*Original Research*

# Differential Transcriptomic Signatures between Early and Late Passages of Bovine Horn Core Carcinoma Culture *In-Vitro*

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**Abstract**

*Squamous cell carcinoma or SCC of horn in bovines (bovine horn core carcinoma) frequently observed in Bos indicus affecting almost 1% of cattle population. Freshly isolated primary epithelial cells may be closely related to the malignant epithelial cells of the tumor. Whole transcriptome sequencing of horn's SCC tissue derived early passage BHCC cells and late passage BHCC cells were done using Ion Torrent PGM$^R$. Comparative expression and analysis of different genes and pathways were observed. Cancer related TGF beta signalling pathway, PI3K/Akt signaling, Ubiquitin mediated proteolysis in early passage BHCC cells and pentose phosphate pathway in late passage BHCC cells were discussed. The cells at later passages could retain the transcriptomic signature of horn cancer.*

**Key words:** Cell Culture, Cancer Pathways, Differential Gene Expression, Horn Cancer, NGS, Transcriptome

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## Introduction

Cell lines *In vitro* usually provide powerful tools for identification of potential molecular targets for therapeutic intervention as well for initial pre-clinical evaluation of novel drug molecules (Feldmann *et al.*, 2009). The results of the research in horn core squamous cell carcinoma cell lines can usually be extrapolated to *in vivo* tumors originated from squamous cells in bovines (Shil *et al.*, 2017). Transcriptomic profiling of the initial passage or low passage cells and Late Passage BHCC cells were attempted at present to study the differences in transcriptome in these two in vitro conditions at molecular level. The differences

will highlight whether in higher passages this cells could retain the transcriptomic profile of parental tumor or not.

**Review of Literature**

Availability and use of *In vitro* cultures of SCC of horn (BHCC) in bovines have been limited. Most of the cell lines are not representative of original squamous cell carcinoma (Cifola *et al.,* 2011). Furthermore, it is always of concern that how extensively alteration happens while in passage for a long term in biological properties of cell lines than the earlier passages (Craven *et al.,* 2006), which rather are enriched in tumour cell component (Cifola *et al.,* 2011) and retains phenotypic, transcriptomics profile of the corresponding tissues from which they derive (Perego *et al.,* 2005; Craven *et al.,* 2006 and Bianchi *et al.,* 2010). Cellular activity within a tissue is evinced by the transcriptome at a specific time (Shil *et al.,* 2017). Genome-wide expression studies can be used to evaluate pathophysiology of complex diseases like cancer (Bianchi *et al.,* 2010). Eukaryotic transcriptome can be best understood by RNA Sequencing (RNA-Seq) by read mapping on to the reference eukaryotic genome (Twine *et al.,* 2011).

SCC of horn of bovines is a squamous cell carcinoma of horn core mucosa with least known genetic landscape, reported only in *Bos indicus* (Shil *et al.,* 2017). This causes heavy economic losses due to subsequent metastasis and death of animal. Till date, the comparison of transcriptome profile between cell culture of early passage and parental tissue of SCC of horn of bovines has been performed (Shil *et al.,* 2017). This study was designed to compare transcriptome profiles in between SCC affected horn tissue derived early passage BHCC cells and late passage BHCC cells sub cultured from that early passage in vitro for 45 cell doublings, using Ion Torrent PGM$^{R}$ sequencing platform.

**Materials and Methods**

**Ethical Approval**

This research work was granted vide approval no. IAEC: 155/2011 of College of Veterinary Science and animal Husbandry, Anand Agricultural University, Anand-388 001, Gujarat.

**Tissue Collection**

Carcinomatous and normal horn core mucosa were collected during corrective surgery in RNAlater® (Thermo Fisher scientific, Massachusetts, USA) from clinically affected (left horn) and normal (right horn) horn of a Kankrej breed of bullock (age 7 years) from Rajkot, Gujarat, India. Necrotic tissues were not collected. Fresh tissues were cut into pea-sized segments and preserved in methods as described by Shil *et al.* (2017).





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**Histopathology**

Horn SCC tissues were processed and observed for histopathological studies as described in Shil *et al.* (2017). The tissues were stained with Haematoxylin and Eosin (H&E); Luna (1968).

**Cell Culture**

Tumour tissues (at 4°c) were mechanically minced in 1 $mm^3$ fragments after removal of adipose tissue. Then primary culture was established and this finite cell line was sub cultured at a split ratio of 1:3 up to $15^{th}$ generation and 45 doublings. The sub culturing was done using methods as described in Shil *et al*. (2017). Similarly tumour tissue explant culture was also performed by standard protocol (Freshney, 2005). By inverted microscope (Leica, Germany), cell morphology was observed in contrast phase, at 40X magnification. Differential trypsinization and dilution cloning were performed as described in Shil *et al.* (2017). These isolated clones were used for RNA-Sequencing purposes for both the cell passages.

**Cell Proliferation and Doubling Time Assay**

Two counts were performed for each passage, in triplicate. For doubling time analysis plating and counting of cells were done as per Shil *et al.* (2017). Software for analysing doubling time (in hour) was used as described in a previous study (Roth, 2006).

**RNA Isolation**

TRIzol$^R$ (Sigma-Aldrich, St. Louis, USA) method as per manufacturer's instructions was used to isolate RNA from early passage (pooled RNA of passage 2 & 3) and late passage (pooled RNA of passage 15, 45 doublings) cells.

**Preparation of Sample and Transcriptome Procedure**

All the protocols starting from mRNA isolation to library preparation were followed as per manufacturer's instructions and as described in Shil *et al*. (2017). The detailed protocol steps can be accessed from Ion Torrent's 'Ion Total RNA-Seq Kit' (Part No.: 4467098) using 316 chip.

**In Silico Gene Expression Analysis**

Sequence reads were generated from cDNA libraries of Early Passage BHCC cells and Late Passage BHCC cells using Ion Torrent PGM chemistry using 316 chips (Koringa *et al.*, 2013). Raw sequence reads (*fastq files) were checked for quality control in Fast QC v0.10.1.The read alignments and calculation of differential expression by Cuffdiff v 2.2.1 were done by methods as described in Shil *et al.* (2017).







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**RNA-Seq Data Normalization**

The raw RNA-Seq read counts for cufflinks transcripts were first $\log_2$ transformed at fragments per kilo base of exon per million reads mapped (FPKM) and then quantile normalized.

**Functional Annotation**

The genes differentially expressed in short term primary culture and the long term late passage was selected for functional categorization. The comparisons between expressed genes which produced Cuffdiff output with ''Q value'' less than 0.01 and ''OK'' marked test status were considered to be differentially expressed. Gene ontology (GO) and pathway analyses of up and down regulated genes by DAVID database (Huang *et al*., 2008). The criteria set for genes to be used in gene set analyses were same as described in Shil *et al.* (2017). Being practically impossible and economically non-feasible to validate all of the genes, we have followed standard procedure to validate NGS data by selecting randomly selected sufficient set of transcripts and concordance of expression pattern has been proved using quantitative real time PCR (Data not shown).

**Results**

**Histopathology of SCC tissue**

The tumour cells were featured with moderately high to abundant eosinophilic cytoplasm, potentially increased nucleus to cytoplasmic ratio, focal presence of intercellular bridges. Keratinized epithelial cells (Fig. 1) and pleomorphism of epithelial cells were seen. Histopathology confirmed squamous cell carcinoma of the horn core epithelium.

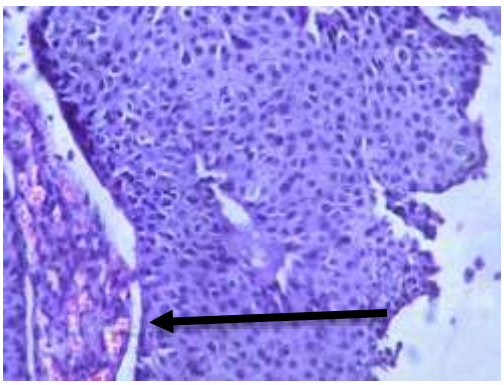

**Fig.1:** Histopathology of BHCC Tumor tissue with H&E stain at 100X (Arrow indicating keratinization of cells

**Isolation of SCC Horn Epithelial Cells**

Primary monolayer culture with finite mitotic lifespan (BHCC early passage cells) was established from the Bullock affected with BHCC of horn (Fig. 2) following the enzymatic dis-aggregation methods as





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described earlier in Shil *et al*. (2017). Multinucleated bizarre tumour cells and spindle to polygonal shaped cells having vesicular nuclei and multiple small nucleoli were frequently seen at later passages of culture (Fig. 3).

