ORIGINAL ARTICLE

Gene Expression Differences Between Cervical Intraepithelial Neoplasia and Cervical Squamous Cell Carcinoma

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ABSTRACT

Introduction: Human papillomaviruses (HPVs) is responsible for 75% of cervical cancer worldwide. Cervical squamous cell carcinoma (CSCC) is introduced by a pre-cancerous infection or cervical intraepithelial neoplasia (CIN) at different stages. We aimed to identify the differences between CIN and CSCC, both compared to normal tissue at gene expression level, which may be useful as targets for treatment. **Methods:** GEO from the NCBI's were analyzed using expression profiling by array for differential gene expression from normal HPV-positive tissue (n=10), high-grade CIN2/3 (n=15), and cervical squamous cell carcinomas (CSCC, n=10). GEO2R and GSEA were used to compare the two groups for identifying significantly differentiated genes and pathways. **Results:** Different expression patterns were detected between CIN and CSCC. MHC class I genes significantly increased in CIN compared to upregulation of MHC class II genes in CSCC. The results showed significant downregulation of Alzheimer's disease genes in CIN while Arachidonic acid metabolism pathway genes were significantly downregulated in CSCC patients. ATF6, VEGFA, CDKN1A and other genes were overlapped between CIN downregulated genes and CSCC upregulated genes. While, CCDC3, TLE2 and AVIL were overlapped between CIN upregulated genes and CSCC downregulated genes. **Conclusion:** In conclusion our data analysis study defined significant differential gene expression changes between pre-cancerous CIN and CSCC, the most interesting results showed some of the genes were upregulated pre-cancerous CIN while downregulated in CSCC and vice versa. These results may recognize and provide evidence for molecular biomarkers changes and prognosis of the infection with CSCC.

Keywords: Human Papilloma Viruses, Cervical Intraepithelial Neoplasi, Cervical Squamous Cell Carcinoma , MHC class I, MHC class II

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INTRODUCTION

Human papilloma viruses (HPVs) basically are the causative agents of all cervical cancers (1). HPV types 16 and 18 are the cause of 75% of cervical cancer cases around the world (2). Cervical cancer infection with relatively 570000 new cases and 311000 deaths in 2018 make it the second most prevalent female malignancy worldwide (3). In developed countries 0.8% of women over the age of 65 may have cervical cancer compared to 1.5% in developing countries (4).

However, invasive cervical squamous cell carcinoma (CSCC), introduced by a pre-invasive stage of the disease which known as cervical intraepithelial neoplasia (CIN) that associated with the human papilloma virus (HPV) infection and integration (5). Three grades of CIN (CIN1, CIN2, CIN3) were found and related to how abnormal cells deeply go into the skin covering the cervix (6). The cervical cancer starts when cervical cells change and become pre-cancerous. It is important to find those cells and treating them before they become cancer (7).

In Iraq 2.1% of total women with malignancies were diagnosed with cervical carcinoma during 1999-2009 in late stage of the disease due to lack of screening programs (8).

The mortality rate with cervical cancer decreased due to the vaccination against HPV (9), the screening and treatment of pre-cancerous lesions also the diagnosis and treatment of invasive cervical cancer may lead to the control and prevention of cervical cancer (10). The

correlation between HPV at different grades of infection and the cervical carcinoma has been well studied (11; 12; 13). In addition, patients with cancer have a lower opportunity of Alzheimer disease compared to non-cancer patients, this inverted correlation between cancer and Alzheimer's disease were concluded by different studies (26,27).

There is a large gap between the prevalence of infection and the occurrence of cervical cancer, but is there any difference at the gene expression level between cervical intraepithelial neoplasia (CIN) and cervical squamous cell carcinoma (CSCC)? This is the type of question that we aimed to answer in this study.

MATERIALS AND METHODS

Data source:
To achieve our aim to determine the differences in gene expression levels between HPV cervical intraepithelial neoplasia (CIN) and HPV cervical squamous cell carcinoma (CSCC),), *in silico* data of 35 frozen sections of tissue samples with or without HPV infection were analyzed by gene array to study gene expression. . Global mRNA profiles were obtained from normal HPV-positive cervical epithelium tissue (n = 10), high-grade precancerous lesions (CIN2/3, n = 15), and cervical squamous cell carcinomas (CSCC, n = 10), using whole human genome oligo microarrays (G4112F, mRNA 4 Ч 44K; Agilent Technologies).

Gene expression (mRNA) data obtained from cervical tissue specimens with or without HPV infection are available from GEO dataset in the NCBI's (National Center for Biotechnology Information) Gene expression Omnibus (GEO) accessible through GEO Series accession number GSE138081 (14).

