Cancer Biology & Therapy

ISSN: 1538-4047 (Print) 1555-8576 (Online) Journal homepage: www.tandfonline.com/journals/kcbt20

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To cite this article: J. Nyagol, Eleonora Leucci, A. Omnis, G. De Falco, C. Tigli, Francesca Sanseverino, M. Torriccelli, N Palummo, L. Pacenti, Rosa Santopietro, D. Spina, P. Gichangi, L. Muchiri, S. Lazzi, Felice Petraglia, Lorenzo Leoncini & Antonio Giordano (2006) The effects of HIV-1 Tat protein on cell cycle during cervical carcinogenesis, Cancer Biology & Therapy, 5:6, 684-690, DOI: 10.4161/cbt.5.6.2907

To link to this article: https://doi.org/10.4161/cbt.5.6.2907

Published online: 10 Apr 2006.

[Cancer Biology & Therapy 5:6, 684-690, June 2006]; ©2006 Landes Bioscience

Research Paper

The Effects of HIV-1 Tat Protein on Cell Cycle during Cervical Carcinogenesis

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Received 05/09/06; Accepted 05/18/06

Previously published online as a *Cancer Biology & Therapy* E-publication: http://www.landesbioscience.com/journals/cbt/abstract.php?id=2907

KEY WORDS

cervical cancer, HPV, HIV-1, Tat, cell cycle regulators, tumor biology, tumor suppressor genes

ACKNOWLEDGEMENTS

See page 689.

ABSTRACT

The role of HPV in the carcinogenesis of intraepithelial and invasive anogenital lesions is currently well established. E6 and E7 oncoproteins of high-risk HPV genotypes are known to inactivate p53 and pRb pathways. Several studies have described an increased prevalence and recurrence of both cervical HPV infection and invasive cervical cancer among HIV-1 positive women compared to HIV-1 negative cases. For these reasons, cervical cancer is considered an AIDS-defining neoplasm. Unlike other AIDS-associated neoplasms, the occurrence of cervical cancer is independent of immune suppression. HIV-1 infection in patients with high grade precancerous lesions and invasive cervical cancers results in a therapy refractory and more aggressive disease phenotype, which is not yet well understood at the molecular level. An upregulation of HPV E6 and E7 gene expressions by HIV-1 proteins such as Tat has been documented by some authors. However, the role of HIV-1 in cervical carcinomas is still unclear. It is already known that HIV-1 Tat protein is able to influence cell cycle progression. Altogether, these facts led us to investigate the effects of Tat on the expression of cell cycle regulator genes. After transfection of HeLa cells with Tat, we analyzed the expression of cell cycle regulators from these cells by IHC and Real-time PCR. A significant reduction in the expression of cell cycle inhibitors of transcription and an increase in the levels of proliferation markers were observed. These results suggest that HIV-1 may enhance cervical carcinogenesis by promoting cell cycle progression. We also found that this HIV-1 Tat-induced cell proliferation was not dependent on the E2F family of transcription factors, and therefore postulate that Sp factors may be involved.

ABBREVIATIONS

HPV, human papilloma virus; HIV-1, human immunodeficiency virus-1; AIDS, acquired immunodeficiency syndrome; IHC, immunohistochemistry, CDC; center for disease control and prevention; WHO, World Health Organization; TBS, tris-buffered saline; M-MLV, moloney-murine leukemia virus; HPRT, hypoxanthine phosphoribosyltransferase; LGSIL, low grade squamous intraepithelial lesions; HGSIL, high grade squamous intraepithelial lesions; CKI, cyclin-dependent kinase inhibitors; VEGFR2, vascular endothelial growth factor receptor 2; KS, Kaposi's sarcoma.

INTRODUCTION

The association between cervical cancer and HPV is well known, with the virus being implicated in approximately 99.7% of cases (reviewed in ref. 1). The HPV family includes more than 130 genotypes of small double-stranded DNA virus; more than twenty-three of which infect the genital tract. According to Lorincz et al.[2] these genotypes can be grouped into high and low risk genotypes according to their potential to induce malignant transformation.

As has been noted by Evander et al.[3] and Giroglou et al.,[4] HPV enters basal epithelial cells of the cervix through micro-abrasions probably using the alpha6beta4-integrin family and heparin sulphate receptors. The lifecycle of the virus is closely linked to the differentiation stages of the keratinocytes, the host cells. HPV replicates in the proliferating basal layer of the epithelium and, as infected cells leave the basal membrane and differentiate, the virus undergoes the productive stage of its lifecycle. Cell transformation occurs when the virus integrates into the cellular genome, leading to loss of function of E1/E2 viral products that are responsible for E6 and E7 repression, according to Chow and Broker.[5] Braun et al.,[6] Scheffner et al.[7] and Dyson et al.[8] demonstrated that E6 and E7 are viral oncogenes that interfere with p53 and pRb functions respectively, causing progression through the cell cycle. In addition, these oncogenes are responsible for the suppression of

interferon expression, thus impairing the antiviral and antiproliferative effects of these molecules, as noted by Massad et al.[9]

