Cancer of the Uterine Cervix and Human Papillomaviruses

Cancer of the uterine cervix is the most common cancer among Indian women. In India, annually about 130,000 women fall prey to cervical cancer which is commonly seen among women of low socio-economic status. The factors that are known to predispose to this disease include poor genital hygiene, early marriage, multiple pregnancies and childbirth, promiscuous sexual life and chronic infection or STD of lower genital tract. A wealth of epidemiologic, clinico-pathologic and molecular biologic data have now established a strong etiologic link between Human Papillomavirus (HPV) infection and cervical cancer. Human Papilloma virus is a small double stranded DNA virus that infects epithelial mucosal and cutaneous tissues. More than 100 genotypes of HPV have been identified and about one third of them have been completely sequenced. The HPV types 16 and 18 are considered as the major ‘high risk’ HPV types (HR-HPVs) as they are found preferentially in cervical and anogenital invasive carcinoma. The prevalence of HPV type 16, has been detected in 70% to 90% of cervical cancer cases in India. Cancer of the uterine cervix is a unique cancer in the sense that it takes several years to develop into an invasive cancer and this cancer is preventable if it is detected early. Thus, the cervical cancer provides an excellent human model for studying the the process of carcinogenesis in vivo.

With the aim of addressing issues related to HPV and cervical cancer, ICPO has built up a unique approach by amalgamating very strong basic, clinical and applied research involving clinical and basic scientists, working within the scope of the present mission to control cervical cancer with an emphasis on its early detection, primary and secondary prevention. Multidisciplinary high quality research involving epidemiological, behavioral, clinical, cytomorphological, cytogenetic, biochemical, virological, immunological and molecular biological aspects are being undertaken in order to understand the natural history, biological behavior and mechanisms of cervical carcinogenesis.
Transcriptional Regulation of Human Papillomavirus Gene Expression in Human cervical Cancer Cells

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The High-risk HPV 16 and HPV 18 are associated with malignant transformation and carcinogenesis. The most widely known factors associated with HPV are the E6 and E7 oncoproteins, which interact with p53 and Rb tumor suppressors respectively. The interaction with these cellular proteins results in disruption of normal physiological process of programmed cell death. Although infection of HPV is essential, it is not sufficient to produce malignant disease. These viral proteins seem to require involvement of host cell genes. Activator Protein (AP-1), which is formed either by a homodimer of Jun proteins or a heterodimer of Jun and Fos proteins derived from host cells has been found to play a pivotal role in transcriptional regulation of viral oncogene expression. Thus AP-1 and other transcription factors such as NF-κB whose DNA binding sequences are present in HPV genome can be exploited as targets for therapeutic interventions. AP-1 and NF-κB are primarily regulated by phosphorylation and redox status of the cells.

Western Blot analysis showing the expression dynamics of different components of AP-1 in different grades of cervical lesions

Upstream Regulatory Region of HPV
Band Supershift Assay showing the different components of AP-1 taking part in the binding activity in cervical lesions

Time-dependent down regulation of AP-1 binding activity in HPV 18 positive HeLa cells in presence of Curcumin

It is well known that the antioxidative drugs and herbal antioxidant agent interfere with the redox status of cells. PDTC, a synthetic antioxidant selectively down regulates HPV-16 gene expression in HPV-16 immortalized human keratinocytes cell lines, HPK1a. We have discovered another effective natural antioxidant, Curcumin acts at transcriptional level and down regulates activity and expression of AP-1 components in HPV-18 positive cervical carcinoma cell line, HeLa. Curcumin has also been found to inhibit the activation of NF-kB and HPV transcription in cervical cancer cells. These observations are being pursued further in the form of proposal to conduct a national clinical evaluation of curcumin in HPV-infected women through a national multi-centric study by DBT.

Apart from curcumin, we are investigating the role of other herbal antioxidative agents, Berberine, Phyllanthus, Myristicin, and Azadirachta which have been shown a wide range of pharmacological effects including antimicrobial, anti-inflammatory effects and anti-proliferative activity against some of the cancer cells.
Carcinoma of the uterine cervix is preceded by a well defined precancerous stage - the dysplastic lesions which may regress to normalcy or in some cases progress to invasive cancer. Infections of Human Papillomavirus (HPV) especially HPV type 16 and 18 is strongly associated with development of this cancer. Recently, we have reported a selective down-regulation of HPV-18 transcription as well as inhibition of AP-1 binding activity which is essential for transcription of viral genes by a herbal anti-oxidant curcumin. It also causes reversal of c-fos/fra-1 transcription to a normal state in tumorigenic HeLa cells and can thus control transcription of pathogenic HPVs during keratinocyte differentiation and progression of cervical cancers.

