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Early Detection and Molecular Targeting of Human Papillomavirus for Control of Cervical Cancer

Bhudev C. Das
Division of Molecular Oncology, Institute of Cytology & Preventive Oncology,
Indian Council of Medical Research (ICMR)
1-7, Sector-39, NOIDA

Abstract

Infection with specific types of high risk human Papillomaviruses (HR-HPVs), particularly the HPV type 16 and HPV 18 are essential for the development of cervical cancer. Since it takes 10-15 years to develop invasive cancer, early detection of HPV and treatment of precancerous lesions can reduce the incidence of cervical cancer significantly. Several new technologies have been developed for HPV screening tests. Early detection of HPV infection provides an opportunity to interfere with the viral persistence, propagation and progression of lesions. Several studies revealed that HPV uses several host cellular protein factors for expression of two viral oncogenes, E6 and E7. Targeting these key host cellular regulatory factors is important to silence the expression of oncogenic HPVs. We have therefore screened a few potent anticancer herbal derivatives such as curcumin, *Emblica Officinalis* and B*ryophyllum Pinnata* which are known to have no or negligible toxic/ adverse effects on human systems in order to discover an effective anti-HPV molecule. All the three herbal products are found to have strong suppression

Correspondence: Bhudev C. Das, Division of Molecular Oncology, Institute of Cytology & Preventive Oncology (ICMR), I-7, Sector-39, NOIDA. Email: bcdas48@hotmail.com. Pran Nath Chhuttani Oration, 2006-2007. Delivered at the Annual Conference of the National Academy of Medical Sciences (NAMSCON, Amritsar), 2007.

activity on HPV oncogene expression. Thus early detection of HPV and its targeting by nontoxic herbal drugs can be an effective approach for controlling and reducing cervical cancer.

Key words: Cervical cancer, Human Papillomavirus (HPV), VIA, VILI, Multiplex PCR, Curcumin, Transcription factor.

Introduction

Cervical cancer is the most common cancer amongst women in developing countries including India. The worldwide annual incidence rate of cervical cancer was estimated to be 493,000 and mortality of 274,000 per year and more than two thirds of them are from developing countries (1). The areas of the highest incidence are sub-Saharan Africa, Latin America, the Caribbean, south central Asia, and Southeast Asia (1). In India more than 130,000 cases, comprising one fourth of total world annual incidence, are diagnosed and about 74,000 deaths occur every year. It constitutes 24% of the total cancer incidence; much ahead of breast cancer in India.

It is now well established that the specific types of oncogenic human papillomaviruses (HPVs) are the major etiological agents associated with the development of cervical cancer (2). In addition, various cofactors such as early marriage, high parity, promiscuity, smoking, and other reproductive tract infections may modify the risk of developing cancer in women following infection with HPV. Of more than 100 HPV types known, HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 71 and 82 have been found to be associated with invasive cervical cancer and hence classified as high risk types (3).

The major reason for high mortality is due to the cervical cancer insidious latent period of 10-15 years when clinical symptoms are not clear for the diagnosis. In the past two and half decades research has produced enormous amount of evidence that indicates nearly all cervical cancer cases are caused by certain specific types of human Papillomavirus (HPV), making it perhaps the first cancer to be recognized as virally induced. This viral association with disease progression provides a unique opportunity to detect HPV infection and associated cellular changes, low or high grade lesions at an early stage of the disease. With the development of simple, reliable and cost effective

detection procedure of HPV and with early and regular screening programs for disease precursors, most of the cervical cancer can be prevented and/ or successfully treated.

Organized screening for cervical pre-cancer and cancer lesions become the most effective way of prevention and treatment of this disease as is evidenced by the experience in developed countries like USA, where screening of women through Pap-Smear had vast impact on reducing (~70%) the incidence and mortality caused by this disease (4). In developing countries like India where cervical cancer is the most serious public health problem among women, such screening programs are not implemented at national level because of large population, high cost, complex procedure and lack of trained manpower and of expertise.

Early detection of high-risk types of HPV is of great importance for identification of clinically latent papillomavirus infections which are highly prevalent in the general population. Reliable diagnosis of HPV would facilitate early identification of high risk population. The techniques available at present for the early detection of cervical pre-cancer and cancerous lesions can be broadly classified in two categories:

1) Conventional Cytological Diagnostics Approaches
2) Molecular Diagnostic Approaches

Conventional Cytological Diagnostics Approaches:

Currently the cervical cancer screening programs are based on conventional cytological and histopathological examinations.