Several studies (by Vernon et al.,[10] Kapiga et al.,[11] Tornesello et al.[12] and Palefsky et al.[13]) have described an increased prevalence of both cervical HPV infection and invasive cervical cancer among HIV-1 positive women compared to HIV-1 negative ones. For this reason, the CDC declared invasive cervical cancer an AIDS-defining neoplasm in 1993. However, cervical cancer differs from other HIV-1 related malignancies because its occurrence is probably independent of immune suppression, and the diagnosis is believed to precede that of HIV-1 in approximately 70% of cases, according to Walker et al.[14]

Table 1 **Classification of the study cases**

	Precancerous lesions		Invasive cervical cancers of various grades		
	Low grade	High grade	I	II	III
HPV genotypes					
Low risk	9	0	0	0	0
High risk	1	10	4	7	5
HIV-1 negative	10	10	4	4	2
HIV-1 positive	0	0	0	3	3
Total	10	10	4	7	5

Although the oncogenic role of HIV-1 in cervical carcinoma is still controversial, it is known that HIV-1 infection correlates with a more aggressive and less therapy-responsive phenotype, as noted by DeFilippis et al.[15] Buonaguro et al[16] have postulated an upregulation of HPV E6 and E7 gene expression by HIV-1 proteins such as Tat. In addition, recent evidence underlines the implication of Tat in the pathogenesis of AIDS-related malignancies by interfering with cellular functions (reviewed in ref. 17). We sought to investigate changes in the expression of cell cycle regulator proteins in HeLa cells transfected with Tat, and in primary cervical cancers positive or negative for HIV-1, with the ultimate goal of stressing the importance of such regulated genes as diagnostic and/or prognostic indicators in the management of HIV-1 infected cervical cancer cases.

Table 2 **The panel of antibodies used for immunohistochemistry**

Antibody	Clone	Source
Rb2/p130	QKM	BioGenex
Ki-67	SP6	NeoMarkers
Cyclin A	6E6	Novocastra
Cyclin E	CYE 05	NeoMarkers
Cyclin D1	SP4	NeoMarkers
p16	16P07	LabVision
p21	EA10	BioGenex
p27	DCS-72.F6	NeoMarkers

MATERIALS AND METHODS

Study cases. Thirty-six 10% formalin-fixed paraffin embedded tissue blocks of both precancerous cervical lesions and invasive cervical cancers were retrieved from the archives of the Department of Pathology, University of Siena, Siena, Italy and the Department of Pathology, Nairobi Hospital, Nairobi, Kenya, respectively (Table 1). Sections from these samples were first stained with *haematoxylin* and *eosin* to confirm the diagnoses and establish the tumor grades according to the WHO guidelines. HIV-1 status was initially tested by enzyme-linked immunosorbent assay on sera of the patients, following counseling of the patients involved and their informed consent, and was later confirmed by PCR testing on the retrieved tissue blocks.

DNA extraction. DNA was extracted from primary cervical carcinoma tissue blocks using digestion buffer, 50 mM Tris-pH 8.5, 1 mM EDTA, pH 8.0 and 0.5% Tween 20 (Sigma) and proteinase-K (Roche) at a final concentration of 500 μg/ml. The lysates were purified using the phenol-chloroform/isoamyl alcohol protocol, and DNA precipitated with one-tenth the volume of 3M sodium acetate (pH 5.2) and isopropanol. Three hundred nanograms of DNA were used in a multiplex reaction to verify DNA integrity and purity.

PCR testing for HIV-1. For HIV-1 testing, a nested PCR was done with outer (Unipol 1/Unipol 2) and inner (Unipol 3/Unipol 4) primers (see Addendum). For each sample, strictly 500 ng of DNA-containing volumes were used as templates in the first PCR reaction with (U1/U2) primers. The products of the first PCR reaction were diluted to 1:1000, from which 2 μl were used for the subsequent PCR reaction with U3/U4 primers. PCR reactions were performed in a final volume of 50 μl, containing 25 μl (for U1/U2 target) or 25 μl (for U3/U4 target) of AmpliTaq Gold PCR Master Mix (Applied Biosystems, NJ, USA), 45pmol of primers (MWG), and either DNA or water for sample and negative controls respectively. The cycling conditions involved 10 minutes activation step for Taq DNA polymerase, followed by 34 cycles each for 15 secondes at 95°C, 25 secondes at 40°C and 15 seconds at 72°C for U1/U2, or 9 cycles each for 15 seconds at 95°C, 25 seconds at 40°C, and 20 seconds at 72°C, followed by 19 cycles each for 15 seconds at 95°C, 25 seconds at 50°C, and 20 seconds at 72°C for U3/U4. The last cycles were followed by a final extension step of 10 minutes at 72°C. The PCR products were analyzed by electrophoresis on 2% agarose gel stained with ethidium bromide.

