Transcriptomic analyses of genes differentially expressed by high-risk and low-risk human papilloma virus E6 oncoproteins

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Abstract Human papilloma virus is the causative agent for cervical cancer with 99 % of cervical cancer cases containing HPV. The high risk HPV-16, 18 and 31 are the major causative agents. The low risk HPV-6, 11 have been reported to cause penile, laryngeal, bronchogenic and oesophageal cancer. Since E6 oncoprotein is frequently over expressed in cancers, we did gene expression studies to compare between the E6 genes of high-risk (HPV18) or low-risk (HPV11) stably transfected in epithelial cell line EPC-2 or mock transfected with the basic vector pCDNA3.1. Microarray studies showed a total of 697 genes showing differential expression between the samples. Genes involved in several key cellular processes such as cell adhesion, angiogenesis, transcription regulation, cell cycle regulation and cell division showed altered expression between the samples. Gene Ontology mapping of 44 genes according cellular pathways revealed 13 pathways namely angiogenesis, alzheimer's, Wnt, p53, interleukin, TGF- β , cadherin, integrin, PI3-kinase, catenin, insulin, chemokine and G protein signalling pathways. The microarray results were confirmed by quantitative real-time PCR for some representative genes like *IFI27*, *CTNNA1*, *OSMR*, *CYP11B1*, *TNFSF13*, *LAMA2* and *COL5A3*. Analysis of differentially expressed genes by high-risk and low-risk HPV E6 proteins might help in identification of potential biomarkers for diagnosis, progression and therapy

of oesophageal cancer. The understanding of mechanisms of activation of these genes as well as the function of gene products will give a further insight into their roles in oesophageal cancer.

Keywords HPV · E6 · Cervical cancer · Microarray · Epithelial cells

Introduction

Human Papilloma Viruses are small, double-stranded DNA viruses that infect mucosal and cutaneous epithelial tissue. They are non-enveloped viruses with icosahedral capsids [21]. In a normal viral life cycle, virions are shed from the infected cell, in case of non-productive life cycle HPV DNA gets integrated into the host genome which results in unregulated expression of viral oncoproteins E6 and E7. Numerous studies have detected HPV DNA integrated in oesophageal cancer specimens [24], implicating HPV infection as a further risk factor in the development of OSCC [5, 41, 50]. There are approximately 118 types of HPV which are classified as high-risk or low-risk types depending on whether they cause malignant tumours or benign warts/lesions respectively [14, 26, 40]. The E6 and E7 proteins from high-risk HPV are sufficient for the induction and maintenance of cellular transformation [6, 31]. The over expression of E6 and E7 proteins within the proliferating cells alters several pathways, ultimately leading to tumour formation [33, 38, 44, 49]. High-risk E6 protein binds and degrades p53 tumour suppressor protein [1, 4, 13, 18, 19, 34, 48].

High-risk HPV E6 oncoproteins can immortalise the host cell. This is achieved by induction of expression of catalytic subunit of Human Telomerase Reverse Transcriptase (hTERT) by forming a complex with E6-associated protein

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(E6AP) and binding to hTERT promoter [7, 9, 10, 30, 45]. Enhanced expression of hTERT prevents telomere shortening at the end of each cell division, since shortening of telomere length in each cell division corresponds to aging process; therefore constitutive expression of hTERT causes the cell to divide indefinitely causing immortalization. High-risk HPV E6 proteins also target several host proteins containing a PDZ domain. The high-risk E6 proteins have a four amino acids PDZ binding motif ETQL at the C-terminus through which it can bind to PDZ (PSD95/Dlg/ZO1) domain containing proteins like MUPP1, hDlg and hScrib through complexation with E6AP [8, 11, 17, 23, 28, 36, 42, 43]. E6AP is a host ubiquitin ligase and it is required by E6 to exert its cellular effects. Since it has been shown that expression of HPV E6 alone can lead to transformation of host cell, we have focussed our studies with E6 only [16, 25, 39]. However, the role of E6 proteins in oesophageal cancer is not very clear since the low-risk HPV11 is the predominant type present in the oesophageal cancer specimens in South Africa [47].

In this study, we have compared the gene expression profiles between E6 protein from high-risk HPV18 and low-risk HPV11 using an Affymetrix gene chip array. We generated an oesophageal epithelial cell line EPC-2 stably transfected with HPV18-E6, HPV11-E6 genes or mock-transfected with the vector only. EPC-2 cell line is HPV-negative; therefore we wanted to determine which genes have altered expression in the presence of high-risk and low-risk HPV E6 proteins compared to control mock-transfected cells. Microarray studies showed that a total 697 genes showed differential expression between the samples which belonged diverse cellular processes. Quantitative real time PCR was done to confirm the microarray data for some selected genes. This is the first report showing the differential expression by low-risk and high-risk HPV E6 in oesophageal cancer.

