

Role and insights of human papillomavirus E5 oncoprotein in cervical carcinogenesis

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Abstract. Cervical cancer is a highly prevalent malignancy affecting the health of women, and a leading cause of cancer-related mortality worldwide, particularly in low- and middle-income countries. The primary cause of cervical is persistent infection with high-risk human papillomavirus (HPV), particularly through the activation of its oncoproteins E6 and E7 that interact with host tumor suppressor genes. Another HPV oncoprotein, E5, has been reported to alter several tumorigenic signaling pathways; however, its precise role in cervical cancer has not yet been fully elucidated. Therefore, the aim of the present study was to perform a comprehensive *in silico* analysis to investigate the HPV-E5 associated hub genes and their associated signaling pathways. Five microarray datasets, namely GSE265111, GSE26888, GSE9750, GSE7803 and GSE5787 were retrieved from the Gene Expression Omnibus (GEO) database using ‘HPV E5’ and ‘cervical cancer’ as search terms. A total of 8,202 differentially expressed genes (DEGs) were common to all five datasets, from which the 10 hub genes, including several nucleoporins (NUPs) were identified. Kaplan-Meier analysis of these genes revealed that the expression of NUP85, NUP88 and NUP-like 1/NUP58 genes was associated with overall survival, suggesting the prognostic significance of these genes. In addition, three pairs of genes, specifically: NUP5 and NUP160; NUP50 and NUP98; and NUP160 and NUP98 were observed to exhibit significant co-occurrence, suggesting that

they may be interdependent and involved in shared biological pathways in cervical cancer pathogenesis. Data from the Human Protein Atlas revealed that NUP50, NUP98 and NUP160 were differentially expressed in HPV-positive and -negative cervical cancer cell lines. The findings of the present study have improved our understanding of the potential role of HPV E5 in cervical carcinogenesis. However, further functional validation is necessary to confirm the findings.

Introduction

Cervical cancer is the most common gynecological malignancy and ranks second in cancer-related mortality among women in India. In addition, according to GLOBOCAN 2022 estimates, cervical cancer is the fourth most common cancer among women worldwide. The estimated age-standardized incidence and mortality rates of cervical cancer per 100,000 females are 662,301 (6.9%) and 348,874 (8.1%), respectively (1). Major contributing factors include persistent infection with high-risk (HR) human papillomavirus (HPV), early onset of sexual activity, long-term use of oral contraceptives (>5 years), multiple sexual partners and lifestyle factors including consumption of alcohol and tobacco, ultraviolet light exposure, poor nutrition and a sedentary lifestyle (2).

HPV plays a major role in the pathogenesis of cervical cancer. There are >200 different subtypes of HPV, of which 40 strains are known to be sexually transmitted and associated with cervical, vulvar, vaginal and anal diseases (3). However, persistent infection with HR HPV subtypes, particularly HPV-16, -18, -31, -33, -35, -45, -52 and -58, leads to the dysregulated proliferation of healthy cervical cells, resulting in pre-malignant lesions that may become malignant over time (4). Among these, HPV-16 and -18 account for >70% of all cervical cancer cases (5).

Population-based studies have shown that the incidence of new cases of cervical cancer has declined over the past 20 years, primarily due to increased cervical cancer screening and vaccination (6,7). Despite World Health Organization recommendations, only 100 countries have included HPV

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vaccination in their national immunization programs (8). The implementation of HPV vaccination has led to significant reductions in HPV infection rates, anogenital wart diagnoses and high-grade cervical lesions among women, and reported to provide herd immunity benefits in boys and older women (9). However, in a number of low- and middle-income countries, including India, the integration of HPV vaccination into national immunization programs has not yet occurred (10,11). A major issue is the affordability of the currently available HPV vaccines.

The HPV genome is approximately eight kilobases in length and consists of double-stranded DNA. It encodes six early genes (E1, E2, E4, E5, E6 and E7) involved in viral replication and oncogenesis, along with two late genes (L1 and L2) contributing to capsid formation. The early genes regulate HPV genome replication, transcription, cell cycle progression, cell signaling and structural modifications in infected cells, while late genes are required for virus propagation (12). A number of studies have demonstrated the impact of E1, E2, E4, E6 and E7 oncoproteins on cervical cancer (13-16). HPV 16 E5 is a hydrophobic protein comprising 83 amino acids that localizes in the cell membrane, endosomes and Golgi apparatus (17). It impairs the degradation of activated epidermal growth factor receptor (EGFR) by interfering with the acidification of late endosomes (18-20). HPV16 E5 downregulates the expression of the tumor suppressor proteins p21 and p27, both of which are cyclin-dependent kinase (CDK) inhibitors that regulate the cell cycle by inhibiting CDK activity.

In vitro studies have demonstrated that HPV16 E5 increases the malignancy of cervical cancer cells by promoting proliferation, migration and actin cytoskeleton-mediated invasion (21). One such study showed that HPV16 infection leads to an increase in the expression levels of the Met receptor, a crucial growth factor receptor involved in tumor cell motility, invasion and metastasis. This upregulation of Met is primarily mediated by the viral oncogene E5, with a lesser contribution from E6. The mechanism by which E5 induces Met expression involves the EGFR. E5 increases EGFR expression at the mRNA level, and this upregulation is implicated in the induction of Met (22). This suggests a complex crosstalk between the EGFR and Met signaling pathways mediated by HPV oncogenes (23). Furthermore, another study proposed that the known antiviral agent rimantadine could be utilized to counteract this resistance mechanism, thereby improving treatment outcomes for patients with head and neck cancer. The study suggested that rimantadine may have the potential to enhance the efficacy of immunotherapy by inhibiting the action of HPV E5 and restoring the sensitivity of tumors to immune checkpoint blockade (24).

Pertinently, novel approaches for improved diagnostics, effective vaccines and increased awareness among women from lower socioeconomic backgrounds are required for effective cervical cancer management. The present study performed an *in silico* analysis focused on the E5 oncoprotein of HPV and explored the hub genes and related signaling pathways that contribute to the progression of cervical cancer. The expression of certain hub genes was validated using reverse transcription-quantitative PCR (RT-qPCR). The findings from this study may provide insights into the molecular etiopathogenesis of cervical cancer, which could

be useful in the development of novel, high-efficacy diagnostics and vaccines.

Materials and methods

Extraction of microarray datasets from the Gene Expression Omnibus (GEO). The GEO database of the National Center for Biotechnology Information (<https://www.ncbi.nlm.nih.gov/geo>) (25) was searched using the terms 'HPV E5' and 'cervical cancer'. The search yielded 24 results, from which five microarray data sets, specifically GSE26888 (26), GSE26511 (27), GSE9750 (28), GSE5787 (29) and GSE7803 (30), were selected for analysis.