PCR testing for HPV and genotyping. To increase the sensitivity of HPV detection, nested PCR was performed using MY09/MY11 as outer and $GP5^+/GP6^+$ as inner primers (addendum). Starting with 500 ng of purified DNA lysates for the first PCR reaction, two microliters of the resulting PCR products were used as templates for the nested PCR amplification. PCR reaction was performed in a final volume of 50 μl. Each PCR mixture contained 25 μl of AmpliTaq Gold PCR Master Mix (Applied Biosystems, NJ USA), 50 pmol of primers, 2 μl of either DNA. The cycling conditions were as follows: a 10 minutes activation step for Taq DNA polymerase, followed by 40 cycles each for 50 seconds at 94°C, 50 seconds at 55°C for MY09/MY11 or 42°C for $GP5^+/GP6^+$ and 50 seconds at 72°C. The last cycle was followed by a final extension step of 10 minutes at 72°C. The PCR products were analyzed by electrophoresis on 2% agarose gel stained with ethidium bromide.

For genotyping, restriction enzyme digestions using RSA I and Dde I were performed with 10 μl of PCR products in each reaction, for at least two hours at 37°C. The products were separated by electrophoresis on 2% agarose gel. The results were also confirmed by DNA sequencing with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Weiterstadt, Germany), with the Big Dye Terminator V1.1 cycle sequencing kit (Applied Biosystems, NJ, USA) using forward ($GP5^+$) and reverse ($GP6^+$) primers respectively. Sequences were compared with the GenBank database as described by Levi et al.[18]

Immunohistochemistry. IHC was performed as described previously by Russo et al.[19] For the primary tumors, the test was done on 4 μm sections mounted on positively charged slides. In the case of Ki-67, the antigen retrieval was done using protease treatment. All the slides were incubated

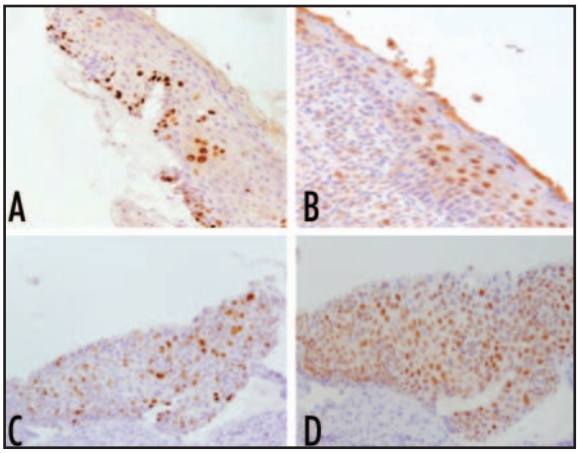

Figure 1. pRb2/p130 and Ki-67/Mib-1 expression in LGSIL and HGSIL. In LGSILs the basal layer of epithelium showed positive staining for proliferating markers as Ki-67 (A) but negative staining for cell cycle inhibitor proteins such as pRb2/p130 (B). In HGSIL, both superficial and basal layers demonstrated positive staining for both markers (C and D).

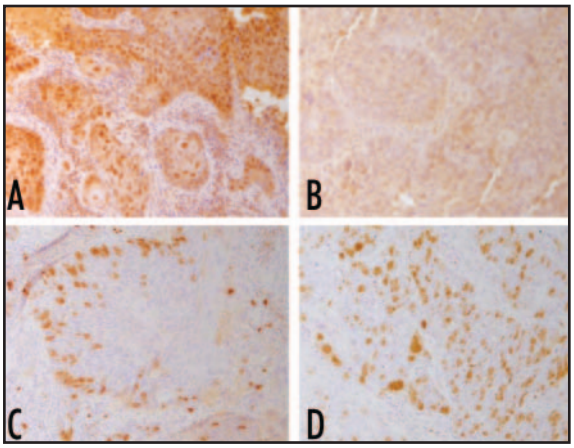

Figure 2. pRb2/p130 and Cyclin A expression in HIV-1 negative and HIV-1 positive invasive cervical cancers. The expression of pRb2 was higher in HIV-1 negative (A) as compared to HIV-1 positive cases (B). Conversely, Cyclin A expression was low in HIV-1 negative (C) compared to HIV-1 positive cases (D).

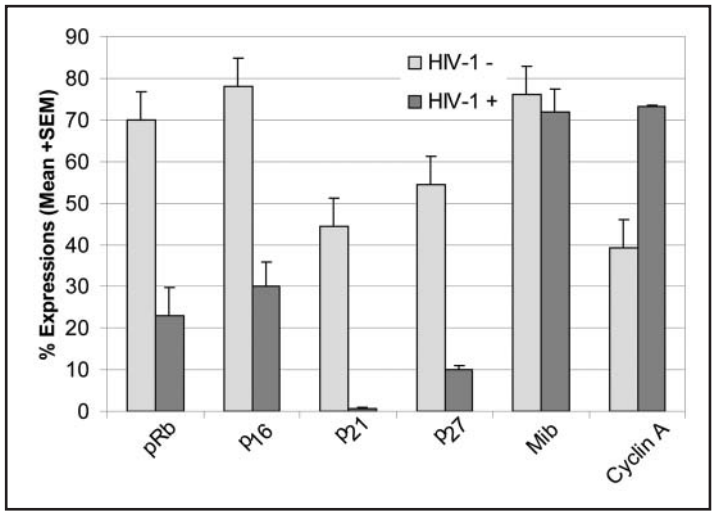

Figure 3. Mean percentage expressions of cell cycle regulator and proliferating markers in HIV-1 negative and HIV-1 positive invasive cervical cancers. The reduced percentage expressions of cell cycle regulator proteins were evident for pRb2/p130, p16, p21 and p27 in series, with increased Cyclin A in HIV-1 positive cases. The percentage expression of Mib-1, though >50%, was not statistically significant between the HIV-1 negative and HIV-1 positive categories.

