

Different Types of Gene Expression Profiles and Pathway Enrichment Analysis of Oral Dysplastic Keratinocyte Cells Caused by Heavy Smoking

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ABSTRACT

Aim/Background: The aim of the study is to carry out survival analysis on the candidate hub genes to check out their effects on Keratinisation of mucous cells are related to the genes which are responsible for OSCC. **Materials and Methods:** Gene expression profiling array for two microarray data sets of keratinized and normal oral tissue has been taken from GEO then DEGs and Inc RNAs are identified in oral cancer using LIMMA R package 15 in bio-conductor followed by KEGG analysis. STRING tool used to construct the PPIN for the sub PPIN, MCODE algorithm used for our work. The prognostic correlation co efficient values of the genes responsible for the keratinisation process in cancers have been studied using TCGA project in The Encyclopedia of RNA Interactomes (ENCORI). Functional annotation and co-expression results done with these CD44, CD58, CD40, VIM, KRT19, MMP1, ANGPTL4, FABP4, ENTPD1, and FCER2 key protein coding genes. **Results:** Hyper-methylated promoters play a crucial role in the inactivation suppressor genes during carcinogenesis and dysregulation of the DEGs are intently related with progression of cancer as functions as reporter genes. The enrichment result of DEGs can reveal the role of some URGs shows various activated levels in keratinocyte cells of heavy smokers and keratinocytes of people who had never smoked. ENTPD1 is connected to the DEGs which are related to TNF receptor, lymphocyte receptor. Ag3 associated with lymphocytes and alterations of genes. **Conclusion:** From this study a hallmark gene ENTPD1 has been identified for oral squamous cell carcinoma along with HNSCC with the seed gene CD44 interconnected with CD40, CD58.

Keywords: Oral squamous cell carcinoma, Head and neck squamous cell carcinoma, Differentially expressed genes, Keratinisation, Hallmark gene.

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INTRODUCTION

Oral cancer is a very aggressive disease, it is the 6th most common disease throughout the world.^[1,2] Carcinogenic overgrowth within the oral cavity of normal cells is major causes of fear. Cancer occurring in the tissues of oral cavity (begins at the lips and extends backwards to the front part of the tonsils) or oro-pharynx (part of the throat) is termed as oral cancer. Oral cavity

consists of Tongue, Lips, Gums and Teeth, Lining of cheeks, Salivary glands, Floor of the mouth, Roof of the mouth (hard palate), Tonsils, Uvula, Larynx. Oral Squamous Cell Carcinoma (OSCC) includes various types of cancers like TSCC, Salivary and maxillary gland cancer, tonsil cancer, larynx cancer, nasopharyngeal cancer head and neck cancer etc.^[3,4] Epidemiologic studies have indicated that bidi, cigarettes smoking may cause major role in etiological factors of OSCC^[5,6] and excessive alcohol in taking, smokeless (areca or tobacco chewing, betel nut chewing,) like carcinogenic agents and human papilloma virus(HPV) plus poor oral hygiene and genetic disposition.^[7-10] Registration of this very fatal disease is not compulsory in India, so the true incidence and mortality may be higher, as many

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cases are unrecorded and loses follow up.^[11] It has been predicted that India's incidence of cancer will increase from 1 million in 2012 to more than 1.7 million in 2035. This indicates that the death rate because of cancer will also increase from 680000 to 1- 2 million in the same period The international agency for research said.^[12] The survey says although multidisciplinary treatments including chemotherapy, radiotherapy and surgery have remarkably improved the survival rate of OSCC, HNSCC, LSCC patients. The etiological fraction of OSCC in men attributable to smoking is around 70%.^[13,14] From 1920's some of the Eminem scientists configured that Cigarette smoking and cancer these two words are interrelated and by the 1950's a casual relationship with lung cancer was truly established.^[14-18] By the time, in 1985, under the auspice of IRAC (International Agency For Research On Cancer) an international group of scientists proved a casual relationship between oral cavity, pharynx, larynx, lungs, pancreas, urinary bladder, urethra, and renal pelvis.^[19] Approx 17 years later in a revised Monograph on Tobacco smoke and involuntary smoking, IARC also included cancers of nasal cavities and nasal sinuses, stomach, oesophagus, stomach, kidney (renal cell carcinoma), liver, uterine cervix and bone marrow (myeloid leukaemia) to the long list of cigarette smoking related carcinoma.^[20] While smoking is an established risk factor for many forms of cancer, the magnitude of the risk varies between studies and in this study we have tried to show some Differentially expressed genes which causes oral dysplasia. Though it is dismal due to rapid metastasis and high regional rate.^[21-24] that's why OSCC malignancy tends to relapse or progress even after treatment within 5 years of span.^[25] Overall, this study will provide convincing up-regulated or down-regulated gene expressions which can be necessary for proper prediction of cancer prognosis. Till date it has become clear that Genes are involved in normal physiological processes, cellular behaviours including cell-cycle regulation, cell proliferation, differentiation inflammation, stress response, migration, invasion, differentiation and apoptosis plus negatively regulate protein expression at the post-transcriptional or translational level.^[26,27] Hypermethylated promoters play a crucial role in the inactivation of tumour suppressor genes during carcinogenesis and dysregulation of the differentially expressed genes are closely related to cancer progression, functioning as tumour oncogenes or suppressors, repressors, enhancers. Previous studies suggest that cigarette smoke influences the development of various microenvironments of cells. A pathway enrichment analysis of DEGs (Differentially expressed genes) can reveal the role of some up-regulated

