Altered Expressions of Matrix Metalloproteinases 2 and 9 in Cervical Carcinoma with high risk HPV-16 and HPV-18 in a South Indian Population: A Pilot Study

Authors
Thirumurugan @ Murali. P1, K. Santha2, R. Ramesh3*, S. Sethupathy2, Vickneshwaran V4

1Associate Professor of Biochemistry, Mother Theresa Post Graduate & Research Institute of Health Sciences, Puducherry
2Professor, Department of Biochemistry, Rajah Muthaiah Medical College, Annamalai University, Chidambram
3Professor, Department of Biochemistry, JIPMER, Puducherry
4Tutor, Department of Biochemistry, JIPMER-Karikal, Puducherry

*Corresponding Author

Abstract
Cervical cancer is one of the commonest cancer affecting women worldwide. HPV types 16 and 18 shows high risk for the development of both squamous cell carcinoma and adenocarcinoma of the cervix. Serum tumor markers are useful in screening cancers and also for assessing the prognosis of the disease. However, no specific tumor markers have been found suitable for the detection of ca cervix. Tumor cells secrete certain enzymes such as Matrix metalloproteinases (MMPs) which degrade the ECM necessary for neovascularization by growth factors such as vascular endothelial growth factors (VEGF). MMP-2 (72 kDa gelatinase) and MMP-9 (92 kDa gelatinase) play a vital role in ECM degradation. The aim of the present study was to determine plasma levels of VEGF, MMP-2 and MMP-9 and their relative gene expression in patients with HPV16 & HPV18 positive Cancer cervix patients in relation to healthy controls.

Keywords: Cervical Carcinoma, MMP9, HPV-16, HPV-16.

Introduction
Cervical cancer is one of the commonest cancer affecting women worldwide and it is second leading cancers among Indian women. The cytological screening of this cancer by pap smear has led to a reduction in the mortality due to ca cervix(1). It has been found that about 5 lakhs women develop ca cervix every year and hence the detection at the early stage of this cancer has been utmost importance(2). Development of vaccine against the human papilloma virus (HPV) has become a major advance to avoid HPV infection in terms of genital warts and precancerous cervical lesions(3–5). Although there are many types of HPV ranging about 100, HPV types 16 and 18 shows high risk for the development of both squamous cell carcinoma and adenocarcinoma of the cervix. In India, more than 80% of them were determinable to HPV 16 and 18. The determination of HPV as screening has shown consistently superior over the cytological methods of screening in terms of sensitivity. Serum tumor markers are useful in screening cancers and also for assessing the
prognosis of the disease\(^6\). However, no specific tumor markers have been found suitable for the detection of ca cancer. Most of the cancers involve stromal invasion followed by metastasis which involve degradation and remodeling of the extracellular matrix (ECM). Tumor cells secrete certain enzymes such as Matrix metalloproteinases (MMPs) which degrade the ECM necessary for neovascularization by growth factors such as vascular endothelial growth factors (VEGF)\(^7\). Although more than 20 MMPs have been found, MMP-2 (72 kDa gelatinase) and MMP-9 (92 kDa gelatinase) are responsible for ECM degradation\(^8,9\). Elevated levels of MMP-9 are found in breast, brain, ovarian, pancreatic, colorectal, bladder, prostate and lung cancers, and melanoma\(^10-12\). Matrix metalloproteinase 9 is considered to be a powerful factor stimulating the secretion of proangiogenic factors such as vascular endothelial growth factor (VEGF), which is widely regarded as one of the most important growth and survival factors affecting the vascular endothelium\(^13,14\). The aim of the present study was to determine plasma levels of VEGF, MMP-2 and MMP-9 and their relative gene expression in patients with HPV16 & HPV18 positive Cancer cervix patients in relation to healthy controls.

Study Subjects

Mode of Selection of Study Subjects

Selection of cases was made with age matched control, as per the ASCCP guidelines. All the study participants were of age group 35-58 years, who attended OPD clinic in the department of obstetrics and gynecology for screening of intra epithelial lesion of cervix to confirm the presence or absence of squamous cell carcinoma or adenocarcinoma which was done by pap smear. The cervical samples were further used to screen for HPV type 16 and 18 by using commercially available probe based High-Risk Human Papillomavirus (HPV) and Genotyping 16 & 18 Real-Time PCR Kit (Liferiver, CA, USA). The carcinoma of cervix subjects were divided into HPV16 and HPV18 based on the results obtained. 90(30 in each group) study subjects were recruited for this cross sectional study who are divided into 3 groups such as control, HPV16 positive cancer cervix and HPV 18 positive cancer cervix subjects. Study subjects with clinical complication such as a history of cervical neoplasia, skin or genital warts, immune-compromised conditions, chronic or acute viral infections, other cancers, and previous operations on uterine cervix and pregnant women were excluded from the study. The venous blood from the study subjects were collected before they were subjected to treatment for ca cervix. All the patients and control subjects were followed through surgery to collect tissue biopsies /cervical scrapings of squamous cervical carcinoma or normal cervical tissue.

