E7 can inhibit pRb tumor suppressing activities by preventing its binding to the E2F transcription factors and promoting its degradation (31,32).

The oncogenic potential of E6 and E7 is dependent upon their constitutive expression, which is required to preserve the malignant state of the infected cell (14); therefore, to overcome this effect has been tried by stabilizing p53 through a number of different strategies, among them, RNAi (7,15,22). However, the results obtained in these works differ in their conclusions; other approaches have determined that the inhibition of E6 is not sufficient in itself to promote p53 activation and that it is also necessary to induce p53 response by additional means, such as genotoxic treatment (33) or p53 antagonists co-suppression (24).

On the other hand, it has been shown that the stabilization and activation of p53 is negligible by inhibiting Mdm2 expression in an HPV-infected cervical cancer cell line (24,30), therefore, although Mdm2 knockdown is enough to upregulate the expression of p53, and downregulate E6 and E7, it is not sufficient to elicit p53 tumor suppressing activities, since no senescence, growth arrest or apoptotic effect was observed.

While the co-suppression of E6 and Mdm2 has been found to potentially increase the apoptotic effect that would be otherwise obtained by silencing only E6 (24); the fact that the knockdown of Mdm2 alone downregulates E6 and E7 in an HPV-infected cell line is somewhat baffling and hints at a not yet fully understood relation between these proteins that may be influenced by previously described Mdm2/E2 transcription activity (21).

In regards to E2, its open reading frame codes a ~42-kDa protein that regulates viral transcription through its binding to DNA palindromic sequences present in several copies in the long controlling region (LCR) of all papillomaviruses (reviewed in ref. 34) and it is also involved in the viral replication cycle by collaborating with E1 and cellular replication factors (35). E2 binds to multiple sites in the regulatory region (URR) controlling the expression of early promoters in a positive (low E2 concentration) or negative (high E2 concentration) way (36). However, the E2 transcriptional role is not completely clear and it seems that its transcription role depends on the HPV serotype.

The E2 protein is the transcriptional activator element for E6 and E7 expression in HPV-16 and, according to Gammoh et al (21), the E2 association with Mdm2 allows it to reach its full transcriptional potential; from this point of view, it makes sense that in knocking down Mdm2, the expression levels of E6 and E7 could be downregulated.

We consider that further research in this regard could improve the opportunity to fully understand the relationship between Mdm2 and HPV oncogene regulation and provide enlightenment to alternative therapy strategies against HPV-infected cervical cancer cells.

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