Besides HPV involvement of several other sexually transmitted agents such as Chlamydia trachomatis, Trichomonas vaginalis, and N. gonorrhea have been suggested as co-factors for cervical carcinogenesis. Talwar et al (2000) developed a polyherbal formulation which inhibited strongly the growth of N. gonorrhea, multi-drug resistant E. coli and various species of Candida. It had virucidal action against HIV-1 and prevented, in progestin sensitized mice, the transmission of Herpes simplex-2 infection by vaginal route. Praneem polyherbal (PPH) dispensed as pessary or tablet have undergone Phase-I safety and Phase-II efficacy studies in 6 centres of ICMR. Basant, an improved next generation polyherbal cream, constitute purified Curcumin, purified extract of Emblica Officinalis (Amla), Quinine hydrochloride, purified saponins from Sapindus mukerosi (Reetha), Mentha citrata oil and Aloe vera gel. These ingredients are formulated in pharmacopically approved excipients glycerol, algic acid, xanthan gum, lactic acid, citric acid, potassium-sodium tartrate, and benzoic acid as preservative. Quality control criteria have been established for each component of the formulation to ensure consistency of the preparation from batch to batch. The inclusion of the various ingredients in our formulation is primarily based on their anti-microbial, anti-inflammatory, anti-HPV and anti-HIV action.
To our knowledge, there is no drug available for attenuation of HPV infection. The formulation Basant has ingredients with potential action on HPV. All ingredients, purified and quality controlled are of plant origin with no known toxicity. The formulation is washable and will be used only topically. In addition, curcumin alone will also be tested for its anti-HPV activity in this clinical evaluation. The Project, therefore, seeks to determine whether Curcumin or Basant, a curcumin-containing polyherbal cream with a wide spectrum antimicrobial action against vaginal pathogens, can eliminate or reduce HPVs in women with cervical dysplasia. The present study will be carried out in 200 women enrolled on the basis of clinical examination, cytology, histo-pathology and presence of HPV as determined and quantitated by molecular techniques. Subjects will be equally divided in two arms of the study; one of these will receive 3 ml Basant cream and the other will have gelatin encapsulated curcumin powder (500 mg) for intra-vaginal application. 100 patients in each arm of the study will be equally subdivided (50 patients) and will receive Basant/curcumin treatment every night for 30 days (excluding menstruation days) and the other group will receive a Placebo vaginal cream/capsule. Repeat clinical examination, cytology, histopathology and HPV viral load assay will be performed within 72h of post treatment following which the placebo and treatment groups will be crossed over so that placebo groups will also receive the Basant/curcumin treatment and vice-versa. Patients will be re-evaluated on above parameters after completion of 30 day treatment period and the data will be analyzed to examine the effect.
Development of HPV Diagnostics for Early Detection of Cervical Cancer

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Unlike other viruses, no sensitive/reliable serological test is available for HPV, which is detected mainly by molecular methods. These molecular methods are generally expensive, time consuming and labor intensive. It is therefore important to develop a simple and cost effective HPV DNA diagnostic procedure/kit for early detection of cancer by identifying women infected with high-risk HPV type 16 and 18. In this context, a simple “Paper Smear” method has been developed for dry collection, transport and storage of cervical smears/scrapes at room temperature for subsequent detection of HPV DNA by a simple PCR assay. The principal objective of this multi-centric project is to evaluate suitability of multiplexing PCR assay for detection of both total HPVs as well as specific HPV types HPV16 and HPV18 infection in cervical exfoliated cells or biopsy specimen.

*Multiplex PCR of Cervical Biopsies  
Samples showing different amplimer of L1 (450 bp), ß- Globin (268 bp) HPV 16 (217 bp), HPV 18 (100 bp). P is positive control DNA, N is negative control DNA. M is Φ x 174 Hae III- digested Molecular weight marker.*
collected as Paper Smear by a single reaction step. To validate the procedure and the assay, multiplex results are being compared with that of separate PCR.

Multiplex PCR was performed with the samples received from four different centers. Control multiplex PCR, which contained DNA from both HPV 16 and 18 human DNA, resulted in amplification of four products i.e. HPV consensus L1, HPV 16, 18 and β-globin as an internal control. 20–25 samples from each center have been analyzed and about 50% to 60% were found to be HPV positive by L1 consensus primers and about 80% of them were positive for HPV type 16. HPV type 18 positivity was not detected in any of the samples. Collection of cervical scrapes as “Paper Smear” was found to be good and adequate from all the centers.