Although *cytology based Pap test* (Papanicolaou-stained smear method) is the universal cervical cancer screening method and has been shown to be effective in reducing cervical cancer incidence in US and other developed countries, in India screening is highly inadequate or non-existing in major part of the country. Recent improvements in liquid based cytology have made the testing more reliable but the tests are still prone to error and not being practiced commonly. *Cervicography* is another technique which uses a photograph of cervical lesion (cervigram), which is then highly magnified and examined. Though this technique is painless, easy to use and provides documentation of area, but it is not sensitive for HPV detection and is limited to only abnormal cellular changes. Another important technique for detection of cervical abnormalities is *colposcopy* which magnifies the cervix

opportunity to interfere with the viral persistence, propagation and progression of lesions. The most common treatment available is the removal of tumorous part by surgery, excision by laser technique and cryosurgery, followed by chemo or radiotherapy. But all these measures are effective against the diseased part of the tissue, not against the HPV that causes the disease. Since the only way to prevent and cure cervical cancer is through effective intervention of viral life cycle within the host cell, different strategies are being developed employed worldwide to restrict and remove HPV infection from the host cell. Molecular studies suggest that HPV uses several host cells signaling pathways for their replication and oncogenes expression.

Host Cell Transcription Factor: The Molecular Target

The tumorigenicity of HPV is dependent upon the aberrant expression of its two early genes E6 and E7(37), the products of which interact and silence the two principal tumor suppressor genes p53 and Rb respectively and interfere with the host cell cycle control, which leads to uncontrolled cell cycle proliferation and neoplasm. The expression of HPV E6 and E7 genes is mainly depended on the availability of host cell transcription factor, Activator Protein-1(AP-1). AP-1 is a very crucial factor for the life cycle of HPV as is evidenced by the several studies where site directed mutagenesis of the AP-1 binding sites on the upstream regulatory region of the HPV genome completely abolishes transcription of URR-driven reporter constructs, either under transient transfection conditions or in stable differentiation-dependent infection assays in organotypic raft cultures (38, 39). Numerous studies have shown that AP-1 is indispensable for HPV transcription regulation and oncogene expression.

What is Activator Protein-1 (AP-1)?

AP-1 is a one transcriptional factor, which is involved in cellular growth, proliferation, differentiation and neoplastic transformation. It is formed by either homodimerization of two jun proteins(c-Jun, JunB, JunD) or heterodimerization of jun and fos proteins(c-Fos, FosB, Fra-1, fra-2) through the "leucine zipper" (40). The upstream regulatory region (URR) of HPV 16 genome contains three crucial binding sites for the AP-1, whereas the HPV 18 URR contains only two binding sites (Fig. 2). Since DNA binding is a necessary prerequisite of transactivation, the expression of different proteins of the jun and fos family is crucial for the activation of

downstream genes regulated by AP-1. Earlier we demonstrated a higher binding activity of AP-1 in both HPV positive and HPV negative cervical cancer cell lines namely HeLa and C33a, respectively, as well as in HPV16 transformed human keratinocytes HPK1a cells (41).

