Transcriptomic comparison of primary bovine horn core carcinoma culture and parental tissue at early stage

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Abstract

Aim: 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. Comparison of gene expression in between horn’s SCC tissue and its early passage primary culture using next generation sequencing was the aim of this study.

Materials and Methods: Whole transcriptome sequencing of horn’s SCC tissue and its early passage cells using Ion Torrent PGM were done. Comparative expression and analysis of different genes and pathways related to cancer and biological processes associated with malignancy, proliferating capacity, differentiation, apoptosis, senescence, adhesion, cohesion, migration, invasion, angiogenesis, and metabolic pathways were identified.

Results: Up-regulated genes in SCC of horn’s early passage cells were involved in transporter activity, catalytic activity, nucleic acid binding transcription factor activity, biogenesis, cellular processes, biological regulation and localization and the down-regulated genes mainly were involved in focal adhesion, extracellular matrix receptor interaction and spliceosome activity.

Conclusion: The experiment revealed similar transcriptomic nature of horn’s SCC tissue and its early passage cells.

Keywords: cummerbund, gene ontology, primary culture, RNA-sequencing, squamous cell carcinoma of horn, transcriptome profiling.

Introduction

Cancer cell lines, in general, are used as a model in testing of anticancer drugs presently used [1,2] as well as in the development of new therapies [3,4]. There is no bovine cell line of squamous cell carcinoma (SCC) origin. This is probably the first ever attempt to develop a SCC cell line of bovine origin. The horn cancer-based cell line can be used as an in vitro model in cancer research to define potential molecular markers as well as for the screening and characterization of cancer therapeutics similar to human lung and breast cancer cell lines [5,6]. The results of the research in cancer cell lines can usually be extrapolated to in vivo tumors originated from squamous cells. Transcriptomic profiling of the initial passage cells and the SCC tissue was attempted in this study to confirm the initial passage cells represent the SCC tissue at molecular level.

Historically, in vitro cultures of SCC of horn (bovine horn core carcinoma [BHCC]) have been limited in availability and scope, compared to those from many other organs such as mammary tumors and endometrial cancer cell lines. Cell lines, those derived from metastases, do not span the range of most of cancer phenotypes, and in particular, are not representative of original SCC [7]. Furthermore, how extensively long-term culture alters the biological properties of cell lines are always of concern [8]. Adaptation of fresh cancerous tissue specimens which grow in vitro as primary cell cultures provides homogeneous cellular material, enriched in tumor cell component [7] and it also retains phenotypic, transcriptomics profile of the corresponding tissues from which they derive [8-10] at the first passages.
Usually, up regulations of genes are involved in proliferation and metabolism. Cellular activity within a tissue is evinced by the transcriptome at a specific time. Pathophysiology of complex diseases, like cancer, can be evaluated by an unbiased method like genome-wide expression studies [10]. RNA sequencing (RNA-Seq) analysis is an affordable accurate and comprehensive tool to analyze transcriptome of complementary DNAs (cDNA) using next generation sequencing (NGS), followed by mapping of reads onto the reference genome making it possible to identify introns, exons, their flanking regions and thus providing an opportunity to understand the complexity of eukaryotic transcriptome [11].

SCC of horn of bovines is a SCC of horn core mucosa with least known genetic landscape, reported only in Bos indicus. This causes heavy economic losses due to subsequent metastasis and death of animal. In India, approximately 1% of the cattle population is affected by this tumor [12], most commonly in working bullocks, sometimes in cows and rarely in bulls, buffaloes, sheep, and goats [13-16]. The incidence of SCC of horns is more frequent in Kankrej breed than other zebu cattle, crossbred or non-descript cattle [17]. From Sumatra [18], Brazil [19], and Iraq [20] few cases were reported. Till date, the comparison of gene expression profile between cell culture and parenteral tissue of SCC of horn of bovines has not been performed. The study was designed to compare gene expression profiles in SCC affected horn tissue and primary cell culture derived from that tumor using Ion Torrent PGM sequencing platform.

Materials and Methods

Ethical approval

Approval for research work 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 RNA-later® (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:

a. 10% neutral buffered formalin for histopathological studies
b. RNA-later® (Sigma-Aldrich, St. Louis, USA) for RNA extraction
c. Dulbecco’s modification of Eagle’s medium (DMEM) (50 ml) (Thermo Fisher Scientific, Massachusetts, USA) with penicillin-streptomycin (500 µl) (Thermo Fisher Scientific, Massachusetts, USA) + amphotericin-B (500 µl) (Thermo Fisher Scientific, Massachusetts, USA) and brought to lab at 0-4°C.

Histopathology

Horn SCC tissues were processed for histopathological studies and paraffin-embedded sections were cut at 5-6 µ thickness with section cutting machine (Leica, Germany) and stained with hematoxylin and cosin (H and E) [21]. The H and E stained sections were observed under light microscope and lesions were observed [21].

Cell culture

After removal of adipose tissue, tumor tissues (at 4°C) were mechanically minced in 1 mm² fragments. Then, the primary culture was established and incubated at 37°C and 5% CO₂ [21]. Similarly, tumor tissue explant culture was also performed by standard protocol [16]. DMEM and Ham’s F12 50/50 mix (DMEM-F12) medium was changed twice weekly and split ratio for cells was 1:3 when cells reached up to 90% confluence. Cell morphology was observed in contrast phase, at 40× magnification, by inverted microscope. The cells were sampled at intervals, resuspended in a freezing medium (80% DMEM, 10% fetal bovine serum, and 10% dimethyl sulfoxide), and stored at −80°C at every two passages for cryopreservation.

Differential trypsinization was used for removal of the fibroblasts which detached sooner than the tumor cells. Isolation of pure population of tumor cells was done by plating approximately 10,000 detached cells in 100 mm Petri dishes and following dilution cloning [22]. These isolated clones were used for RNA-Seq purposes.

Cell proliferation and doubling time assay

Two counts were performed for each passage, in triplicate. For doubling time analysis, plating of cells in triplicate onto 6-well plates at a concentration of 2.5 × 10⁵ cells/well in DMEM-F12 were done. After 24, 48 and 72 h, cells were collected after trypsinization and counted in a Neubauer chamber. Doubling time (in hour) was calculated as described in a previous study [23].

RNA isolation

TRIzol (Sigma-Aldrich, St. Louis, USA) method as per manufacturer’s instructions was used to isolate RNA from early passage cells of SCC of horns (pooled RNA of passage 2 and 3) and parental SCC tissue.