Results of the multiplex reactions will also be compared with the results of hybrid capture II (HC2) system for an aliquot of about 100 samples to assess the efficacy and reliability of both techniques in detecting HPVs and for quality control. Once the validation process is over, ICPO is planning to conduct a wet workshop for a week for all participating centers to increase human resource base.
Molecular Markers for the Detection and Progression of Cervical Cancer

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The biological behavior of invasive cervical cancer is not always predictable. The issue gets more complicated in case of cervical pre-cancers where prognosis of the natural history and clinical course of the lesion is always a challenge. Biological markers represent signals in a continuum of events between start of carcinogenesis and final expression of clinical disease. It is also clear that mere determination of HPV is not sufficient for assessing or explaining tumor progression in the uterine cervix. Most of the data available molecular markers of cervical cancer are sporadic from different centers reporting only one or two markers. Such data is always difficult to follow and validate. The present study is designed as a multicentric one and with 4 leading centres involved. Understanding the genetics and epigenetics of cervical cancer will form the basis of new screening and diagnostic tests for the early detection of cervical cancer, and will help in identifying patients at risk of aggressive disease and characterization of subtype pathological changes.

The present study is designed to analyze the expression of select markers which have been shown in isolated studies to be of relevance in HPV-mediated carcinogenesis in various stages and grades of cervical cancer and establish their clinical correlations. These include apoptosis-related proteins bcl2 and bax, transcription factor NF-κB and AP-1, cell cycle regulators, p53, p16, cyclin D1, E2F1, pRb, HPV16/18 and expression of E6 and E7 genes as molecular markers of cancer.

More than 150 samples of the pre-malignant malignant lesions were collected and expression of various cellular markers of the NF-κB and AP-1 family and the apoptotic and cell cycle markers p53, bcl2 and cyclin D1 were analyzed using immunohistochemistry.
κB family member p50, showed a very low or negligible cytoplasmic expression in normal and pre-malignant tissues while a very intense nuclear expression of p50 was observed in malignant tissues. In contrast, the p65 was found to exhibit uniformly a steady state level of low to moderate cytoplasmic expression from normal controls to pre-malignant to cancer. A very low or negligible expression of c-jun was observed in normal and pre-malignant tissues, while a very strong immunoreactivity of c-jun antibody was observed in malignant tissues. Among the fos family members, c-fos showed a gradual increase in expression as severity of lesion increased. While it showed a very low expression in normal samples and a moderate expression in premalignant lesions, the severe dysplastic lesion (HSIL) and tumor tissues showed a very intense staining. Fra-1, showed a very high expression in normal cervical tissues and it gradually decreased with the increasing severity of the lesion and was almost absent in invasive tumor. p53 expressions were found to be significantly higher in invasive tumour as compared to normal and pre-invasive samples. The normal cervical tissue showed negative immunoreactivity for bcl-2 antibody. The HSIL and LSIIL cases showed mild to moderate bcl-2 positivity. The invasive carcinoma cases showed intense bcl-2 expression. The presence of bcl-2 protein in the cancerous cases seems to be an over-expression.

Immunoblotting experiments were performed to analyze the level of expression of NF-κB family of proteins in cervical tissues comprising malignant, pre-malignant and normal controls. Interestingly enough the analysis of expression profile of p50 and p65 proteins in nuclear extracts from different grades of cervical tissue specimens’ demonstrated gradual increase in the p50 expression as the lesion progressed to cancer. In contrast, the p65 was found to exhibit uniformly a steady state level of low to moderate expression during cancer progression. A negligible expression of p50 was observed in normal and pre-malignant tissues, while a very high expression of p50 was observed in malignant tissues. Comprehensive data analysis to correlate all the experimental parameters with HPV and clinicopathological features of the tumor is in the process.

Work on the NOTCH signaling pathway has now been initiated along with the analysis of LOH, mRNA expression dynamics of p16, Notch-1, E2F and Bax transcripts to further correlate with these markers with disease progression.
Cancer of the Uterine Cervix and Human Papillomaviruses

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Cancer of cervix shows a highly variable well-characterized developmental pattern spanning 10-15 years. Thus, it represents an excellent in vivo model to study sequential changes occurring at cellular and molecular level during progression of cancer. Infection with high risk HPV types, HPV 16 and 18 has been considered as a primary and major risk factor playing a key role in the development of cervical cancer. Besides the immunological aspects, development of cervical cancer involves differential regulation machineries involving two principal oncogenes, E6 and E7 (early genes). The constitutive expression of these two early genes of high risk HPVs (Type 16 and 18) is responsible for tumorigenic transformation and is mainly dependent on the host transcription factors such as AP-1 and NF-κB, which play an indispensable role in viral and host gene expression. The project is designed to analyze and identify battery of genes that are differentially expressed during cervical carcinogenesis using commercially available DNA chip/microarrays as well as customized macroarrays of genes shown to be associated with cervical carcinogenesis. In addition, study is designed to identify genetic markers in specific chromosomal regions of interest using (CA)\textsubscript{n} repeats.