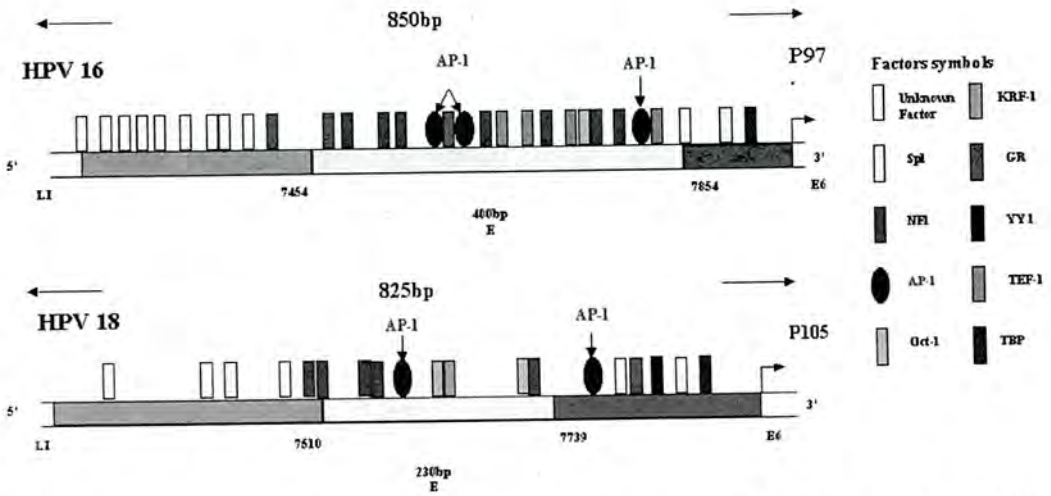

Figure 2: Schematic diagram of upstream regulatory region (URR) of HPV 18 and HPV 16. Transcription factors binding sites in URR of HPV.

In a previous study, we had demonstrated that the transactivation and DNA binding affinity of AP-1 can be modulated by alterations of the intracellular redox status if the cells are treated with a potent antioxidative agent, pyrrolidine dithiocarbamate (PDTC) (41), which blocked not only the transcription of endogenous cellular NFkB–responsive genes such as the monocyte chemoattractant protein 1 (MCP-1) gene, (42) but also selectively suppressed HPV16 expression. Although PDTC induced elevated binding of the AP-1 transcription factor to its cognate recognition sites within the viral regulatory region, it was revealed that the blockage of HPV transcription was mainly due to antioxidant-induced reconstitution of the AP-1 transcription complex (41).

Since the therapeutic utility of synthetic antioxidants such as PDTC is limited due to their high toxicity and other side effects and since there is no

chemotherapeutics available for the treatment of HPV, we have investigated the role of a potential antioxidative agent Curcumin (diferuloylmethane), an active component of the perennial herb turmeric in transcription regulation of HPV. The cell line used for the experiment is HPV18-positive cervical carcinoma cells, HeLa. Treatment was done, in different concentrations of curcumin (50 μM, 100 μM and 200μM) for a fixed duration of 1 hr as well as for different time periods (15 min, 30 min, 1 hr, 2 hr, 3 hr, 4 hr, 5 hr, 6 hr, 12 hr and 24 hr).

Curcumin selectively suppresses HPV18 transcription in cervical cancer cells, HeLa

The results revealed a time and concentration-dependent decrease in HPV18 expression (Fig. 3). A nontoxic dose of 100μM curcumin was finalized for further experiments as verified by MTT 3- (4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetra-zolium bromide assay, which showed no cell death, indicating nontoxicity of the dose. A slight decline in HPV18-specific mRNA expression was observed after incubation of HeLa