Preparation of sample and transcriptome procedure

All the protocols starting from mRNA isolation to library preparation were followed as per manufacturer’s instructions. 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 cells and parental SCC horn tissue using Ion Torrent PGM chemistry using 316 chips [24]. Raw sequence reads (*.fastq files) were checked for quality control in FastQC v0.10.1. To avoid low quality data negatively influencing downstream analysis, the reads were trimmed and low quality sequences were filtered using PRINSEQ-lite.
version 0.20.2 with default parameters in Linux. This quality checked reads were aligned to the bosTau7.fa build of the cow genome (http://hgdownloadtest.cse.ucsc.edu/goldenPath/bosTau7/chromosomes/) using GMAP [25] and Samtools allowing for unique non-gapped alignments to the genome. The default parameters for the GMAP method were used.

The resultant *.sam files were converted to *.bam files with Samtools then *.sorted.bam files were used in Cufflinks v 2.2.1. The resulting Cufflinks assemblies of all samples were combined together using Cuffcompare v 2.2.1. The differential expression was calculated by Cuffdiff based on transcript abundances [26]. Cuffdiff v 2.2.1 was then employed on the combined transcripts to identify differentially expressed genes/transcripts.

**RNA-Seq data normalization**

The raw RNA-Seq read counts for cufflinks transcripts were first log, transformed at fragments per kilobase of exon per million reads mapped (FPKM) and then quantile normalized.

**Functional annotation**

The genes differentially expressed in SCC horn tissue and the short-term primary culture was selected for functional categorization. The comparisons between expressed genes which produced Cuffdiff output with “Q value” <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 [27] and PANTHER database [28] were done, respectively. Gene set analyses were done in terms of biological processes, molecular function, and cellular component. The list of differentially expressed genes having >5 FPKM value and log2 fold change value above 2 (based on FPKM ratio), p=0.05 and false discovery rate (FDR) value 5% were chosen.

Whole transcriptome analysis using NGS will identify several thousands of genes which are deregulated in number of cancer-related pathways. Since the depth of sequencing for each gene varies because of inherent methodology involved in NGS, it is globally accepted protocol to validate data obtained by this methodology via randomly selecting few of the genes through quantitative real-time polymerase chain reaction (PCR) [29,30]. Since it is practically impossible to validate all of the genes found in NGS-based study as well as it is economically non-feasible approach to study all identified genes, we have followed standard procedure to validate NGS data by selecting randomly selected sufficiently large set of transcripts and proved concordance of expression pattern using quantitative real-time PCR (Data not shown).

**Results**

**Histopathology of SCC tissue**

The tumor cells were tightly cohesive, featured with moderately high to abundant eosinophilic cytoplasm. The nucleus to cytoplasmic ratio was potentially increased with nuclei showing frequent prominent nucleoli. Mitotic activity was abundant including atypical forms such as ring and tripolar configurations. Intercellular bridges were focally present. Keratinization of individual epithelial cells (Figure-1a) and pleomorphic epithelial cells with enlarged nuclei (Figure-1b) were seen. Histopathology confirmed SCC of the horn core epithelium.

**Isolation of SCC horn epithelial cells**

Primary monolayer culture with finite mitotic lifespan (SCC early passage cells) was established from the bullock affected with SCC of horn (Figure-2) following the enzymatic disaggregation methods as described earlier [22]. By the first week, tumor cells were seen rounding up and growing throughout the T-25/T-75 flask (Figure-3) among the normal stromal fibroblasts that grew in parallel.

**Growth curve and population doubling time analysis**

Population doubling time ascertained around 28.1 h (Figure-4), and cell viability ranged from 85% to 94%. The culture success rate was 90%.

**Transcriptomic comparison between SCC horn tissue and its early passage cells**

The total number of genes differentially overexpressed in SCC horn tissue were 717 (8.40% of total genes expressed) compared to early passage cells; 150 genes (1.76% of total genes expressed) were differentially up-regulated which had more than 2-fold Log$_{2}$ value with maximum value of 6.03-fold change. There were 746 genes (8.74% of total genes expressed) which had differential over-expression in early passage cells than SCC horn tissue, 248 genes (2.90% of total genes expressed) had more than 2-fold Log$_{2}$ value with maximum Log$_{2}$ value 7.02. In this comparison, 5219 genes (~38% of total genes no., i.e., 14513 no.) showed no expression at the terms of FPKM in both the samples 1600 genes had more than 5 FPKM value in early passage cells.

**Genes overexpressed in SCC early passage cells and SCC horn tissue**

Density plot and dispersion plot were derived for this comparison, respectively. Density plot assessed the distributions of FPKM scores across samples. Among the differentially expressed genes maximum genes had FPKM value between Log$_{10}$ 0.2 and Log$_{10}$ 2. Distribution of genes in SCC horn tissue ranged from Log$_{10}$ 0.2 to Log$_{10}$ 3.7 and for early passage SCC cells, it was Log$_{10}$ 0.7 to Log$_{10}$ 3.7. Dispersion plot showed normal dispersion of genes across samples. N-Myc downstream regulated 1, integrin alpha 6, TP53 apoptosis effector (PERP), eukaryotic translation initiation factor 4 A1 (A1EIF4A1), desmoplakin, etc., genes were up-regulated (up-to 2-fold FPKM value) in SCC horn tissue compared to SCC early passage cells. Up-regulated genes (up to 2-fold FPKM value) in horn SCC early passage cells compared to parental tissue were coiled-coil domain containing 69 (CCDC69), CCDC94, Sec61 gamma subunit (SEC61G), Paladin,
Hedgehog (Hh) receptor patched homolog 1 (PTCH1), Armadillo repeat containing X-linked 2 and thioredoxin, etc.

GO category of the genes differentially expressed above 2 log2 fold change in SCC early passage cells compared to SCC horn tissue to be of calcium channel activity, calcium ion binding, protein phosphatase Type 2A activity and extracellular matrix (ECM) binding as per DAVID database (Table-1). The genes which were up-regulated in SCC horn tissue compared to its early passage cells showed major histocompatibility complex (MHC) Class I protein binding, MHC protein binding, procollagen proline 4-dioxygenase activity, peptidyl-proline dioxygenase activity, pro-collagen-proline dioxygenase activity, and protein disulfide isomerase activity.

The percentage of genes which showed up-regulation in SCC horn tissue than SCC early passage cells was 1.76%. Genes up-regulated (≥2-fold) in SCC horn tissue as compared to horn SCC early passage cells were involved in biogenesis, apoptotic response and response to stimulus in biological processes; structural molecular activity and translation regulator activity in molecular function; cell part, organelle and macromolecular complex in cellular component and the up-regulated genes (≥2-fold) in horn SCC early passage cells were involved in cellular process, metabolic process, biological regulation in biological processes; catalytic activity, enzyme regulator activity, binding in molecular function; membrane, extracellular region in cellular component as per PANTHER database.