Materials and methods

Maintenance of cell line

Epithelial cell line, EPC2-hTERT, was routinely maintained in Keratinocyte Serum Free Medium (KSFM) (Invitrogen Inc., Carlsbad, CA, USA) containing 1 ng/ml of epidermal growth factor (EGF), 50 µg/ml bovine pituitary extract (BPE) and 100U/ml of penicillin and streptomycin at 37 °C in a humidified chamber.

Stable transfection of cells

Oesophageal cancer cell line, EPC-2, was transfected using Fugene®6 Transfection Reagent (Roche Diagnostics, Indianapolis, USA). For transfection, 2 µg each of

pcDNA3 Flag, pcDNA3 Flag-11E6 or pcDNA3 Flag-18E6 was mixed with Fugene®6: KSFM and incubated at room temperature for 20 min. The mixture was then added drop wise to the cells and incubated for 5 h at 37 °C. Thereafter, the medium was replaced with fresh KSFM and cells allowed to grow for 96 h, after which G418 (Sigma, St. Louis, MO, USA) was added to a final concentration of 30 µg/ml to select for the transfected cells.

Extraction of Genomic DNA and PCR detection of E6 gene and mRNA

For genomic DNA extraction, 1.5×10^6 cells were taken and pelleted by centrifugation at 1.5 krpm for 5 min. The supernatant was discarded and pellet washed with 1 ml of ice-cold PBS. The pellet was then resuspended in 600 µl of digestion buffer (100 mM NaCl, 10 mM Tris-Cl pH 8.0, 25 mM EDTA pH 8.0, 0.5 % SDS and 0.1 mg/ml of Proteinase K). The samples were then kept overnight at 50 °C in a water bath. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added and mixed thoroughly by vortexing. The samples were then centrifuged at 10.0 krpm for 10 min and the upper aqueous layer transferred to a fresh tube. To this, 2 volumes of 100 % ethanol and ½ volumes of 7.5 M ammonium acetate was added following which DNA was recovered by centrifugation at 13.0 krpm for 5 min. The DNA pellet was washed with 70 % ethanol, dried and dissolved in 10 mM Tris-EDTA (pH 8.0) and DNA concentration determined spectrophotometrically.

PCR mixture used to amplify HPV11-E6 and HPV18-E6 genes consisted of GoTaq® Flexi Buffer, 1.5 mM MgCl₂, 0.2 mM dNTP and 20 pmol each of forward and reverse primers, 1U of GoTaq® DNA polymerase and 200 ng of genomic DNA in a total volume of 25 µl. For HPV18-E6, forward primer was 5'-GCGACCCTACAAGCTACCTG-3' and reverse primer was 5'-GAGTCGTTCTGTCGTGCTC-3'. PCR conditions were 95 °C-5 min, (94 °C-30 s, 66 °C-30 s and 72 °C-45 s) × 35 cycles, 72 °C-7 min. For HPV11-E6, forward primer was 5'-CCTCCACGTCTGCAACATC-3' and reverse primer was 5'-ATCTCTGCGGTGGT-CAGTG-3'. PCR conditions were 95 °C-5 min, (94 °C-30 s, 60 °C-30 s and 72 °C-30 s) × 30 cycles, 72 °C-7 min.

For detection of E6 mRNA, total RNA was extracted from the three cell lines using QiaZol (Qiagen, Valencia, CA, USA); 1 µg of total RNA was used to convert to cDNA in a 20 µl reaction using ImpromII reverse transcriptase (Promega, Wisconsin, MI, USA) according to manufacturer's protocol. The reaction was carried out at 42 °C for 50 min; the enzyme was then heat inactivated by heating to 70 °C for 10 min. From the reaction mixture, 2 µl of cDNA was used for RT-PCR. For HPV11-E6 mRNA, forward primer is 5'-CCTCCACGTCTGCAACATC-3' and reverse primer is 5'-

ATCTCTGCGGTGGTCACTG-3'. For HPV11-E6 mRNA, forward primer was 5'-CCAGAAACCGTTGAATCCAG-3' and reverse primer was 5'-GAGTCGTTCTGTCGTGCTC-3'. PCR conditions for HPV11-E6 mRNA were 95 °C-10 min, (95 °C-10 s, 62 °C-5 s and 72 °C-10 s) × 45 cycles. The PCR conditions for HPV18-E6 mRNA were 95 °C-10 min, (95 °C-10 s, 55 °C-5 s and 72 °C-10 s) × 45 cycles.

Quantitative real time PCR was done to check E6 transcript levels using SYBRmix (Kapa Biosystems) in a Mini-Opticon Thermal cycler (BIO-RAD, Hercules, CA, USA). The qRT-PCR conditions were same as above. The experimental control was β -actin. The E6 gene expression was normalized against the β -actin control.