The GSE26888 dataset contains expression data from HeLa cells transiently transfected with control or SON small interfering RNA (three control and three transfected samples) for 66 h, followed by RNA isolation and profiling using an Affymetrix GeneChip Human Genome U133A 2.0 array (26). The GSE26511 dataset includes transcriptome profiles from 20 patients with negative and 19 patients with positive lymph node status (27). GSE9750 comprises transcriptomic data from 33 primary tumors, nine cervical cancer cell lines and 24 normal cervical epithelium samples (28). The GSE5787 dataset consists of 33 biopsies from 11 patients, with each tumor sampled from two to five different regions, and subjected to RNA extraction and hybridization using Affymetrix U133 Plus 2.0 oligonucleotide chips (29). GSE7803 contains 10 normal cervical epithelium samples, seven high-grade intraepithelial lesions and 21 invasive squamous cell carcinoma samples, all obtained via laser capture microdissection. For each sample, two rounds of T7-based linear RNA amplification were performed using the Arcturus RiboAmp kit, followed by analysis with the Affymetrix HG_U133A array (30).

The present study included samples from cervical cancer cell lines and patients with cervical cancer confirmed to be infected with HPV, and excluded samples from patients with other comorbidities or without HPV infection. A schematic overview of the analysis pipeline is shown in Fig. 1.

Analysis using the GEO2R tool. The differentially expressed genes (DEGs) between HPV-positive cervical cancer tissues or cells and normal tissues or cells were identified using the GEO2R tool (<https://www.ncbi.nlm.nih.gov/geo/geo2r/>) based on thresholds of $-\log_{10}(\text{P-value}) < 0.05$ on the y-axis and $-\log_2(\text{fold change}) < -1$ and > 1 on the x-axis (25).

Intersection analysis. The DEGs common to these five microarray datasets were selected by intersection analysis performed by the construction of a Venn diagram using an online Venn diagram tool (<https://bioinformatics.psb.ugent.be/webtools/venn/>).

Gene enrichment analysis. Gene enrichment analysis and functional annotation were performed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) version 2021 (31). Furthermore, Gene Ontology (GO) analysis (32) was performed to categorize the biological roles of the DEGs into biological processes (BP), cellular components (CC) and molecular functions (MF) (33). In addition, pathway analysis was conducted using the Kyoto Encyclopedia of Genes

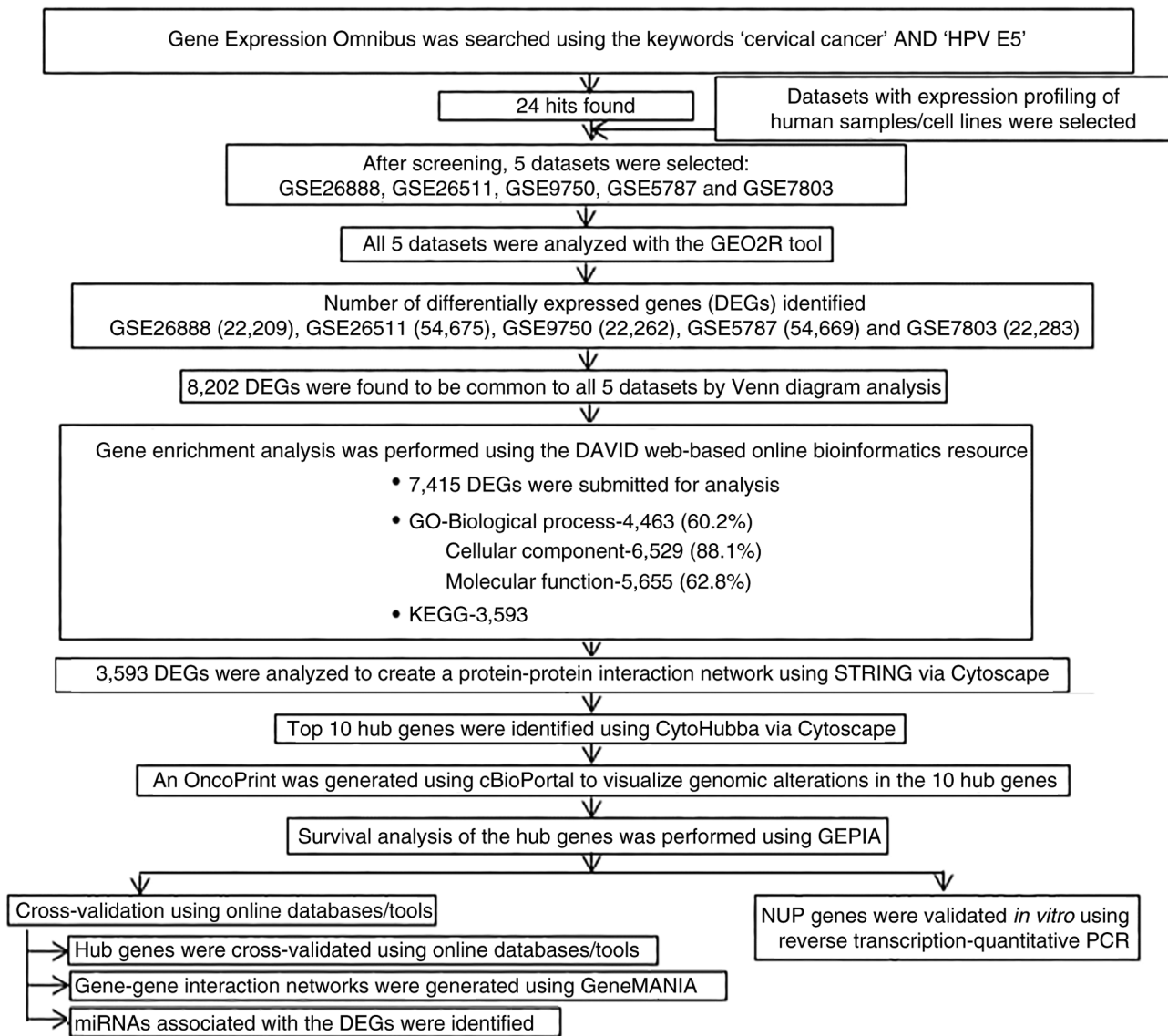


Figure 1. Flow chart of the analyses performed in the present study. HPV, human papillomavirus; DAVID, Database for Annotation, Visualization, and Integrated Discovery; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; STRING, Search Tool for the Retrieval of Interacting Genes; GEPIA, Gene Expression Profiling Interactive Analysis; miRNAs, microRNAs; NUP, nucleoporin.

and Genomes (KEGG) database to identify significantly enriched signaling pathways ($P < 0.05$) (34).

Protein-protein interaction (PPI) network and hub gene analysis. The PPI network of DEGs was generated using the Search Tool for the Retrieval of Interacting Genes (STRING) database and visualized with Cytoscape software (version 3.9.1) (35). Key genes and sub-networks within the PPI network were identified using the CytoHubba plugin for Cytoscape (36). Among the 11 topological analysis methods available in CytoHubba, Maximal Clique Centrality (MCC) was selected due to its superior performance. Without the newly proposed method, MCC outperforms the others by identifying more essential proteins among the top-ranked results, both in high-degree and low-degree protein groups among other 11 methods (36). MCC was applied as a filter with the following parameters: Degree cut off, 2; maximum depth, 100; node score cut off, 0.2; and k-core, 2. This identified the top 10 significant hub genes:

Nucleoporin (NUP)160, NUP85, NUP205, SEC13 homolog, nuclear pore and COPII coat complex component (SEC13), NUP50, NUP37, NUP98, NUP88, NUP-like 1 (NUP1; also known as NUP58) and NUP133. cBioportal (37) was used to check the mutual exclusivity or co-occurrence of these genes, and an OncoPrint of the 10 hub genes in cervical cancer was constructed. Gene Card was used to find out the function of the key genes (38). Kaplan-Meier survival analysis of the hub genes was performed using Gene Expression Profiling Interactive Analysis (GEPIA2) (39). In GEPIA2 was provided the gene expression analysis for survival analysis based on tumor (altered group) and normal (unaltered group) samples from the TCGA and GTEx database.