With the goal of identifying genes with a differential pattern of expression between cervical carcinomas and normal cervix, GeneChip Human Genome Focus Arrays were used which analyzed the expression level of ~ 8,500 transcripts and variants including 8,400 well characterized human genes. Although, there have been limited studies on the expression genomics of cervical cancer, and most of the published reports are focused on tissue culture cell lines. We performed a pilot study on three HPV positive samples from patients with invasive cervical carcinoma and one sample (used as control) from subject undergoing hysterectomy for non-neoplastic condition for microarray study. The ontology of differential

Molecular Genetic Basis of Cancer: Analysis of Genetic Alteration and Transcriptional Profiling of Genes During Cervical Carcinogenesis

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Harsimrut Kaur

Scatter plot showing up-regulated (orange) and down-regulated genes (green) in cervical cancer tissues with respect to normal cervix
expression of genes involved in cervical cancer was compared to control in different biological processes (physiological, cellular, development related, regulatory and behavioral process, viral life cycle and obsolete biological processes). It was observed that out of total, the most significant ontologies of genes were up-regulated in physiological process (48%) and in cellular process (36.27%). In addition, the results identify several genes that were significantly up and down regulated in cervical cancer in some important regulatory pathways triggered by HPV transformation like MAPK signalling pathway, JAK-STAT signalling pathway, Cytokine-cytokine receptor interaction pathway. Further analysis in a larger group of cervical cancer patients and control will be done for confirmation and elucidation of specific marker genes in different clinical stages of cervical cancer.
INFECTION of “high risk” types HPV is the major cause of the development of cervical cancer. Even having persistent HPV infection, only 10 to 30%, especially with oncogenic types lead to the development of high-grade cervical intraepithelial neoplasia (CIN) that progress to invasive carcinoma. Factors that contribute viral persistence have not been elucidated. It is very likely that the host immune responses play a major role in the onset and persistence of HPV infection. Inherited polymorphisms in immunomodulatory genes may contribute to the variations in immune function and genetic susceptibility for complex disease like cancer. Tumor necrosis factor α (TNFα), a cytokine to have a potent immunomodulatory activity, is induced by bacterial lipopolysaccharide, mitogens and viruses. The aim of the present study was to find polymorphisms in TNFα promoter region in pre-cancerous and cancerous lesions of uterine cervix, which could be interesting not only as genetic marker but also for their involvement in clearance of HPV infection.

We have collected 130 cervical cancer tissue biopsies from LNJP and Safdarjung Hospital, New Delhi and 50 healthy control blood samples. Genomic DNA has been extracted from all the samples. HPV status is also checked for all the samples. There are 10 reported SNPs (single nucleotide polymorphism) in the promoter region of TNFα gene. We have designed primers and optimized PCR mainly for two loci (-308) G/A and -238 G/A. Denatured HPLC (DHPLC) methodology was employed for screening of the polymorphisms at -308 and -238 loci followed by PCR-RFLP. A total of 129 cases and 45 control samples have been screened for both loci. Analysis of these loci revealed that -308 locus is more polymorphic than -238 but larger sample size is needed for further analysis.
The prognosis of invasive cervical cancer is generally poor. Therefore, the dissection of molecular events that mediates the pathological changes from non-invasive to invasive tumors is of considerable importance. Clinico-epidemiological studies suggested role of multiple risk factors including infections of HPV. It has been found to be an infection with certain high-risk HPV subtypes substantially increases the relative risk for cervical cancer but involvement of host genetic events appear to play a role in the process of cervical carcinogenesis. In any kind of cancer, one or many candidate genes involved in cell cycle control, signal transduction, DNA repair, cell to cell communication, tumor suppressor pathways are found to be mutated, deleted, methylated or polymorphic or found as combination of any of these two. Emerging concept is variation in nucleotide sequence either in coding or non-coding sequences could have correlation with the cancer incidence. So it is important to evaluate the nucleotide variations in those candidate genes involved in the development of the disease.

Since in cervical cancer, precancerous stage is well defined but conversion of precancerous stage to neoplasia is variable, the aim of this study was to evaluate whether single nucleotide polymorphisms (SNPs) in Cyclin D1 gene (CCND1), an important gene in cell cycle regulatory pathway may shed new light to understand the disease manifestation and the biological significance of the molecular markers involved in these cancer. We have collected 130 cervical cancer tissue biopsies from LNJP and Safdarjung Hospital, New Delhi and 50 healthy control blood samples. Genomic DNA has been extracted from all the samples. HPV status is also checked for all the samples.