with the respective primary antibodies for 1 hour at room temperature, rinsed with TBST and then incubated with secondary antibody (ABC kit-DAKO Cytomation) for another 30 minutes. The sections were rinsed with TBST and streptavidin peroxidase was applied, followed by incubation for another 30 minutes. The sections were rinsed and then stained with DAB for 7 minutes. The sections were then rinsed again, counter-stained with Meyers' haematoxylin and mounted. The test was done for Rb2/p130, Ki-67, cyclin A, cyclin D, cyclin E, p16, p21, and p27, with the antibodies shown in Table 2. All the dilutions were made at 1:50. The stains were independently assessed by two experienced pathologists (LL, RS) as mean percentages of positive cells expressing each parameter. In each case, complete cell counts were evaluated in five different fields at 40X and positive cells were expressed as mean percentages of the total cells in the fields. The inter-observer correlation coefficient was more than 90%.

Cell culture and transfections. HeLa cells, a cervical carcinoma cell line, were grown in D-MEM supplemented with 10% FBS, 2 mM L-glutamine and antibiotics at concentrations of 1%. The cells were transfected with pcDNA3 alone or pcDNA3-Tat using calcium phosphate (Invitrogen, Milan, Italy) and harvested 48 hours after transfection. Tat expression in transfected cells was confirmed by Real-time PCR using the primers shown in the addendum. After this log phase growth, HeLa cells were also smeared on positively charged slides, fixed in cold acetone for one hour and then stained.

RNA extraction and real-time PCR. RNA was extracted from HeLa cells transfected with either pcDNA3-Tat or pcDNA3 alone using TRIzol (Invitrogen, Italy). DNase (Promega, Milan, Italy) treated RNA (500 ng) was retro-transcribed using M-MLV (Ambion, Austin, TX, USA) and random hexamers according to the manufacturer's instructions. Equal volumes of cDNA for each sample were then subjected to Real-time PCR using DNA engine Opticon 2 (MJ research, CA) and SYBR GREEN I (Finnzyme, Finland).

Primers used for real-time (see Addendum) were designed between two exons of the gene of interest using Autoprime software, available at www.autoprime.de. HPRT was used as a reference gene and relative quantification was performed using the comparative $\Delta\Delta C_T$ method.

Luciferase assay. For the luciferase assay, 2 μg of each plasmid was used for cell transfection. Cells were harvested 48 hours after transfection and subjected to luciferase assay. Reactions were normalized by using renilla luciferase activity. The luciferase assay was performed according to the manufacturer's instruction (Promega, Milan, Italy). The artificial E2F promoter containing three consecutive E2F consensus binding sites linked to a luciferase reporter gene has been described previously by Lukas et al.[20]

Statistical analysis. For the invasive primary tumors, correlations of IHC expression were analyzed using the SYSTAT programme. The Kruskal-Wallis one-way analysis was performed to test significant association between expression of the cell cycle regulator proteins and HIV-1 status. Conversely, inter-parametric correlation was tested by the Pearson correlation matrix, holding HIV-1 status and histological classifications as distinct and separate constants. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Histology, tumor grade, HPV and HIV-1 status. On histological confirmation, ten precancerous lesions were classified as LGSIL, and another ten as HGSIL. The age ranged from 18–53 years. The LGSIL were associated with HPV types 6, 11, 42 and 16, while the HGSIL were found to be associated only with HPV types 16 and 18.

The remaining 16 cases were invasive cervical squamous cell carcinoma of varied grades, as depicted in Table 1. Among the invasive cervical carcinomas,

the age range was 25–60 years and six out of the 16 cases were associated with HIV-1 infection, both on serological and PCR analysis. All the invasive cervical carcinoma cases were of high-grade tumors and tested positive for high risk HPVs. Of these, ten cases were associated with HPV 18 and six cases with HPV 16.

Significant statistical association between HIV-1 status and HPV genotypes could not be established in our study, due to the small number of HIV-1 positive cases obtained.

Expression of cell cycle regulatory proteins in primary tumors. We investigated the expression levels of both proliferation and CKI markers by IHC in precancerous and in invasive cervical cancers that were either HIV-1 negative or HIV-1 positive.