The gene symbol names associated with data set were pulled from each GPL file and merged with its GSE read using the R merge function. Gene expression data were analyzed to determine the gene expression differences between CIN and CSCC tissue samples compared to normal control tissue specimens.

Data analysis:

Expressed genes were subjected to data analysis using:

1. GEO2R, NCBI (ncbi.nlm.nih.gov/geo/geo2r/). GEO2R was used to compare two or more groups of samples in order to identify genes that are differentially expressed across experimental conditions (CIN or CSCC tissue samples compared to normal tissue), based on the R programming language that provides tools for the analysis of high-throughput genomic data. Results are presented as a table of genes ordered by significance.
2. GSEA (Gene Set Enrichment Analysis) (15). GSEA is a computational method that determines whether a priorily defined set of genes shows statistically significant, concordant differences between two biological states.

RESULTS

Upregulation of MHC class I antigen presentation components in cervical intraepithelial neoplasia (CIN), grade 2-3:
The results obtained from gene expression at mRNA level in HPV cervical intraepithelial neoplasia (CIN), grade 2-3 compared to normal tissue revealed that 1084 genes were significantly upregulated in HPV CIN patients while 1469 genes were significantly downregulated at p value ≤ 0.05 and fold change (FC) ≥1.5 set as the threshold criteria.

Significant genes (p value ≤ 0.05) were further analyzed using GSEA (gene set enrichment analysis), Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were achieved for the differentially expressed genes.

The results showed only four from nine gene sets pathways significantly enriched at nominal p value < 5% in correlation to HPV CIN upregulated genes (antigen processing and presentation, cell adhesion molecules CAMS, natural killer cell mediated cytotoxicity, and chemokine signaling pathway) as shown in (Figure 1-A). In order to identify which genes, have the highest impact in CIN, leading edge analysis of the differentially expressed genes was performed in GSEA to determine which subsets of genes contributing the most to the enrichment signal of a given gene sets leading edge or

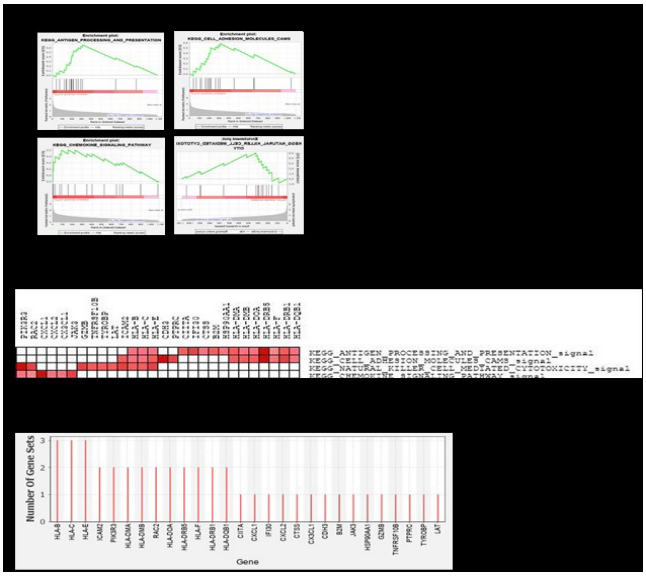

Figure 1. Pathways correlated to upregulated gene expression identified in comparison between HPV CIN patients and normal control. A. Enrichment plot, of four pathways significantly enriched at nominal p value < 5%. B. Heat map for the overlapped significant genes in different pathways. C. Gene in Subsets output from leading edge analysis showing the upregulation of MHC class I antigen presentation components in cervical intraepithelial neoplasia (CIN

core enrichment. The results showed the upregulation of MHC class I antigen presentation components (HLA-B, HLA-C and HLA-E) in cervical intraepithelial neoplasia (CIN) these three overlapped genes enriched in three of four gene sets pathways (Figure 1-B and C).

Downregulation of Alzheimer disease (AD) genes expression in cervical intraepithelial neoplasia (CIN), grade 2-3:

Our results showed that 1469 genes were significantly downregulated at p value ≤ 0.05 and fold change (FC) ≥1.5 set as the threshold criteria. Significant genes were analyzed using GSEA.

The results showed that Alzheimer's disease (AD) gene set from ten gene sets were significantly enriched at nominal p value <5% as shown in figure 2.