Processing of Blood Samples for ELISA

The blood samples collected were centrifuged at 3500 rpm for 15 minutes at room temperature. The supernatant containing serum was aliquoted and stored at -40°C till analysis. These plasma samples were used for the assay of serum parameters such as VEGF, MMP2 and MMP9 by using commercially available ELISA (RayBio, Norcross, GA, US A) kits.

Processing of Tissue Samples for Real-time Gene Expression:
The cervical tissue samples were collected from the control (histrectomy patients) and HPV 16 and HPV18 positive cancer cervix patients and the tissue biopsy was immersed in Trizol solution (medox, India) and stored at -80°C till analysis. The sample was homogenized using trizol and the homogenate was used for RNA isolation using commercially available RNA isolation kit (Roche, Indianapolis, IN). Purity and RNA concentration was assessed by measuring the absorbance at 260 and 280nm using Nanodrop 2000 (Thermo Scientific, United States). 1 ug of RNA was converted into cDNA by using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Thermofischer scientific, USA). The gene-specific oligonucleotide primers (Integrated DNA Technologies) for Vascular endothelial growth factor A (VEGFA) -
gcagcttgagtaacagca, Matrix metallopeptidase 2 (MMP2)- FP- gaggtaatcttagttacaccgtgc and RP- ccagcagacaccagttgc, MMP9- FP- gaacaatctccgacag and RP- gggccgcagtgtaaaca and betα acin (ACTB) – FP- ccagcagacccaggtaga and ccaagcctacaggtag were used. CFX96 Real-Time PCR Detection System was used for evaluating the gene expression levels by using SYBR green master mix (Roche, Indianapolis, IN) using the kits protocol for thermal conditions. Signals were normalized to the housekeeping gene β-actin as the endogenous internal control. Relative fold change was calculated by 2-ΔΔCt method using Livak’s method[15].

Statistical Analysis
The values are expressed as mean ± S.D/SEM for parametric data and median (interquartile range) for non parametric data and analysed using statistical package for social sciences (SPSS), version 19.0 software. Post hoc Analysis of variance with Tukey was used to compare the means in parametric data. For non parametric data, Kruskal-Wallis test was used. Pearson’s/ spearman’s correlation was employed to assess the correlation between the molecular markers with the pregnancy outcome. Statistical significance was presumed if a null hypothesis could be rejected at a p value of ≤ 0.05.

Results
Patient Recruitment and Plasma Levels of Matrix Metalloproteinases and Angiogenic Factor in Cervical Carcinoma with HPV 16/18

The study was carried out with 90 study participants, after obtaining informed consent from the women with cervical carcinoma (50) and the control women (50). The cervical carcinoma patients were further divided into Human papilloma virus positive for type 16 (25) and HPV positive for type 18 (25). From the study subjects venous blood was collected before they were involved into the treatment. From the same study subjects, cervical biopsy was collected randomly from 10 patients from each group and stored under appropriate condition. The plasma levels of MMP2, MMP9 and VEGF were found to be elevated in the cervical carcinoma with HPV 16 & 18 patients when compared to the control subjects (Table 1).

The Tissue Biopsy Expressions of Matrix Metalloproteinase, Apoptotic and Angiogenic Factor in Cervical Carcinoma Patients with HPV 16 & 18.
It has been found that the gene expression of MMP-2 (Fig 1) and MMP-9 (Fig 1,2) were increased in HPV 16 and HPV 18 positive cervical carcinoma when compared to the control tissue. The mRNA expression of VEGF showed a significant increase in HPV16 and HPV19 positive cervical carcinoma when compared to the control tissue. (Fig 3)

Table 1: Plasma levels of MMP2, MMP9 and VEGF markers in cervical carcinoma with HPV 16 & 18

<table>
<thead>
<tr>
<th>S:No</th>
<th>Parameters</th>
<th>Control (n=50)</th>
<th>Cx HPV 16 (n=25)</th>
<th>Cx HPV 18 (n=25)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MMP-2 (ng/ml)</td>
<td>308.7±334.6</td>
<td>1260.2 ± 556.7</td>
<td>1581.4 ± 300.5</td>
</tr>
<tr>
<td>2</td>
<td>MMP-9 (µg/ml)</td>
<td>4.5±1.9</td>
<td>6.6±2.2</td>
<td>6.7±2.2</td>
</tr>
<tr>
<td>3</td>
<td>VEGF (pg/ml)</td>
<td>13.52 ± 14.3</td>
<td>42.09 ± 23.45</td>
<td>38.8 ± 21.27</td>
</tr>
</tbody>
</table>

Data are reported as mean ± standard deviation (S.D). Statistically significant differences (P < 0.05, one way ANOVA with Tukey post hoc) are indicated as follows: * vs control (C; n=30) women; (Significant differences between other groups are not indicated). MMP-2: Matrix metalloproteinase 2, MMP-9 Matrix metalloproteinases 9, VEGF: Vascular endothelial
growth factor. P<0.05 were considered significant. Cx HPV16: Cervical cancer with Human papilloma virus positive for typing 16. Cx HPV18: Cervical cancer with Human papilloma virus positive for typing 18.

