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HPV-induced carcinogenesis of the uterine cervix is associated with reduced serum ATRA level

V.M. Berlin Grace [a,b,*], S. Niranjali Devaraj [c], M. Radhakrishnan Pillai [d], Halagowder Devaraj [b]

[a] Department of Biotechnology, KITS, Deemed University, Karunya Nagar, Coimbatore-641 114, India
[b] Unit of BioChemistry, Dept. of Zoology, University of Madras, Guindy Campus, Chennai-25, India
[c] Department of BioChemistry and Molecular Biology, University of Madras, Guindy Campus, Chennai-25, India
[d] Rajiv Gandhi Centre for Biotechnology, Trivandrum, India

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Abstract

Objective. In uterine cervical cancer, certain oncogenic HPV types are considered as key etiologic factor. But the progression of HPV associated cervical precancerous lesions depends on many other factors such as oncogenes, immune system, anti-viral factors etc. This study is therefore focused on the effect of an important dietary anti-viral factor called All Trans Retinoic Acid (ATRA) on the development of HPV associated cervical cancer as it is found higher in poor socioeconomic people.

Method. We analyzed a total population of 130 including control subjects who have no complaints of uterine cervical lesions and the HPV-6/11, 16/18 infected cases of low grade squamous intraepithelial lesions [SIL], high grade squamous intraepithelial lesions [HSIL], and invasive cancers, for serum ATRA level. This study also focused to find out the association of serum ATRA level with the proliferation status in terms of proliferating cell nuclear antigen (PCNA) expression as it is an anti-proliferation agent and with the grades of cervical lesions, using SPSS statistical package.

Results. The results showed a highly significant negative association for serum ATRA level with different stages of cervical lesions ($F = 3.305$; $P = 0.000$) by one-way ANOVA and with intensity of PCNA expression ($r = -0.825$; $P < 0.01$) by Pearson's correlation test. A highly significant association was observed for the PCNA expression with the grades of cervical lesions too ($F = 37.89$; $P = 0.000$). Further, we found from our data that all the invasive cancer cases were infected with HPV-16/18 and none with HPV-6/11. Hence, we analyzed the association of serum ATRA level with HPV-16/18 infected preinvasive cases in developing invasiveness, by Fisher's Exact Test, using Graph Pad Prism as shown in Table 1. The results show an odds ratio (OR) of 36.93 and a relative risk (RR) of 4.99 with an 95% interval being 2.896 to 8.603, which is significant at the level of $P = 0.0001$ for the reduced [<0.6 μg/ml] serum ATRA level in developing invasive cancer in HPV-16/18 infected preinvasive cases.

Conclusion. All these results suggest that the serum ATRA level highly influences the progression of cervical lesions to invasive cancer and can be therefore aimed as a marker for progression in combination with HPV-16/18, which helps to enhance the modalities of therapy towards cost effectiveness.

Keywords: Serum ATRA level; Progression and diagnostic marker; HPV associated cervical lesions; PCNA expression

Introduction

Worldwide, cervical cancer is still the second leading cause of cancer death in women [1], and the incidence of uterine cervical cancer is higher in low socioeconomic people in the developing countries [2]. In India, the incidence is ranging from 15.4 to 46.5 per l00,000 women and is 15% of the world's cervical cancer [3,4]. HPV has been implicated as the primary cause of cervical dysplasia and cervical cancer [5]. Surplus evidences are available worldwide, to point out certain oncogenic HPV types as major causative factor for uterine cervical cancer with different onco-potency [6]. However, evidences are available for the regression of cervical precancerous lesions too [7,8] and clearance of HPV naturally

Abbreviations: HPV, human papilloma virus; ATRA, all trans retinoic acid-active metabolite of vitamin A.

* Corresponding author. Department of Biotechnology, KITS, Deemed University, Karunya Nagar, Coimbatore-641 114, India.
E-mail address: berlsdg@yahoo.com (V.M. Berlin Grace).

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or upon treatment [9]. In this study, we have selected the cases infected either with low-risk HPV-6/11 or high-risk type HPV-16/18 and controls subjects, who have no complaints of cervical lesions and are not infected with either of HPV types 6, 11, 16 and 18.

Epidemiologic studies of patients with SIL and cervical cancer have suggested that the low intake of vitamin A is associated with risk [10,11]. It is also reported that the retinoids, active metabolites of vitamin A, can halt the progression of disease in premalignant lesions of the cervix and are therefore effective in prevention of tumor development [12]. Liu et al. (1995) also reported an association of high plasma vitamin A level with regression of cervical dysplasia, especially in HPV-16-positive women [13]. Nagata et al. (1999) reported from his follow-up study that the rate of progression of cancer in situ or cervical cancer was 4.5 times higher in women with the lower serum retinol levels than in women with higher serum retinol levels, suggesting an association of low serum retinol level with development of cervical cancer [14]. These reports suggest that there is a need for additional factors like ATRA for the HPV infection to develop cervical cancer as it has both anti-viral and anti-proliferation functions through its receptors (RAR and RXR) mediated cell signaling pathways [15,16]. A decrease in the ATRA receptor level has been observed in cervical carcinogenesis [17], which may in turn be associated with the decrease in circulating ATRA level. It is thought that the anti-carcinogenic effects of preformed vitamin A (all-*trans*-retinol) in these cases are mediated by its conversion to retinoic acid. Retinoic acid, via its binding to nuclear receptors, may induce cell differentiation, inhibit proliferation, and/or induce apoptosis [15,16].