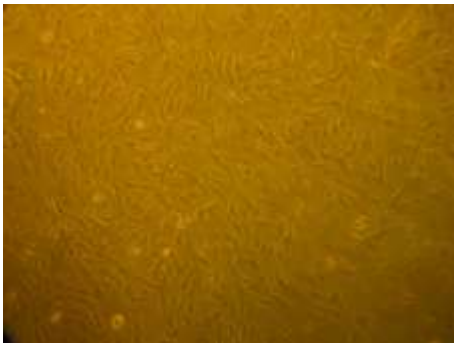

**Fig. 2:** Primary monolayer culture of BHCC early passage cells with phase contrast photomicrograph at 40X.

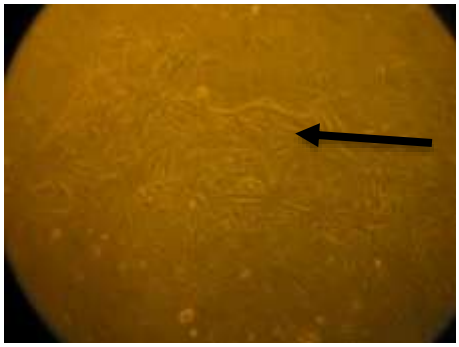

**Fig. 3:** Monolayer culture of BHCC late passage cells with phase contrast photomicrograph at 40X. (Arrow indicates multinucleated cells)

**Growth Curve and Population Doubling Time Analysis**

Population doubling time ascertained around 28.1 hour and 58.51 hour respectively for passage 2 (P2) and passage 15(P15) of horn cancer cells (Fig. 4 and Fig. 5) and cell viability ranged from 85 to 94%. Marked increase in the time required for cell doubling were observed and it showed an enlarged, flattened cellular morphology at P15 and remained viable in culture.

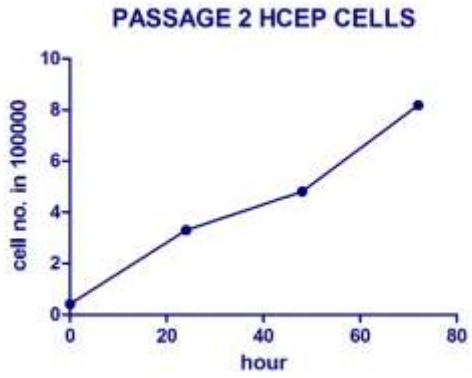


**Fig. 4:** Growth curve of BHCC cells at P2

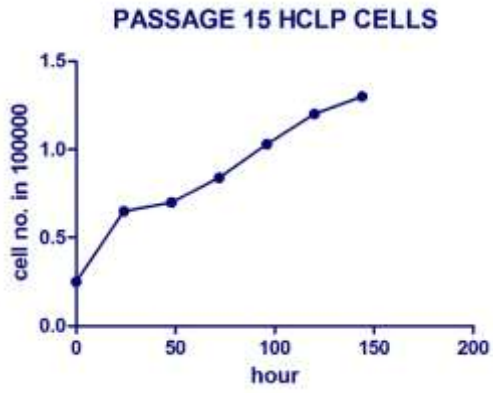


**Fig. 5:** Growth curve of BHCC cells at P15

**Transcriptomic Comparison between Early Passage BHCC cells and Late Passage BHCC cells**

Quality checking of reads of both the early passage BHCC cells (HCEP) and late passage BHCC cells (HCLP) were done by FASTQC (Table 1). The total number of genes (having FPKM value more than five) differentially over expressed in early passage BHCC cells was 308 no. and in HCLP, 340 no. genes were differentially up-regulated over HCEP. In this comparison 12125 genes (∽83.55% of total genes no. i.e.







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14512 no.) showed no expression at the terms of fragments per kilobase of transcript per million mapped reads (FPKM) in both the samples.

**Table 1:** Quality checking of reads by FASTQC

| Category | HCEP | HCLP |
|---|---|---|
| Total Sequences | 1581227 | 1018138 |
| Sequences flagged as poor quality | 0 | 0 |
| Sequence length | 8-481 | 8-619 |
| %GC | 50 | 50 |

**Genes over Expressed in Early Passage BHCC Cells and Late Passage BHCC Cells**

Mapping statistics of sequenced reads by Gmap-Cufflinks were derived (Table 2).

**Table 2:** Mapping statistics of sequenced reads by Gmap-Cufflinks

| | HCEP | HCLP |
|---|---|---|
| Mapping Percentage | 59.5%. | 62.5%. |
| Total Reads | 1581227 | 974421.5 |
| Mapped Reads | 941601 | 608928 |

Results obtained from Cuff-Diff (*diff) from two Transcript assembly (By Cufflinks) obtained from two consequent reads of HCEP and HCLP were visualized by 'CummeRbund' package in R environment (Goff *et al.,* 2013). Density plot & dispersion plot were derived for this comparison respectively (Fig. 6 & Fig. 7). Density plot assessed the distributions of FPKM scores across samples. Amongst the differentially expressed genes maximum genes had FPKM value between $Log_{10}$ 1 and $Log_{10}$ 3. Dispersion plot showed normal dispersion of genes across samples (Fig. 7).

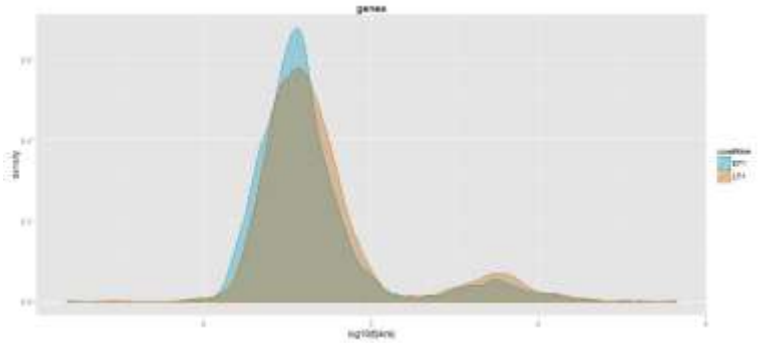

**Fig. 6:** Density plot of genes of HCEP & HCLP based on transformed FPKM value







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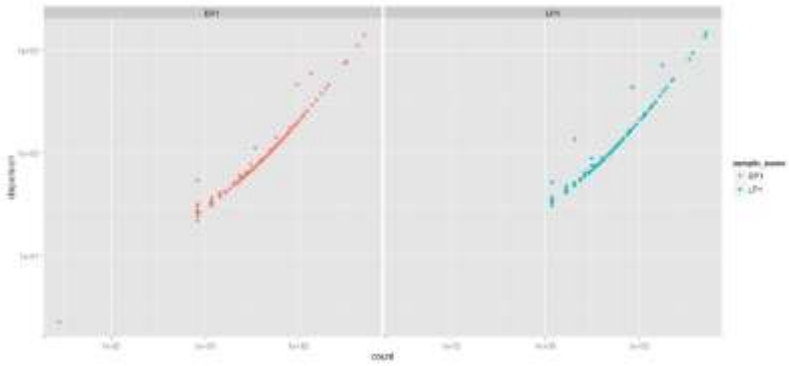

**Fig. 7:** Dispersion plot of Genes of HCEP & HCLP

Gene ontology category of the genes (having FPKM value >5) differentially expressed above 2 $\log_2$ fold change in early passage BHCC cells compared to late passage BHCC cells found to be of extracellular exosome, focal adhesion, proteinaceous extracellular matrix etc. as per DAVID database (Table 3).

**Table 3:** GO of up-regulated genes (≥2 fold) in HCEP than HCLP

| Term | Count | % | P Value | Official Gene Symbol | Fold Enrichment | FDR |
|---|---|---|---|---|---|---|
| GO:0070062~extracellular exosome | 21 | 42 | 0 | ACTB, EPDR1, EFEMP2, LUM, S100A11, GJA1, NID2, CAPN2, TIMP3, HSPH1, PFN2, HSP90B1, APP, BGN, EZR, SERBP1, COL1A2, RHOA, COL6A1, RPL7A, LAMC1 | 3.12 | 0 |
| GO:0005925~focal adhesion | 8 | 16 | 0 | ACTB, HSP90B1, EZR, RHOA, CD99, GJA1, RPL7A, CAPN2 | 8.1 | 0.05 |
| GO:0005578~proteinaceous extracellular matrix | 6 | 12 | 0 | BGN, LUM, COL3A1, FBN1, COL6A1, TIMP3 | 10.79 | 0.24 |
| GO:0005615~extracellular space | 10 | 20 | 0 | APP, EZR, LUM, COL3A1, FBN1, S100A11, COL1A2, PTN, COL1A1, TIMP3 | 3.39 | 2.31 |
| GO:0005829~cytosol | 10 | 20 | 0 | ACTB, HSPH1, HSP90B1, EZR, MAP1B, RHOA, GJA1, DPYD, CAPN2, PPP2R2A | 3.14 | 3.85 |
| GO:0005584~collagen type I trimer | 2 | 4 | 0 | COL1A2, COL1A1 | 322.1 | 6.97 |
| GO:0005768~endosome | 4 | 8 | 0 | APP, EZR, SNX5, RHOA | 9.13 | 10.15 |
| GO:0005604~basement membrane | 3 | 6 | 0.01 | NID2, LAMC1, TIMP3 | 18.94 | 11.76 |
| GO:0005856~cytoskeleton | 4 | 8 | 0.01 | ACTB, PFN2, CALD1, RHOA | 8.05 | 14.01 |
| GO:0005581~collagen trimer | 3 | 6 | 0.01 | COL3A1, COL1A2, COL1A1 | 16.95 | 14.36 |
| GO:0035253~ciliary rootlet | 2 | 4 | 0.02 | APP, KIF5B | 80.52 | 25.11 |
| GO:0005764~lysosome | 3 | 6 | 0.06 | EPDR1, GJA1, CAPN2 | 6.85 | 56.88 |
| GO:0043209~myelin sheath | 3 | 6 | 0.06 | ACTB, EZR, CLTC | 6.85 | 56.88 |
| GO:0005874~microtubule | 3 | 6 | 0.07 | HSPH1, KIF5B, MAP1B | 6.31 | 62.25 |

The genes which were up-regulated in late passage BHCC cells compared to its early passage BHCC cells showed membrane, endoplasmic reticulum exit site, catalytic step 2 spliceosome etc. (Table 4).