Human Protein Atlas (HPA) analysis. The HPA (<https://www.proteinatlas.org/>) leverages data from The Cancer Genome Atlas database and uses various techniques to analyze protein expression and localization in various cancer tissues (40). In

the present study, the HPA database was used to examine the expression of NUP50, NUP98 and NUP160 between tumor and normal tissues as determined by immunohistochemical (IHC) analysis. IHC data were available for NUP50 (antibody ID: HPA047162) but not for NUP98 or NUP160.

GeneMANIA analysis. The GeneMANIA database was used to explore the gene-gene interaction and PPI networks of NUP genes (41). It is an online tool that integrates multiple types of biological interactions and annotations to predict associations between genes.

Gene-microRNA (miRNA/mir) interaction network analysis. A gene-miRNA interaction network analysis was conducted to identify miRNAs that closely interact with the target genes. This analysis was conducted by inputting the genes of interest into NetworkAnalyst a web-based tool that integrates multiple biological interaction databases (42).

Validation of in-silico based key hub genes in cervical cancer cells

Cervical cancer cell lines and maintenance. The human cervical cancer cell lines used in this study were HPV16-positive SiHa, HPV18-positive HeLa and HPV-negative C33A. These cell lines were procured from the National Centre for Cell Science cell repository in Pune, India. The C33A and HeLa cells were maintained in Minimum Essential Medium and SiHa cells were maintained in Dulbecco's Modified Eagle's Medium (cat nos. 11095-072 and 11965-084; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (cat. no. 16000-044; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin (cat. no. 15070-063; Gibco; Thermo Fisher Scientific, Inc.) at 37°C in 5% CO₂.

mRNA expression: RNA extraction and RT-qPCR. Among the top 10 genes, NUP50, NUP98 and NUP160 exhibited significant co-occurrence. To examine the gene expression patterns of these three genes, total RNA was extracted from the C33a, SiHa and HeLa cervical cancer cell lines using the TRIzol®-chloroform method (cat. no. 10296010; Invitrogen; Thermo Fisher Scientific, Inc.). DNase I (Fermentas; Thermo Fisher Scientific, Inc.) was used to remove any DNA contamination (Fig. S1). In addition, RNA purity and concentration were measured using a DS-11 Spectrometer (DeNovix) according to the manufacturer's protocol (priming for 5 min at 25°C, reverse transcription for 20 min at 46°C, RT inactivation for 1 min at 95°C). cDNA was then synthesized from the RNA using a cDNA synthesis kit as per manufacture instructions (cat. no. 1708840; Bio-Rad Laboratories, Inc.).

Subsequently, qPCR was performed to assess the expression of NUP50, NUP98 and NUP160 using SYBR Green PCR chemistry (cat. no. 1725121; Bio-Rad Laboratories, Inc.) on a CFX96™ Real-Time System (C1000™ Thermal Cycler; Bio-Rad Laboratories, Inc.). Primers were designed using Primer 3 software and their sequences are shown in Table SI. The qPCR thermocycling conditions were as follows. Initial denaturation at 95°C for 5 min and 56°C for 1 min, followed by 40 cycles of 95°C for 10 sec, 65°C for 5 sec, 95°C for 50 sec, and a final extension at 72°C for 1 min. The expression levels of NUP50, NUP98 and NUP160 were quantified using the 2^{-ΔΔC_q} method, with normalization to β-actin (43).

Statistical analysis. Quantitative data are presented as the mean ± SD and all experiments were performed in triplicates. SPSS20.0 software (IBM Corp.) was used to perform the statistical analysis. Differences in quantitative data were compared by one-way ANOVA followed by post-hoc analysis using Tukey's test. P<0.05 was considered to indicate statistical significance.

Results

Identification of DEGs. The five datasets included in the analysis comprised GSE26511, GSE7803 and GSE5787 with data on human cervical cancer cases, GSE26888 with data on cervical cancer cell lines, and GSE9750 with data on both human cervical cancer cases and cell lines. Analysis with the GEO2R tool revealed a total of 176,098 DEGs in these five datasets. The dysregulated genes in each data set are shown as volcano plots (Fig. 2). Intersection analysis revealed a total of 8,202 DEGs were common to all five datasets, as depicted in a Venn diagram (Fig. 3).

Identification of enriched genes and pathways. The 8,202 DEGs were subjected to functional annotation using the DAVID online database to identify enriched genes and pathways. Among these DEGs, 5,655 (62.8%) were enriched in 19 MF categories (Table SII), 4,463 (60.2%) were enriched in 40 BPs (Table SIII) and 6,529 (88.1%) were enriched in 22 CCs (Table SIV). In addition, 3,593 DEGs were enriched in 175 KEGG pathways, as depicted in Table SV.

PPI network and hub gene identification. The 3,593 DEGs enriched in KEGG pathways were further analyzed by the construction of a PPI network using the STRING plugin in Cytoscape. The PPI network comprised 1,679 proteins, corresponding to the DEGs, and was further analyzed using another Cytoscape plugin, CytoHubba, to identify the top 10 hub proteins. These comprised NUP160, NUP85, NUP205, SEC13, NUP50, NUP37, NUP98, NUP88, NUPL1 and NUP133. These hub proteins are illustrated in a PPI network (Fig. S2), with decreasing order of significance represented by a color gradient from red to orange to yellow. The corresponding genes are involved in several signaling pathways and processes, including 'transport of the SLBP independent mature mRNA', 'cell cycle, mitotic', 'EML4 and NUDC in mitotic spindle formation' and 'gene silencing by RNA', as shown in Table I.

Co-occurrence of identified hub genes. The top 10 hub genes were further analyzed using cBioPortal to determine their mutual exclusivity or co-occurrence. Significant co-occurrence was observed for three gene pairs, namely NUP50 and NUP98, NUP160 and NUP50, and NUP160 and NUP98 (P<0.001; Table II).