Testing precancerous lesions by immunohistochemistry, we found a consistent pattern in the cell cycle regulator protein expression. In low grade precancerous lesions, we found that the basal layer stained positive for proliferation markers such as Ki-67 and cyclin A and negative for p16, p21, p27, pRb2/p130; while the superficial layer was composed of cells demonstrating inverse immunophenotypes for these markers (Fig. 1A and B). In high grade lesions, however, this pattern was different, as both basal and superficial layers showed elevated levels of positive cells staining for Ki-67, cyclin A and pRb2/p130 (Fig. 1C and D). In addition, the expression of p16 was quite diffusely positive in high-grade precancerous lesions (on average >50% of the cells were positive), while the expression of p21 and p27 in these tumors was only regionally positive (5–50% of the cells were positive).

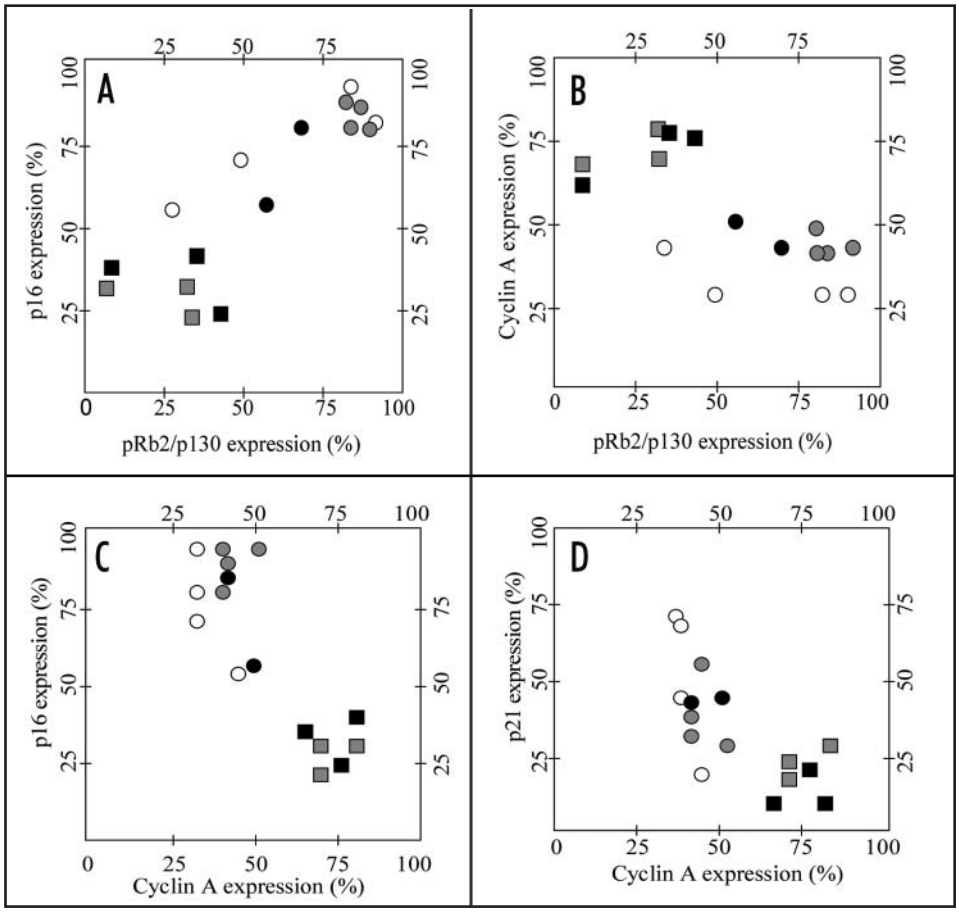

Figure 4. Correlation of the percentage expressions of the cell cycle regulator and proliferating markers in HIV-1 negative (circles) and HIV-1 positive cancers (squares) with varied tumor grades (grade I, /grade II and /grade III). Direct correlation was demonstrated only between pRb2/p130 and p16 (A), while inverse correlations were demonstrated between pRb2 and Cyclin A (B), p16 and Cyclin A (C) and p21 and Cyclin A (D).

HIV-1 positive cases as compared to the HIV-1 negative cases showed comparable decreased expression of pRb2/p130 ($p = 0.02$), p16 ($p = 0.001$) and p21 ($p = 0.004$), and increased expression of cyclin A ($p = 0.001$); (Fig. 2A–D). Using Kruskal-Wallis one-way analysis, the difference in Mib-1 expression was not statistically significant between the two categories ($p = 0.40$); (Fig. 3). Cumulatively, in both HIV-1 negative and HIV-1 positive cases, direct correlation was obtained between expression of p16 and pRb2/p130 ($r = 0.87$, $p < 0.001$) and inverse correlations between Cyclin A and pRb2/p130 ($r = -0.75$, $p = 0.011$), p16 and Cyclin A ($r = -0.88$, $p < 0.001$) and between p21 and Cyclin A ($r = -0.77$, $p = 0.007$), using the Pearson correlation matrix (Figs. 4A–D).

With the exception of the elevated expression of Cyclin A in high grade tumors ($p = 0.041$), there was no statistically significant difference in the expression of other parameters studied and different tumor grades in HIV-1 positive and HIV-1 negative tumors.