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**Table 4:** GO of genes up-regulated (≥2 Fold) in HCLP than HCEP

| Term | Count | % | P Value | Official Gene Symbol | Fold Enrichment | FDR |
|---|---|---|---|---|---|---|
| GO:0016020~membrane | 9 | 23.68 | 0 | ALDOA, EIF3D, CKAP5, KLC1, HNRNPF, ILK, RPS11, DDX5, HNRNPH1 | 4.05 | 1.14 |
| GO:0070971~endoplasmic reticulum exit site | 2 | 5.26 | 0.01 | TMED5, PDCD6 | 135.62 | 13.86 |
| GO:0071013~catalytic step 2 spliceosome | 3 | 7.89 | 0.01 | HNRNPF, DDX5, HNRNPH1 | 14.36 | 16.79 |
| GO:0070062~extracellular exosome | 10 | 26.31 | 0.06 | ALDOA, CYB5R3, ERP44, TALDO1, CD44, RPS11, DDX5, PDCD6, ITM2C, PCOLCE2 | 1.88 | 49.95 |

**Table 5:** KEGG pathway of genes up-regulated (≥2 Fold) in SCC Early Passage BHCC cells significantly over Late Passage cells

| Term | P Value | Official Gene Symbol | Fold change | Fold Enrichment | FDR |
|---|---|---|---|---|---|
| bta04510:Focal adhesion | 0 | ACTB | -2.77 | 8.6 | 0.02 |
| | | COL3A1 | -3.51 | | |
| | | RHOA | -2.44 | | |
| | | COL1A2 | -3.07 | | |
| | | COL6A1 | -2.07 | | |
| | | COL1A1 | -2.21 | | |
| | | LAMC1 | -2.11 | | |
| | | CAPN2 | -2.09 | | |
| bta04974:Protein digestion and absorption | 0 | SLC38A2 | -2.02 | 13.3 | 0.48 |
| | | COL3A1 | -3.51 | | |
| | | COL1A2 | -3.07 | | |
| | | COL6A1 | -2.07 | | |
| | | COL1A1 | -2.21 | | |
| bta04512:ECM-receptor interaction | 0 | COL3A1 | -3.51 | 12.8 | 0.55 |
| | | COL1A1 | -2.21 | | |
| | | COL1A2 | -3.07 | | |
| | | COL6A1 | -2.07 | | |
| | | LAMC1 | -2.11 | | |
| bta04611:Platelet activation | 0 | ACTB | -2.77 | 8.8 | 2.27 |
| | | COL1A2 | -3.07 | | |
| | | COL3A1 | -3.51 | | |
| | | RHOA | -2.44 | | |
| | | COL1A1 | -2.21 | | |
| bta04151:PI3K-Akt signalling pathway | 0 | HSP90B1 | -3.05 | 4.5 | 3.65 |
| | | COL6A1 | -2.07 | | |
| | | PPP2R2A | -3.04 | | |
| | | COL3A1 | -3.51 | | |
| | | COL1A2 | -3.07 | | |
| | | COL1A1 | -2.21 | | |
| | | LAMC1 | -2.11 | | |
| bta05205:Proteoglycans in cancer | 0.01 | ACTB | -2.77 | 5.5 | 11.46 |
| | | TIMP3 | -2.04 | | |
| | | EZR | -3.54 | | |







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| | | | | | |
|---|---|---|---|---|---|
| | | LUM | -3.23 | | |
| | | RHOA | -2.44 | | |
| bta05146:Amoebiasis | 0.01 | COL3A1 | -3.51 | 8 | 12.72 |
| | | LAMC1 | -2.11 | | |
| | | COL1A2 | -3.07 | | |
| | | COL1A1 | -2.21 | | |
| bta04670:Leukocyte transendothelial migration | 0.01 | ACTB | -2.77 | 7.5 | 14.81 |
| | | EZR | -3.54 | | |
| | | CD99 | -2.21 | | |
| | | RHOA | -2.44 | | |
| bta04141:Protein processing in endoplasmic reticulum | 0.04 | HSPH1 | -2.23 | 5.3 | 33.24 |
| | | SEC61G | -2.52 | | |
| | | HSP90B1 | -3.05 | | |
| | | CAPN2 | -2.09 | | |
| bta05100:Bacterial invasion of epithelial cells | 0.04 | ACTB | -2.77 | 8.7 | 38.68 |
| | | RHOA | -2.44 | | |
| | | CLTC | -2.03 | | |
| bta04810:Regulation of actin cytoskeleton | 0.06 | ACTB | -2.77 | 4.2 | 51.56 |
| | | PFN2 | -2.983 | | |
| | | EZR | -3.54 | | |
| | | RHOA | -2.44 | | |

There were few pathways like focal adhesion, protein digestion & absorption, ECM receptor interaction etc in 5 FDR limit for differentially up regulated genes (Official gene symbols) (having FPKM value >5) in early passage BHCC cells compared to late passage BHCC cells in Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathways (Table 5). Surprisingly most of the genes which showed top fold change (within first twenty) were detected by DAVID during pathway analysis. Pentose phosphate pathway was the only pathway under 5 FDR value shown by the genes which were up-regulated in late passage BHCC cells than early passage BHCC cells (Table 6).

**Table 6:** KEGG pathway of genes up-regulated (≥2 Fold) in HCLP than HCEP

| Term | Count | P Value | Genes | Fold Change | Fold Enrichment | FDR |
|---|---|---|---|---|---|---|
| bta00030:Pentose phosphate pathway | 2 | 0.05 | ALDOA | 2.35444 | 35.1 | 39.56 |
| | | | TALDO1 | 2.54398 | | |

**Discussion**

Reliable prediction of a cancer's grade and stage is very important as it can provide useful information for cancer mechanism studies as well as for selection of the most appropriate treatment plans. In this study, we compared gene expression profiles of the two conditions i.e. *In vitro* cancer cells at their early passages and at their late passages to identify the changes in gene expression *In vitro* with time, so that it could be extrapolated to that of *In vivo* changes during the course of the horn cancer progression. The early passage BHCC cells grew & survived well for the first fifteen passages without difficulties. The cellular compositions were homogeneous and were of morphological characteristics typical of squamous cell





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epithelium. Maximum value of differential gene expression in early passage BHCC cells was 3.54 fold changes (all genes >5 FPKM value) as compared to late passage cells. The genes which were differentially up regulated in early passage BHCC cells were also found to be involved in other carcinomas as well (Table 7).

**Table 7:** Implications of genes mined from KEGG pathway & upregulated in SCC early passage BHCC cells significantly over late passage cells