There was no pathway in 5 FDR limit, but the lowest FDR value was found at transforming growth factor (TGF) beta signaling pathway and ribosomal pathway for differentially up-regulated genes in SCC early passage cells compared to SCC horn tissue in Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (Table-2). Surprisingly, most of the genes which showed top fold change (within first 20) were not detected by DAVID during pathway analysis. Focal adhesion, ECM-receptor interaction, thyroid cancer, and pathways in cancer were shown by the genes which were up-regulated in SCC horn tissue than SCC early passage cells (Table-3).

Genes up-regulated in SCC early passage cells compared to SCC horn tissue were involved in fibroblast growth factor signaling pathway, wnt signaling pathway, vascular endothelial growth factor signaling pathway, apoptosis signaling pathway and p53 signaling, epidermal growth factor receptor, cell cycle,
inflammatory pathways mediated by chemokine and cytokine, etc., as per PANTHER database.

KEGG pathway of all genes, expressed in SCC early passage cells showed to be involved in focal adhesion, transforming growth factor TGF-beta signaling pathway, ubiquitin mediated proteolysis, pathways in cancer, prostate cancer mechanism within 5 FDR value (Table-4). KEGG pathways such as thyroid cancer, focal adhesion, small cell lung cancer, pathways in cancer, prostate cancer and spliceosome were shown to be involved when all the common genes (≥5 FPKM) between SCC horn tissue and SCC early passage cells compared in DAVID (Table-5). To unveil the genes involved in horn cancer pathogenesis, both in-vivo and in-vitro genes were mined from common pathways up to 5 FDR (Table-6).

Genes that were uniquely expressed in SCC early passage cells as compared to SCC horn tissue showed involvement in metabolic and cellular process in biological processes; binding, catalytic activity in molecular function; heterotrimeric G protein signaling G alpha pathway, Huntington disease, endothelin signaling pathway, angiogenesis, interleukin signaling pathway, etc., in pathway as per PANTHER database.

High proliferative and antiapoptotic potential are related to the up-regulation of growth hormone receptor and calmodulins [31]. The top 20 genes which were found to be up-regulated in SCC early passage cells in comparison to SCC horn tissue were investigated to have roles in other cancers as well as SCC in human and domestic animals (Table-7) [32-61] and vice versa (Table-8) [62-95].
In this study, we compared gene expression profiles of the two conditions, i.e., in vivo cancer tissue and in vitro cancer cells at their early passages. The growth and survival rate of SCC early passage cells were good and it grew for the first few
Table-4: KEGG pathway of all genes expressing ≥5 FPKM in SCC early passage cells.

| Term                               | Count | FDR     | %       | p value |
|------------------------------------|-------|---------|---------|---------|
| bta04510:Focal adhesion             | 48    | 4.06E-06| 3.292181| 3.33E-09|
| bta04810:Regulation of actin cytoskeleton | 40    | 0.042391| 2.743484| 3.47E-05|
| bta04350:TGF-beta signaling pathway | 22    | 0.0641  | 1.508916| 5.25E-05|
| bta04512:ECM-receptor interaction  | 21    | 0.091836| 1.440329| 7.52E-05|
| bta04520:Adherens junction         | 18    | 0.596801| 1.234568| 4.90E-04|
| bta04120:Ubiquitin mediated proteolysis | 28    | 0.923001| 1.920439| 7.59E-04|
| bta05010:Alzheimer's disease       | 30    | 2.493014| 2.057613| 0.002065|
| bta03010:Ribosome                  | 19    | 3.36116 | 1.303155| 0.002775|
| bta05200:Pathways in cancer        | 49    | 3.410855| 3.360768| 0.002838|
| bta05215:Prostate cancer           | 18    | 7.802359| 1.234568| 0.00663 |
| bta05016:Huntington's disease      | 30    | 7.998577| 2.057613| 0.006803|
| bta04670:Leukocyte transendothelial migration | 22    | 8.071079| 1.508916| 0.006867|
| bta04114:Oocyte meiosis            | 21    | 12.05085| 1.440329| 0.01046 |
| bta00640:Propanoate metabolism     | 9     | 15.5853 | 0.617284| 0.013778|
| bta03050:Proteasome               | 11    | 17.3639 | 0.754458| 0.015496|
| bta04270:Vascular smooth muscle contraction | 20    | 17.64442| 1.371742| 0.015771|
| bta04530:Tight junction            | 22    | 19.73693| 1.508916| 0.017843|
| bta03040:Spliceosome              | 22    | 19.73693| 1.508916| 0.017843|
| bta00112:ARVC                     | 14    | 20.06711| 0.960219| 0.018174|
| bta05211:Renal cell carcinoma      | 14    | 20.06711| 0.960219| 0.018174|
| bta04540:Gap junction              | 16    | 20.36185| 1.097394| 0.018471|
| bta05212:Pancreatic cancer         | 14    | 24.78525| 1.097394| 0.018471|
| bta05012:Parkinson's disease       | 22    | 28.04137| 1.508916| 0.02699 |
| bta05210:Colorectal cancer         | 16    | 31.81445| 1.097394| 0.030871|
| bta05414:Dilated cardiomyopathy    | 15    | 32.44796| 1.371742| 0.031584|
| bta04360:Axon guidance             | 20    | 32.68997| 1.371742| 0.031907|
| bta04110:Cell cycle                | 21    | 35.81972| 1.440329| 0.035663|
| bta05410:HCM                      | 14    | 38.06963| 0.960219| 0.03942 |
| bta04150:mtOR signaling pathway    | 11    | 39.72862| 0.754458| 0.040613|
| bta05222:Small cell lung cancer    | 15    | 40.92982| 1.028807| 0.042193|
| bta04720:Long-term potentiation    | 12    | 43.26753| 0.823045| 0.045355|
| bta04142:Lysosome                 | 19    | 48.66196| 1.303155| 0.053134|
| bta05213:Endometrial cancer        | 10    | 55.76984| 0.685871| 0.064618|
| bta04666:Fc gamma R-mediated phagocytosis | 15    | 59.04148| 1.028807| 0.070491|
| bta00520:Amino sugar and nucleotide sugar metabolism | 9 | 60.46497| 0.617284| 0.073175|
| bta05220:Chronic myeloid leukemia   | 13    | 69.07887| 0.891632| 0.091639|
| bta00190:Oxidative phosphorylation | 20    | 70.92517| 1.371742| 0.096207|