PCR-RFLP methodology was employed for screening of CCND1-A870G polymorphism in different grades of cervical cancer tissue and controls. Final confirmation was done by sequencing. A total of 50 samples each of cervical cancer and control blood samples were analyzed. Further analysis with larger sample size will give the actual genotyping frequencies of this SNP in our population.
Multimodal Screening Tools for Early Detection of Cervical Cancer and Precancerous Lesions

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Precancerous lesions of the cervix can be identified well before the development of invasive cancers through cytology screening (Pap smear) or visual inspection methods. Many of the developed countries have registered a marked decline in the incidence and mortality from cervical cancer through regular screening of women using cytology methods. In developing countries like India, due to lack of resources and trained manpower, cytology screening at population level is not feasible. ICPO took the pioneering role in developing alternative modalities of visual inspection of cervical precancer and cancer and along with international agencies like IARC, JHPIEGO and PATH established a method of visual inspection using acetic acid (VIA) as a feasible strategy for early detection. Present project is formulated to study the usefulness of VIA for early detection of overt cancer and precancerous lesions and compare it to conventional cytological Pap test, colposcopy and histopathology.

A total 1,020 women underwent cytology screening of which 518 (51.7%) had negative or inflammatory smears, 162 (16.1%) ASCUS/AGUS, 168 (16.7%) LSIL, 104 (10.3%) HSIL and 68 (6.7%) invasive cancer cytology. All these women underwent simultaneous colposcopy and directed biopsy, wherever a total of 195 biopsy proven CIN-II plus lesions were detected. A total of 968 women underwent VIA screening of which 270 (27.8%) were VIA positive. Of the 195 biopsy proven CIN-II plus lesion, 158 showed VIA positivity. Sensitivity of VIA was 87.2% and specificity was 89.2%.
The knowledge about the natural history of HPV infection of uterine cervix and about the factors which govern its persistence and progression in some women is limited at present. An insight into the relationship between the natural history of HPV infection and development of precancerous and cancerous lesions of cervix can lead to development of more efficient and meaningful cervical cancer screening program by combining cytology screening with high risk HPV testing in a specific group of patients. The study might also prove useful in deciding future strategies on cancer immunotherapy and prophylaxis. The major objectives of the study are to assess the prevalence of high risk HPV infection among Indian women in reproductive age group, to elucidate the rate of regression/ persistence/ progression of HPV and to study the risk factors associated with HPV persistence.

The earlier work done in this project where cervical scrapes from 580 women were tested, revealed HPV DNA in 20.5% cases. However, there was a very low high-risk HPV (16 &18) positivity in the target women (with cytological diagnoses of within normal limits, Benign cellular changes and ASCUS/ LSIL) which was not commensurate with the existing

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<th>Cytodanosis</th>
<th>HPV L1 +</th>
<th>HPV 16 +</th>
<th>HPV 18+</th>
<th>HPV 11+</th>
<th>HPV 6+</th>
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<td>BCC-143</td>
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<td><strong>TOTAL-361</strong></td>
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literature. The technique of PCR was re-standardised and a fresh sample of 361 women was tested for HPV with new high risk probes. Of these 72 (19.9%) were positive for HPV with L1 consensus primer. Further testing for high risk HPV types (16 & 18), yielded HPV16 in 60 (16.6%) and HPV18 in 2 (0.6%) cases. When these high risk HPV positive cases were stratified according to their cytological diagnoses, 60 (16.6%) of these were found to belong to the target group (cytologically normal/ benign cellular changes/ASCUS/LSIL). These have been enrolled for the follow up studies to determine the progression/ regression/ persistence of HPV and various risk factors associated with progression/ persistence.
INSTITUTE generated a huge amount of clinico-epidemiological data during the part II study carried out by the institute for cervical dysplasia. Epidemiological information relating to socio-demographic, reproductive factors, personal hygiene, sexual practices, obstetric history and smoking habits were collected by trained social workers. The main aim of the present study is to develop probability models by logistic regression method through Forward and Backward methods to define the risk factors associated with the development of pre-cancerous and cancerous lesions of uterine cervix. In addition, the study is designed to develop scoring system by identifying the high-risk group of pre-cancerous and cancerous lesions of uterine cervix and to establish its validity. The comparison of the two models was evaluated in terms of various indicators. Both the models identified certain reproductive factors such as early age at start of sexual activity, poor genital hygiene, sexual behavior and low socio-economic status as risk factors. Backward model identified a few additional factors compared to forward model. Backward model revealed slightly higher odds ratios compared to forward model for some of the factors but with wider confidence intervals. However, sensitivity, specificity and other indices remained same for both the models. The model had the highest agreement with the predicted factors for invasive cancer cases followed by the other two groups (Mild/Moderate, Severe/CIS). It is appropriate to examine both type of models and choose among them on the basis of interpretability and ease of variable acquisition.