| S. No. | Official Gene Symbol | Implications of the Genes in Cancer |
|---|---|---|
| 1 | ACTB | De-regulated in various cancers as in breast, prostate, ovarian cancers and its up-regulation is associated with invasiveness and metastasis; Guo et al (2012). |
| 2 | CAPN2 | Calpain 2 promotes tumour cell growth both *in vitro* and *in vivo* through the PI3K-Akt-FoxO-$p27^{Kip1}$ signalling cascade; Ho et al (2012). |
| 3 | COL3A1 | Highly up-regulated in colorectal cancer patient by deep sequencing; Wu et al (2012). Promotes CRC cell proliferation by stimulating PI3K-AKT signalling; Wang X-Q et al (2016).COL3A1 might affect cell migration or invasion through MAPK signalling pathway; Yuan et al (2017). |
| 4 | RHOA | RhoA is needed for cancer cell extravasation; in vitro, can stimulate transformation and plays a key role in EMT; Parri and Chiarugi (2010). |
| 5 | COL1A2 | Enhanced expression as metastasis-associated gene signature was observed in diffuse type gastric cancers; Jinawath et al (2004).Higher COL1A1 and COL1A2 expression levels were related to lower overall survival in gastric cancer patients; Li et al (2016). COL1A2, involved in epithelial to mesenchymal transition and angiogenesis; Tamilzhalagan et al (2017). |
| 6 | COL6A1 | COL6A1 upregulation indicates concurrence with inactivation of other tumour suppressors and may represent a signal of poor prognosis; Wan et al (2015). Overexpression was associated with accelerated cell proliferation via JAK-STATs pathway and elevated vitality in prostate cancer cells; Zhu Y-P (2015).Knock-down of COL6A1 suppresses the metastatic ability of cancer cells; Hou et al (2016). |
| 7 | COL1A1 | COL1A1 usually upregulated in invasive HCC; Hayashi et al (2014). COL1A1 has also been reported to regulate proliferation and migration in gastric cancer cells; Li et al (2016). Regulates cell death of cervical cells via Caspase-3/PI3K/AKT pathways, abundant COL1A1 inhibit the cell death; Liu et al (2017). |
| 8 | LAMC1 | Silencing of LAMC1 significantly inhibited cell migration and invasion in cancer cells, and LAMC1 functions to promote metastasis; Nishikawa et al (2014). Overexpression of LAMC1 was associated with tumour growth and invasion; Zhang et al (2017). |
| 9 | SLC38A2 | It increases in cancer cells as a response to amino acid starved condition; Broer et al (2015). |
| 10 | HSP90B1 | Acts as molecular chaperones with roles in stabilizing and folding other proteins and in lung cancer adenocarcinoma tissues it has been shown to be upregulated than the non-malignant tissues; Wu et al (2015). |
| 11 | PPP2R2A | A potential tumour suppressor gene; Cheng et al (2011). |
| 12 | LUM | High levels are associated with a poor prognosis in some cancers and a better prognosis in others; Nikitovic et al (2014). |
| 13 | TIMP3 | Loss of TIMP3 correlates with advanced-stage disease and poor prognosis in colorectal, breast, brain, bladder and particularly head and neck squamous cell carcinoma (HNSCC); Jackson et al (2016). |
| 14 | EZR | High expression correlates with poor outcome in cancer patients; Li et al (2015). |
| 15 | PFN2 | EMT contributes to tumour invasion and metastasis in various cancers; Cui et al (2016). |
| 16 | CD99 | The invasive cervical squamous cell carcinoma showed significantly increased expression of CD99; Zhou et al (2014). |
| 17 | HSPH1 | Upregulation is associated with poor outcome in many cancers; Yang et al (2015). |
| 18 | CLTC | Associated oncogene to Breast cancer; Yao et al (2015). |







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The unique genes expressed in early passage BHCC cells showed TGF beta signalling, ubiquitin mediated proteolysis, insulin signalling pathway etc. (Table 8). GO of unique genes showed these were involved in focal adhesion, extracellular exosome, cytosol etc. (Table 9).

**Table 8:** KEGG Pathway of all genes uniquely expressed (FPKM >5) in HCEP

| Term | Count | P Value | Fold Enrichment | FDR |
|---|---|---|---|---|
| bta04350:TGF-beta signalling pathway | 14 | 0 | 2.79 | 1.69 |
| bta05161:Hepatitis B | 20 | 0 | 2.23 | 1.82 |
| bta04120:Ubiquitin mediated proteolysis | 18 | 0 | 2.19 | 4.19 |
| bta04910:Insulin signaling pathway | 18 | 0 | 2.19 | 4.19 |
| bta00640:Propanoate metabolism | 7 | 0 | 4.45 | 4.9 |
| bta03040:Spliceosome | 17 | 0 | 2.18 | 5.85 |
| bta04921:Oxytocin signaling pathway | 19 | 0 | 2.05 | 6.14 |
| bta05205:Proteoglycans in cancer | 23 | 0 | 1.87 | 6.76 |
| bta04022:cGMP-PKG signaling pathway | 20 | 0 | 1.98 | 7.02 |
| bta00020:Citrate cycle (TCA cycle) | 7 | 0 | 3.85 | 10.04 |
| bta05016:Huntington's disease | 22 | 0 | 1.83 | 10.72 |
| bta04390:Hippo signaling pathway | 18 | 0 | 1.97 | 11.58 |
| bta04152:AMPK signaling pathway | 15 | 0 | 2.07 | 15.8 |
| bta04141:Protein processing in endoplasmic reticulum | 19 | 0 | 1.86 | 15.95 |
| bta01200:Carbon metabolism | 14 | 0 | 2.12 | 16.66 |
| bta04510:Focal adhesion | 22 | 0 | 1.75 | 16.71 |
| bta05211:Renal cell carcinoma | 10 | 0 | 2.5 | 20.06 |
| bta04114:Oocyte meiosis | 14 | 0 | 2.07 | 20.18 |
| bta03015:mRNA surveillance pathway | 12 | 0 | 2.23 | 20.83 |
| bta03008:Ribosome biogenesis in eukaryotes | 11 | 0 | 2.3 | 23.1 |
| bta00670:One carbon pool by folate | 5 | 0 | 4.59 | 23.65 |
| bta05012:Parkinson's disease | 17 | 0 | 1.85 | 24.03 |
| bta05010:Alzheimer's disease | 19 | 0 | 1.76 | 24.67 |
| bta01130:Biosynthesis of antibiotics | 21 | 0 | 1.68 | 26.86 |
| bta01100:Metabolic pathways | 92 | 0 | 1.22 | 29.23 |
| bta04261:Adrenergic signalling in cardiomyocytes | 16 | 0 | 1.84 | 29.96 |
| bta05220:Chronic myeloid leukaemia | 10 | 0 | 2.26 | 33.57 |
| bta04666:Fc gamma R-mediated phagocytosis | 11 | 0 | 2.14 | 34.11 |
| bta05230:Central carbon metabolism in cancer | 9 | 0 | 2.4 | 34.6 |
| bta04110:Cell cycle | 14 | 0 | 1.85 | 39.82 |
| bta00240:Pyrimidine metabolism | 12 | 0 | 1.96 | 41.8 |
| bta04144:Endocytosis | 24 | 0 | 1.52 | 42.68 |
| bta00190:Oxidative phosphorylation | 15 | 0 | 1.77 | 43.55 |
| bta04068:FoxO signalling pathway | 14 | 0.1 | 1.75 | 52.27 |
| bta04810:Regulation of actin cytoskeleton | 20 | 0.1 | 1.55 | 52.95 |
| bta03050:Proteasome | 7 | 0.1 | 2.51 | 53.42 |
| bta00562:Inositol phosphate metabolism | 9 | 0.1 | 2.09 | 57.39 |
| bta04150:mTOR signalling pathway | 8 | 0.1 | 2.24 | 57.56 |
| bta04070:Phosphatidylinositol signalling system | 11 | 0.1 | 1.85 | 61.75 |
| bta04915:Estrogen signalling pathway | 11 | 0.1 | 1.85 | 61.75 |
| bta05215:Prostate cancer | 10 | 0.1 | 1.92 | 63.41 |
| bta04722:Neurotrophin signalling pathway | 13 | 0.1 | 1.72 | 63.46 |
| bta04550:Signaling pathways regulating pluripotency of stem cells | 14 | 0.1 | 1.66 | 64.62 |
| bta04919:Thyroid hormone signalling pathway | 12 | 0.1 | 1.75 | 65.69 |
| bta04611:Platelet activation | 13 | 0.1 | 1.69 | 67.02 |
| bta05222:Small cell lung cancer | 10 | 0.1 | 1.88 | 67.75 |
| bta05166:HTLV-I infection | 23 | 0.1 | 1.41 | 70.89 |
| bta05212:Pancreatic cancer | 8 | 0.1 | 2.03 | 73.15 |
| bta04720:Long-term potentiation | 8 | 0.1 | 2.03 | 73.15 |
| bta03013:RNA transport | 15 | 0.1 | 1.56 | 74.52 |





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**Table 9:** GO of all genes uniquely expressed (FPKM >5) in HCEP