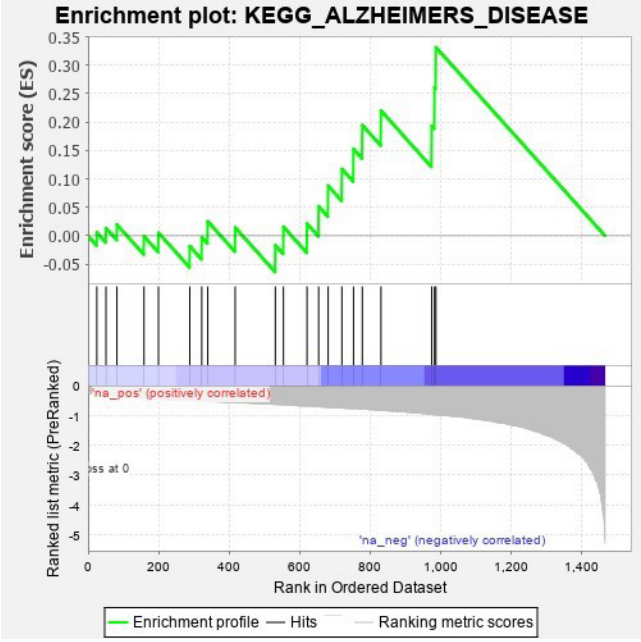

Figure 2 : Pathways correlated to downregulated gene expression identified in comparison between HPV CIN patients and normal control. A. Enrichment plot, of Alzheimer disease pathway significantly enriched at nominal p value < 5%.

Upregulation of MHC class II antigen presentation components in cervical squamous cell carcinoma (CSCC)

Results obtained from gene expression at mRNA level in HPV cervical squamous cell carcinoma (CSCC) compared to normal tissue revealed that 1930 genes were significantly upregulated in HPV CSCC patients while 1962 genes were significantly downregulated at p value ≤ 0.05 and fold change (FC) ≥1.5 set as the threshold criteria.

Significant genes analyzed using GSEA and pathway analysis were achieved for the differentially expressed genes.

Our results revealed twenty one gene sets from thirty seven gene sets pathways significantly enriched at nominal p value < 5% in correlation to HPV CSCC upregulated genes (allograft rejection, autoimmune thyroid disease, antigen processing and presentation, type I diabetes mellitus, graft versus host disease, viral replication, viral myocarditis, DNA replication, intestinal immune network for IGA production, systemic lupus erythematosus, cell adhesion molecules CAMS, leishmania infection, natural killer cell mediated cytotoxicity, cell cycle, toll like receptor signaling pathway, endocytosis, pyrimidine metabolism, purine metabolism, oocyte meiosis progesterone mediated oocyte maturation, leukocyte transendothelial migration, and spliceosome) as shown in Figure 3-A.

The results of leading edge analysis indicating the upregulation of MHC class II antigen presentation components (HLA-DOA, HLA-DRB5, HLA-DRB4, HLA-DRA, HLA-DRB3, HLA-DRB1, HLA-DPA1, HLA-DQB1, HLA-DMA and HLA-DPB1) in cervical squamous cell carcinoma (CSCC), these ten overlapped genes enriched in ten of twenty one gene sets pathways (Figure 3-B and C).

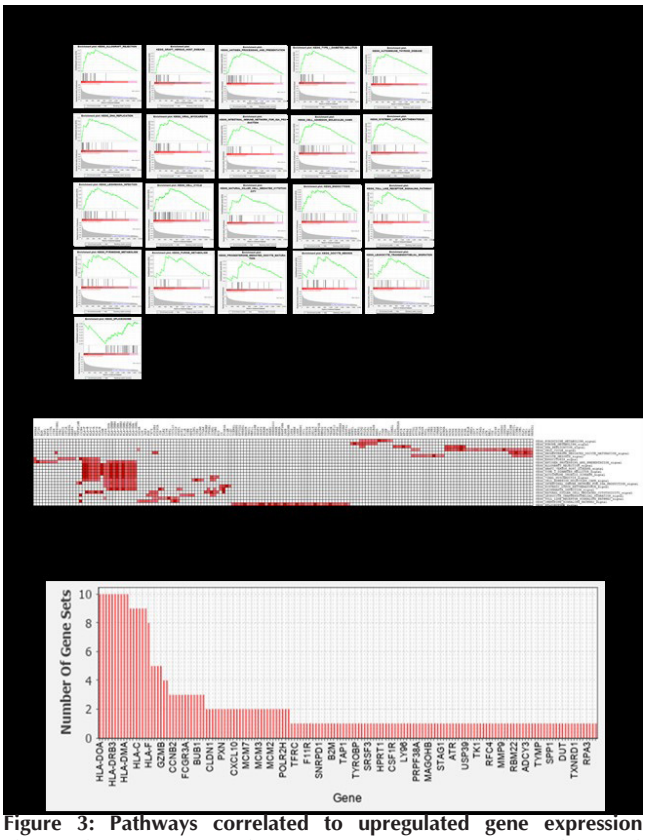

Figure 3: Pathways correlated to upregulated gene expression identified in comparison between HPV CSCC patients and normal control. A. Enrichment plot, of twenty-one pathways significantly enriched at nominal p value < 5%. B. Heat map for the overlapped significant genes in different pathways, and C. Gene in Subsets output from leading edge analysis showing the upregulation of MHC class II antigen presentation components in cervical squamous cell carcinoma (CSCC).