Preparation of cell lysates and Western blotting

5×10^6 HPV11E6-EPC2, HPV18E6-EPC2 and mock-transfected EPC2 cells were harvested and pelleted by centrifugation at 2500 rpm for 5 min. The supernatant was discarded and the pellet resuspended in 150 μ l of lysis buffer (10 mM HEPES pH 8.0, 10 mM NaCl, 0.5 mM EGTA, 0.1 mM EDTA, 1 μ g/ml each of pepstatin A, leupeptin and aprotinin and 0.1 % NP-40). The lysis was done for 15 min on ice and the cell lysate was then centrifuged at 13,000 rpm for 5 min, the supernatant was collected which represented the cytoplasmic extract. The protein estimation was done for the extracts using Bicinchoninic acid (BCA) kit (Pierce Biosciences). 50 μ g of extract was separated on a 10 % SDS-PAGE and transferred to a nitrocellulose membrane (Amersham Biosciences, Uppsala, Sweden) and kept in blocking solution overnight at 4 °C. The membrane was then washed and probed with anti-Flag (1:1000) and anti-p53 (1:500) antibody (Pierce Biosciences), washed and incubated with secondary anti-mouse HRPO (horseradish peroxidase). The blot was developed using enhanced chemiluminescence (ECL) kit. The blot was stripped and probed using anti-tubulin (1:2000) antibody.

Microarray hybridisation and data analysis

Total RNA from mock-transfected, HPV11-E6 and HPV18-E6 cells were extracted using QiaZol and purified using Qiagen RNeasy MinElute Cleanup kit. Then, double-stranded cDNA was synthesized using the One-Cycle cDNA synthesis Kit (Affymetrix, Santa Clara, CA, USA). The cRNA, performed by in vitro transcription, was biotinylated using the IVT Labeling Kit according to the manufacturer's recommendations. The quantity and purity of biotinylated cRNA was determined spectrophotometrically and an aliquot of sample was checked by gel electrophoresis. The biotinylated cRNA was then fragmented and hybridised to the Affymetrix Human Genome-U133 Plus 2.0 GeneChips according to manufacturer's protocol. Then the arrays were

washed, stained with streptavidin-phycoerythrin and scanned with a GeneChip Scanner 3000 (Affymetrix). The scanned array images were processed with GeneChip Operating software (GCOS) and CEL files were extracted. Quality control, normalisation and proper hybridisation was assessed using GCOS. Gene Ontology mapping of selected genes were done using Panther database. Microarray analysis was done on triplicates for each sample group.

Quantitative real time PCR analysis

The microarray data was validated using quantitative real-time PCR (qRT-PCR) analysis of a few representative genes. From the samples, 5 μ g of total RNA was isolated and converted to cDNA as described above. For real-time PCR, 2 μ l of the cDNA was used for mRNA amplification using KAPA SYBR FAST PCR Universal Kit (Kapa Biosystems) in a Mini-Opticon Thermal Cycler (BIO-RAD) with the specific forward and reverse primers (Supplementary Table S1 for the list of primers). Glyceraldehyde-3-phosphate dehydrogenase house-keeping gene was used as an internal control for qRT-PCR. Melting curve analysis was done to confirm the specificity and purity of the amplified product.

Three independent PCR reactions were done for each of the genes. The relative expression of the target gene was calculated using the $2^{-\Delta C_T}$ (comparative threshold cycle) method. Relative expression of target gene = $2^{-\Delta C_T}$; $\Delta C_T = C_{T(\text{target gene})} - C_{T(\text{internal control GAPDH})}$. Student's *t* test was done to evaluate the results of qRT-PCR with *p* value < 0.05 was considered as significant and *p* < 0.001 considered highly significant.

Results

Confirming the stable transfection of EPC-2 cells

To detect the E6 gene within the EPC-2 cells, genomic DNA was isolated from mock, HPV11-E6 and HPV18-E6-transfected cells as described in "Materials and methods" section. The presence of E6 gene was confirmed by PCR using 200 ng of genomic DNA as described in Methods. The PCR amplification of HPV11-E6 and HPV18-E6 gave fragments of 112 bp and 423 bp respectively (Fig. 1a) which was of expected size. The E6 mRNA was detected using PCR of the cDNA obtained from transfected cells as described in Methods. HPV11-E6 and HPV18-E6 gave amplified products of 112 bp and 127 bp respectively (Fig. 1b) which was of expected size. The E6 expression in HPV11 and HPV18 was further validated using quantitative Real time PCR and normalized against β -actin control (Fig. 1c).