Count denotes gene count. ARVC=Arrhythmogenic right ventricular cardiomyopathy, KEGG=Kyoto encyclopedia of genes and genomes, SCC=Squamous cell carcinoma, HCM=Hypertrophic cardiomyopathy, FPKM=Fragments per kilobase of exon per million, FDR=False discovery rate, ECM=Extracellular matrix.

passages without difficulties. The cellular compositions were homogeneous and were of morphological characteristics typical of squamous cell epithelium. These findings are more or less similar to previously described studies [31] that indicated that early passage cell cultures expressed genes similar to in vivo gene expression pattern. Hence, it could be used for in vitro investigation of transcriptomic alteration in cancers. Maximum value of differential gene expression in SCC early passage cells was 6.02-fold changes as compared to parental tissue. CCDC94 a dose-dependent modifier of the anti-apoptotic function of B-cell lymphoma 2 gene found to be up-regulated [96] in SCC early passage cells; PTCH1 overexpression might indicate invasive behavior of metastatic cells [97]; low Hh signaling [98] (Table-9). PTCH-1 overexpression in many epithelial-derived cancers correlates to overexpression of other “Hh pathway” members [99] and promotion of an alternate epidermal cell fate decision that potentiates SCC formation [100]. Netrin 4 overexpression might have control on reduced angiogenesis and metastasis [101]; high SATB homeobox 1 expression might have helped to promote cell cycle progression, proliferation, migration and increased invasive capability with strong expression of Vimentin (2750.61 FPKM) but low or lost E-cadherin (CDH1) expression - A pivotal event for epithelial to mesenchymal transition EMT [102]. EIF41A, X-linked gene overexpression along with EIF2A gene (fold change −0.56) downregulation shows improved cell proliferation as EIF2A gene is a negative regulator of protein translation, RPS7 gene overexpression (fold change −0.88) might have role in cancer cell cycle proliferation and cell cycle progression in BHCC early passage cells [103]. 14-3-3 gamma was not expressed in BHCC early passage cells denoting that 14-3-3 gamma might not have control on reduced angiogenesis and metastasis [101]; high SATB homeobox 1 expression might have helped to promote cell cycle progression, proliferation, migration and increased invasive capability with strong expression of Vimentin (2750.61 FPKM) but low or lost E-cadherin (CDH1) expression - A pivotal event for epithelial to mesenchymal transition EMT [102]. EIF41A, X-linked gene overexpression along with EIF2A gene (fold change −0.56) downregulation shows improved cell proliferation as EIF2A gene is a negative regulator of protein translation, RPS7 gene overexpression (fold change −0.88) might have role in cancer cell cycle proliferation and cell cycle progression in BHCC early passage cells [103]. 14-3-3 gamma was not expressed in BHCC early passage cells denoting that 14-3-3 gamma might not be working at transcriptional level, but 14-3-3 theta which was found to be increased (fold change 0.30) might had a positive effect on tumor cell adhesion and growth [104]. In correlation to that Stratifin or 14-3-3 sigma was not expressed in BHCC early passage cells...
cells. Cyclin D1 (FPKM in BHCC early passage cells is ~86) which usually acts as an active switch for regulation of continuous cell cycle progression, had almost same expression in two samples, revealing the possible cycle chain in between these key players. Phosphoserine phosphatase [105]; inorganic pyrophosphatase 2 subunit B, epsilon isoform (PPP2R5E) is a potential tumor suppressor gene [106], PP2, phosphatase 2 subunit B isoform alpha (PPP2R2A) is one of the four major Ser/Thr phosphatases and a key regulator of cellular growth and cancer development by antagonizing protein kinases in human cancers. Protein phosphatases are involved in the suppression of protein kinases and can play a regulatory role in cancer cells. It has been postulated that protein phosphatases are involved in the suppression of cellular growth and cancer development by antagonizing protein kinases in human cancers. Protein phosphatase 2 subunit B isofrom alpha (PPP2R2A) is one of the four major Ser/Thr phosphatases and is a potential tumor suppressor gene [106], PP2, regulatory subunit B, epsilon isoform (PPP2R5E)

### Table-5: KEGG pathway of all common genes (≥5 FPKM) in between SCC horn tissue and SCC early passage cells.

| Term                          | Count | FDR     | %     | p value  |
|-------------------------------|-------|---------|-------|----------|
| bta04510:Focal adhesion       | 32    | 2.52E-06| 4.878049 | 2.12E-09 |
| bta04810:Regulation of actin cytoskeleton | 26    | 0.014261| 3.963415 | 3.963415 |
| bta04512:ECM-receptor interaction | 15    | 0.027243| 2.286585 | 2.30E-05 |
| bta05200:Pathways in cancer   | 31    | 0.450733| 4.72561  | 3.81E-04 |
| bta05215:Prostate cancer      | 13    | 1.366685| 1.981707 | 0.00115989 |
| bta05412:ARVC                 | 11    | 1.968263| 1.676829 | 0.00167511 |
| bta04670:Leukocyte transendothelial migration | 15    | 2.062266| 2.286585 | 0.001755881 |
| bta04520:Adherens junction    | 11    | 2.482973| 1.676829 | 0.002118237 |
| bta04120:Ubiquitin mediated proteolysis | 15    | 10.18146 | 2.286585 | 0.00915052 |
| bta04530: Tight junction       | 14    | 11.1892  | 2.134146 | 0.009957604 |
| bta03040:Spliceosome           | 14    | 11.1892  | 2.134146 | 0.009957604 |
| bta04350: TGF-beta signaling pathway | 10    | 21.36772 | 1.52439  | 0.020069312 |
| bta05213: Endometrial cancer   | 7     | 35.30036 | 1.067073 | 0.036055226 |
| bta05216: Thyroid cancer       | 5     | 40.50522 | 0.762195 | 0.049576494 |
| bta05414: Dilated cardiomyopathy | 9    | 41.73228 | 1.371951 | 0.049020477 |
| bta03110: Lysine degradation   | 6     | 44.89775 | 0.914634 | 0.049576494 |
| bta05211: Renal cell carcinoma | 8     | 45.78755 | 1.219512 | 0.049576494 |
| bta04540: Gap junction         | 12    | 47.42947 | 1.829268 | 0.052785299 |
| bta04110: Cell cycle           | 9     | 48.09441 | 1.829268 | 0.052785299 |
| bta05222: Small cell lung cancer | 6     | 56.59508 | 1.371951 | 0.053801616 |
| bta05010: Alzheimer's disease  | 14    | 53.2995  | 2.134146 | 0.062196631 |
| bta05210: Colorectal cancer    | 9     | 56.59508 | 1.371951 | 0.067966842 |
| bta03010: Ribosome             | 9     | 56.59508 | 1.371951 | 0.067966842 |
| bta05410: HCM                  | 8     | 61.65301 | 1.219512 | 0.077654962 |
| bta04720: Long-term potentiation| 7     | 64.27834 | 1.067073 | 0.083155068 |
| bta04722: Neurotrophin signaling pathway | 11    | 64.64354 | 1.676829 | 0.0839493 |