The oncogenic HPV-16/18 was found to develop invasive cancer by disrupting the cell cycle and apoptosis by various mechanisms including the dysfunction of p53 and retinoblastoma proteins [18]. Several reports have shown the therapeutic effect of ATRA in uterine cervical lesions including invasive cancer [12], suggesting that the serum ATRA level may have a key role in prevention of oncogenic HPV from developing invasive cancer. Further, it is unclear whether sufficient conversion of all-*trans*-retinol to retinoic acid in vivo occurs to make a significant anti-carcinogenic. Our study is therefore focused to assay the level of ATRA in the serum of cases associated with HPV (6/11, 16/18) and controls to find out their association with the grades of cervical lesions. Identification of such carcinogenic risk, associated with HPV types, is an important step in the process of developing a combination HPV vaccine for the prevention of cervical neoplasia.

ATRA has been shown to possess both the properties of anti-proliferation and differentiation in vitro and in vivo [19,20]. It is also reported that the PCNA could participate in the transcriptional control and DNA repair in a fashion analogous to that of TFIIH, by interacting with RAR [21], which suggest a strong influence of serum ATRA level on cell proliferation which can be indexed by PCNA expression. Hence, attempts were also made in this study to analyze the statistical correlation of the level of serum ATRA with the cell proliferation status indexed by PCNA overexpression too.

Materials and methods

Study subjects

A total of 130 samples including control subjects who underwent total hysterectomy due to complaints other than uterine cervical lesions such as uterine fibroids, endometriosis, etc. and cases of low socioeconomic status with different grades of cervical lesions were included in this study. The biopsy and blood samples of the cases were collected from the OP following the institute's IRB approval of the study protocol, after verifying their abnormal Pap smear report, whereas the blood and cervical epithelium tissue of control subjects were obtained from the surgical ward after verifying their basic screening report including normal Pap smear. All these study samples were kindly provided by the Institute of Obstetrics and Gynecology (IOG) and Research Institute of Madras Medical College, Chennai. Approval for this study was obtained from the Institute's ethical board, and the case record is maintained effectively. Informed consent was obtained from controls as well as cases and completed a standardized, self-administered questionnaire pertaining to basic demographic data, sexual behavior, contraceptive practices, and number of children, age, etc. and a gynecologic examination that included a Pap smear test.

All the paraffin-embedded biopsies were then histopathologically classified based on the Bethesda system of classification (1988) by hematoxylin–eosin staining method upon consultation with chief pathologist, IOG, Chennai, India. Thus, the study subjects along with their age range are grouped as follows:

Group I: 20 Control subjects (40–60 years)
Group II: 20 Low grade squamous intraepithelial lesions (36–50 years) [CIN 1 and mild dysplasia]
Group III: 30 High grade squamous intraepithelial lesions (39–55 years) [CIN 2, CIN 3, carcinoma in situ, moderate and severe dysplasia]
Group IV: 60 Invasive cancer (42–63 years) [FIGO stages I to IV].

DNA isolation from paraffin embedded tissues

About 10–15 sections of 10 μm size were taken using microtome in an ependorff tube and dewaxed by xylene wash. Sections were then rehydrated with 100% ethanol and added 1 ml of cell lysis buffer along with proteinase K after centrifugation. The tubes were incubated at 55° for 2 days, and then the DNA was extracted using phenol:chloroform:isoamyl alcohol (25:24:1) mixture. Extreme care was taken to avoid cross-contamination during sectioning, by cleaning the microtome blade with absolute ethanol in between each block cutting and during DNA extraction, by using disposable ependorff tubes, microtips, etc. for each sample. The DNA was then precipitated from the clear aqueous phase by adding 7.5 M ammonium acetate and 100% ice-cold ethanol. The quality of DNA isolated was checked in 1% agarose gel for PCR to be carried out and was quantified using UV spectrophotometer.

Polymerase chain reaction

PCR was carried out to test the presence of HPV types using type-specific primers for both high-risk types HPV (16/18) and low-risk types HPV (6/11) based on the methods described earlier [22,23]. Briefly, PCR was carried out in a study population of 130 with type-specific primers and PCR amplification kit (supplied by Bangalore GENEI). A reaction mixture of 50 μl was prepared using 1× PCR buffer with 3.5 mM $MgCl_2$, 1.25 U Taq DNA polymerase enzyme, 200 μM of each dNTP (dATP, dGTP, dCTP, and dTTP), 1 μM each of primer (forward and reverse) and 2–3 μg of genomic DNA per 50 μl reaction mixture. The volume was made up to 50 μl with sterile distilled water. Reaction mixture without DNA template was used as negative control and that with known DNA template of known molecular size, supplied along with the kit was used as positive control which yielded the PCR product of expected size. Thirty cycles of PCR were carried out for HPV-16/18 and that of forty for HPV-6/11. PCR products were stored at 4°C for further analysis. The PCR products were analyzed by electrophoresis in 2% agarose gel along with ethidium bromide. A molecular weight marker of 100-bp range (supplied by Bangalore GENEI) was also run simultaneously to verify the molecular size of the PCR products. The