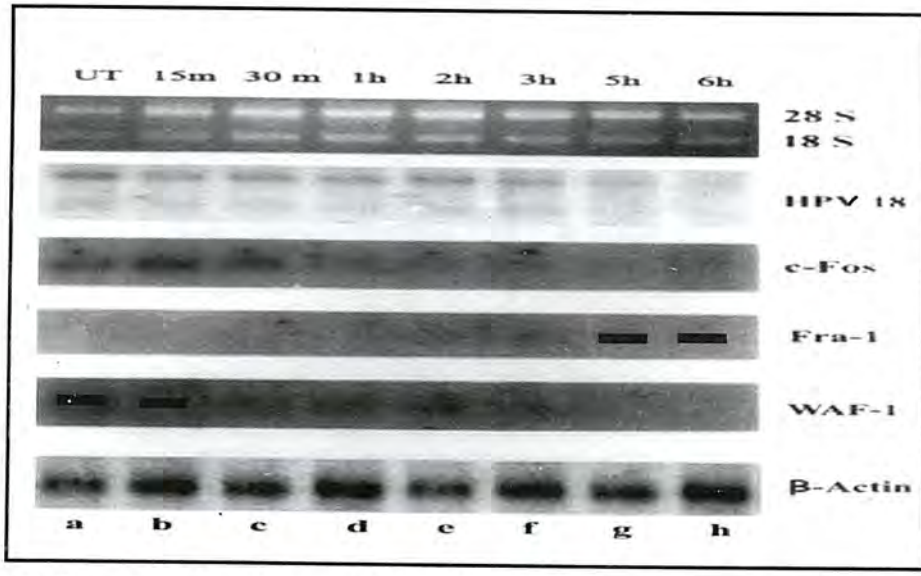

Figure 3: Time-dependent down regulation of HPV 18 transcription in presence of Curcumin. Northern blot analysis showing selective down regulation of HPV18, c-Fos and WAF-1 mRNA expression and up-regulation of Fra-1 mRNA expression after time dependent treatment of 100μm Curcumin in HeLa cells. Lane a, untreated cells; lane b to h, 15min, 30 min, 1h, 2h, 3h, 5h and 6h of incubation in the presence of 100μm Curcumin respectively. To confirm equal RNA loading, the filters were reincubated with β-actin probes. The positions of the 28S and 18S rRNAs are indicated.

cells with 100 μM curcumin for 2.5 hr, and by 4–5 hr the HPV18 expression was completely abolished (Fig. 3). This abolition of HPV transcription was found to be selective and virus-specific and not the consequence of a general transcriptional blockage, since curcumin has no effect on the expression of the endogenous control gene β-actin (Fig. 3). Since the disappearance of HPV18 mRNA follows roughly the same kinetics as observed for HPV16, with the half life of its mRNA being 2.5 hr (41), curcumin interference seems to act at the level of initiation of transcription. This is also borne out by our findings that the disappearance of viral mRNA follows similar kinetics (approximately 2.5 hr) as observed after treatment of HeLa cells with a nonspecific transcription inhibitor, actinomycin D. The viral mRNA, however, started reappearing by 8–9 hr and regained its normal expression by 12 hr (data not shown) possibly because of complete metabolization of the drug.

Curcumin downregulates the AP-1, but not the SP-1, transcription factor

Incubation of HeLa cells with 100 μM curcumin for different durations indicated time-dependent down-regulation of AP-1 binding activity. The AP-1 binding activity started declining after about 2 hr, by which time HPV18 transcription also showed a decline, and was completely abolished by 4–5 hr (Fig. 4). To investigate the specificity of the effect of curcumin on AP-1, we analyzed its effect on other transcription factors such as SP-1. But we could not find any inhibitory effect of curcumin on the SP-1 transcription factor.

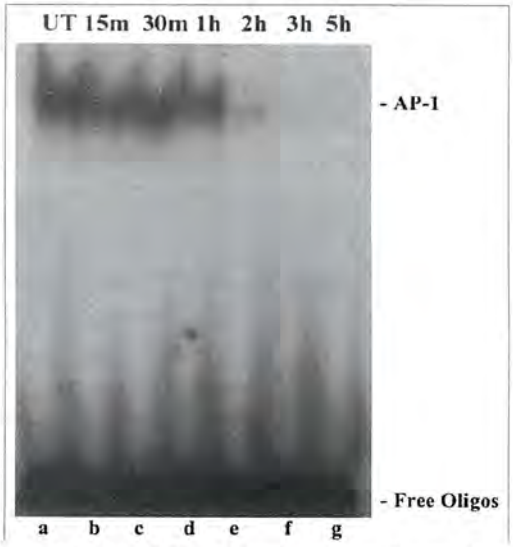

Figure 4: EMSA showing time dependent down-regulation in binding activity in HPV-18 positive HeLa cell extracts following curcumin treatment for different time periods. Lane a, untreated cells; lane b to g 15min, 30min, 1h, 2h, 3h and 5h of incubation in the presence of 100 mM Curcumin respectively. The positions of the specific retarded bands are indicated.