Count denotes gene count. HCM=Hypertrophic cardiomyopathy, FDR=False discovery rate, KEGG=Kyoto encyclopedia of genes and genomes, FPKM=Frags per kilobase of exon per million, SCC=Squamous cell carcinoma, TGF=Transforming growth factor, ARVC=Arrhythmogenic right ventricular cardiomyopathy

### Table-6: Genes common in pathways up to 5 FDR between SCC horn tissue and SCC early passage cells.

| KEGG pathway term                          | FDR     | Genes                                                                 |
|--------------------------------------------|---------|----------------------------------------------------------------------|
| bta04510:Focal adhesion                    | 2.5165E+00 | TTN1, COL3A1, ITGB1, CTNNB1, MYL9, VCL, ACTG1, CDC42, ITGAV, ILK, COL6A2, COL6A1, THBS2, PIK3R2, FN1, ACTB, COL4A1, ACTN4, PPP1CB, FNBL, FNLA, LAMA4, PPP1CA, CCND1, ITGA6, ITGAV5, JUN, COL1A2, PDGFR, RAP1A, PDGFRB, COL1A1, CRK |
| bta04810:Regulation of actin cytoskeleton   | 0.142   | RDX, PIP5K1A, ITGB1, MYL9, VCL, ACTG1, CDC42, ITGA6, EZR, GSN, ITGAV, MSN, FGFR2, FN1, APC, PIK3R2, ACTB, ACTN4, PPP1CB, ARCP1A, PPP1CA, ITGA6, ITGAV5, CFL1, PDGFB, PDGFRB, CRK, PIP4K2C |
| bta04512:ECM-receptor interaction          | 0.272   | COL4A1, COL3A1, ITGB1, ITGB4, SDC1, LAMA4, ITGAV, CD44, ITGAV5, ITGAV6, COL6A2, COL1A2, COL6A1, COL1A1, THBS2, FN1 |
| bta05200:Pathways in cancer                | 0.4507  | HSP90A2B, TGF, MMP2, ITGB1, CTNNB1, CDC42, ITGAV, MYC, FGFR2, FN1, APC, PIK3R2, COL4A1, HSP90A1A1, EPS1A, CREBBP, SMAD4, CTNN1A1, STAT3, LAMA4, HSP90B2, CCND1, CDKNA1A, HIF1A, ITGAV6, NCOA4, JUN, PDGFRB, PDGFB, JAK1, CRK |
| bta05215:Prostate cancer                   | 1.3666  | HSP90A1B1, HSP90A1A1, CREBBP, CTNNB1, CCND1, HSP90B1, CDKNA1A, AT1F4, PDGFR, CREB3L2, CREB3L1, PDGFRB, PIK3R2 |
| bta05412:ARVC                              | 1.9682  | ACTB, ACTG1, ACTN4, ITGAV6, ITGAV5, ITGAV, LMNA, DSP, G1A1, CTNN1A1, ITGB1, CTNNB1 |
| bta04670:Leukocyte transendothelial migration | 2.0622 | ACTB, ACTN4, GNA2A, GNA1A, CTNN1A, MMP2, ITGB1, VCL, MYL9, CTNNB1, ACTG1, CDC42, EZR, RAP1A, MSN, PIK3R2 |
| bta04520:Adherens junction                 | 2.4829  | ACTB, ACTG1, CDC42, PVR1, ACTN4, PTPFR, CREBBP, SMAD4, CTNN1A1, SNA1A2, VCL, CTNNB1 |

ARVC=Arrhythmogenic right ventricular cardiomyopathy, FDR=False discovery rate, KEGG=Kyoto Encyclopedia of Genes and Genomes, SCC=Squamous cell carcinoma, ECM=Extracellular matrix
### Table 7: Functions of highly expressed genes in SCC early passage cells in comparison to SCC horn tissue.

| Gene ID (ENSBTAG) | Gene title               | Name                                      | FPKM EP | FPKM HCT | Log₂ fold change | Roles and implications in cancer of human and other |
|-------------------|--------------------------|-------------------------------------------|---------|----------|------------------|---------------------------------------------------|
| 00000002834       | CCDC69                   | Coiled-coil domain containing 69          | 318.123 | 2.3446   | +7.084           | Expressed in various cancer cell lines such as HeLa, U2OS and MDA-MB-231, exogenous expression of CCDC69 in HeLa cells destabilized microtubules and disrupted the formation of bipolar mitotic spindles [32] |
| 00000012830       | CCDC94                   | Coiled-coil domain containing 94          | 842.151 | 10.503   | +6.325           | Avoids DNA damaging apoptosis in zebra-fish [33] |
| 00000014971       | SEC61G                   | Sec61 gamma subunit                       | 4614.43 | 62.285   | +6.211           | Proto-oncogene required for tumor cell survival in GBM, involved in the cytoprotective ER stress-adaptive response to the tumor microenvironment [34] |
| 00000008583       | KIAA1274                 | Paladin                                   | 207.836 | 2.808    | +6.209           | Vascular-restricted expression in human brain, astrocytoma, and glioblastomas. Paladin expression is reactivated during pathological tumor angiogenesis in the adult [35] |
| 00000048213       | PTCH1                    | Hh receptor patched homolog 1, Uncharacterized protein | 92.8954 | 1.2552   | +6.209           | Inversely correlated with the metastatic potential of colon cancer cell lines, high expression associated with low Hh signaling [36] |
| 0000003183        | NTN4                     | Netrin 4                                  | 123.963 | 2.010    | +5.946           | Anti angiogenic effect, over expression could decrease tumor growth [37] |
| 00000010232       | NDUFS5                   | NADH dehydrogenase (ubiquinone) Fe-S protein 5, 15 kDa (NADH-coenzyme Q reductase) | 1206.46 | 24.433   | +5.625           | Highly expressed in endometrial cancer [38,85] |
| 00000019417       | ARM CX2                  | Armadillo repeat containing, X-linked 2   | 145.59  | 2.950    | +5.624           | Might have a role in tumor suppression, role in development and tissue integrity [39] |
| 00000021158       | SATB1                    | SATB homeobox 1                           | 161.255 | 3.7353   | +5.431           | High levels of SATB1 expression facilitate CRC and are associated with poor prognosis, promotes breast cancer metastasis, EMT marker in prostrate cancer [40] |
| 0000003130        | CHRNA3                   | Cholinergic receptor, nicotinic, alpha 3 (neuronal) | 1615.84 | 43.60    | +5.211           | Polymorphism associated with high chance for NSCLC [41,85] |
| 00000017633       | EIF1AX                   | Eukaryotic translation initiation factor 1A, X linked | 509.347 | 13.759   | +5.210           | Mutation is having protective role in uveal melanoma, over expressed in metastatic prostate cancer [42,43] |
| 00000002428       | PPA2                     | Pyrophosphatase (inorganic) 2             | 255.495 | 6.903    | +5.209           | Significantly increased in LnMPCa tissues, supplies increased energy requirement in metastasis cells [44,45] |
| 00000000753       | PIAS4                    | Protein inhibitor of activated STAT, 4     | 582.593 | 17.174   | +5.084           | Necessary for proficient DNA repair of DSBs, promotes BRCA1 SUMOylation and DNA repair [46,47] |
| 00000013081       | PSPH                     | Phosphoserine phosphatase                 | 516.471 | 17.942   | +4.847           | Up-regulated in CRC, increased expression in non-small-cell lung cancer corresponds to clinical response. Suppression inhibited proliferation, tumor formation of MDAMB-468 and MCF10 cells respectively [48,49] |
| 00000002953       | TXN                      | Thioredoxin                               | 3783.39 | 136.25   | +4.795           | Promote cell growth, induces VEGF, PTEN, angiogenesis and inhibit apoptosis in tumor cells [50,51] |