The E6 protein expression in the stable transfected cells were confirmed using anti-Flag antibodies (Fig. 1d). Both HPV11 and HPV18 E6 proteins were expressed to similar levels. β -tubulin was the loading control. The expression of E6 protein was also indirectly confirmed using p53, since high-risk E6 proteins are known to degrade p53 protein. The western blot of p53 protein showed that in the presence of HPV18E6 protein, the level of p53 was markedly reduced compared to mock-transfected EPC2 (Fig. 1e) while in case of HPV11E6 there was no reduction in p53 levels. These results suggest that E6 gene gets transcribed and translated inside the EPC2 cells.

Microarray analysis of genes differentially expressed in HPV18-E6, HPV11-E6 and mock transfected cells

Microarray analysis was done using Affymetrix Human Genome U133 Plus 2.0 Arrays to screen for the 47000 transcripts in human epithelial cells EPC-2 expressing E6 protein of either low-risk (HPV11) or high-risk (HPV18) or

control mock-transfected cells using triplicates for each sample group. The studies revealed a total of 697 genes showing ± 1.5 fold changes or more between the three samples. The genes showing significant changes ($p \leq 0.001$) were chosen for analysis. We identified 69 genes differentially expressing between HPV11E6-EPC2 and mock-EPC2, 315 genes between HPV18E6-EPC2 and mock-EPC2 and 302 genes between HPV18E6-EPC2 and HPV11E6-EPC2 (Fig. 2). The gene ontology mapping of 44 genes revealed physiologically important cellular pathways like angiogenesis, Alzheimer disease presenilin pathway, interleukin, integrin, insulin, chemokine, G protein, p53, Wnt, TGF- β , PI-3 kinase, PDGF (platelet derived growth factor) and cadherin signalling pathways (Fig. 3).

Genes showing differential expression between HPV18E6-EPC2 and mock-EPC2

Gene expression analysis showed that out of total 315 genes, 217 genes were upregulated and 98 genes down-regulated between HPV18E6-EPC2 and mock-transfected

Fig. 1 PCR detection of the E6 gene. **a** PCR OF E6 gene from the genomic DNA of mock-transfected EPC2 (E), HPV11E6-EPC2 (11) and HPV18E6-EPC2 (18), M represents 100 bp DNA molecular weight marker. 1.5×10^6 cells were used to isolate genomic DNA; 200 ng of genomic DNA was used for PCR. **b** Quantitative Real time PCR of E6 mRNA from the transfected EPC2 cells. Lanes are same as before. Total RNA was isolated from the three cell types, 1 μ g of total

RNA was used to synthesize cDNA which was used for qRT-PCR. **c** Graphical representation of E6 gene expression through qRT-PCR normalized against β -actin control. **d** Western blot of E6 protein using anti-Flag antibodies. **e** The western blot of p53 indirectly showing the expression of E6 protein and β -tubulin as loading control. 50 μ g of total protein was used for loading onto a 10 % SDS-PAGE. The lanes are same as before

Fig. 2 The genes upregulated and downregulated between mock-EPC2, HPV11E6-EPC2 and HPV18E6-EPC2. The genes showing differential expression between mock-transfected EPC2, HPV11E6-transfected EPC2 and HPV18E6-transfected EPC2 are shown as a Venn diagram. 217 genes were up- and 98 downregulated in HPV18E6-EPC2 compared to mock-EPC2, 211 genes were up- and 91 genes downregulated in HPV18E6-EPC2 compared to HPV11E6-EPC2 whereas 27 genes were up- and 42 genes downregulated in HPV11E6-EPC2 compared to mock-EPC2. These results are shown as Venn diagram

EPC2 cells. Some of the representative genes involved in apoptosis and cell cycle regulation, signal transduction, cell adhesion, metabolism and transcription regulation like α -catenin, CDC25A, STAT1, GPX3, IFI27, CELSR1, COL5A3, SIRPB1, SP3 etc. showed altered expression between the two samples (Table 1). The complete list of genes between the two samples is given as supplementary Table S2. The genes showing downregulation are indicated with minus sign.

Fig. 3 Gene Ontology mapping of genes according to different cellular pathways. Gene ontology mapping of 44 genes were done according to different biological pathways which revealed 13 different pathways namely angiogenesis, Alzheimer disease presenilin pathway, interleukin, integrin, insulin, chemokine, G protein, p53, Wnt, TGF- β , PI-3 kinase, PDGF and cadherin signalling pathways. The results are shown in the form of a broken pie chart

Genes showing differential expression between HPV11E6-EPC2 and mock transfected EPC2

Out of the total 69 genes, 27 genes showed an increase while 42 genes showed a decrease in HPV11E6-EPC2 cells compared to mock-transfected EPC2 cells. Representative genes were grouped according to their roles in apoptosis and cell cycle, signal transduction, cell adhesion, metabolism and transcription regulation namely HEATR4, GPR22, CDC25C, TRIM42, FOXI1 etc. (Table 2). The complete list of genes showing differential expression between the samples is shown as supplementary Table S3.

