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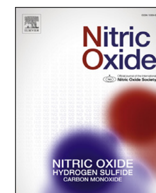

Correlation of plasma nitrite/nitrate levels and inducible nitric oxide gene expression among women with cervical abnormalities and cancer

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A B S T R A C T

Cervical cancer is caused by infection with high risk human papillomavirus (HR-HPV). Inducible nitric oxide synthase (iNOS), a soluble factor involved in chronic inflammation, may modulate cervical cancer risk among HPV infected women. The aim of the study was to measure and correlate plasma nitrite/nitrate levels with tissue specific expression of iNOS mRNA among women with different grades of cervical lesions and cervical cancer. Tissue biopsy and plasma specimens were collected from 120 women with cervical neoplasia or cancer (ASCUS, LSIL, HSIL and invasive cancer) and 35 women without cervical abnormalities. Inducible nitric oxide synthase (iNOS) mRNA from biopsy and plasma nitrite/nitrate levels of the same study subjects were measured. Single nucleotide polymorphism (SNP) analysis was performed on the promoter region and Ser608Leu (rs2297518) in exon 16 of the iNOS gene. Differences in iNOS gene expression and plasma nitrite/nitrate levels were compared across disease stage using linear and logistic regression analysis. Compared to normal controls, women diagnosed with HSIL or invasive cancer had a significantly higher concentration of plasma nitrite/nitrate and a higher median fold-change in iNOS mRNA gene expression. Genotyping of the promoter region showed three different variations: A pentanucleotide repeat (CCTTT) n, −1026T > G (rs2779249) and a novel variant −1153T > A. These variants were associated with increased levels of plasma nitrite/nitrate across all disease stages. The higher expression of iNOS mRNA and plasma nitrite/nitrate among women with pre-cancerous lesions suggests a role for nitric oxide in the natural history of cervical cancer.

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1. Introduction

Cancer of the cervix uteri is the fourth most common cancer diagnosis and the second most leading cause of cancer mortality among women, with an estimated 529,409 new cases and 274,883 deaths in 2008 worldwide with nearly 86% occurring in developing countries [1]. India reports the highest number of cases (134,000) and deaths (73,000) worldwide, representing one-quarter of the cervix cancer burden globally [2]. Infection with high risk types of the human papillomavirus (HR-HPV) is the major etiological factor for cervical cancer [3]. Persistence of HR-HPV is the major risk factor for cervical carcinogenesis and failure of the host immune response to respond adequately to HPV infection is a recognized driver of viral persistence and progression to cancer. Persistent viral infection has been associated with chronic inflammation, an important and recognized cofactor in cancer progression [4,5]. During inflammation a variety of inflammatory cells are activated,

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which trigger oxidant-generating enzymes such as inducible nitric oxide synthase (iNOS) [6].

Nitric oxide (NO) is a soluble factor generated during inflammation in epithelial cells and leukocytes and is controlled by inducible nitric oxide synthase (iNOS). In response to pro-inflammatory cytokine signaling due to infection or other stimuli, short-term nitric oxide production is associated with cytotoxicity, facilitating resolution of an infection. Prolonged production of nitric oxide due to the presence of a chronic inflammatory state and elevated iNOS activity has been shown to potentially facilitate carcinogenesis in both animal and human tumors [7–10]. Over expression of iNOS has also been demonstrated in a variety of human carcinomas [11–19]. Nitric oxide exposure increased DNA double strand breaks in cultured HPV-infected cells with HPV E6/E7-mediated down regulation of p53 and pRb [20,21]. Significant increases in nitric oxide metabolites were also reported in high-risk HPV positive cervical intraepithelial neoplasia (CIN) lesions compared to HPV positive controls [22]. Elevated levels of nitric oxide metabolites in cervical fluid were also associated with HR-HPV persistence in a longitudinal study [23].

While available data strongly suggest that physiologically high doses of NO could promote malignant progression of HPV infected cells, there is limited information on differences in cervical iNOS expression among women with cervical lesions, as well as information on factors modulating iNOS expression. The present study was designed to measure the iNOS expression in different lesions of the cervix and genotype iNOS promoter gene polymorphisms (SNPs and TRNPs), and Exon 16 S608L (rs2297518) polymorphism that were reported elsewhere [24–27] and evaluate their associations HPV associated cervical cancer in study subjects from the state Andhra Pradesh, India.