(Contd...)
### Table-7: Continued...

| Gene ID (ENSBTAG) | Gene title Name | FPKM EP | FPKM HCT | Log₂ fold change | Roles and implications in cancer of human and other |
|-------------------|-----------------|---------|----------|-------------------|--------------------------------------------------|
| 00000015522       | MRPS31 Mitochondrial ribosomal protein S31 | 12.604  | 310.988  | +4.624            | Up-regulated in human breast cancer, CRC and found in 77% of all types of cancer [52,53,85] |
| 00000045742       | CSH12orf75 Chromosome 12 open reading frame 75 | 6.018  | 148.477  | +4.624            | Highly expressed in granulosa cells and membrane associated granulosa cells before ovulation in cattle [54] |
| 0000009405        | TRPC4 Transient receptor potential cation channel, subfamily C, member 4 | 2.091  | 51.582   | +4.624            | Highly expressed in NSCLC, LNCaP cells activating store operated channel calcium influx factor [55,56] |
| 0000008636        | PDE4B Phosphodiesterase 4B, cAMP-specific | 1.6824  | 41.5034  | +4.624            | Highly expressed in diffuse large BCL, expression of it avoids CAMP mediated apoptosis. Induces angiogenesis and cell proliferation in lung cancer cell line [57,58] |
| 0000008294        | KCNJ2 Potassium inwardly-rectifying channel, subfamily J, member 2 | 1.4278  | 35.2224  | +4.624            | Expressed in medullloblastoma with poor clinical outcome, avoids apoptosis and induces cell proliferation in oral cancer also. Increased expression in papillary thyroid cancer [59-61] |

**Table-8: Functions of highly expressed genes in SCC horn tissue in comparison to SCC early passage cells.**

| Gene ID (ENSBTAG) | Gene title Name | FPKM HCT | FPKM EP | Log₂ fold change | Roles and implications in cancer of human and other |
|-------------------|-----------------|----------|---------|-------------------|--------------------------------------------------|
| 00000000711       | NDRG1 N-Myc downstream regulated 1 | 30.4749  | 2001.28 | −6.03715         | Regulated by androgens, acts as metastasis suppressor and negatively correlated with it, found to be down regulated in various cancers, prostate cancer [62,63] |
| 00000017266       | ITGA6 Integrin, alpha 6 | 21.8316  | 835.447 | −5.2580          | Prostate tumors persistently express ITGA6, linked to increased tumor cell invasion, migration, and metastasis. Increased adhesion in AML cells [64,65] |
| 00000020097       | PERP PERP, TP53 apoptosis effector | 47.026   | 1624.07 | −5.11001         | Tumor suppressor. Loss induces tumorigenesis, cell survival, and desmosome loss by enhancing inflammatory set of genes in SCCs [66,67] |
| 0000000132        | EIF4A1 Eukaryotic translation initiation factor 4A1 | 54.0897  | 1613.66 | −4.8988          | Associated with highly metastasizing melanoma. Overexpression is an early marker for metastasizing hepatocellular carcinoma and NSCLC [68,69] |
| 00000015106       | DSP Desmoplakin | 63.9491  | 1837.68 | −4.8448          | Loss of desmoplakin, a cell adhesion molecule, has been implicated in breast cancer metastasis [70] |
| 00000047330       | FABP5 Fatty acid binding protein 5 (psoriasis associated) | 51.7861  | 1255.27 | −4.5992          | Involved in cell survival and growth, enhances cell proliferation and anchorage-independent growth in prostate and breast cancer cells [71,72] |
| 00000012447       | PPP1CB Protein phosphatase 1, catalytic subunit, beta isozyme | 34.5396  | 764.459 | −4.4681          | Enhances proliferation and colony formation in leukemia cell line, expressed in SS cancer cell lines [73,74] |
| 00000010365       | SQRDL Sulphide quinone reductase-like (yeast) | 57.2255  | 1206.17 | −4.3976          | Under expressed in ductal breast carcinoma, but down regulation reduce cell growth and induce apoptosis in breast cancer cell line [75,76] |