Genes showing differential expression between HPV18E6-EPC2 and HPV11E6-EPC2

Analysis of gene expression showed that 302 genes showed differential expression, out of which 211 genes showed an increase while 91 genes showed a decrease in HPV18E6-EPC2 compared to HPV11E6-EPC2. The full list of genes is given in supplementary Table S4. Some of the genes were functionally grouped according to their roles in apoptosis and cell cycle, signal transduction, cell adhesion, metabolism and transcription regulation (Table 3).

Quantitative real time PCR of some genes to confirm the microarray data

To confirm the microarray data, quantitative real time PCR was done for some representative genes like interferon

Table 1 Gene expression in HPV18E6-EPC2 compared to mock-EPC2 according to functions

Gene title	Gene symbol	Fold change
Up regulated genes		
Cell adhesion and cytoskeleton		
C-type lectin domain family 2, member D	CLEC2D	4.6
Catenin (cadherin-associated protein), alpha 1	CTNNA1	3.7
Regulator of G-protein signaling 4	RGS4	2.8
Cell cycle and apoptosis		
Hematopoietic SH2 domain containing	HSH2D	4.5
Promyelocytic leukemia	PML	4.3
Anaphase promoting complex subunit 13	ANAPC13	3.5
Pituitary tumor-transforming 2	PTTG2	3.8
v-rel reticuloendotheliosis viral oncogene homolog	v-REL	2.9
Retinoblastoma binding protein 6	RBBP6	2.5
Cell division cycle 25 homolog A (S. pombe)	CDC25A	2.3
B-cell CLL/lymphoma 11B	BCL11B	1.9
Cell division cycle and apoptosis regulator 1	CCAR1	1.6
asp (abnormal spindle) homolog, microcephaly associated associated	ASPM	1.3
Cell division cycle associated 7	CDCA7	1.2
Cell surface receptors and Signal transduction		
Signal transducer and activator of transcription 1	STAT1	3.9
EF-hand domain family, member A2	EFHA2	3.2
G protein-coupled receptor 39	GPR39	3.0
Phosphatidylinositol-4-phosphate 5-kinase, type I, alpha	PIP5K1A	2.9
Oncostatin M receptor	OSMR	2.5
Phosphoinositide-3-kinase, regulatory subunit 1 (p85 alpha)	PI3KR1	1.8
Epidermal growth factor receptor	EGFR	1.3
Rho GDP dissociation inhibitor (GDI) alpha	ARHGDIA	1.4
Metabolism		
UDP glycosyltransferase 8	UGT8	5.5
Cytochrome P450, family 1, subfamily B, polypeptide 1	CYP1B1	2.3
Glutathione peroxidase 3 (plasma)	GPX3	2.1
Eukaryotic translation initiation factor 2, subunit 3 gamma	EIF2S3	3.5
Transcription regulation		
Jumonji domain containing 2C	JMJD2C	4.2
Mesenchyme homeobox 2	MEOX2	4.1
Leucine rich repeat (in FLII) interacting protein 1	LRRFIP1	3.6
Ring finger protein 180	IL7	3.3
Nuclear receptor interacting protein 1	RNF180	1.9
Dicer1	NRIP1	1.7
Nuclear factor I/B	DICER1	1.5
Endothelial cell growth factor 1	ECGF1	1.4
Immune response		
Interferon, alpha-inducible protein 27	IFI27	5.6
Chemokine (C-X-C motif) ligand 11	CXCL11	4.2
Interferon, alpha-inducible protein 6	IFI6	2.4
Interleukin 7	IL7	2.2
Chemokine (C-X-C motif) ligand 10	CXCL10	1.3
Down regulated genes		
Cell adhesion and cytoskeleton		
Fibrous sheath interacting protein 1	FSIP1	-4.8