genes displaying differing activated levels in dysplastic keratinocyte cells of heavy smokers and keratinocytes of people who had never smoked.

MATERIALS AND METHODS

Identification of DEGs from Public Microarray data

The public gene expression profiles of GSE54861 ID: 200054861 downloaded from the Gene Expression Omnibus (GEO, <http://www.ncbi.nlm.nih.gov/geo>). These profiles were deposited by Chia H. lee in 2015 and overall design of the data is: Total RNA was collected from DOK and HOK cells followed by gene expression microarray analysis like boxplot Figure 2. Comprised four samples from Homo Sapiens, those are GSM1325302 HOK(1), GSM1325303 HOK(2), GSM1325304 DOK(1), GSM1325305 DOK (2) and The dataset was analysed with R BIOCONDUCTOR packages, and raw datasets were normalised based on the PREPROCESSCORE package and the DEGs were screened out via the LIMMA package through the cut-off criteria of adjusted P -value < 0.05 .

Functional and pathway enrichment analysis

Dennis G Jr *et al.* Described DAVID (<https://david.ncifcrf.gov/>) as an integrative and systematic approach to learning. It is a systematic functional annotation tool that allows researchers to unveil the biological fruitfulness behind a large list of genes.^[28,29] We have used DAVID to perform functional and pathway enrichment analysis followed by GO (Gene ontology) analysis. GO includes 3 functional ontologies CC (cellular component), MF (molecular function), and BP (biological process) and KEGG (Kyoto Encyclopaedia of Genes and genomes) pathway enrichment analysis.^[29-31] are carried out for the down-regulated and up-regulated genes separately. Statistical significance is regarded as $P < 0.05$.

Protein-protein interaction network construction and module analysis

In order of priority the molecular mechanisms of the key genes responsible for the dysplastic keratinocyte in oral cavity, responsible for heavy smoking, the online search tool STRING database (<https://stringdb.org/>) is used to construct a PPI / protein-protein interaction network of interacting genes to predict interactive relationships among common DEGs encoding proteins. The minimum interaction score > 0.4 is required to construct the PPI network. Subsequently, according to the connection degree by CYTOSCAPE software the hub genes are selected.

RESULTS

Identification of DEGs

Compared with Normal keratinocyte cells of oral cavity, a total of 12,242 DEGs were identified in human oral squamous cells of heavy smoker consisting of 16 up-regulated and 15 down-regulated genes. The enlisted genes are accumulated in Table 1.

GO functional annotation and pathway enrichment

In GO functional annotation and pathway enrichment analysis there were no enriched categories of Down-regulated genes in DAVID. Total important terms for up-regulated genes are listed in Table 2.

In the CC ontology, only one enriched category satisfied the cut-off criteria ($P < 0.05$) and we found that the majority of enriched categories were relevant to extracellular components, such as cornified envelope (6 genes). In the Biological process ontology, the regulation of inflammatory immune response items constituted the majority of enriched GO categories, including keratinization (2 genes), peptide cross linking (1 gene), keratinocyte differentiation (two genes), epidermal cell differentiation (two genes), skin development (4 genes), epithelial cell differentiation (4 genes), epithelium development (6 genes), tissue development (3 genes). The second majority of enriched categories were associated with structural molecular activity consisting more than 3 genes and fold

enrichment is 14.52. Furthermore, the KEGG pathways of upregulated genes are mainly involved in cancer (Table 2) and included glutathione metabolism, PPAR signalling pathway, Cytochrome-p450 drug metabolism, chemical pathways of cancer, microRNAs involved

Table 1: The significant up-regulated and down-regulated genes.