Identification of significantly expressed hub genes in cervical cancer development and prognosis. To underscore the clinical significance of the identified hub genes in cervical cancer progression, an OncoPrint was created using the cBioPortal online tool. The analysis revealed various genetic alterations, including deletions, amplifications, missense mutations,

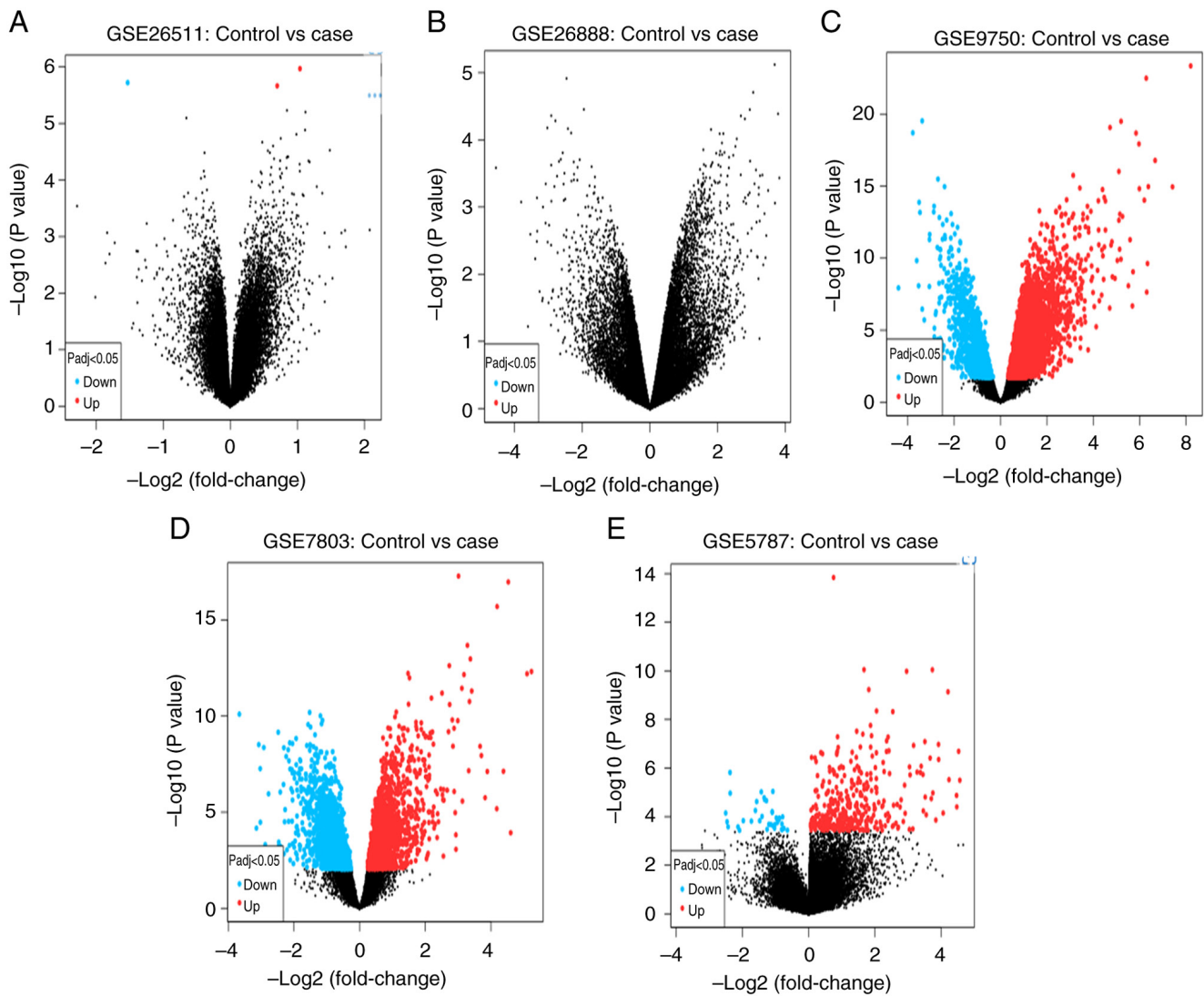


Figure 2. Volcano plots showing the differentially expressed genes in five cervical cancer datasets. Volcano plots for the (A) GSE26511, (B) GSE26888, (C) GSE9750, (D) GSE7803 and (E) GSE5787 datasets. Red dots indicate significantly upregulated genes, blue dots indicate significantly downregulated genes, and black dots represent genes with no significant change in expression. Padj, adjusted P-value.

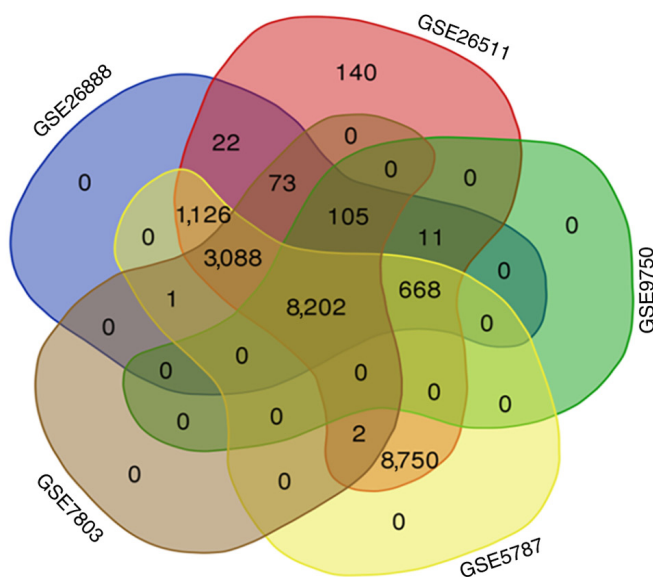


Figure 3. Venn diagram of the differentially expressed genes in five cervical cancer data sets: GSE26511, GSE26888, GSE9750, GSE7803 and GSE5787.

mRNA upregulations and downregulations for each hub gene (Fig. 4A). Among the top 10 hub genes, NUP133 had the highest frequency of genetic alterations in cervical cancer (26%) followed by NUP98 (14%). However, overall and progression-free survival analyses of these 10 genes combined did not yield statistically significant results (Fig. 4B and C). Therefore, a survival analysis was performed for each hub gene individually. The results showed that three genes, namely NUP85 ($P=0.047$), NUP88 ($P=0.023$) and NUPL1/NUP58 ($P=0.00038$) were significantly associated with prognosis in cervical squamous cell carcinoma and endocervical adenocarcinoma, where high NUP85 and NUP88 and low NUPL1 are associated with a good prognosis (Fig. S3).

Gene expression analysis of NUP50, NUP98 and NUP160 in cervical cancer cell lines. RT-qPCR was performed to validate the expression of the genes that were shown to have a significant mutual co-occurrence. The results revealed that the expression levels of NUP50, NUP98 and NUP160 genes were significantly upregulated in the HPV-positive SiHa and HeLa

Table I. Top 10 hub genes and their pathways.