Based on detailed epidemiological information collected at the enrolment of subjects into the study, factors associated with development of pre-cancerous and cancerous lesions of cervix were evaluated. All the risk factors were dichotomized into risk and non-risk levels. Two scoring methodologies were employed to score levels in the risk factors. In the former procedure unity and zero scores were assigned while in latter the scores were based on the value of odds ratios (multiplied by ten and rounded off to ‘zero’ or nearest integer of five). Total scores were computed for each subject and were compared between case and control groups. Various performance statistics were computed. The sensitivity computations revealed that the scoring methodology enabled identification of 64.8%, 73.9%, and 87.1% of mild / moderate dysplasia, severe dysplasia/CIS and invasive cancer cases at a cut-off score of <6 vs >=6. Similarly, in the other approach, identification was to the extent of 81.8%, 84.1% and 93.5% respectively for the above groups with cut off score of >300 vs <=300. The risk scoring system appears to have potential for assisting and targeting of screening population. The effectiveness and efficiency of the finding have to be tested in the field conditions. This would further help towards enrichment, modification and improvement of the strategy.
Cytological Diagnosis, Menopausal Status and HPV Infection in Postmenopausal Women

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**Investigator**: Veena Kashyap  
**Staff**: Dinesh Kumar

The cancer cervix occupies either the top rank or is the second among cancer in women in developing countries. It usually affects women between the age of 50 and 55 years. Mature women who are not sexually active are seen to be at low risk of cervical carcinoma, present another set of circumstances that needs to be screened for the identification of cytologic abnormalities. It is now recognized that HPV infection always precedes cervical cancer and is a causative agent for the development of cervical cancer. The aim of this study is to screen postmenopausal women in the age group of 51 years to 65 years for menopausal status, cytological diagnosis and for HPV infection by cytology as well as latent infection by molecular techniques and to compare the prevalence of HPV infection with perimenopausal women in the age group of 45 to 50 years.

During the year, one thousand Pap smears were screened for this study. Out of them, 110 (11%) were postmenopausal women and 177 (17.7%) were perimenopausal women. Cytological changes of human papillomavirus were reported in 10 (9%) postmenopausal women and 20 (11.2%) perimenopausal women. Malignancy was about three fold (2.7%) higher in postmenopausal women. Atypical cells of undetermined Significance (ASCUS) and Atypical Glandular cells of Undetermined Significance (AGUS) were also observed only in perimenopausal women. Detection of HPV infection by PCR will be performed on these samples to corroborate the findings.
DEVELOPMENT of invasive cervical carcinoma is primarily dependent on the type of infecting HPV, duration of persistence of HPV infection. Recently, the variations in the sequence of HPV have also been attributed to degree of disease manifestation. HPV variants can be defined as having less than 2% sequence variation in their genome compared to the prototype. Sequence variations in HPV may affect virus assembly, immunologic recognition by the host, p53 degradation and immortalization activity. Nucleic acid sequencing data have shown that numerous HPV 16 variants do exist worldwide. In vitro analyses have indicated that naturally occurring variants HPV 16 differ in biological and biochemical properties. Sequence variation of such viral genes could therefore lead to proteins with altered biological function resulting in different natural histories and clinical outcomes of the disease. The present study is organized to determine the HPV variants specific to Indian population to develop effective prophylactic and therapeutic vaccines against cervical cancer.

HPV 16 is the major oncogenic HPV type most prevalent worldwide and specifically in India where more than 90% prevalence of HPV 16 alone have been reported. Since this virus type is the focus of most prophylactic and therapeutic vaccine development efforts, identification of HPV 16 variant leading to detection of intra type HPV 16 and amino acid variation would be important for generation of specific immune response in the context of vaccine development, trial and treatment strategies. The most common variations found so far are in ORFs E2, E6, E7 and LCR. Sequence variation in LCR may change the transcriptional activity of HPV. Also sequence variation in one or more of the protein of E2, E4, E5, E6 and E7 may result in amino acid change that could alter the biological function or the binding affinity of HLA presenting viral peptides, thus increasing the risk of carcinogenesis.

HPV 16 was detected in 34 out of 74 biopsy specimens of cervical carcinomas collected from the patients attending the Gynaecology Department of Lok Nayak hospital, New Delhi and Jawaharlal Nehru Medical College, Aligarh (U.P.). More samples of HPV are being acquired and their E6, E7 and LCR regions are being sequenced and analyzed.
CURRENTLY two prophylactic vaccines against HPV and cervical cancer - made by Merck & Co., New Jersey, USA and GlaxoSmithKline (GSK) Biologicals of Rixensart, Belgium have completed phase II trials and are in phase III clinical trials in several countries. These recombinant vaccines are based on virus-like-particles (VLPs) of capsid proteins of HPVs. The relatively high cost of VLP vaccine production and distribution, the expected type-specificity of their protection, absence of clinical efficacy data with respect to Indian population, and India-specific variations in HPV types are major issues of particular concern for implementation of similar vaccine program in developing countries like India, where cancer of the uterine cervix and infection of HPV, particularly the high risk type 16 is the highest in the world. One of the second-generation vaccines that could address the limitations of the current VLP vaccines and will be suitable for India should be “Genetic Vaccines” or “DNA Vaccines”. The DNA vaccine has a large number of additional advantages over conventional vaccines. An important feature of DNA-based immunization is the in situ production of the expressed protein(s), mimicking in this respect a partial viral infection. So the antigenic potential of this vaccine candidate is very high as they generate both cell-mediated and humoral responses. It is cost-effective to produce and purify these vaccines in large-scale and cold chain is also not essential for their storage and transport. With this background we have initiated work to develop DNA vaccine against India specific HPV 16 variants and prototype.