Table 1 continued

Gene title	Gene symbol	Fold change
Myosin, light chain 7, regulatory	MYL7	−4.7
Cadherin, EGF LAG seven-pass G-type receptor	CELSR1	−3.0
Laminin, alpha 2	LAMA2	−2.7
Collagen, type V, alpha 3	COL5A3	−2.6
Transgelin	TAGLN	−2.5
L1 cell adhesion molecule	L1CAM	−1.8
Myosin, light chain 9, regulatory	MYL9	−1.7
Parvin, beta	PARVB	−1.7
Keratin 4	KRT4	−1.5
Cell cycle and Apoptosis		
Tumor necrosis factor (ligand) superfamily, member 13	TNFSF13	−3.9
Cordon-bleu homolog (mouse)	COBL	−3.4
c-mer proto-oncogene tyrosine kinase	MERTK	−3.5
Cell surface receptors and Signal transduction		
Signal-regulatory protein beta 1	SIRPB1	−3.3
Ryanodine receptor 1 (skeletal)	RYR1	−2.3
Adrenergic, alpha-1B-, receptor	ADRA1B	−2.3
Insulin receptor	INSR	−2.1
Metabolism		
Arylsulfatase D	ARSD	−5.7
Peptidyl arginine deiminase, type IV	PADI4	−4.6
Glutamate dehydrogenase 2	GLUD2	−4.2
Ubiquitin specific peptidase 29	USP29	−3.6
Uncoupling protein 2 (mitochondrial, proton carrier)	UCP2	−3.4
UDP glucuronosyltransferase 1 family, polypeptide A6	UGT1A6	−3.1
Aldehyde dehydrogenase 5 family, member A1	ALDH1A1	−2.9
Dehydrogenase/reductase (SDR family) member 2	DHRS2	−1.8
Transcription regulation		
Zinc finger protein 804B	ZNF804B	−4.4
Zinc finger protein 251	ZNF251	−3.9
Zinc finger protein 542	ZNF542	−3.4
Zinc finger protein 568	ZNF568	−3.2
Double homeobox, 4	DUX4	−2.7
Activating transcription factor 5	ATF5	−2.7
Sp3 transcription factor	SP3	−2.3

inducible protein 27 (*IFI27*), catennin α 1 (*CTNNA1*), oncostatin M receptor (*OSMR*), cytochrome p450 1B1 (*CYP1B1*), tumour necrosis factor superfamily ligand 13 (*TNFSF13*), laminin α 2 (*LAMA2*) and collagen type V α 3 (*COL5A3*). The results are plotted for the mock-transfected EPC2, HPV11E6-transfected EPC2 and HPV18E6-transfected EPC2 as fold change (Fig. 4). The *IFI27* gene showed an increase of 5.5 fold for HPV18E6-EPC2 (18E6) compared to mock transfected-EPC2 (mock) which is given a value of 1.0 (Fig. 4). *CTNNA1* showed a decrease of 0.8 fold for 18E6, *OSMR* showed an increase of 1.74 fold for 18E6, *CYP1B1* had an increase of 0.76 fold for 18E6, *LAMA2* showed a decrease of 0.24 fold for 18E6,

TNFSF13 showed a decrease of 0.84 fold while *COL5A3* exhibited 0.95 fold reduction for 18E6 compared to mock (Fig. 4). All the genes showed correlation with the microarray data except *CTNNA1* which showed an opposite trend to the microarray data.

Discussion

In this study we have shown the effect of E6 oncoproteins from high-risk HPV18 and low-risk HPV11 types on the global gene expression profiles in the EPC2 cells. We have used the Human Genome U133 Plus 2.0 Array which has

Table 2 Gene expression in HPV11E6-EPC2 compared to mock-EPC2 according to functions

Gene title	Gene symbol	Fold change
Up regulated genes		
Cell adhesion and cytoskeleton		
Keratin 3	KRT3	3.5
Neuronal growth regulator 1	NEGR1	3.0
Cell cycle and Apoptosis		
HEAT repeat containing 4	HEATR4	4.9
Melanoma antigen family member 2, 3, 5	BAGE	4.6
Peroxisome proliferator-activated receptor alpha	PPARa	2.3
Cell surface receptors and Signal transduction		
G-protein coupled receptor 22	GPR22	5.1
CD69	CD69	3.8
Retinal G-protein coupled receptor	RGR	3.0
Neutrophil cytosolic factor 4	NCF4	2.0
Down regulated genes		
Cell adhesion and cytoskeleton		
Neuregulin 1	NRG1	-4.2
Similar to coxsackie virus and adenovirus receptor precursor		-3.9
Cadherin, EGF LAG seven-pass G-type receptor 1	CELSR1	-3.1
Apolipoprotein A-I	APO I	-2.9
Endomucin	EMCN	-1.4
Cell cycle and Apoptosis		
Cytochrome b-561 domain containing 2	CYB561D2	-4.1
CDK5 regulatory subunit associated protein 1-like 1	CDKAL1	-4.1
Cell division cycle 25 homolog C (S. pombe)	CDC25C	-3.3
Vasohibin 1	VASH1	-2.4
Cell surface receptors and Signal transduction		
IQ motif containing GTPase activating protein 3	IQGAP3	-5.1
Phosphodiesterase 1A, calmodulin-dependent	PDE1A	-3.2
RPA interacting protein	RPAIN	-3.1
Dopamine receptor D3	DRD3	-2.4
Metabolism		
ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 5	ST8SIA	-4.1
NHS-like 2	NHSL2	-3.3
Ankyrin repeat and SOCS box-containing 14	ASB14	-3.1
Peroxisome biogenesis factor 26	PEX26	-2.4
Transcription regulation		
Tripartite motif-containing 42	TRIM42	-4.2
Forkhead box I-1	FOXI-1	-4.0
Distal-less homeobox 6	DLX6	-3.8