Gene name	Pathways
NUP160	- Transport of the SLBP-independent mature mRNA - EML4 and NUDC in mitotic spindle formation - Cell cycle, mitotic
NUP85	- Transport of the SLBP independent mature mRNA - EML4 and NUDC in mitotic spindle formation - Separation of sister chromatids
NUP205	- Transport of the SLBP independent mature mRNA - Cell cycle, mitotic - Gene silencing by RNA
SEC13 homolog, nuclear pore and COPII coat complex component	- Transport of the SLBP independent mature mRNA - EML4 and NUDC in mitotic spindle formation - wtCFTR and delta508-CFTR traffic/generic schema (norm and CF)
NUP50	- Transport of the SLBP independent mature mRNA - Cell cycle, mitotic - Gene silencing by RNA
NUP37	- Transport of the SLBP independent mature mRNA - EML4 and NUDC in mitotic spindle formation - Cell cycle, mitotic
NUP98	- Transport of the SLBP independent mature mRNA - EML4 and NUDC in mitotic spindle formation - Cell cycle, mitotic
NUP88	- Transport of the SLBP independent mature mRNA - Cell cycle, mitotic - Gene silencing by RNA
NUP-like protein 1/NUP58	- Transport of the SLBP independent mature mRNA - Cell cycle, mitotic - Gene silencing by RNA
NUP133	- Transport of the SLBP independent mature mRNA - EML4 and NUDC in mitotic spindle formation - Cell cycle, mitotic

NUP, nucleoporin; SLBP, stem-loop binding protein; EML4, echinoderm microtubule-associated protein-like 4; NUDC, nuclear distribution protein C; SEC13, secretory 13; COPII, coat protein complex II; wtCFTR, wild-type cystic fibrosis transmembrane conductance regulator.

cell lines compared with those in the HPV-negative C33A cell line (Fig. 5). These findings clearly indicate an association between the expression of these genes and HPV-positive cervical cancer.

Immunohistochemistry: Analysis using HPA data. IHC staining images from the HPA were analyzed to compare protein expression between normal and cervical cancer tissues (Fig. S4A). A total of 374 and 383 samples were included in the comparative analysis. The results revealed that NUP50 was highly expressed in the glandular and squamous epithelial cells of normal and cervical cancer tissues. However, in cervical cancer cell lines, SiHa, CaSki, and HeLa (HPV-positive) exhibited lower NUP50 mRNA expression compared with those in the HPV-negative cervical cancer cell line C33A. At the protein level, NUP50 expression was downregulated in HPV-positive cervical cancer cell lines, including HeLa, SiHa, ME-180 and MS751 (Fig. S5A).

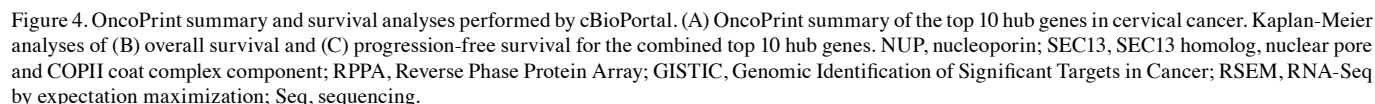
For NUP98 and NUP160, data for IHC analysis were unavailable (Fig. S4B and C). However, mRNA expression analysis revealed that NUP98 was slightly downregulated in HPV-positive cervical cancer cell lines compared with that in HPV-negative cervical cancer cells, C33A, with expression decreasing in the order SiHa, followed by ME-180, HeLa, CaSki, and MS751. At the protein level, NUP98 followed a similar downregulation pattern, in the SiHa, HeLa, MS751 and ME-180 cell lines (Fig. S5B). By contrast, NUP160 exhibited high expression at the RNA and protein levels in HPV-positive cervical cancer cell lines compared with those in HPV-negative cervical cancer cells (Fig. S5C). These findings suggest that the regulation of NUP50, NUP98 and NUP160 is heterogeneous in HPV-mediated cervical carcinogenesis.

PPI analysis of key genes NUP50, NUP98 and NUP160 using GeneMANIA. A total of 20 functionally similar

Table II. Co-occurrences among the top 10 hub genes as shown by mutual exclusivity analysis.

Gene A	Gene B	Neither	A not B	B not A	Both	Log ₂ odds ratio	P-value	^a q- value	Tendency
NUP98	NUP50	277	3	4	3	>3	<0.001	0.008	Co-occurrence
NUP98	NUP160	274	3	7	3	>3	<0.001	0.013	Co-occurrence
NUP50	NUP160	273	4	7	3	>3	<0.001	0.015	Co-occurrence
NUP37	NUP153	277	0	9	1	>3	0.035	0.373	Co-occurrence
NUP133	SEC13	280	5	1	1	>3	0.041	0.373	Co-occurrence
NUP58	NUP98	277	4	5	1	>3	0.101	0.39	Co-occurrence
NUP58	NUP50	276	4	6	1	>3	0.117	0.39	Co-occurrence
NUP133	NUP88	276	5	5	1	>3	0.12	0.39	Co-occurrence
NUP133	NUP98	276	5	5	1	>3	0.12	0.39	Co-occurrence
NUP88	NUP98	276	5	5	1	>3	0.12	0.39	Co-occurrence
NUP133	NUP205	276	5	5	1	>3	0.12	0.39	Co-occurrence
NUP88	NUP205	276	5	5	1	>3	0.12	0.39	Co-occurrence
NUP98	NUP205	276	5	5	1	>3	0.12	0.39	Co-occurrence
NUP133	NUP50	275	5	6	1	>3	0.139	0.39	Co-occurrence
NUP88	NUP50	275	5	6	1	>3	0.139	0.39	Co-occurrence
NUP50	NUP205	275	6	5	1	>3	0.139	0.39	Co-occurrence
NUP58	NUP160	273	4	9	1	2.923	0.164	0.414	Co-occurrence
NUP205	NUP153	272	5	9	1	2.596	0.193	0.414	Co-occurrence
NUP133	NUP160	272	5	9	1	2.596	0.193	0.414	Co-occurrence
NUP88	NUP160	272	5	9	1	2.596	0.193	0.414	Co-occurrence
NUP205	NUP160	272	5	9	1	2.596	0.193	0.414	Co-occurrence
NUP153	NUP160	268	9	9	1	1.726	0.303	0.619	Co-occurrence
NUP58	NUP153	272	5	10	0	<-3	1	1	Mutual exclusivity
NUP37	NUP205	280	1	6	0	<-3	1	1	Mutual exclusivity
NUP133	NUP58	276	6	5	0	<-3	1	1	Mutual exclusivity
NUP58	NUP88	276	5	6	0	<-3	1	1	Mutual exclusivity
NUP58	NUP205	276	5	6	0	<-3	1	1	Mutual exclusivity
NUP50	NUP153	270	7	10	0	<-3	1	1	Mutual exclusivity
NUP133	NUP153	271	6	10	0	<-3	1	1	Mutual exclusivity
NUP88	NUP153	271	6	10	0	<-3	1	1	Mutual exclusivity
NUP98	NUP153	271	6	10	0	<-3	1	1	Mutual exclusivity
SEC13	NUP153	275	2	10	0	<-3	1	1	Mutual exclusivity
SEC13	NUP160	275	2	10	0	<-3	1	1	Mutual exclusivity
NUP37	NUP160	276	1	10	0	<-3	1	1	Mutual exclusivity
NUP58	NUP37	281	5	1	0	<-3	1	1	Mutual exclusivity
NUP133	NUP37	280	6	1	0	<-3	1	1	Mutual exclusivity
NUP88	NUP37	280	6	1	0	<-3	1	1	Mutual exclusivity
NUP98	NUP37	280	6	1	0	<-3	1	1	Mutual exclusivity
NUP50	SEC13	278	7	2	0	<-3	1	1	Mutual exclusivity
NUP37	NUP50	279	1	7	0	<-3	1	1	Mutual exclusivity
NUP58	SEC13	280	5	2	0	<-3	1	1	Mutual exclusivity
NUP88	SEC13	279	6	2	0	<-3	1	1	Mutual exclusivity
NUP98	SEC13	279	6	2	0	<-3	1	1	Mutual exclusivity
SEC13	NUP205	279	2	6	0	<-3	1	1	Mutual exclusivity
NUP37	SEC13	284	1	2	0	<-3	1	1	Mutual exclusivity

^aBenjamini-Hochberg false discovery rate correction. NUP, nucleoporin; SEC13, SEC13 homolog, nuclear pore and COPII coat complex component.