| Term | Count | P Value | Fold Enrichment | FDR |
|---|---|---|---|---|
| GO:0070062~extracellular exosome | 209 | 0 | 1.57 | 0 |
| GO:0005925~focal adhesion | 54 | 0 | 2.77 | 0 |
| GO:0005730~nucleolus | 75 | 0 | 2.02 | 0 |
| GO:0005737~cytoplasm | 258 | 0 | 1.35 | 0 |
| GO:0043209~myelin sheath | 26 | 0 | 3 | 0 |
| GO:0016020~membrane | 92 | 0 | 1.66 | 0 |
| GO:0005654~nucleoplasm | 120 | 0 | 1.49 | 0.01 |
| GO:0005829~cytosol | 95 | 0 | 1.51 | 0.07 |
| GO:0005634~nucleus | 218 | 0 | 1.24 | 0.51 |
| GO:0016607~nuclear speck | 19 | 0 | 2.56 | 0.6 |
| GO:0005794~Golgi apparatus | 53 | 0 | 1.64 | 0.71 |
| GO:0005913~cell-cell adherens junction | 15 | 0 | 2.66 | 2.01 |
| GO:0010494~cytoplasmic stress granule | 8 | 0 | 4.34 | 2.71 |
| GO:0005769~early endosome | 19 | 0 | 2.21 | 3.29 |
| GO:0000932~cytoplasmic mRNA processing body | 11 | 0 | 2.99 | 4.73 |
| GO:0030027~lamellipodium | 16 | 0 | 2.33 | 4.95 |
| GO:0005813~centrosome | 32 | 0.01 | 1.62 | 11.54 |
| GO:0005815~microtubule organizing centre | 12 | 0.01 | 2.47 | 11.87 |
| GO:0000139~Golgi membrane | 23 | 0.01 | 1.73 | 18.86 |
| GO:0036513~Derlin-1 retrotranslocation complex | 4 | 0.01 | 7.24 | 19.47 |
| GO:0008250~oligosaccharyltransferase complex | 4 | 0.01 | 7.24 | 19.47 |
| GO:0005802~trans-Golgi network | 14 | 0.01 | 2.11 | 19.7 |
| GO:0005789~endoplasmic reticulum membrane | 34 | 0.02 | 1.52 | 21.62 |
| GO:0005739~mitochondrion | 71 | 0.02 | 1.3 | 22.62 |
| GO:0030904~retromer complex | 5 | 0.02 | 4.79 | 22.99 |
| GO:0005681~spliceosomal complex | 9 | 0.02 | 2.67 | 23.69 |
| GO:0015630~microtubule cytoskeleton | 11 | 0.02 | 2.33 | 24.39 |
| GO:0005844~polysome | 6 | 0.02 | 3.76 | 24.84 |
| GO:0016281~eukaryotic translation initiation factor 4F complex | 3 | 0.02 | 12.22 | 26.55 |
| GO:0005759~mitochondrial matrix | 16 | 0.02 | 1.88 | 29.39 |
| GO:0016363~nuclear matrix | 9 | 0.02 | 2.53 | 30.5 |
| GO:0031012~extracellular matrix | 14 | 0.03 | 1.97 | 31.46 |
| GO:0005774~vacuolar membrane | 4 | 0.03 | 5.92 | 32.32 |
| GO:0005764~lysosome | 16 | 0.03 | 1.85 | 32.33 |
| GO:0042470~melanosome | 10 | 0.03 | 2.29 | 35.06 |
| GO:0055038~recycling endosome membrane | 6 | 0.03 | 3.37 | 35.97 |
| GO:0031965~nuclear membrane | 15 | 0.03 | 1.84 | 39.44 |
| GO:0030529~intracellular ribonucleoprotein complex | 9 | 0.03 | 2.36 | 40.66 |
| GO:0005743~mitochondrial inner membrane | 27 | 0.04 | 1.51 | 42.09 |
| GO:0005938~cell cortex | 10 | 0.04 | 2.2 | 42.16 |
| GO:0005791~rough endoplasmic reticulum | 5 | 0.04 | 3.7 | 47.2 |
| GO:0031902~late endosome membrane | 7 | 0.05 | 2.65 | 49.91 |
| GO:0005786~signal recognition particle, endoplasmic reticulum targeting | 3 | 0.05 | 8.15 | 51.34 |

The unique genes expressed in later passage (HCLP) cells showed to be involved in metabolic pathways, lysosome and oxidative phosphorylation up to 5 FDR limit in KEGG pathway (Table 10).







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**Table 10:** KEGG Pathway of all genes uniquely expressed (FPKM >5) in HCLP

| Term | Count | P Value | Fold Enrichment | FDR |
|---|---|---|---|---|
| bta01100:Metabolic pathways | 63 | 0 | 1.49 | 0.95 |
| bta00190:Oxidative phosphorylation | 14 | 0 | 2.95 | 1.1 |
| bta04142:Lysosome | 13 | 0 | 3.04 | 1.38 |
| bta00983:Drug metabolism - other enzymes | 6 | 0.01 | 4.92 | 8.37 |
| bta03013:RNA transport | 13 | 0.01 | 2.41 | 9.15 |
| bta05134:Legionellosis | 7 | 0.01 | 3.62 | 14.41 |
| bta05164:Influenza A | 13 | 0.01 | 2.22 | 16.59 |
| bta05016:Huntington's disease | 14 | 0.02 | 2.08 | 19.71 |
| bta05010:Alzheimer's disease | 13 | 0.02 | 2.15 | 20.01 |
| bta00020:Citrate cycle (TCA cycle) | 5 | 0.02 | 4.92 | 20.29 |
| bta05162:Measles | 11 | 0.02 | 2.32 | 22.76 |
| bta01130:Biosynthesis of antibiotics | 14 | 0.02 | 2.01 | 24.78 |
| bta03040:Spliceosome | 10 | 0.03 | 2.29 | 32.62 |
| bta01200:Carbon metabolism | 9 | 0.03 | 2.44 | 33.1 |
| bta05152:Tuberculosis | 12 | 0.04 | 1.96 | 42.82 |
| bta05200:Pathways in cancer | 21 | 0.05 | 1.55 | 48.39 |
| bta05168:Herpes simplex infection | 12 | 0.06 | 1.86 | 52.43 |
| bta04966:Collecting duct acid secretion | 4 | 0.06 | 4.37 | 55.77 |
| bta04620:Toll-like receptor signalling pathway | 8 | 0.06 | 2.25 | 57.45 |
| bta05202:Transcriptional misregulation in cancer | 11 | 0.07 | 1.87 | 60.66 |
| bta05012:Parkinson's disease | 10 | 0.07 | 1.94 | 61.6 |
| bta03020:RNA polymerase | 4 | 0.08 | 3.93 | 65.45 |
| bta05160:Hepatitis C | 9 | 0.08 | 2 | 66.09 |
| bta00330:Arginine and proline metabolism | 5 | 0.09 | 2.95 | 69.25 |

**Table 11:** GO of all genes uniquely expressed (FPKM >5) in HCLP

| Term | Count | P Value | Fold Enrichment | FDR |
|---|---|---|---|---|
| GO:0070062~extracellular exosome | 113 | 0 | 1.52 | 0 |
| GO:0043209~myelin sheath | 16 | 0 | 3.31 | 0.14 |
| GO:0005913~cell-cell adherens junction | 10 | 0 | 3.17 | 5.74 |
| GO:0005829~cytosol | 52 | 0 | 1.48 | 6.12 |
| GO:0005925~focal adhesion | 21 | 0.01 | 1.93 | 9.08 |
| GO:0005875~microtubule associated complex | 4 | 0.01 | 9.72 | 9.33 |
| GO:0048471~perinuclear region of cytoplasm | 23 | 0.01 | 1.82 | 11.26 |
| GO:0044615~nuclear pore nuclear basket | 3 | 0.01 | 17.5 | 14.26 |
| GO:0005794~Golgi apparatus | 29 | 0.01 | 1.6 | 18.86 |
| GO:0055038~recycling endosome membrane | 5 | 0.02 | 5.03 | 20.62 |
| GO:0005730~nucleolus | 32 | 0.02 | 1.54 | 20.9 |
| GO:0005737~cytoplasm | 127 | 0.02 | 1.19 | 23.23 |
| GO:0016607~nuclear speck | 10 | 0.02 | 2.41 | 27.95 |
| GO:0030136~clathrin-coated vesicle | 5 | 0.03 | 4.17 | 35.41 |
| GO:0005783~endoplasmic reticulum | 26 | 0.04 | 1.53 | 39.66 |
| GO:0000276~mitochondrial proton-transporting ATP synthase complex, coupling factor F(o) | 3 | 0.04 | 9.72 | 40.09 |
| GO:0030027~lamellipodium | 9 | 0.04 | 2.34 | 42.2 |
| GO:0031965~nuclear membrane | 10 | 0.04 | 2.19 | 42.84 |
| GO:0044297~cell body | 5 | 0.04 | 3.84 | 43.56 |
| GO:0072546~ER membrane protein complex | 3 | 0.04 | 8.75 | 46.67 |
| GO:0005793~endoplasmic reticulum-Golgi intermediate compartment | 5 | 0.05 | 3.65 | 49.04 |







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| | | | | |
|---|---|---|---|---|
| GO:0016363~nuclear matrix | 6 | 0.05 | 3.02 | 49.71 |
| GO:0042405~nuclear inclusion body | 3 | 0.05 | 7.96 | 52.99 |
| GO:0016020~membrane | 41 | 0.06 | 1.33 | 54.84 |
| GO:0005639~integral component of nuclear inner membrane | 3 | 0.06 | 7.29 | 58.93 |
| GO:0005776~autophagosome | 5 | 0.06 | 3.31 | 59.72 |
| GO:0005739~mitochondrion | 40 | 0.07 | 1.32 | 61.85 |
| GO:0000139~Golgi membrane | 13 | 0.07 | 1.75 | 64.3 |
| GO:0030426~growth cone | 5 | 0.07 | 3.17 | 64.73 |
| GO:0005789~endoplasmic reticulum membrane | 19 | 0.08 | 1.52 | 67.87 |
| GO:0045121~membrane raft | 8 | 0.08 | 2.12 | 70.44 |
| GO:0005765~lysosomal membrane | 11 | 0.09 | 1.78 | 73.52 |
| GO:0030017~sarcomere | 3 | 0.09 | 5.83 | 73.97 |
| GO:0042470~melanosome | 6 | 0.1 | 2.47 | 75.57 |
| GO:0000346~transcription export complex | 2 | 0.1 | 19.45 | 76.9 |
| GO:0005787~signal peptidase complex | 2 | 0.1 | 19.45 | 76.9 |