**EP=SCC early passage cells, HCT=SCC horn tissue, FPKM=FFPKM=Fragments per kilobase of exon per million.**
| Gene ID (ENSBTAG) | Gene title | Name | FPKM EP | Log₂ fold change | Roles and implications in cancer of human and other |
|------------------|------------|------|---------|------------------|----------------------------------------------------|
| 00000011969      | HSPB1      | Heat shock 27 kDa protein 1 | 137.28 | −4.3349 | Involved in DNA repair, recombination, anti-apoptotic activity in HeLa cells, in most of human cancers, high levels indicate presence of metastatic tissues. Low levels are associated with resistance [77,78] |
| 00000011488      | PRPB8      | PRPB pre-mRNA processing factor B homolog (S. cerevisiae) | 230.594 | 11.6561 | −4.3062 | Associated with spliceosome pathway, tumor suppressor in myeloid malignancies [79,80] |
| 00000012927      | ALDOA      | Aldolase A, fructose-bisphosphate, mRNA | 1162.91 | 60.5281 | −4.2639 | Promote lung cancer metastasis, invasion capability [81,82] |
| 00000015107      | SLCA6A1    | Solute carrier family 16, member 1 (monocarboxylic acid transporter 1) | 465.287 | 28.411 | −4.0336 | Positively associated with cell survival, negatively with mir-124 in medulloblastoma [83] |
| 00000021035      | CTSK       | Cathepsin K, mRNA | 917.7 | 56.0406 | −4.0334 | Inconsistent expression in horn cancer tissue in bovine, involved in Hh signaling and pre-osteoclast to osteoclast differentiation in breast cancer [84,86] |
| 00000010793      | CCDC80     | CCDC80, mRNA | 393.218 | 24.1296 | −4.0264 | Tumor suppressor, down regulated in thyroid carcinomas [87] |
| 00000013315      | ATP5B      | ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide, mRNA | 853.925 | 53.4692 | −3.9973 | Overexpressed and associated with poor survival in breast cancer. High ATP5B mRNA expression in ovarian cancer was associated with worse OS [88] |
| 00000003418      | MSN        | Moesin (MSN), mRNA | 339.836 | 22.2957 | −3.93 | High levels associated with poor breast cancer survival, by increased metastasis, invasion and EMT changes [89] |
| 00000008409      | MYC        | V-myc myelocytomatosis viral oncogene homolog (avian) | 596.739 | 41.8222 | −3.8347 | Correlated with distant metastasis, aggressive breast cancer. Induces genome instability [90] |
| 00000021523      | STAT3      | Signal transducer and activator of transcription 3 (acute-phase response factor), mRNA | 566.044 | 39.895 | −3.8266 | Associated with increased angiogenesis, metastasis, immune signaling and inflammation in basal like breast cancers [91,92] |
| 00000008611      | IGFBP4     | Insulin-like growth factor binding protein 4 | 627.819 | 44.5065 | −3.8182 | Antagonist of wnt beta catenin signaling pathway, higher in metastatic RCC. Increases invasion, cell proliferation in glioma [93,94] |
| 00000007606      | HNRNPU     | Heterogeneous nuclear ribonucleoprotein U (scaffold attachment factor A), mRNA | 386.733 | 28.0595 | −3.7847 | Involved in spliceosome pathway in causing prostate cancer [95] |

**Table-8: Continued...**

expression are usually downregulated in cancer tissue and represses cell viability and growth promoting apoptosis in cells as a target of MicroRNA-23a (miR-23a) [107]. MiR-23a overexpression decreases PPP2R5E expression but as the cells were good and healthy by their phenotypes so we cannot support this hypothesis for our cell line. Glutaminase which indicates faster growth rate and change in Warburg effect [108] was increased (0.33-fold change) (not shown in table) in cells though, MYC oncogenic transcription factor expression in BHCC early passage cells was lower than BHCC tissue, and there was no expression of MiR-23a/b which are usually suppressed by MYC [109]. Solute carrier family 7A5, phosphoglycerate dehydrogenase decreased in cells, ACACA expression remained almost same, but ACLY expression was 1.5-fold lower in cells (Table-10). SERBP1 expression was also lower in cells by 1.5-fold. Moderate secretory carrier membrane proteins 3 expressions suggested a universal role in membrane traffic at the plasma membrane [110,111].

Cytoplasmic serine hydroxymethyltransferase 1 (SHMT1) and thymidylate synthase genes of the *de novo* thymidylate biosynthesis pathway were found to be increased in early passage cells than BHCC tissue, but SHMT2 was not expressed in cells [110,112,113]. Tumor protein 53-induced nuclear protein 1, apoptosis activating factor-1 was found to be increased in
Table 9: Expression of genes that are usually altered in cancer and involved in cancer pathways.

| Official gene symbol | SCC horn tissue FPKM | SCC early passage cells FPKM | Log2 (fold change) | Official gene symbol | SCC horn tissue FPKM | SCC early passage cells FPKM | Log2 (fold change) | Official gene symbol | SCC horn tissue FPKM | SCC early passage cells FPKM | Log2 (fold change) |
|----------------------|----------------------|-----------------------------|--------------------|----------------------|----------------------|-----------------------------|--------------------|----------------------|----------------------|-----------------------------|--------------------|
| **Tumor suppressor genes** [114] | | | | | | | | | | | |
| TGFBR2 | 37.681 | 116.199 | 1.62466 | TGFBR1 | 43.997 | 180.905 | 2.03972 | TGFBR1 | 43.997 | 180.905 | 2.03972 |
| TGFBR1 | 31.946 | 53.198 | −0.62321 | CTGF | 1237.8 | 1486.66 | 0.26426 | CTGF | 1237.8 | 1486.66 | 0.26426 |
| TGFBR2 | 37.681 | 116.199 | 1.62466 | TERT | 0 | 53.039 | ∞ | TERT | 0 | 53.039 | ∞ |
| **Apoptosis** [114] | | | | | | | | | | | |
| CDKs [114] | | | | | | | | | | | |
| CDK16 | 59.044 | 58.262 | −0.01924 | CDC2 | 21.499 | 106.068 | 2.30264 | CDC2 | 21.499 | 106.068 | 2.30264 |
| **Genes highly expressed in cell, tumor** [114] | | | | | | | | | | | |
| NME1 | 20.3035 | 65.978 | | TNFAIP8L1 | −0.03833 | 0.96166 | 18.0213 | TNFAIP8L1 | −0.03833 | 0.96166 | 18.0213 |
| **Genes expressed in immortal cell lines** [114] | | | | | | | | | | | |
| CDC26 | 0 | 276.369 | Infinity | MET | 4.43146 | 18.2192 | 2.03961 | MET | 4.43146 | 18.2192 | 2.03961 |
| **Oncogenes** [114] | | | | | | | | | | | |
| TPX2 | 109.221 | 354.565 | 1.6988 | TPX2 | 109.221 | 354.565 | 1.6988 | TPX2 | 109.221 | 354.565 | 1.6988 |
| **Gli pathway** [114] | | | | | | | | | | | |
| SCAMP3 | 135.784 | 139.585 | 0.03983 | SCAMP3 | 135.784 | 139.585 | 0.03983 | SCAMP3 | 135.784 | 139.585 | 0.03983 |
| NAMPT | 19.696 | 69.401 | 1.81721 | NAMPT | 19.696 | 69.401 | 1.81721 | NAMPT | 19.696 | 69.401 | 1.81721 |
| AKTIP | 61.472 | 42.971 | −0.5163 | AKTIP | 61.472 | 42.971 | −0.5163 | AKTIP | 61.472 | 42.971 | −0.5163 |
| CTSC | 337.90 | 48.657 | −2.80156 | CTSC | 337.90 | 48.657 | −2.80156 | CTSC | 337.90 | 48.657 | −2.80156 |
| LAMTOR5 | 65.978 | 203.589 | 1.62537 | LAMTOR5 | 65.978 | 203.589 | 1.62537 | LAMTOR5 | 65.978 | 203.589 | 1.62537 |
| LAMTOR4 | 9.0 | 207.586 | ∞ | LAMTOR4 | 9.0 | 207.586 | ∞ | LAMTOR4 | 9.0 | 207.586 | ∞ |
| aEBP1 | 269.61 | 95.0135 | −1.50469 | aEBP1 | 269.61 | 95.0135 | −1.50469 | aEBP1 | 269.61 | 95.0135 | −1.50469 |
| RPS6K4A | 26.142 | 29.3143 | 0.165186 | RPS6K4A | 26.142 | 29.3143 | 0.165186 | RPS6K4A | 26.142 | 29.3143 | 0.165186 |
| RPS6KB1 | 69.345 | 183.303 | 1.40236 | RPS6KB1 | 69.345 | 183.303 | 1.40236 | RPS6KB1 | 69.345 | 183.303 | 1.40236 |