Tat increases mRNA levels of proliferation markers and decreases levels of cell cycle inhibitors. Hypothesizing that the above mentioned effects on the expression of cell cycle markers could be mediated by Tat, we analyzed both Tat- and empty vector-transfected HeLa cells for differences in expression of proliferation markers (Fig. 5) by IHC. The results of the IHC were confirmed by Real-time PCR, where decreases in mRNA levels of p16 and p27 were seen, accompanied by a less evident twofold decrease in p21 levels. For the cyclins, we observed a 4.5 fold increase in cyclin A and a weak decrease in cyclin D and cyclin E levels. The level of pRb2/p130 mRNA transcript in HeLa-Tat was also downregulated compared to HeLa-pcDNA3 alone (Fig. 6). These results were consistent with the above-described findings in primary tumors.

The increase in proliferation is independent of E2F factors. The expression of genes required for progression through the cell cycle is highly modulated through a regulatory axis containing the E2F transcription factor and pRb families. Expecting an increase in cell proliferation based on the increased expression and release of the E2F family by Tat, we measured the activity of a promoter responsive to E2F in the presence or in absence of Tat. Interestingly, our results demonstrated a strong reduction of the promoter activity in Tat-transfected cells, which was dose dependent (Fig. 7). We obtained the same results with soluble Tat (data not shown).

DISCUSSION

As has been noted by Motoyama et al.[21] and Wu et al.,[22] cervical cancer is the second most common malignancy among women worldwide. Since 1993, cervical cancer has been considered an AIDS defining malignancy, due to the high recurrence rate of invasive cervical cancer and the more aggressive phenotype manifested by HIV-1 positive women, as demonstrated by Hawes et al.[23] While it is generally assumed that HIV-1 plays an oncogenic role in cancer development through interference with immune functions, recent insights into molecular pathogenesis have shown that HIV-1 proteins can directly promote cancer growth by interfering with cellular functions (according to Vanegas et al.[24]). For instance, Tat has been reported by Albini et al[25] as promoting angiogenesis through interaction with VEGFR2, stimulating the growth of spindle cells in KS.

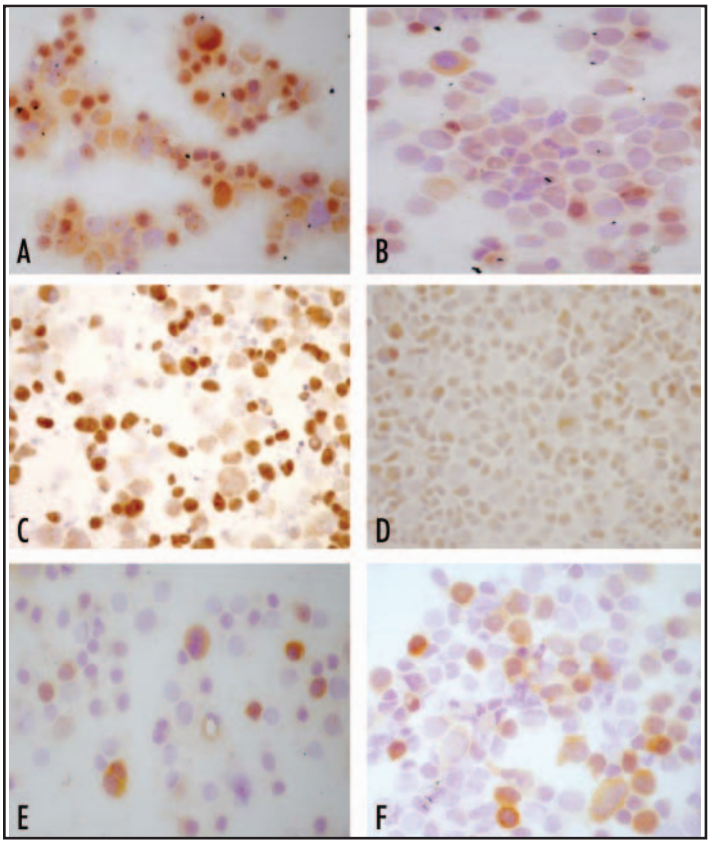

Figure 5. pRb2/p130, p27 and Cyclin A expressions in HeLa and HeLa-pcDNA3-Tat cells. The expression of pRb2/p130 observed in HeLa cells transfected with the vector alone (A) was reduced in comparison to HeLa cells transfected with Tat (B). A similar observation was made regarding the expression of p27 in HeLa cells (C) compared to HeLa-pcDNA3-Tat (D). The expression of Cyclin A was low in HeLa cells (E) but increased in HeLa-Tat (F).