Downregulation of Arachidonic acid metabolism (AA) genes expression in cervical squamous cell carcinoma (CSCC):

The results obtained from significantly downregulated

1962 genes at p value ≤ 0.05 and fold change (FC) ≥1.5 set as the threshold criteria, the data analysis revealed that from twenty nine gene sets, only one gene sets (Arachidonic acid metabolism) are significantly enriched at nominal p value 0.005 as shown in figure 4-A.

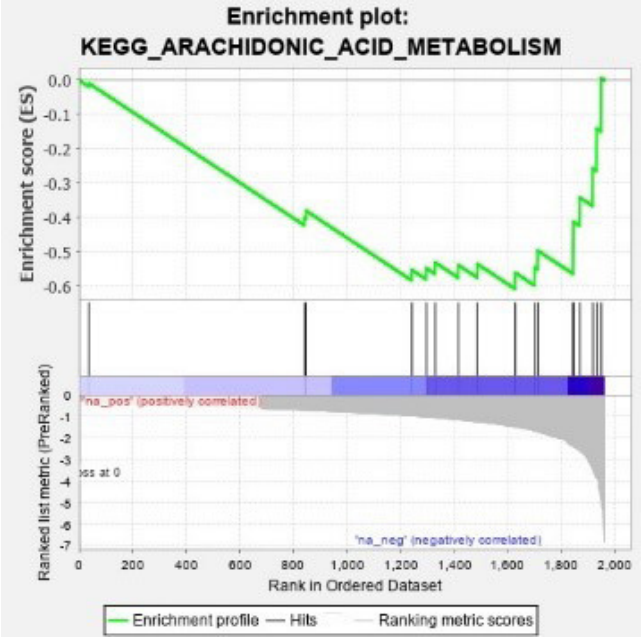

Figure 4: Pathway correlated to downregulated gene expression identified in comparison between HPV CSCC patients and normal control. A. Enrichment plot, of Arachidonic acid metabolism pathway (AA) significantly enriched at nominal p value 0.005.

Overlap between significantly differentially upregulated and downregulated genes in CIN and CSCC infection: A Venn diagram were used to show all possible logical relations of significantly differentially deregulated mRNA (upregulated and downregulated genes) in both CIN and CSCC infections (Figure 5).

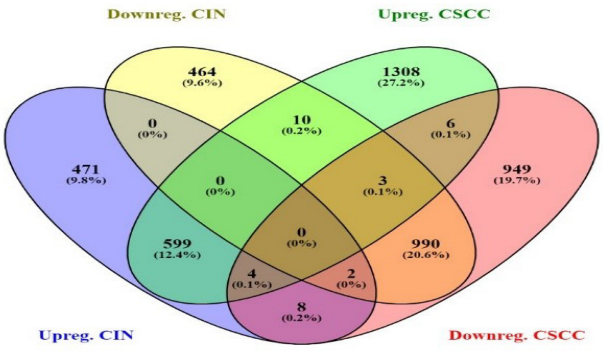

Figure 5: Venn diagram of significantly differentially deregulated gene expression (upregulated and downregulated) in sample groups. CIN = cervical intraepithelial neoplasia, CSCC = cervical squamous cell carcinoma, Downreg. =Downregulated, Upreg. = Upregulated.

This study revealed that 10 common genes were downregulated in CIN patients but upregulated in CSCC patients (KPNA4, VEGFA, ATF6, SPAG9, PSMD2, PCYT1A, CDKN1A, RABL3, MSI2, NIT2). In addition, 8 common genes were upregulated in CIN but downregulated in CSCC patients (CCDC3, LOC100190986, MPPED2, NR2F2, TLE2, ZNF446, ZNF429, AVIL).

DISCUSSION

The present study demonstrates the upregulation of MHC class I in cervical intraepithelial neoplasia grade 2-3 (CIN), while in cervical squamous cell carcinoma (CSCC) is associated with increase expression of MHC class II.