(Contd...)
### Table 9: Continued...

| Official gene symbol | SCC horn tissue FPKM | SCC early passage cells FPKM | Log2 (fold change) | Official gene symbol | SCC horn tissue FPKM | SCC early passage cells FPKM | Log2 (fold change) |
|----------------------|----------------------|-----------------------------|--------------------|----------------------|----------------------|-----------------------------|--------------------|
| RPS6KC1              | 27.447               | 19.9144                     | −0.4628            | FOXJ2                | 29.0679              | 29.878                      | 0.039683           |
| BCL2L13              | 12.845               | 118.832                     | 3.20963            | PRKAR1A              | 534.133              | 67.9277                     | −2.9753            |
| Oncogenes [114]      |                      |                             |                    | PRKAR2A              | 141.262              | 62.2342                     | −1.18295           |
| METTL13              | 8.588                | 35.31                       | 2.03968            | TGFBI                | 381.889              | 71.3708                     | −2.41975           |
| PDGFRα               | 55.2793              | 12.8642                     | −2.10337           | THBS2                | 295.379              | 41.8762                     | −2.81836           |
| ARNTL                | 5.52379              | 39.7419                     | 2.84693            | CKAP2                | 70.1512              | 112.865                     | 0.686055           |

Table 9: Official gene symbol, SCC horn tissue FPKM=FPKM of SCC horn tissue, SCC early passage cells FPKM=FPKM of SCC early passage cells, Log2 (fold change)=Log2 of fold change.

### Table 10: Genes commonly deregulated in cancer.

| Official gene symbol | SCC horn tissue FPKM | SCC early passage cells FPKM | Log2 (fold change) |
|----------------------|----------------------|-----------------------------|--------------------|
| IPO7                 | 330.60               | 70.3061                     | −2.2333            |
| FKBP10               | 125.032              | 34.2715                     | −1.86721           |
| PRC1                 | 97.974               | 30.2115                     | −1.69731           |
| FNDC3B               | 79.1106              | 25.3438                     | −1.64224           |
| ILF3                 | 79.5314              | 25.8148                     | −1.62332           |
| ACLY                 | 121.74               | 41.7097                     | −1.54534           |
| ADAM12               | 69.750               | 29.6667                     | −1.23336           |
| PSMB2                | 315.665              | 139.101                     | −1.1822            |
| EIF2AK1              | 56.175               | 31.7431                     | −0.8348            |
| NME1                 | 264.99               | 163.488                     | 0.69677            |
| ADAM10               | 58.022               | 39.761                      | −0.54523           |
| ANP32E               | 196.546              | 138.547                     | −0.50440           |
| HRNRPL               | 43.4377              | 31.5166                     | −0.4628            |
| FAM49B               | 148.32               | 107.624                     | −0.4627            |
| EIF2S2               | 396.41               | 344.378                     | −0.2030            |
| KDEL3R               | 213.373              | 202.472                     | −0.0756            |
| SPP1                 | 909.522              | 965.956                     | 0.08684            |
| UTP18                | 44.7713              | 50.2058                     | 0.165273           |
| ZBTB1                | 42.3114              | 49.9708                     | 0.23228            |

Table 10: Genes commonly deregulated in cancer, SCC=Squamous cell carcinoma, FPKM=FPKM per kilobase of exon per million, TGF=Transforming growth factor.
BHCC early passage cells (>1-fold change) along with effector genes such as caspase 6 (CASP6) and caspase 9 (CASP9) (>2-fold change) but in contrast cytochrome C was not found to be expressed and the genes CASP3, CASP8 were not detected [114]. The above discussion denotes a number of key players in pathogenesis of SCC of horns in bovines which showed resemblance with human cancer studies in expression profiling.

Conclusion

The signaling pathway investigation in this first culture based approach revealed that many of the cancer-related pathways reported in the literatures for other carcinomas may also be held responsible for SCC of horn in bovines. Cells from bovine horn SCC surgical specimens may be adapted in vitro with high efficiency, independently from any clinicopathological characteristics.

Low-passage horn cancer cell lines would still closely reflect the phenotype of the horn cancer cells in vitro bypassing the obstacle for obtaining more detailed insights into the diversity of phenotypic and molecular changes occurring in horn cancer cells. Our result based on the pathway analysis suggested that primary culture of horn cancer in-vitro may serve as the model for SCC of horns in cattle.

This transcriptome-based approach demonstrates that epithelial cultures isolated from primary horn SCC retain complex characteristics of the malignant tissue. Thus, the opportunity for basic and clinical application of functional cells derived from SCC horn tissue, instead of a few immortal cell lines should not be missed.

Authors’ Contributions

SS: Carried out laboratory experiment and written manuscript as part of MVSc. in Animal Genetics and Breeding. RSJ: Helped in manuscript correction. CGJ: Conceptualized the project. AKP: Helped in bioinformatics work. SJJ: Helped in NGS work. SK: Helped in manuscript writing. BR: Helped in bioinformatics work. PGK: Helped in NGS work and sample collection. DNR: Helped in manuscript correction and improvement. All authors read and approved the final manuscript.

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Competing Interests

The authors declare that they have no competing interests.

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