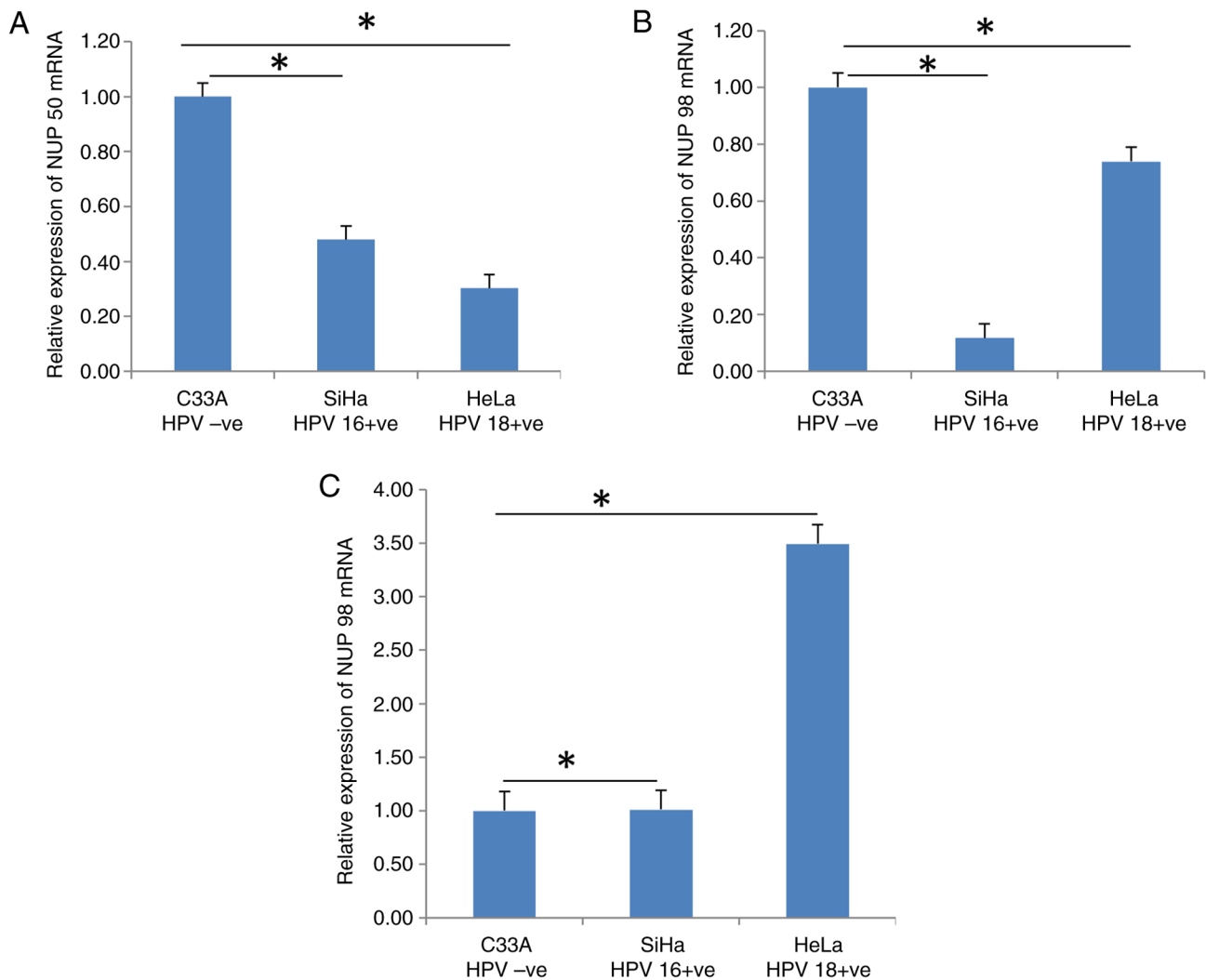


Figure 5. NUP50, NUP98 and NUP160 mRNA expression in cell lines with different HPV status. (A) NUP50, (B) NUP98 and (C) NUP160 expression in the SiHa and HeLa HPV-positive cell lines relative to those in the C33a HPV-negative cell line. *P<0.05. HPV, human papillomavirus; NUP, nucleoporin.

genes were identified for each key gene using GeneMANIA (Fig. 6). In the network visualization, the central node represents the key gene, and the predicted genes are arranged in the surrounding circle, highlights physical interactions, co-expression, co-localization, genetic interactions, and pathway associations, and indicated that the key NUP genes influence the immune system. Functional enrichment analysis revealed that these genes are primarily associated with platelet a-granule function and endopeptidase activity, aligning with previous studies on the functional pathways of age-related proteins (44,45). Notably, the release of platelet a-granules increases during blood coagulation, which has been identified as a key functional pathway in age-related protein networks.

Regulatory interaction network analysis. Gene-miRNA interaction networks were generated using NetworkAnalyst. The constructed candidate gene-miRNA network revealed that hsa-mir-16-5p was associated with the expression of NUP50, NUP98 and NUP160 simultaneously (Fig. 7). In addition, hsa-mir-4646-5p and hsa-mir-204-3p were indicated to regulate the expression of NUP50 and NUP98, while hsa-mir-93-3p

and hsa-mir-324-5p were indicated to regulate NUP50 and NUP160 expression.

Discussion

Oncoproteins are central to HR-HPV pathogenesis. Among the six early HPV genes, E6 and E7 have been well characterized, while the roles of other early genes, particularly E5, remain underexplored. E6 and E7 have been shown to disrupt cell cycle regulation in infected cells by targeting retinoblastoma protein and p53 (46), thereby blocking p53-dependent cell-cycle checkpoints and promoting unregulated cancerous growth (47). *In vivo* studies suggest that HPV E5 supports the oncogenic activities of E6 and E7, thereby contributing to malignant progression (48,49). E5 is active during the early stages of viral infection (50). Further HPV E5 has been observed to activate mitogen-activated protein kinase (MAPK), p38 and ERK1/2 in human keratinocytes in an EGF-independent manner (51). This MAPK activation upregulates the transcription of c-fos and c-jun, which in turn stimulates the transcription of E6 and E7 (52-54).