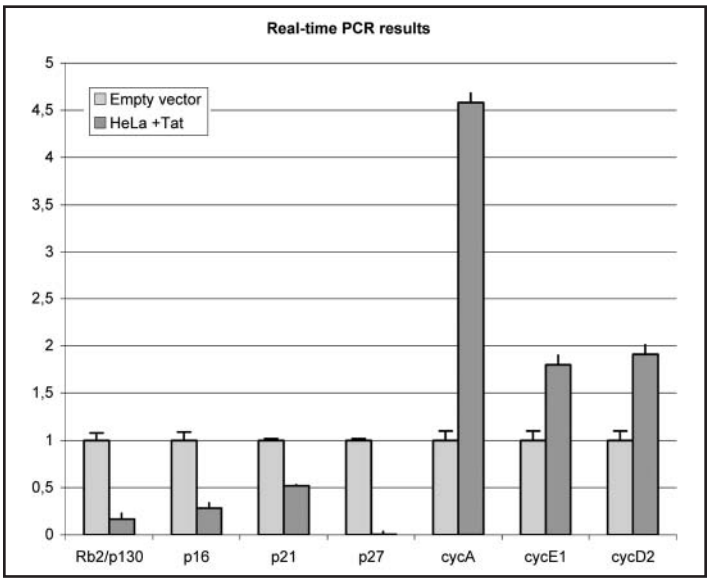

Figure 6. Expression of mRNA of the cell cycle regulator and proliferation markers in HeLa and HeLa-pcDNA-3-Tat. The figure shows relative changes between HeLa and HeLa-pcDNA3-Tat cells in gene expression of cell cycle regulators and proliferating markers, which are consistent with descriptions for the primary invasive cervical cancers (Fig. 3).

We have previously demonstrated (Bellan et al.[26]) an interaction between HIV-1 Tat protein and pRb2/p130, which may be a cofactor in AIDS related malignancies, such as lymphomas or KS.

In HIV-1 negative cervical carcinomas, the mechanism of malignant transformation is mainly due to the integration of HPV DNA into the genome of the host cells, with consequent disruption of E2 negative control on E6/E7 viral oncogenes. The synergistic effects of E6-p53 interaction and E7-Rb complex formation lead to functional inactivation, deregulated cell cycle control, and (according to Wang et al.[27]) is solely believed to be critical of HPV-induced cervical carcinogenesis. The high levels of pRb2/p130 expression as observed in our study cases of high grade precancerous lesions and HIV-1 negative primary tumors therefore suggests that pRb2/p130 may be inactivated, probably through phosphorylation or interaction with E7 protein. This was also in line with the findings of high expression of p16 in high-grade precancerous lesions and HIV-1 negative tumors. Rb represses the transcription of p16 gene, for which an inverse expression pattern exists in high-risk HPV infection, due to the E7 inactivation of Rb and its family members such as pRb2/p130. In addition, CDK inhibitors implicated in the control of G_0/G_1 transition, such as p21 and p27, are both bound and inactivated by HPV E7 proteins, as has been noted by Polyack et al28 and Snyder et al.[29]

With the intervention of HIV-1, Rb2/p130 mRNA may be shut down, with a consequent significant decrease in protein expression, demonstrating that Tat introduces a further block on the pRb2/p130 pathway at the transcriptional level. An upregulation of HPV E6 and E7 gene expressions by HIV-1 Tat has also been reported by Strickler et al[30] and La Ruche et al.[31] The end-point in cervical carcinogenesis and disease progression of this Tat-induced oncogene upregulation is, however, currently ill-defined. Whether or not HIV-1 Tat protein changes the architecture of pRb2/p130-E7 interaction is currently unknown.

We found a significant downregulation of p16 in HIV-1 positive compared to the HIV-1 negative cases, where overexpression of p16 has been reported. Whether this was due to the effect of Tat, rather than a negative feedback control mechanism secondary to pRb2/p130 inactivation, needs further investigation. p21 and p27 expression were also reduced in HIV-1 positive cases.

The results of studies on the effects of Tat on the cell cycle are still conflicting, probably because Tat acts in a dose-dependent manner and its effects are strictly cell type-dependent. For example, Regulier et al.[32] and Luker and Leib[33] demonstrated that Tat exerts a negative effect on cell growth in neural cells in CNS, while Bettaccini et al.[34] has noted mitogenic effects on mammary and amniotic epithelial cells. We found out that HIV-1 Tat protein was able to speed up proliferation of HeLa cells through increase in cyclin A expression and downregulation of CKIs. It is well known that Cyclin A is implicated in the control of S phase and progression into mitosis (according to Girard et al,[35] Pagano et al,[36] Minshull et al.[37] and Lehner and O'Farrel[38]), and that CKI play central roles in blocking cell cycle progression (review in ref. 39). Consistently, a lack of statistically significant association between the expression of Mib-1 and HIV-1 positivity was supported by the more rapid cell cycle transition of HeLa cells transfected with HIV-1 Tat protein, as demonstrated by cell counts and in accordance with a previous report by Conaldi et al.[40]

We expected activation of E2F responsive promoter, due to the important role played by E2Fs during cell cycle progression (reviewed in ref. 41), but when we tested the activity of E2F promoter