**Table 12:** Top 20 genes (FPKM>5) Up-regulated in HCEP (left side of table) and up-regulated in HCLP (right side of table) by Cuffdiff (EP1, LP1 denotes FPKM value of HCEP and HCLP respectively)

| Up in HCEP | EP1 | LP1 | log2(fold change) | Up in HCLP | EP1 | LP1 | log2(fold change) |
|---|---|---|---|---|---|---|---|
| EZR | 512.3 | 43.75 | -3.54 | ATP13A3 | 7.66 | 151.92 | 4.3 |
| COL3A1 | 965.7 | 84.38 | -3.51 | NFE2L2 | 7.99 | 147.82 | 4.2 |
| COX7A2L | 224.76 | 21.47 | -3.38 | DDX5 | 20.38 | 200.08 | 3.29 |
| LUM | 239.23 | 25.35 | -3.23 | SEC63 | 9.45 | 91.4 | 3.27 |
| COL1A2 | 2830.52 | 335.57 | -3.07 | TIMM17B | 178.26 | 1622.23 | 3.18 |
| HSP90B1 | 190.33 | 22.88 | -3.05 | PLS3 | 9.04 | 81.42 | 3.17 |
| PPP2R2A | 159.82 | 19.32 | -3.04 | GANAB | 4.52 | 40.71 | 3.16 |
| PFN2 | 115.06 | 14.55 | -2.98 | CXCL6 | 14.11 | 121.23 | 3.1 |
| CNEP1R1 | 168.53 | 22.54 | -2.9 | EIF4G2 | 20.62 | 174.26 | 3.07 |
| CALD1 | 3332.75 | 476.36 | -2.8 | PRKAR1A | 49.8 | 397.73 | 2.99 |
| SNX5 | 30941.7 | 4426.91 | -2.8 | GRIPAP1 | 40.77 | 309.14 | 2.92 |
| ACTB | 1780.91 | 260.94 | -2.77 | TMED5 | 31.97 | 241.54 | 2.91 |
| SCAP | 312.25 | 46.4 | -2.75 | RALYL | 25272.7 | 187060 | 2.88 |
| SERBP1 | 4062 | 625.36 | -2.69 | BICD2 | 10.27 | 70.53 | 2.77 |
| KIF5B | 99.38 | 15.42 | -2.68 | SLC25A6 | 182.7 | 1230.47 | 2.75 |
| XPO1 | 56.29 | 8.81 | -2.67 | ADNP | 9.52 | 63.65 | 2.74 |
| SS18 | 78.34 | 12.58 | -2.63 | CKAP5 | 33129 | 201671 | 2.6 |
| EFEMP2 | 535.81 | 87.88 | -2.6 | TALDO1 | 102.57 | 598.23 | 2.54 |
| GJA1 | 31.53 | 5.24 | -2.58 | HNRNPH1 | 10.98 | 61.09 | 2.47 |
| DPYD | 483115 | 83200 | -2.53 | ADAMTS2 | 28.85 | 159.31 | 2.46 |

*For all the genes, 'Official Gene Symbols' were used in this article during discussion*

Oxidative phosphorylation pathway might be activated in response to DNA damage (Yadav *et al.*, 2015). Lysosome pathway was activated might be through LAMP2 (FPKM=58.07) in later passages indicating cells might not be apoptotic resistant; Halaby (2015). Genes found to be upregulated in early passage BHCC cells, were involved in PI3K-AkT signalling pathway, indicating enhanced glycolysis and cell growth and survival (Liu *et al.*, 2009).





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**Table 13:** Top 20 genes (FPKM>5) Up-regulated in HCEP (left side of table) and up-regulated in HCLP (right side of table) and their implications in various cancers

| Up in HCEP | Implications | Up in HCLP | Implications |
|---|---|---|---|
| EZR | Promotes tumor metastasis; Konstantinovsky et al (2012) | ATP13A3 | Polyamine transport in human pancreatic cancers; Madan et al (2016). |
| COL3A1 | Up-regulated in colorectal cancer; Wu et al (2012) | NFE2L2 | Protect cells from oxidative damage; Chen et al (2009). |
| COX7A2L | Up regulated in a breast cancer cell line; Pathiraja et al (2010) | DDX5 | DDX5 and ErbB2 upregulates miR-21 and promotes cell invasion; Wang et al (2011). |
| LUM | Altered the expression and activity of MMP-14; Pietraszek et al (2013) | SEC63 | Overexpressed in short survivors of canine OS; Selvarajah et al (2009). |
| COL1A2 | Reported in Cis- and one of Dox-resistant ovarian cancer cell line; Januchowski et al (2014). | TIMM17B | Expressed in all cancer tissues. |
| HSP90B1 | It has been shown to be upregulated in lung cancer adenocarcinoma tissues; Wu et al (2013). | PLS3 | Aberrantly expressed in invasive front of the CRC tumor (Battle et al (2012). |
| PPP2R2A | Therapeutic target in NSCLC therapy; Shen et al (2014). | GANAB | Plays negative roles, in the regulation of cell growth, cell migration and invasion; Chiu et al (2011). |
| PFN2 | Might regulate the N-WASP/Arp2/3 signalling pathway; MA et al (2011). | CXCL6 | Responsible for vasculogenesis; Yuzhalin and Kutikhin (2014). |
| CNEP1R1 | No known association. | EIF4G2 | Associated with decreased metastatic progression; Silvera et al (2010). |
| UTRN | Found to be up-regulated in various types of dystrophies as breast cancer, | PRKAR1A | Bone tumor suppressor gene; Molyneux et al (2010). |
| CALD1 | Commonly deregulated in cancer; Zheng et al (2004). | GRIPAP1 | Increased in hepatocellular carcinoma aberrantly; Hodo et al (2010). |
| SNX5 | Tumorigenesis of the thyroid gland was tightly associated with the abundance of SNX5/Snx5; Ara et al (2012). | TMED5 | Significantly upregulated in superficial bladder cancer; Scaravilli et al (2014). |
| ACTB | Up-regulation with invasiveness and metastasis in different cancers; Guo et al (2013). | RALYL | More expressed in invasive colon cancer cell line than non-invasive colon cancer cell line; Zhang et al (2013). |
| SCAP | Avoid lipotoxicity in fastest growing tumor cells; Williams et al (2013). | BICD2 | Overexpression of it is responsible for cellular proliferation; Raaijmakers et al. (2012). |
| SERBP1 | Are essential for cancer cell growth and tumorigenesis; Williams et al (2013). | SLC25A6 | Expressed profoundly in prostate cancer cells than benign epithelium; Tomlins et al (2007). |
| KIF5B | Acts as a driver mutation of lung adenocarcinoma; Ju et al (2012). | ADNP | Up regulation increases cell viability; Castorina et al (2012) |
| XPO1 | Overexpressed in hematologic and nonhematologic tumors; Walker et al (2013) | CKAP5 | Predicted for chromosomal instability when found in aberrant expression; Fukasawa (2007). |
| SS18 | Promotes cell proliferation through MiR-17; Minami et al (2014). | TALDO1 | Up regulated in late-stage human colon-cancer tissue; Kočevar et al (2012). |
| EFEMP2 | Promotes oncogenic potential of gliomas cancer cell growth and metastasis; Wang et al (2015). | HNRNPH1 | Strong HNRNPH1 levels were significantly associated with poorer tumor differentiation; Sun et al (2016). |
| GJA1 | Maintains cell differentiation & prevents transformation into cancer cells; Falck and Livan (2013). | ADAMTS2 | It has anti-angiogenic properties; Dubail et al (2010). |