47000 transcripts and 54000 probe sets. The microarray studies revealed 697 genes showing significant changes between mock-EPC2, HPV11E6-EPC2 and HPV18E6-EPC2 cells. The Gene Ontology mapping of 44 genes revealed 13 different signalling pathways; angiogenesis, alzhemier's, Wnt, p53, interleukin, TGF- β , cadherin, integrin, PI3-kinase, catennin, insulin, chemokine and G protein signalling pathways. From the microarray data, seven genes were selected for further confirmation by qRT-PCR,

namely *IFI27*, *CTNNA1*, *OSMR*, *CYP11B1*, *TNFSF13*, *LAMA2* and *COL5A3*.

IFI27 is an interferon inducible protein that has been reported to be upregulated in skin psoriasis and in some epithelial cancers [2]. It was also shown to have increased expression in squamous cell carcinoma of the skin [32]. In our studies *IFI27* was found to be upregulated in 18-E6 compared to mock or 11-E6. The increased expression of *IFI27* is likely to enhance the cytotoxic activity against

Table 3 Gene expression in HPV18E6-EPC2 compared to HPV11E6-EPC2 according to functions

Gene title	Gene symbol	Fold change
Up regulated genes		
Cell adhesion and cytoskeleton		
Catennin alpha 1	CTNNA1	4.6
Glycoprotein nmb	GPNMB	2.6
Transmembrane protein 47	TMEM47	2.4
Integrin beta 6	ITGB6	1.9
Paxillin	PXN	1.7
Collagen type 1 alpha 1	COL1A1	1.6
Cell cycle and apoptosis		
Wingless type, member 16	WNT16	5.3
Pvt1 oncogene homolog	PVT1	3.3
Myc induced nuclear antigen	MINA	4.4
Cell division cycle 14 homolog A (S. Cerevisae)	CDC14A	2.6
Cyclin dependent kinase inhibitor 1C (Kip2)	CDKN1C	2.4
Tumour protein p63	TP63	2.1
Phosphatase and tensin homolog	PTEN	1.8
XIAP associated factor 1	XAF1	1.8
B cell CLL/lymphoma 11B	BCL11B	1.6
Cell division cycle 25 homolog A (S. Pombe)	CDC25A	1.5
Abnormal spindle associated, microcephaly associated (Drosophila)	ASPM	1.5
Cell surface receptors and Signal Transduction		
Ras GEF domain family, member 1A	RASGEF1A	7.2
G protein coupled receptor 135	GPR135	4.0
Fibroblast growth factor receptor 2	FGFR2	3.8
Notch homolog 2	NOTCH2	2.4
EPH receptor B2	EPHB2	2.3
Signal transducer and activator of transcription	STAT1	2.1
Transcription regulation		
Ring finger protein 180	RNF180	5.5
Alx homeobox 1	ALX1	5.1
Highly divergent homeobox	HDX	4.3
Zinc finger protein 326	ZNF326	3.0
Interferon regulatory factor 6	IRF6	2.8
Zinc finger protein 667	ZNF667	2.7
Zinc finger protein 711	ZNF711	2.1
Peroxisome proliferator-activated receptor gamma, coactivator 1 beta	PPARGC1B	1.8
SMAD family, member 3	SMAD3	1.6
Forkhead box O3	FOXO3	1.5
Metabolism		
Aldehyde dehydrogenase 1 family, member A1	ALDH1A1	4.5
UDP glycosyl transferase 8	UGT8	4.1
NADPH oxidase 4	NOX4	3.4
Cytochrome P450 family 1, subfamily B1	CYP1B1	2.0
Acyl CoA dehydrogenase family, member 10	ACAD10	1.9
Glutathione peroxidase 7	GPX7	1.6
Ubiquitin specific peptidase 31	USP31	1.5
Immune response		
Chemokine ligand 20	CCL20	4.2
Chemokine ligand 11	CXCL11	4.0