Given the relevance of HPV E5, the bioinformatics analysis in the present study was focused on its role in cervical cancer.

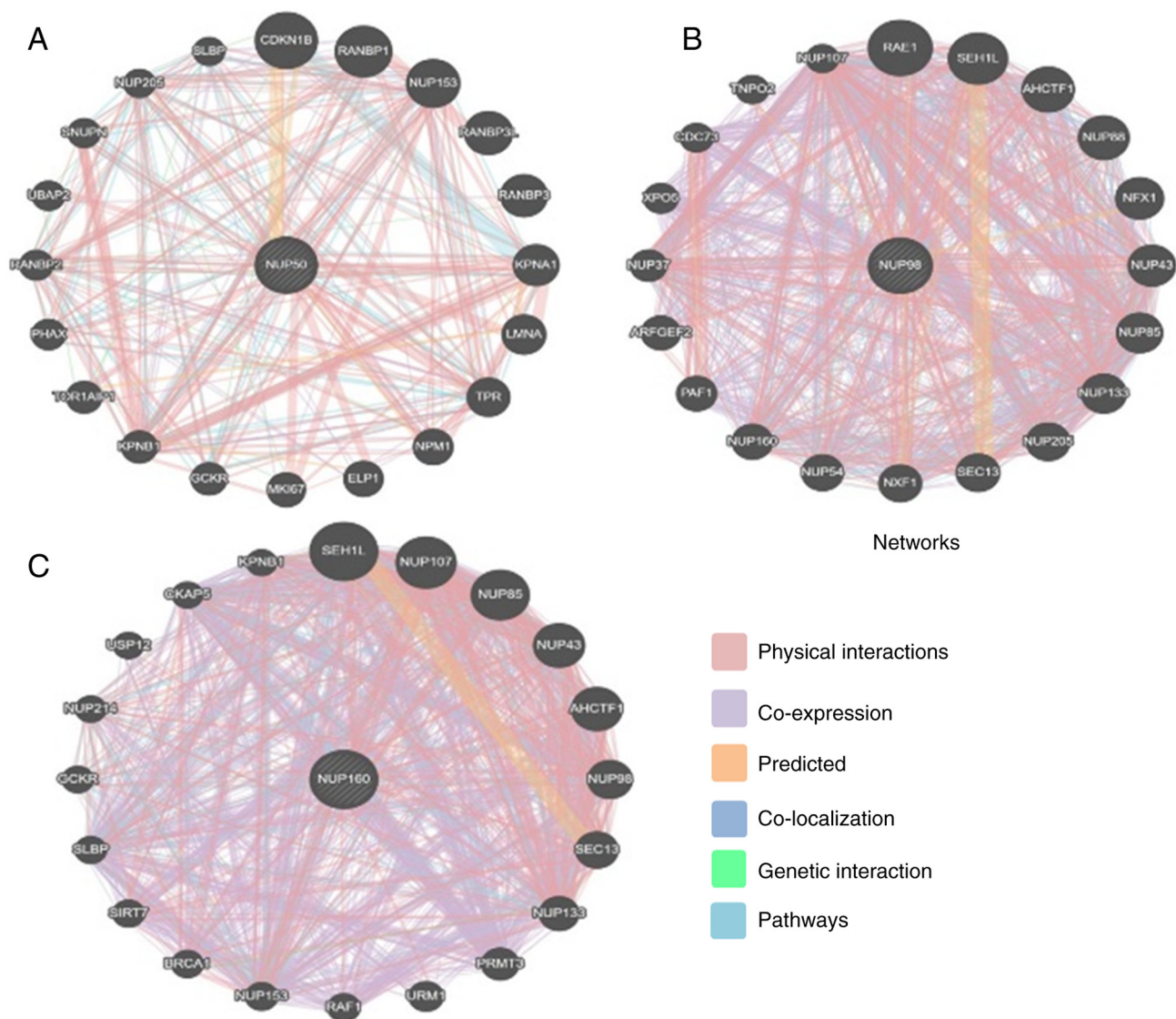


Figure 6. Gene-gene interaction networks for NUP genes generated using GeneMANIA. Networks for (A) NUP50, (B) NUP98 and (C) NUP160. NUP, nucleoporin.

A comprehensive bioinformatics analysis of the microarray datasets GSE26888, GSE26511, GSE9750, GSE5787 and GSE7803 was performed, which led to the identification of 8,202 DEGs. Functional enrichment analysis showed that these DEGs are associated with 19 MFs, 40 BPs and 22 CCs. Out of the 19 MFs, top five were 'kinase', 'activator', 'transferase', 'tyrosine-protein kinase' and 'RNA-binding'. Major BPs included 'host-virus interaction', 'protein transport', 'biological rhythms', 'apoptosis' and 'innate immunity', while major CCs included 'cytoplasm', 'nucleus', 'Golgi apparatus', 'mitochondrion' and 'cytoskeleton'. In addition, KEGG pathway analysis revealed that the DEGs were involved in 175 pathways, with the most notable being the 'MAPK signaling pathway', 'PI3K-Akt signaling pathway' and 'pathways in cancer.' Finally, 10 hub genes, namely NUP160, NUP85, NUP205, SEC13, NUP50, NUP37, NUP98, NUP88, NUP1 and NUP133, were found to be significantly associated with HPV E5 oncoprotein in cervical cancer pathogenesis. Notably, these hub genes are also implicated in important biological pathways such as 'transport of the SLBP independent mature

mRNA', 'cell cycle, mitotic', 'EML4 and NUDC in mitotic spindle formation' and 'gene silencing by RNA'.

The roles of some of these hub genes in different cancers have been reported previously. For example, NUP205 has been shown to increase nuclear c-Myc levels, thereby promoting the proliferation of lung cancer cells (55). In papillary thyroid cancer, HOTAIR-mediated metastatic progression has been observed to be regulated by the miR-488-5p/NUP205 axis (56). Furthermore, in colorectal cancer, the expression of NUP205 in tissues adjacent to the cancer has been suggested to be a predictor of relapse risk (57). The roles of NUP50 and NUP153 in breast cancer and bladder cancer have also been reported in previous studies (58,59). Elevated NUP37 expression has been associated with a low survival rate in patients with gastric cancer (59). NUP88 has been shown to inhibit the Notch signaling pathway in lymphatic leukemia by blocking the nuclear export of the recombination signal-binding component of this pathway (60). Furthermore, the upregulation of NUP88 overexpression has been observed in cases of highly aggressive breast cancers and cervical cancer cases (61,62).