2. Materials and methods

2.1. Study population

Patients without dysplasia or with pre-cancerous and cancerous lesions attending the gynecology department of the local government maternity hospital, Petlaburz, Hyderabad and Mediciti hospitals, a non-profitable private organization were recruited in the study. Patients with newly diagnosed, histopathologically confirmed, and untreated cervical cancer were recruited through Mehdi Nawaz Jung (MNJ), a government cancer hospital in Hyderabad, Andhra Pradesh, India. Women who were 21 years or older, had an intact uterus, no previous diagnosis of diabetes, hypertension or any kind of cancer, and who were mentally competent to give their consent were selected for the study. Written informed consent was obtained by signature or thumbprint.

After consent, women responded to a brief questionnaire designed to assess demographic information including detailed information on age, economic status (<100 rupees/day vs. >100 rupees/day), and tobacco consumption (smoking (Biri/cigarette/Hukka) or chewing Tobacco (Pan, pan masala or gutka) regularly (at least once a day). A detailed protocol of the entire project describing the sample collection and storage including methodologies and benefit of the work was approved by the ethics committee of the participating hospitals.

2.2. Specimen collection

Tissues were collected from all consenting women with cervical abnormality who were referred for colposcopy. At the time of examination, an approximately 3–4 mm punch biopsy was taken and snap frozen in liquid nitrogen for DNA and RNA studies. In addition, 3 ml of whole blood was drawn for biochemical assays.

Exfoliated cell specimens were categorized by disease grade ASC-US (atypical squamous cells of undetermined significance), LSIL (low squamous intraepithelial lesion), and HSIL (high squamous intraepithelial lesion) by cytology. Invasive cancers were diagnosed through histology.

Control samples were collected from in-patients who were admitted to the hospital to undergo a hysterectomy for other gynecological problems but who had a normal cervix. Two days prior to the operation the patient consent was taken and a 3 ml blood sample was drawn for biochemical assays. On the day of operation a small piece of cervix was immediately collected after the cervix was removed and snap frozen in liquid nitrogen. Simultaneously a small piece of tissue was added in vial of buffered formalin and sent to the pathology department for reporting. All of the control cervical samples were histopathologically confirmed as normal.

2.3. HPV testing and PCR based methods

DNA was extracted from the cervical specimen using standard protocols. The cervical punch biopsy (approximately 50 mg) was pulverized using liquid nitrogen, suspended in 1 ml of digestion buffer (10 mM NaCl, 10 mM Tris-Cl pH 8, 25 mM EDTA, 0.5% SDS and 0.1 mg/ml proteinase K), and extracted using standard phenol: chloroform methods [33]. The isolated DNA was subjected to GP5+/GP6+ HPV PCR amplification as described elsewhere [34]. Briefly, 40 cycles of amplification was carried out in a reaction volume of 25 μl containing 50 mM-KCl, 10 mM-Tris-HCl pH 8.3, 200 μM of each dNTP, 3.5 mM-$MgCl_2$, 1 U thermostable DNA polymerase (AmpliTaq, MBI Fermentas) and 25 pmol of each primer of GP5 +/6 +. Each cycle included a 1 min denaturation step at 94 °C, an annealing step to 40 °C for 2 min and a chain elongation step to 72 °C for 1 min. The first cycle was preceded by a 4 min denaturation at 94 °C and the last cycle was extended by a 4 min elongation at 72 °C. The end products were analyzed by gel electrophoresis. The samples with the band at 150 bp were considered positive for HPV. Stringent care was taken to separate pre-PCR and post-PCR procedures to avoid any chances of contamination and false positive results. Positive and negative controls were included in every batch of the experiment. DNA quality was confirmed by amplification of a housekeeping gene (β-globin). The HPV result was considered acceptable only if the β-globin for the sample was positive.

2.4. Plasma nitrite/nitrate level measurement

Nitrite levels were detected in plasma of the patients of different lesions (normal, ASCUS, LSIL, HSIL) and invasive cancer using the Griess reagent (modified),Sigma, Catalog Number (G4410) [35,36]. Briefly, 100 ul of the plasma was first reduced at 37 °C for 20 min using 10 ul nitrate reductase. The sample was then mixed with an equal volume of Griess reagent and incubated for 10 min at room temperature and measured in ELISA reader (Bio-Rad) at 540 nm. The values were calculated based on the standard curve generated by using different concentration of sodium nitrite. All the samples were tested in triplicate and controls were included in every test. Values are expressed in μmolar concentration. Plasma nitrite/nitrate values were log-transformed for statistical analyses.

2.5. iNOS mRNA expression measurement

Total RNA was extracted from cervical biopsy specimens using a total RNA isolation kit (Axyprep, Axygen) following the manufacturer's protocol. Quality and quantity of the RNA was measured using a Nanodrop spectrophotometer. A total of 1ug of mRNA was

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