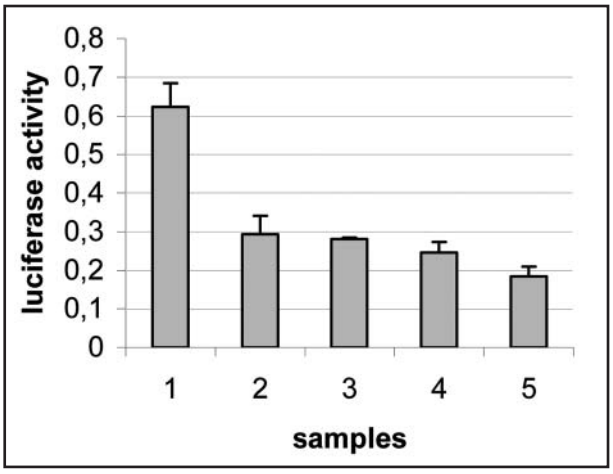

Figure 7. Luciferase assay: lane 1: HeLa-pcDNA3; lane 2: cells transfected with 250ng of pcDNA3-Tat; lane 3: cells transfected with 500ng of pcDNA3-Tat; lane 4: cells transfected with 2\mu\g of pcDNA3-Tat; lane 3: cells transfected with 4\mu\g of pcDNA3-Tat. Firefly luciferase activity was normalized on Renilla luciferase activity and error was calculated using standard deviation between duplicates.

using a luciferase reporter, we found inactivation. We justify this inactivation by the fact that Tat is able to bind to E2F4 in the region corresponding to amino acids 1-84, which is conserved between E2Fs and is responsible for heterodimerization. This Tat-E2Fs interaction may lead to subsequent sequestering of E2F and consequent promoter inactivation (according to Ambrosino et al.[42]). We therefore believe that the speeding up of proliferation in this case was not dependent on E2Fs release but could be dependent on Sp family members, whose transcription factors comprise proteins that bind to promoter sequences rich in GC, namely GC-boxes (reviewed in ref. 43). It has already been reported (by Bartusel et al.[44] and Muller et al.[45]) that Sp1 responsive elements are present in promoter sequences of cyclin A1 and cyclin D1, and that Tat increases the affinity of Sp1 for GC-boxes (according to Seve et al.[46]).

Thus we conclude that HIV-1 Tat plays an important oncogenic role during HPV carcinogenesis by favoring cell proliferation, as has been demonstrated by the increase in cyclin A levels, and that this action cannot be exerted through E2Fs, but possibly by Sp1 transcription factor. Finally, because of the diverse mechanism of oncogenic role of HIV-1 in cervical carcinomas, knowledge of this mechanism and the consequent downstream changes in the expression of cell cycle markers may help in therapeutic design in settings with a high prevalence of HIV-1 infections.

Addendum

Primer sets used for PCR reactions.

Cyclin A REV: 5' AGG CTT CAA AGT ACC TGT GTG 3', Cyclin A FW: 5' TTG ATC CAA CGT GCA GAA 3'; Cyclin D REV: 5' AGG AGC TGC TGC AAA TGG 3', Cyclin D FW: 5' GCG GAT GAT CTG TTT GTT CTC 3'; Cyclin E REV: 5' AGA TTG CAG AGC TGT TGG 3'; Cyclin E FW: 5' GAA ATG ATA CAA GGC CGA AG 3'; p21 REV: 5' TCA CTG TCT TGT ACC CTT GTG 3', p21 FW: 5'TGG TAG AAA TCT GTC ATG CTG 3'; p27 REV: 5' GCA ACC GAC GAT TCT TCT AC 3', p27 FW: 5' AAT TCG AGC TGT TTA CGT TTG 3'; p16 REV: 5' GGA AGG TCC CTC AGA CAT C 3'; p16 FW: 5' GCA GTT GTG GCC GTG TAG 3'; Rb2/p130 REV: 5' TGC TCC TCC CAC ACC TAC 3', Rb2/p130 FW: 5' GCA TCC ATA TTT GCC TGT 3'; Tat REV: 5' GGA GGT GGG TTG CTT TGA TA 3', Tat FW: 5' GGA AGC ATC CAG GAA GTC AG 3', HPRT REV: 5' AGC CAG ACT TTG TTG GAT TTG 3', HPRT FW: 5' TTT ACT GGC GAT GTC AAT AGG 3'; Unipol 1: 5' AGT GGA TTC ATA GAA GCA GAA GT 3', Unipol 2: 5' CCC CTA TTC CTC CCC TTC TTT TAA AA 3'; Unipol 3: 5' GAA ACA GGA MRR GAG ACA GC 3', Unipol 4: 5' TTA ATD GMT TCC ACT ACT CCT TG 3'; MY09: 5' CGT CCM ARR GGA WAC TGA TC 3', MY11: 5' GCM CAG GGW CAT AAY AAT GG 3' GP5+: 5' TTT GTT ACT GTG GTA GAT ACT AC 3', GP6+: 5' AA AAA TAA ACT GTA AAT CAT ATT C 3'.

Acknowledgements

We thank Maurizio Orlandini (Dept. Mol. Biol., University of Siena) for the kind donation of Plasmid containing renilla luciferase and HeLa cells. This work was supported by the Italian Ministry for Education, Universities and Research (MIUR), Rome- Italy, the University of Siena, Siena-Italy and Sbarro Institute of Medical Research, Philadelphia, USA.

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