Curcumin downregulates c-fos, but upregulates fra-1 expression

When we analyzed the protein expression pattern of c-fos following

100μM curcumin treatment for different durations (15 min, 30 min, 1 hr, 2 hr, 3 hr and 5 hr), it first showed a decrease in c-fos expression by 2 hr and further decreased gradually in the following hours until it was completely abolished by 4–5 hr. In contrast, fra-1, which was almost nil in untreated HeLa cells, showed its clear appearance by the same time period of 2 hr (when c-fos had started declining) and by 4–5 hr, the fra-1 expression reached its peak (Fig. 5). It is very interesting to note that even though the c-fos mRNA level became almost nil after about 2 hr of curcumin treatment the c-fos protein remains there up to about 3–4 hr. This may be because the pre-existing c-fos protein pool remains for a few hours and is then gradually metabolized, although the transcriptional machinery is blocked by 2 hr of curcumin treatment. Additional experiments were also carried out to detect variation in other AP-1 components after curcumin treatment but no major change in AP-1 composition could be discerned. We also checked the change in composition of the AP-1 binding activity after 100μM curcumin treatment. Since AP-1 binding activity starts decreasing by 2 hr and becomes nil after around 4–5 hr, we could analyze the AP-1 composition only at 1 hr of curcumin treatment. We found that JunB remains the major component, but involvement of c-fos becomes almost nil. A very small amount of Fra1 and Fra2 reappears, but the rest of the components showed no change in their involvement. The results revealed a time dependent down regulation of c-fos expression matching the down regulation of AP-1 activity, but more interestingly, curcumin up-regulated Fra-1 expression to a level generally observed in normal cervical cells. This further strongly strengthens the possible role of Fra-1 in tumor suppression. Thus the inverse correlation of expression dynamics between c-fos and jun B within the AP-1 complex appears to play a crucial role during development and tumorigenic progression of cervical cancer.

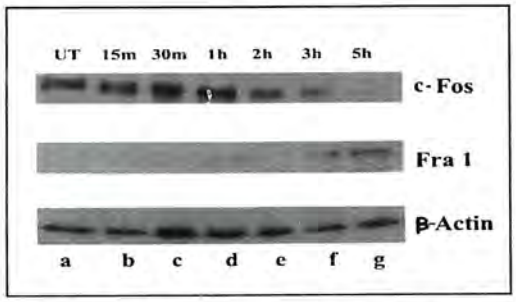

Figure 5: Expression of c-Fos and Fra-1 proteins after time dependent treatment of 100μm curcumin in HeLa cells. Lane a, untreated cells; lane b to g, 15 min, 30 min, 1h, 2h, 3h and 5h of incubation in the presence of 100μm curcumin respectively.

The most significant finding of our study is the selective downregulation of HPV18 transcription in HeLa cells following curcumin treatment. This is

the first time it has been demonstrated that expression of HPV can be specifically suppressed by an antioxidant of herbal origin. It follows almost the same kinetics as revealed by treatment with a transcription inhibitor actinomycin D; therefore it is clear that curcumin acts at the level of initiation of transcription, since downregulation of HPV transcription begins by 2.5 hr, which is also estimated to be approximately the half-life of the HPV18-specific mRNA.

The observation that curcumin-induced suppression of HPV18 expression is paralleled by concomitant abolition of AP-1 binding indicates that AP-1 is indispensable for efficient viral gene expression. This is in sharp contrast to our earlier observation with a synthetic antioxidant, PDTC, which did suppress HPV expression but increased the binding activity of the AP-1 complex. This has been shown to be caused either by changing the composition of AP-1 and/or by posttranslational modifications (41). But curcumin appears to follow a different pathway; it does not depend much on the alteration in AP-1 composition, but it acts at the transcriptional level and downregulates the expression of its components. Although the therapeutic potential of curcumin is implicit, the mechanism(s) of curcumin-induced inhibition of AP-1 binding is not yet clearly understood. It is known that DNA binding of the Jun/Fos complex could be modulated by redox regulation of a single conserved cysteine residue located in the DNA binding domain (43). But it is also possible that curcumin may work in other ways than just as an antioxidant that brings about changes in the redox status of the cell. Whether there exists a curcumin responsive receptor/ element or whether curcumin can activate/inhibit some cellular protein(s) or protein kinases that in turn can interact with Jun/Fos binding, is not known. It is possible that curcumin can act through inhibition of JNK; a c-Jun N-terminal kinase needed for AP-1 activation (44). Another possibility is that curcumin may directly inhibit formation of the jun-fos DNA complex. Similarly, we have investigated anti HPV activity of two other herbal extracts, *Bryophyllum Pinnata* and *Emblica officinalis* on HPV-18 positive cells, HeLa.

Emblica Officinalis suppress HPV18 transcription in HeLa cells.

To analyze the effect of *Emblica Officinalis* on HPV 18 transcription we incubated HPV 18-positive cervical carcinoma cells, HeLa with different concentration of *Emblica Officinalis*

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