While India-specific HPV 16 variants analysis is in progress at our institute, we have initiated in vitro work with Cottontail Rabbit Papillomavirus (CRPV) strain to establish the animal model. The CRPV-pLAI plasmid having the full length Cottontail Rabbit Papillomavirus genome was received on request. The plasmid was successfully transformed into E.coli DH5α strain. The correct transformants were selected and plasmid DNA was isolated. The isolated plasmid was digested with SalI restriction enzyme so as to release the ~8.0 kb CRPV genome and the vector backbone of 3.5 kb. Lane 1: λ Hind III Digest marker; Lane 2: CRPV-pLAI digested with SalI; Lane 3: CRPV-pLAI undigested.

Development of Prophylactic DNA-Based Genetic Vaccine Against Human Papillomavirus Type 16 and 18

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Expression of Targeted Ribozyme Against HPV 16 E6 and E7 Genes in Cervical Cancer Cell Lines

Team Leader : R. Suresh Kumar
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The biology of HPV genome reveals the existence of seven early and two late genes, which are E1 to E7 and L1, L2 respectively and other regulatory regions which are termed as URR. Comparing the functional aspect of these HPV genes, E6 and E7 genes are found to be indispensable for carcinogenesis. E6 gene product binds with p53 and abrogates the function and ubiquitin-mediated proteolysis of p53 protein and E7 gene product binds directly or indirectly with Rb and suppresses its function. Thus, E6 & E7 deregulate the cell cycle and cell proliferation and therefore, are viewed as probable targets for therapeutic interventions against HPV-induced carcinogenesis. Present study is designed to develop a strategy to knock-down these genes using catalytic RNA (ribozyme), which possesses a high specificity in its targeting capability. In this technique, hammerhead ribozyme from satellite RNA of Tobacco ringspot virus is engineered to target any RNA molecule of interest to inactivate and down regulate its function. Hammerhead ribozyme is one of the well characterized and successfully used ribozyme-based therapeutics are also under clinical trials.

Cloning of HPV16 E6-E7 Gene

Cloning of target E6 and E7 genes in pGEM T vector - Amplified PCR product of E6 and E7 and E6-E7 together is represented in 2% Agarose gel showing 480 base pair product of E6, and 870 bp product of E6-E7. Lane 1 & 8 – φX 174 Hae III digested molecular weight marker; Lane 2, 3-E6; Lane 4, 5-E7 and Lane 6, 7 E6-E7 together
For designing of ribozyme against E6, E7 genes and target site validation, the E6 and E7 gene sequences were folded using Zuker’s RNA Folding software program and analyzed with at least twelve different structures for suboptimal folding pattern. Minimum energy levels of folding patterns were preferred for target selection. The folding also done using the ribozyme appended E6, E7 gene folding and checked with different energy levels for the ribozyme accessibility to the selected target site.

Designing of ribozyme and cloning of E6 & E7 target genes have been done. The catalytic RNA has also been cloned in eukaryotic expression (pol II) vector. Construction of pol III-based expression cassette which produces high copy number transcripts of ribozyme for stable transfectants, is underway.

Sequencing of library of E6-E7 clones for the correct orientation respective to T7 promoter

Structural folding of native E6 RNA of HPV16 (only one of the sub-optimal structure is represented). Probable targets are selected comparing many sub-optimal structure

Ribozyme appended with HPV-E6 RNA and binding of ribozyme to its correct sequence
Clinical Trial of Polyherbal Neem Cream and Tablet- ‘Praneem’ in Women with HPV Infection

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Praneem polyherbal cream and a polyherbal tablet which have inhibitory action on a wide spectrum of genital tract pathogens such as Herpes Simplex-2, Chlamydia trachomatis have been developed for intravaginal use. Besides HPV, involvement of other sexually transmitted agents such as Chlamydia trachomatis, N. gonorrhoea, HSV-2 has been suggested as co-factors for cervical carcinogenesis. In view of this fact, the effect of this herbal antimicrobial tablet is being investigated in women with HPV infection. A study has therefore been planned to see the efficacy of this tablet in eliminating the persistent HPV infection in women carrying this infection.