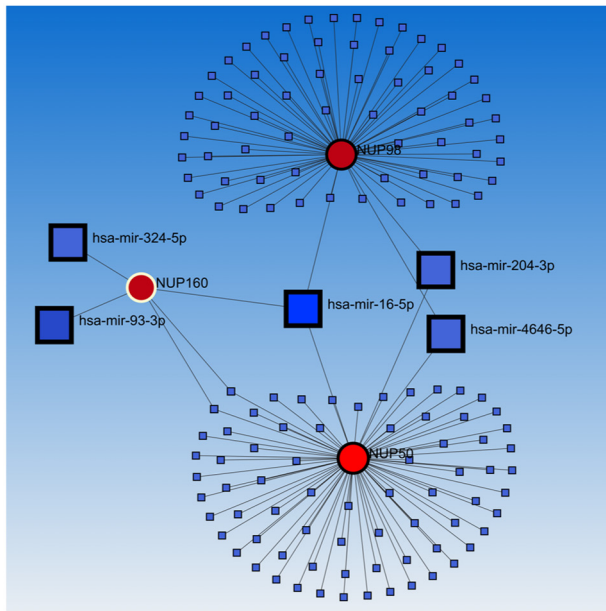


Figure 7. NUP-miRNA interaction network. The red circles represent NUPs and the blue squares represent miRNAs. NUP, nucleoporin; miRNA/mir, microRNA.

Lastly, NUP85 expression has been reported to be upregulated in pancreatic tumors (63).

Analysis of the hub genes using cBioPortal revealed significant co-occurrence for three sets of genes, namely: NUP50 and NUP98; NUP160 and NUP50; and NUP160 and NUP98, suggesting that variations in these genes may not occur in the same samples but might be present in different cancer cohorts, indicating their involvement in multiple pathways for cancer initiation and development (64,65). The observed co-occurrence supports the hypothesis that these genes do not act independently, and their protein products are likely to be interdependent and involved in shared biological pathways for the execution of specific cellular and molecular functions. To validate this finding and to investigate the relationship between these genes in cervical cancer, the expression levels of the NUP50, NUP98, and NUP160 genes in three cervical cancer lines were determined by RT-qPCR. The results revealed that all three genes were significantly upregulated in the HPV-positive cell lines HeLa and SiHa compared with the HPV-negative cell line C33A. NUP50 is a nucleoporin notably localized in the nuclear pore complex and the nucleoplasm. The deletion of NUP50 has been shown to cause abnormalities in the expression of p27Kip1, a CDK inhibitor protein that regulates cell cycle progression and cell proliferation, particularly during development of the neuroepithelium (65). The upregulation of NUP50 observed in HeLa and SiHa cells may contribute to the dysregulated growth of these cells through the dysregulation of this pathway via interaction with the HPV E5 protein.

NUP98 plays a regulatory role in gene expression, particularly for genes associated with development regulation and the cell cycle. In addition to being a component of the nuclear pore complex, its presence or absence in the nucleoplasm appears to modulate transcriptional activity (66). The dynamics of NUP50, NUP98 and NUP160 show HPV strain-specific

expression, which warrants validation in further functional studies.

In addition, an OncoPrint analysis performed using cBioPortal was conducted to observe the type of mutations, including deep deletions, amplifications and missense mutations, and mRNA expression changes of these hub genes in cervical cancer. Among these, NUP133 showed the highest frequency of genetic alterations (26%), followed by NUP98 (14%) and NUP85 (13%). However, while overall survival and progression-free survival analyses of the combined top 10 hub genes did not show a significant association, survival analyses of individual hub genes demonstrated that NUP85, NUP88 and NUPL1/NUP58 were significantly associated with an improved prognosis in cervical cancer.

Cross-validation was performed using the bioinformatics tools HPA and GeneMANIA. The expression of the identified key genes was examined in eight cervical cancer cell lines using HPA data, which revealed that their expression was altered in the presence of HPV; however, patient IHC data was incomplete, with the exception of that for NUP50. The GeneMANIA analysis was performed to highlight physical interactions, co-expression, co-localization, genetic interactions, and pathway associations, and indicated that the key NUP genes influence the immune system (67).

The *in silico* and RT-qPCR expression analyses performed in the present study suggest that the HPV E5 oncoprotein may play a role in cervical carcinogenesis by modulating the expression of the identified NUP genes. However, this requires validation using functional assays to elucidate the precise role of HPV E5 in the molecular etiopathogenesis of cervical cancer.

To the best of our knowledge, this is the first study to implicate NUPs as being significantly involved in HPV E5-mediated cervical cancer progression. NUPs are essential components of the nuclear pore complex and regulate the flow of various molecules across the nuclear envelope by interacting with importin and exportin proteins (59). The findings of the present study may be helpful in the development of potential biomarkers for the early diagnosis of cervical cancer and the identification of novel vaccine targets. However, the publicly available datasets used in this study may contain inherent experimental variations that could not be accounted for. Despite this limitation, the data generated and prior evidence suggest that the role of the E5 oncogene in cervical cancer is noteworthy and merits further exploration.

While the current study provides valuable insights through the integrative analysis of five transcriptomic datasets, it is important to acknowledge certain limitations. Firstly, the analysis provide a collective interpretation of all datasets to maintain consistency with the overarching study objectives. As a result, individual dataset-level differences and pathway enrichment analyses specific to dataset pairs were not performed. Although this integrative approach was advantageous for identifying broader trends, it limited the ability to uncover dataset-specific biological variations and enriched pathways that may offer additional mechanistic insights. Secondly, the current study used the GEO2R tool to perform the differential gene expression analysis. While efficient for pre-processed datasets, this tool does not offer the statistical robustness of DESeq2, which provides superior normalization,

improved handling of low-expression genes, and accounts more effectively for batch effects; however, its application requires a specific raw data file format, which was not available for the current study. The inability to use DESeq2 may have limited the precision of the identification of DEGs and hub genes, particularly in complex RNA-seq datasets. Future studies may benefit from the use of DESeq2 or similar tools to enhance analytical depth, particularly where raw count data is available. Finally, to validate the results of this study, detailed *in vitro*, *in vivo* and *ex vivo* will be essential in the future. Addressing the limitations of the present study through dataset-specific enrichment analysis, more advanced statistical methods and comprehensive preclinical research represents an important direction for future research.

Although various studies have shown HPV E5 oncoprotein to be responsible for initial virus propagation, there is a paucity of evidence regarding its exact role in cervical cancer pathogenesis. The present study highlights the importance of HPV E5 in the development of cervical cancer. Publicly available online databases were searched and an integrated bioinformatics analysis was performed, which revealed 10 hub genes, of which three, namely NUP50, NUP98 and NUP160, were shown to be significantly associated with an improved prognosis in cervical cancer. These findings highlight the role of nucleoporins in cervical cancer development; however, they require functional validation, to gain a more comprehensive understanding of the molecular mechanism and potentially aid in the development of novel diagnostics and vaccines.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

JR contributed to study conception and design, quality control of data and algorithms, analysis and interpretation of data, manuscript preparation and manuscript editing. SS contributed to the quality control of data and algorithms, analysis and interpretation of data, manuscript preparation and manuscript editing. SK contributed to manuscript preparation/drafting, analysis and interpretation of data, manuscript critical review and editing. EG contributed to manuscript drafting, analysis and interpretation of data, critical review and editing. AK contributed to manuscript drafting, analysis and interpretation of data and critical review. KK contributed to analysis and interpretation of data, manuscript preparation, editing and review, supervision and final approval of the version to be published. SH contributed to the conception and study design,

manuscript editing, manuscript review and supervision. All authors read and approved the final manuscript. JR and SS confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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