***N.B.*** *- for all the genes, 'Official Gene Symbols' were used in this article during discussion.*





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**Table 14:** Expression of Genes that are usually altered in cancer and involved in cancer pathways

| Official Gene Symbol | BHCC early passage cells FPKM | BHCC Late passage cells FPKM | $Log_2$ (fold change) | | Official Gene Symbol | BHCC early passage cells FPKM | BHCC Late passage cells FPKM | $Log_2$ (fold change) | | Official Gene Symbol | BHCC early passage cells FPKM | BHCC Late passage cells FPKM | $Log_2$ (fold change) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Genes involved in TGF beta Pathway;Vogelstein et al (2004) | | | | | Tumour suppressor Genes ;Vogelstein et al (2004) | | | | | Apoptosis ;Vogelstein et al (2004) | | | |
| TGFB2 | 26.42 | 0 | ∞ | | PTCH1 | 49.82 | 0 | ∞ | | CDK2AP1 | 171.38 | 0 | ∞ |
| TGFBR1 | 77.12 | 207.73 | 1.42 | | ZFHX4 | 7.63 | 0 | ∞ | | CDK14 | 48.07 | 0 | ∞ |
| TGFBI | 122.84 | 0 | ∞ | | SDHB | 135.43 | 0 | ∞ | | CDKN1A | 30.71 | 0 | ∞ |
| TGFB1I1 | 97.08 | 0 | ∞ | | TP53INP1 | 3.077 | 0 | ∞ | | RPS7 | 913.697 | 1824.83 | 0.997975 |
| CTGF | 439.80 | 195.46 | -1.17 | | TP53BP1 | 21.22 | 0 | ∞ | | TNFRSF1B | 79.91 | 0 | ∞ |
| PTCH1 | 49.24 | 0 | ∞ | | WTIP | 55.65 | 0 | ∞ | | TNFRSF19 | 93.69 | 0 | ∞ |
| TERT | 261.68 | 0 | ∞ | | STK25 | 162.89 | 0 | ∞ | | WDR44 | 28.03 | 37.77 | 0.43 |
| CDKs;Vogelstein et al (2004) | | | | | GSTK1 | 66.07 | 0 | ∞ | | WDR45L | 129.75 | 0 | ∞ |
| CDKN1A | 30.71 | 0 | ∞ | | CTSC | 30.84 | 36.30 | 0.23 | | WDR48 | 6.85 | 0 | ∞ |
| CDK16 | 42.31 | 0 | ∞ | | RB1 | 3.68 | 0 | ∞ | | APAF1 | 5.58 | 0 | ∞ |
| CDK2AP1 | 171.38 | 0 | ∞ | | RNF130 | 56.15 | 0 | ∞ | | TNFAIP8L1 | 33.49 | 0 | ∞ |
| CDK14 | 48.07 | 0 | ∞ | | ZNF189 | 11.22 | 0 | ∞ | | TNFRSF1B | 79.91 | 0 | ∞ |
| Highly Expressed in cell, tumour ;Vogelstein et al (2004) | | | | | RNF11 | 11.36 | 0 | ∞ | | TNFRSF19 | 93.69 | 0 | ∞ |
| SPARC | 834.44 | 240.84 | -1.79 | | RNF13 | 26.45 | 0 | ∞ | | C1QTNF3 | 34.34 | 0 | ∞ |
| Expressed in immortal cell lines ;Vogelstein et al (2004) | | | | | CDKN1A | 30.71 | 0 | ∞ | | TNFAIP8L1 | 33.49 | 0 | ∞ |
| TOP1 | 17.69 | 0 | ∞ | | SMAD4 | 32.09 | 0 | ∞ | | APC pathway ;Vogelstein et al (2004) | | | |
| PCNA | 6.50 | 0 | ∞ | | Stability Genes ;Vogelstein et al (2004) | | | | | LRP12 | 12.40 | 0 | ∞ |
| CDC26 | 98.80 | 0 | ∞ | | ATM | 1.31 | 0 | ∞ | | LRP4 | 37.38 | 0 | ∞ |
| CDC2L1 | 30.33 | 0 | ∞ | | ATMIN | 1.60 | 0 | ∞ | | APC | 2.29 | 6.21 | 1.43 |
| CDC27 | 3.10 | 0 | ∞ | | BRCA1 | 7.38 | 0 | ∞ | | MYC | 39.53 | 0 | ∞ |
| Tumour suppressor Genes ;Vogelstein et al (2004) | | | | | Oncogenes ;Vogelstein et al (2004) | | | | | CCND1 | 268.12 | 0 | ∞ |
| APC | 2.29 | 6.21 | 1.43 | | MET | 8.41 | 0 | ∞ | | Hh pathway ;Vogelstein et al (2004) | | | |
| EXT1 | 47.40 | 43.48 | -0.12 | | METTL13 | 3.47 | 0 | ∞ | | ARNTL | 19.92 | 0 | ∞ |
| EXT2 | 153.62 | 117.74 | -0.38 | | PDGFRA | 5.03 | 22.69 | 2.17 | | | | | |
| Official Gene Symbol | BHCC early passage cells FPKM | BHCC Late passage cells FPKM | $Log_2$ (fold change) | | Official Gene Symbol | BHCC early passage cells FPKM | BHCC Late passage cells FPKM | $Log_2$ (fold change) | | Official Gene Symbol | BHCC early passage cells FPKM | BHCC Late passage cells FPKM | $Log_2$ (fold change) |

*(-) $Log_2$ (fold) change means the FPKM value in the SCC early passage is more than SCC late passage cells*



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| GLi pathway ;Vogelstein et al (2004) | | | | | List of genes that are usually altered in cancer; Dawany et al (2011) | | | | | List of genes that are usually altered in cancer ; Dawany et al (2011) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| EXT1 | 47.40 | 43.48 | -0.12 | | KLF10 | 27.51 | 0 | ∞ | | AOX1 | 21.11 | 0 | ∞ |
| EXT2 | 153.62 | 117.74 | -0.38 | | KLF5 | 43.57 | 0 | ∞ | | BUB1 | 10.40 | 0 | ∞ |
| PTCH1 | 49.82 | 0 | ∞ | | KLF6 | 252.19 | 0 | ∞ | | NME1 | 165.79 | 227.82 | 0.45 |
| CCND1 | 268.12 | 0 | ∞ | | TPX2 | 22.71 | 19.58 | -0.21 | | PCDH18 | 22.56 | 0 | ∞ |
| PI3K Pathway ;Vogelstein et al (2004) | | | | | ACAT1 | 30.08 | 120.84 | 2.00 | | PCDH17 | 30.15 | 0 | ∞ |
| SCAMP3 | 154.18 | 57.93 | -1.41 | | CDC27 | 3.10 | 0 | ∞ | | PCDH7 | 9.71 | 0 | ∞ |
| NAMPT | 9.89 | 0 | ∞ | | CDC2L1 | 30.33 | 0 | ∞ | | ABCA3 | 72.12 | 0 | ∞ |
| AKTIP | 11.27 | 0 | ∞ | | CDC26 | 98.80 | 0 | ∞ | | NMT1 | 32.95 | 0 | ∞ |
| CTSC | 30.84 | 36.30 | 0.23 | | MCM3AP | 30.32 | 0 | ∞ | | PRC1 | 12.68 | 0 | ∞ |
| LAMTOR5 | 106.87 | 0 | ∞ | | SERBP1 | 147.84 | 580.47 | 1.97 | | PTTG1IP | 61.87 | 55.06 | -0.16 |
| LAMTOR4 | 288.78 | 0 | ∞ | | NRBP1 | 124.95 | 0 | ∞ | | SHMT1 | 37.85 | 0 | ∞ |
| AEBP1 | 185.28 | 70.39 | -1.39 | | CIRBP | 36.85 | 34.13 | -0.11 | | RRM2 | 12.32 | 0 | ∞ |
| RPS6KA4 | 78.77 | 0 | ∞ | | CDH13 | 31.31 | 107.92 | 1.78 | | TOP1 | 17.69 | 0 | ∞ |
| RPS6KB1 | 50.64 | 0 | ∞ | | COL4A1 | 115.36 | 0 | ∞ | | SCFD1 | 64.60 | 0 | ∞ |
| RPS6KC1 | 6.87 | 0 | ∞ | | ENO1 | 1078.51 | 875.52 | -0.30 | | NAP1L4 | 44.80 | 34.43 | -0.38 |
| BCL2L13 | 230.30 | 0 | ∞ | | RBFOX2 | 14.73 | 0 | ∞ | | SPP1 | 406.17 | 241.48 | -0.75 |
| Deregulated Genes | | | | | FOXN3 | 7.47 | 0 | ∞ | | CCNE2 | 30.64 | 0 | ∞ |
| VEGFA | 0 | 455.899 | ∞ | | FOXJ2 | 14.37 | 0 | ∞ | | CCNY | 40.99 | 0 | ∞ |
| CDH13 | 31.316 | 107.92 | 1.785 | | PRKAR1A | 49.80 | 397.73 | 2.99 | | TRMT10A | 14.48 | 0 | ∞ |
| HIF1A | 8.69781 | 10.0303 | 0.205644 | | PRKAR2A | 57.27 | 0 | ∞ | | ARHGAP24 | 61.85 | 0 | ∞ |
| | | | | | TGFBI | 122.84 | 0 | ∞ | | New cancer genes ; Dawany et al (2011) | | | |
| | | | | | TGFBR1 | 77.12 | 207.73 | 1.42 | | ITM2B | 275.07 | 89.05 | -1.62 |
| | | | | | THBS2 | 60.03 | 29.90 | -1.00 | | NUP205 | 22.91 | 0 | ∞ |
| | | | | | CKAP2 | 28.03 | 30.93 | 0.14 | | FAT1 | 88.43 | 45.18 | -0.96 |
| | | | | | UBE2C | 111.56 | 0 | ∞ | | ITM2C | 46.47 | 228.24 | 2.29 |