Table 3 continued

Gene title	Gene symbol	Fold change
Interferon alpha inducible protein 6	IFI6	2.4
Interferon induced protein 44	IFI44	2.0
Interferon gamma inducible protein 16	IFI16	1.5
Downregulated genes		
Cell adhesion and cytoskeleton		
C-type lectin like 1	CLEC1	−4.4
Collagen type V, alpha 3	COL5A3	−2.4
L1 cell adhesion molecule	LCAM	−1.6
Cadherin 2, type1 N-cadherin	CDH2	−1.5
Cell surface receptors and signal transduction		
Neurotrophic tyrosine receptor kinase 2	NTRK2	−4.2
c-GMP dependent protein kinase, type 1	PRKG1	−3.0
G protein coupled receptor 22	PRKG1	−3.8
Glycophorin A	GYPA	−2.8
Insulin receptor	INSR	−2.0
Transcription regulation		
Leucine rich repeat neuronal 1	LRRN1	−4.7
Zinc finger protein 257	ZNF257	−3.9
Zinc finger protein 542	ZNF542	−2.9
Sp3 transcription factor	SP3	−2.6
Metabolism		
Arylsulfatase D	ARSD	−4.3
Uncoupling protein 2	UCP2	−3.8
Aldehyde dehydrogenase family 5, member A1	ALDH5A1	−3.7
Arylsulfatase F	ARSF	−3.2
Immune response		
CD22 molecule	CD22	−3.2

The genes are grouped according to their cellular functions

tumour cells and thereby help in containment of the tumour. *OSMR* bind to its ligand oncostatin M (OSM), the latter function to inhibit cell growth by the induction of apoptosis. Ectopic expression of *OSMR* in lung adenocarcinoma cells resulted in reduced cell death by acting as decoy receptor to remove OSM [20], conversely blocking the *OSMR* expression through siRNA enhanced sensitivity of the cells towards OSM. Furthermore, *OSMR* has been found to be upregulated in majority of ovarian carcinomas and is associated with the malignant phenotype [29]. From the microarray and qRT-PCR data, we have observed an increase in *OSMR* expression in 18E6 compared to 11E6 and the mock; this could be a strategy used by E6 protein to induce tumour formation in epithelial cells. *CTNNA1* has been shown to be downregulated in human squamous cell carcinoma of the oesophagus [15, 27], our microarray data showed upregulation of the gene while the qRT-PCR showed a downregulation in 11E6 and further downregulation in 18E6 compared to mock which suggest that 18E6 might be more potent in inhibiting the *CTNNA1* expression

than 11E6. *CYP1B1* is a metabolic enzyme involved in degradation of environmental carcinogens like benzo(α)-pyrene and other polycyclic hydrocarbons (PAHs) present in environmental pollution and cigarette smoke which forms DNA adducts [22], and converts them to soluble products which are excreted out. Elevated *CYP1B1* expression may result in a greater load of reactive oxygen intermediates (ROMs) causing genotoxic stress and tumour initiation [12, 37]. There has been no report on the role of *LAMA2* in cancer development; however from our studies we have observed a downregulation of *LAMA2* expression. The gene product encodes a protein involved in cell–cell and cell–matrix adhesion, therefore downregulation of *LAMA2* might help in the systemic spread of cancerous cells. In the case of *TNFSF13*, their role in cancer is not clear but other family members APRIL (a proliferation inducing ligand) and BAFF (B cell activating factor) play a role in chronic lymphocytic leukemia (CLL) [35] by increasing the survival of leukemic cells through blocking the apoptosis. The shRNA mediated blocking of APRIL

Fig. 4 Quantitative Real time PCR of genes. The quantitative real time PCR of genes *IFI27*, *CTNNA1*, *OSMR*, *CYP1B1*, *TNFSF13*, *LAMA2* and *COL5A3* were done from the RNA of HPV18E6-EPC2, HPV11E6-EPC2 and mock-EPC2 cells. The results are shown as fold change compared to mock EPC2 which is given a value of 1. The graph represents the mean $\pm$ standard deviation for three independent experiments. The * indicates significant ($p < 0.05$) and ** indicates highly significant differences ($p < 0.001$) compared to control mock-EPC2 cells

resulted in reduced growth of pancreatic tumour cells [46]. From our studies we observe an increase in 11E6 and a decrease in 18E6 compared to the mock. Thus, the E6 proteins from the two types of HPV are likely to differentially regulate *TNFSF13* expression resulting in different outcomes. *COL5A3* encodes for a cell adhesion protein. The *COL5A3* expression was downregulated in 11E6 and further downregulated in 18E6. Although there has been no report about the role of *COL5A3* in cancer, *COL5A2* has been shown to be downregulated in squamous cell carcinoma of the uterine cervix [3].

This is the first report showing global gene expression profiles by high-risk (HPV18) and low-risk (HPV11) E6 oncoproteins. The genes identified by microarray and qRT-PCR could be used as biomarkers for diagnosis and progression of oesophageal cancer as well as therapeutic targets. Analysis of the gene expression could give an insight about the role of E6 proteins, especially the low-risk types in oesophageal cancer. It could help to identify potential drug targets for the therapy of oesophageal cancer as well as identification of molecules involved in the advanced stages of the disease.

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