MHC class I present on the cell surface of all nucleated cells and they are presenting antigens to CD8+ cytotoxic T cells. In addition, MHC class II molecules present antigens to CD4+ T cells the expression is through antigen presenting cells such as B cells, macrophages, and dendritic cells (16; 17; 18; 19), both T cells play a major role in the elimination of human papillomavirus (HPV) tumors (20).

Our results are similar to the results obtained by Gameiro et al, 2017, who indicate the increase in mRNA levels of different MHC class I components in both HPV positive tumors of both head and neck and cervical compared with normal control tissue (21). MHC class I is essential for CD8+ cytotoxic T cells responses for the eradication of HPV infected and cancer cells (22).

Nevertheless, another research by Gameiro et al, 2019 revealed a significant increase in mRNA of MHC class II genes which is found to be similar to our results (HLA-DRB5, HLA-DRA, HLA-DRB1, HLA-DPA1, HLA-DQB1, HLA-DPB1) in HPV positive patient compared to HPV negative patients or normal (23). However, the activated CD4+ T cells is crucial to sustain CD8+ T cell function during chronic infection and over anti-tumor response (24; 25).

Our results showed not only the differences in the expression of upregulated genes between CIN and CSCC patients samples, additionally there is a difference in the expression of downregulated genes.

In cervical intraepithelial neoplasia (CIN), Alzheimer's disease (AD) genes expression was significantly downregulated, our results showed an increase in AD in pre-cancerous infection (CIN). No published study found that correlate AD with CIN infection while, a correlation between Alzheimer's disease and decreased risk of cancer were found by Roe et al, in 2010 (26). Nonetheless, some people may have gene mutations such as p53 gene, that lead to an increased risk of cancer and decreased risk of Alzheimer's disease. (27)

Nevertheless, other reported results found that patients with prevalent clinical AD develop incident cancer at a slower rate (28).

Smoking-related cancers (oral cavity and pharynx, lip, pancreas, lung/bronchus, larynx, cervix, kidney/ renal pelvis, bladder, esophagus, and stomach) (29), negatively correlated with Alzheimer's disease (27) than non-smoking related cancers (30).

Our data analysis results in cervical squamous cell carcinoma (CSCC) patients indicate the downregulation of arachidonic acid metabolism gene expression at nominal p value 0.005.

Lipid metabolism have an essential role in cancer development (31; 32), and phospholipids in cell membrane participate in metabolic process and signal transduction pathways (33).

Recent study by Kori and Yalcin Arga in 2018 found that protein deregulation was connected to metabolism pathway in cervical cancer and AA metabolism was the key pathway. Moreover, they suggest important changes in cell metabolism during disease progression (34).

Furthermore, gene enrichment analysis study by Kashyap et al, 2009 determined significant downregulation of AA metabolism pathway genes in esophageal squamous cell carcinoma (ESCC) (35). In addition, another study recommended that altered expression of AA metabolism pathway is modulated during carcinogenesis and may contributed to esophageal squamous cell carcinogenesis (36).

Our study found ten common genes were downregulated in CIN patients but upregulated in CSCC patients (KPNA4, VEGFA, ATF6, SPAG9, PSMD2, PCYT1A, CDKN1A, RABL3, MSI2, NIT2). Furthermore, eight common genes were upregulated in CIN but downregulated in CSCC patients (CCDC3, LOC100190986, MPPED2, NR2F2, TLE2, ZNF446, ZNF429, AVIL). These overlapped genes were significantly involved in cancer-associated pathways in our study, for example, ATF6 gene is downregulated in CIN but it is upregulated in CSCC patients. Recent study revealed higher expression of ATF6 in cervical cancer cells and its expression could increase cell growth, migration, autophagy through ER stress and MAPK signaling in cervical cancer (37).

In this study other overlapped gene is TLE2 which is upregulated in CIN while it is downregulated in CSCC. Different studies correlated the downregulation of TLE2 gene expression with different types of cancer such as bladder cancer (38), and poorer survival outcome in pancreatic cancer patients data (39).

CONCLUSION

In summary our data analysis study identifies and shed some light on the differences in gene expression between HPV cervical intraepithelial neoplasia grade 2-3 (CIN) or pre-cancerous stage and cervical squamous cell carcinoma (CSCC). The present results may provide evidence for biomarkers to monitor prognosis of the infection with CSCC, which may help in better controlling the disease. However, further studies to analyze the role of genes that downregulated in precancerous stage (CIN) but upregulated in CSCC such as (KPNA4, VEGFA, ATF6) and vice versa genes that upregulated in CIN but downregulated in cancer stage CSCC such as (CCDC3, NR2F2, TLE2) using HPV cell culture lines or HPV infected tissues. Those genes might act as key regulators and a disease markers or a potential therapeutic targets. Additional in-depth investigation is needed.

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