Clinical trials are in progress in collaboration with Lok Nayak Hospital, New Delhi, in Out Patients Department, women attending with gynaecologic complaint of genital infections causing abnormal discharge were recruited for the purpose of the study. About 249 women have been screened for the presence of HPV sequences by PCR and 11 women with HPV 16 positivity were given the Praneem tablet for 4 weeks. Two patients discontinued the treatment because of unrelated reasons. Of the six women analyzed 3 showed complete clearance of HPV while two patients felt relieved. These patients are also being tested for any change in the HPV viral load by real time PCR. At the same time, new patients are also being recruited for the placebo or control arm to analyze the spontaneous clearance of HPV infection from the cervix.
Cancer of the Uterine Cervix and Human Papillomaviruses

Awareness Surveys of Cervical Cancer among Physicians and School Teachers of Noida

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Ashok Seghal

CONTROL of cancer of uterine cervix forms a major thrust area of National Cancer Control Programmed through secondary prevention approach. But there are severe constraints concerning organized mass scale screening in India, hence the alternatives need to be worked out. Before 1960s, when wide scale application of cytology screening was absent in West, much of the mortality from cervical cancer could be brought down through health education of masses, training of professionals and changing the health care seeking behavior of women. these issues are in active focus again in view of the present circumstances. There is a definite possibility of carrying out primary prevention approach by modifying life style. Various factors have been identified as directly or indirectly associated with the development of cervical cancer and it is possible to modify variables associated with life style through health education, which could help to bring down the incidence of cervical cancer. As India has limited resources, WHO has also recommended promotion of health education for carcinoma cervix, it’s signs, symptoms, for it’s curability. This is also proved by the study of the cancer registry of Barshi, India, which has developed a methodology which includes education of the population about likely symptoms of cancer, and motivation of symptomatic individuals to undergo medical investigation. It is conceived that action to raise awareness of symptoms of cancer, and to encourage medical consultation, should form an important initial component of cervical-cancer control programme. At the same time it is also necessary that every physician who is dealing with female patient must be aware of looking at the cervix by doing per speculum examination in at least all symptomatic women with carcinoma cervix. It is important to determine the level of awareness of both the health care providers and recipients and to develop interventional tools and to study it’s impact. Therefore, in the present study, awareness of physicians regarding knowledge of risk factors and...
alternative strategies of screening for cervical cancer will be assessed. Awareness will be created by providing handouts on appropriate strategies and to assess the extent of impact through evidence based practice. In conjunction, the knowledge of female teachers on cancer cervix and the impact of health educational intervention will be evaluated.

The self administrated questionnaires for both teachers and physicians have been developed and pre-tested before launching the study. The standard Performa of teachers is being used in the 10 randomly selected schools of Noida. So far 69 teachers have been interviewed and Performae were filled of the target sample size of 600. A sample of 300 general practioners was selected for the awareness survey. Till date 20 physicians have already been interviewed.
Magnivisualizer – A Low Cost Tool for Early Detection of Cervical Cancer by Visual Inspection

**Team Leader**: Aditya Parasari

**Investigators**: Veena Singh, Ashok Seghal

**PRESENT** project was formulated to develop an improved version of Magnivisualizer, a low-cost device to perform cervical examination in low-resource settings and to examine the efficacy of the instrument for detecting the pre-cancerous and cancerous lesions of uterine cervix. This instrument is required for those Primary Healthcare settings where there is no colposcopy facility and even electricity is not available. In VIA tests, Magnivisualizer enhances the sensitivity to detect pre-cancerous lesions and by distinguishing metaplastic epithelium from neoplastic epithelium, improves the specificity reduces the rate of unnecessary biopsies.

We have developed a low-cost, portable, battery-operated version of Magnivisualizer for detection of uterine cervical lesions. The light-house was developed with anodized aluminum body. A photographic quality halogen bulb of 12 volt and 100 watt mercury reflector was fitted as light source. A removable condenser and an 80B filter was attached for giving optical white light of 5500K having full visible spectrum of light. A provision was made in the design for adjustable magnifying lens of 1+, 2+, and 4+ dioptr value. A polarizer can also be fitted with the magnifying lenses for better visualization. The instrument is designed in such a way that any type of filter, condenser, and magnifying lens can be easily interchanged. This instrument can be operated on 12 volts 7.5 Ah battery having auto cut charger provided. Shoulder hanging battery case is also provided to carry it easily.

Twenty pieces of the instrument were prepared. Four instruments were given to Gynecologists of LNJP hospital. Six instruments were given to paramedical staff of this institute and they are being trained by the gynecologist of our institute for visualizing the cervix in the OPD of different Delhi-based hospitals. This instrument is found to be very user-friendly as per the feedback received.