**Fig 1:** mRNA expression of matrix metallo proteinase 2 in HPV 16 and HPV 18 positive cervical carcinoma patients.

mRNA expression of MMP2 in cervical biopsy samples obtained from control cervix (C; n = 10), HPV16 positive carcinoma of cervix (Ca-HPV16; n = 10) and HPV18 positive carcinoma of cervix (Ca-HPV18; n = 10). Total RNA was isolated from frozen samples using tissue RNA isolation kit (Roche, Inc.) and converted to cDNA (Invitrogen, Inc.) followed by amplification of target gene using SYBR green master mix (Roche, Inc,) and quantification by relative fold change (Livak method) as described in Materials and Methods. The bar diagram demonstrates the distribution of quantified placental mRNA expression of relative ratio of MMP2 to β-actin among the study groups (n=10/group. Data are reported as mean ± standard error of mean (SEM). Statistically significant differences (P < 0.05, one way ANOVA with tukey post hoc) are indicated as * vs control samples.

**Fig 2:** Protein expression of matrix metallo proteinase 9 in HPV 16 and HPV 18 positive cervical carcinoma patients by western blotting.
Protein expressions MMP9 in cervical tissue biopsy samples obtained from control women (C; n = 10), HPV16 positive carcinoma of cervix (Ca-HPV16; n = 10) and HPV18 positive carcinoma of cervix (Ca-HPV18; n = 10). The tissue homogenate was obtained from cervical tissue biopsies using RIPA buffer with protease inhibitor cocktail (Roche, Inc.) as describe in Materials and Methods. Representative image with bar diagram depicts the ratio of protein expression of MMP-9 with the β-actin among the study groups (n=10/group). Data are reported as mean ± standard error of mean (SEM). Statistically significant differences (P < 0.05, one way ANOVA with tukey post hoc) was indicated as * vs control.

Methods

**Fig 3:** mRNA expression of vascular endothelial growth factor (VEGF) in HPV 16 and HPV 18 positive cervical carcinoma patients.

mRNA expression of VEGF in cervical biopsy samples obtained from control cervix (C; n = 10), HPV16 positive carcinoma of cervix (Ca-HPV16; n = 10) and HPV18 positive carcinoma of cervix (Ca-HPV18; n = 10). Total RNA was isolated from frozen samples using tissue RNA isolation kit (Roche, Inc.) and converted to cDNA (Invitrogen, Inc.) followed by amplification of target gene using SYBR green master mix (Roche, Inc,) and quantification by relative fold change (Livak method) as described in Materials and Methods. The bar diagram demonstrates the distribution of quantified placental mRNA expression of relative ratio of VEGF to β-actin among the study groups (n=10/group. Data are reported as mean ± standard error of mean (SEM). Statistically significant differences (P < 0.05, one way ANOVA with tukey post hoc) are indicated as * vs control samples.

Discussion

Despite widespread availability of HPV vaccines, Ca cervix is one of the major causes of cancer-related death in women worldwide(1). It has been proved that the enhanced activity of the MMPs, and VEGF by various analytes is strongly linked to a number of tumors(16–20). Vascular endothelial growth factor is considered to be an important factor in blood vessel formation (angiogenesis), which is closely connected with tumor progression and metastasis(21). It has been indicated recently that MMP-9 may be potential markers of ovarian and breast cancers(22). In the present study, we investigated the usefulness of VEGF and MMP-2 and MMP-9 separately in patients with Ca cervix with HPV positive for type 16 and 18. Our results showed statistically significantly higher concentrations of VEGF, MMP-2 & MMP-9 (tested parameters) in patients with Ca positive for HPV16 and HPV18 when
compared to the healthy participants. We also found a significant increase in gene expression of VEGF, MMP-2 and MMP-9. Similar results as been observed in a study by Li et al\textsuperscript{(17)}. Another study demonstrated by Guo et al\textsuperscript{(23)} where MMP-9 expression was associated with lymph node metastasis, suggested an invasive potential in early stages of ca cervix. Another study has demonstrated the statistical significance of VEGF expression in Ca cervix tissues\textsuperscript{(24)}. We have found in our study that there is an increase in the markers as well the mRNA expressions of MMP2, MMP9 and VEGF in cervical carcinoma of with HPV16 and 18.

**Conclusions**

In summary, to the authors’ knowledge, our report is the first to evaluate the plasma levels and, what is more important, the diagnostic usefulness of such an extensive analysis of VEGF, MMP2 and MMP-9in Ca cervix with HPV 16/18 subtypes, not only independently but especially in combination with both established cervical tumor markers. Almost all parameters showed high usefulness in detecting tumor development.

**References**

15. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta