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**Table 15:** Genes commonly deregulated in cancer

| Official Gene Symbol | BHCC early passage cells FPKM | BHCC Late passage cells FPKM | $Log_2$ (fold change) | | Official Gene Symbol | BHCC early passage cells FPKM | BHCC Late passage cells FPKM | $Log_2$ (fold change) |
|---|---|---|---|---|---|---|---|---|
| Genes Up Regulated In Most Cancers; Lu et al (2007) | | | | | IQGAP3 | 19.53 | 0 | ∞ |
| | | | | | ZBTB11 | 48.75 | 217.82 | 2.15 |
| IPO7 | 22.23 | 22.12 | -0.007 | | RPN2 | 567.59 | 0 | ∞ |
| FKBP10 | 46.24 | 0 | ∞ | | IPO4 | 56.71 | 0 | ∞ |
| PRC1 | 12.68 | 0 | ∞ | | FARP1 | 61.25 | 28.34 | -1.11 |
| FNDC3B | 8.64 | 21.79 | 1.33 | | TMEM41B | 12.41 | 0 | ∞ |
| ILF3 | 30.55 | 0 | ∞ | | TTLL4 | 53.34 | 0 | ∞ |
| ACLY | 20.25 | 0 | ∞ | | GEMIN6 | 72.15 | 0 | ∞ |
| ADAM12 | 51.21 | 0 | ∞ | | CALU | 223.52 | 144.92 | -0.62 |
| PSMB2 | 88.15 | 0 | ∞ | | SNX10 | 9.03 | 0 | ∞ |
| EIF2AK1 | 22.07 | 0 | ∞ | | RBAK | 91.86 | 0 | ∞ |
| NME1 | 165.79 | 227.82 | 0.45 | | EPRS | 16.74 | 20.49 | 0.29 |
| ADAM10 | 17.86 | 45.27 | 1.34 | | PGK1 | 131.69 | 178.24 | 0.43 |
| ANP32E | 28.43 | 0 | ∞ | | WISP2 | 50.45 | 109.36 | 1.11 |
| HNRPLL | 11.07 | 0 | ∞ | | Commonly Down regulated Genes in most Cancers ; Lu et al (2007)& Koringa et al (2013) | | | |
| FAM49B | 45.34 | 0 | ∞ | | ERBB2IP | 12.58 | 0 | ∞ |
| EIF2S2 | 184.77 | 71.12 | -1.37 | | DHRS4 | 75.15 | 197.00 | 1.39 |
| KDELR3 | 156.46 | 91.10 | -0.78 | | SERPINE1 | 1619 | 836 | -0.95 |
| SPP1 | 406.17 | 241.48 | -0.75 | | SERPINF1 | 0 | 138.66 | ∞ |
| UTP18 | 17.13 | 0 | ∞ | | SERPINH1 | 579.00 | 0 | ∞ |
| ZBTB1 | 48.75 | 217.82 | 2.15 | | SERPINE2 | 56 | 14 | -1.94 |
| Other Deregulated Genes | | | | | | | | |
| LAMC1 | 29.2673 | 6.75588 | -2.11508 | | CCDC69 | 400 | 121 | -1.71 |
| LAMB1 | 69.2363 | 21.6097 | -1.67985 | | MMP2 | 308.657 | 270.341 | -0.191228 |
| FAM83D | 41.0108 | 140.937 | 1.78097 | | PKM | 52.2968 | 93.4699 | 0.837778 |
| STAT3 | 14.3005 | 20.507 | 0.520055 | | QSOX1 | 0 | 144.507 | ∞ |
| VIM | 2646.27 | 4117.63 | 0.637854 | | CD44 | 24.2322 | 133.35 | 2.46022 |

(-) $Log_2$ (fold change) means the FPKM value in the SCC early passage is more than SCC late passage cells







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Enhanced expression of CDH13 (Table 14) in association with AKT3 in late passages BHCC cells denotes decreased cell motility & increased ROS production (Takeuchi *et al.,* 2002).

Increased TGFBR1 expression denotes less cellular proliferation in later passages (HCLP) (Pasche *et al.,* 2014). Down regulation of HSP90B1 (Table 12) in HCLP might indicate overexpression of miR-223, subsequent decrease in cell viability, cell cycle & increase in apoptosis *In-vitro* (Li *et al.,* 2012). In contrast to the above finding FAM83D (Table 15) expression was found to be increased in late passage cells (HCLP), which would indicate an increased cell proliferation and cell motility, similarly expression of another mesenchymal marker VIM (Vimentin) (Table 15) was found to be increased in later passages (HCLP), but the expression of FBXW7 (a tumour suppressor) was not found in both the cell passages (Wang *et al.,* 2013). CCDC69 (Table 15) was down regulated in late passages (HCLP) along with RHOA (Table 5) may indicate dysregulation of integrity in central spindles in cells thereby in cell proliferation (Pal *et al.,* 2010). Increase in VEGFA (Table 14) and HIF1A (Table 14) in later passages (HCLP) found in our study also indicate production of endogenous ROS and chances of increased angiogenesis but MMP2 (Table 15) expression was found to be decreased. Decrease in expression of SERPINE1 (Table 15) in later passages also indicate towards less migratory/invasive behavior (Klein *et al.,* 2012). This suggests us that horn cancer at advanced stages also being controlled by Leptin molecule in terms of invasiveness & metastasis as in breast cancer (Strong *et al.,* 2015). Increased ROS can lead to "non-specific" damage of macromolecules such as DNA, proteins and lipids; Liou and Storz (2010).

In later passage (HCLP) cells, NDUFA4 (FPKM 289.36), might acted as a saviour to reduce ROS production by shifting mitochondrial respiration to anaerobic glycolysis due to hypoxia condition via induction of HIF1A in cell culture, it also probably helped in colony formation of cells preventing autophagy. NDUFA4 has a role in mediating the Warburg effect, though aerobic glycolysis is a less efficient method for producing ATP but reprogrammed metabolism might support the synthesis of macromolecules required for rapid proliferation (Minton *et al.,* 2016). Down-regulation of LAMC1 & LAMB1 (Table 15) are usually associated with decreased invasiveness in GBM as described by Haas (2010), Koringa *et al.* (2013) also found LAM as signature molecule in horn cancer tissue in bovines. COL1A1 and COL1A2 (Table 5) was also identified by Koringa *et al.* (2013) in horn cancer tissue as in our study in both stages of cell passages it was found to be expressed and COL1A2 was associated with tumour size and depth of invasion (Li *et al.,* 2016). These two genes can be thought of as prognostic markers after further study.

Tumour cells at later stages usually have high energy demand and oxidative stress to cope up with uncontrolled growth, Pentose Phosphate Pathway (PPP); a glucose metabolic pathway helps by providing both antioxidant defence and reductive biosynthesis. Certain mutations in tumour suppressors & oncogenes also help in Warburg effect or oxidative phosphorylation i.e. not detrimental to cancer cells (Jiang *et al.,*






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2014). During oxidative stress, cancer cells will shut down the glycolytic pathway and thus increase glucose flux through the PPP to produce more NADPH for antioxidant defence and to reduce ROS while simultaneously generating high levels of nucleotides for DNA synthesis and repair (Patra & Hay, 2014).

PKM (Table 15) expression was increased in later passages (HCLP) along with STAT3 (Table 15) expression, which is most important molecular signature for promoting cancer progression. PKM dependent histone modifications were absent in later passages (HCLP) unlike STAT3. Based on gene expression profile it was evident that the cells were getting energy regeneration through enhanced PKM activity (Dong *et al.*, 2016). QSOX-1 gene (Table 15) was enhanced in later passages (HCLP) and which in turn enhanced ROS activation and this in turn might drive EMT (Lake and Faigel, 2014). The late passage (HCLP) also expressed CD44 (Table 15) which was almost two fold than the early passage (HCEP) might indicate early stemness in these cells (Sacks & Barbolina, 2015). From the pathway analysis it is eminent that there is a reprogramming shift in the glucose utilization, in early passage BHCC cells it was regulated by PI3K-AkT pathway but by PPP in later passages (HCLP). That may be due the facts that to increase NADPH production, cells can acquire the ability to increase PPP flux by suppressing glycolysis and redirecting glycolytic intermediates into the PPP. Increased expression of ALDOA in late passages (HCLP) may also indicate TGFB induced metastasis and stress response (Ji *et al.*, 2016).

The above discussion denotes a no. of key players controlling the growth pattern and properties of the cancer cells in these two passages.

**Conclusion**

The signalling pathway investigation in this culture based approach revealed many of the cancer-related pathways that could be operative while in diseased condition, at the onset & at the advanced stages of horn cancer malignancy as well. The cells at later passages became more advanced towards EMT like phenomenon and might have acquired stemness. These genes or signalling pathways can easily be targeted for chemotherapy, vaccine production in future to reduce the risk of horn cancer or use of certain panel of genes as prognostic marker for horn cancer in bovines. This finite cell line represents a unique and relevant model which mimics stages in horn core intra-epithelial neoplasia and progression to invasive cancer. Future work for validation of these data in a larger series and in a prospective cohort may determine the clinical applicability of this approach in future biomarker discovery and disease stratification in SCC of horn.

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**Conflict of Interests**

Authors have no conflicts of interest.

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