Upregulated genes			Downregulated genes		
Gene. symbol	logFC	adj.P.Val	Gene. symbol	logFC	adj.P.Val
PI3	7.12	4.65E-20	KLK5	-5.91	3.02E-19
ANGPTL4	6.45	1.23E-19	OLR1	-5.69	3.02E-19
SPRR2F	6.05	1.43E-19	IGFBP3	-5.46	3.02E-19
SPRR2A	5.99	1.43E-19	KLK5	-5.88	7.52E-19
SPRR2D	6.03	1.43E-19	KRT15	-5.17	9.90E-19
SPRR3	6.22	1.43E-19	PLA2G16	-5.09	1.04E-18
VIM	5.87	3.02E-19	ADIRF	-4.88	1.36E-18
KRT6B	6.09	3.02E-19	IGFBP3	-4.74	5.76E-18
KRT19	6.96	3.02E-19	GPX2	-4.22	1.51E-17
LGALS7	5.29	5.57E-19	IRX3	-4.11	4.07E-17
LGALS7B	5.9	5.72E-19	UCA1	-3.77	4.70E-17
SPRR2E	5.11	9.24E-19	DHRS3	-4.34	4.70E-17
LGALS7B	5.24	1.04E-18	CTSH	-3.83	6.44E-17
VIM	5.49	1.04E-18	STMN3	-3.58	9.39E-17
MGST1	5.12	1.39E-18	RPS4Y1	-4.11	1.01E-16
H19	4.92	1.06E-18			

Table 2: Functional annotation and enrichment analysis of DEGs. And list of related genes of Biological Process, Molecular Function and Cellular Component from GO analytic tool.

Term and GO ID	Count	Fold enrichment	Up or Down	Raw P value	Genes
BIOLOGICAL PROCESS					
keratinization (GO:0031424)	5	> 100	up	1.77E-10	LCE1E, LCE6A
peptide cross-linking (GO:0018149)	5	> 100	up	1.26E-06	TGM5,
keratinocyte differentiation (GO:0030216)	5	79.46	up	3.48E-09	UGCG, LCE1E,
epidermal cell differentiation (GO:0009913)	5	53.3	up	2.42E-08	PTPRQ, UGCG
skin development (GO:0043588)	5	36.99	up	1.43E-07	SRF, PSEN1, GBA, TP63
epidermis development (GO:0008544)	5	30.76	up	3.51E-07	FST, BCL2, CTNNA1, FZD6
epithelial cell differentiation (GO:0030855)	5	19.43	up	2.44E-07	UGCG, NF2, SMAD4, THRB,
epithelium development (GO:0060429)	5	10.65	up	7.89E-06	EPHA2, IFT20
tissue development (GO:0009888)	5	7.51	up	9.66E-06	OXCT1, SATB2, SERPINH1, SPTLC2, IMPAD1, SULF1
anatomical structure development (GO:0048856)	1.00E+01	3.43	up	2.89E-05	
GO molecular function complete Homo sapiens					
structural molecule activity (GO:0005198)	7	14.52	up	3.88E-03	rpsQ, rpsB, rplB
GO Cellular component complete Homo sapiens					
cornified envelope (GO:0001533)	5	>100	up	1.55E-13	EVPL, DSC3, DSG3, A0A224ANV7, Q0KG49

in cancer, Epstein-Barr virus infection. The other enriched portions comprised items related to cancer development, which included chemical carcinogenesis-DNA ADDUCTS, microRNAs in cancer.

(Differentially expressed genes). Highlighted genes are significantly differentially expressed at a default adjusted *p*-value cutoff of 0.05 (red = upregulated, blue = downregulated) in the Figure 1.

The nodes represent the genes and the edges represent the corresponding PPI pairs. A total of 37 genes were integrated into the network.

PPI network construction, module analysis and hub gene selection

Protein-protein interaction networks are constructed on the basis of the STRING database and are displayed in Figure 3. Most of the differentially expressed genes and related genes are disconnected in the network. When from the settings Degree ≥ 4 is set as the cut-off criteria, among 37 DEGs and related genes, NINE genes in the ppi network is selected as hub genes: CD44, CD58, CD40, VIM, KRT19, MMP1, ANGPTL4, FABP4. These hub genes might play a sensitive role in keratinisation of dysplastic cells due to heavy smoking. Subsequently, only one significant cluster was identified from the PPI network by MCODE, and consisted of 10 nodes (CD44, CD58, CD40, VIM, KRT19, MMP1, ANGPTL4, FABP4, ENTPD1, FCER2) and ELEVEN edges. Furthermore, identified as the seed gene in this cluster is CD44.

This module consisted 10 of nodes (CD44, CD58, CD40, VIM, KRT19, MMP1, ANGPTL4, FABP4,

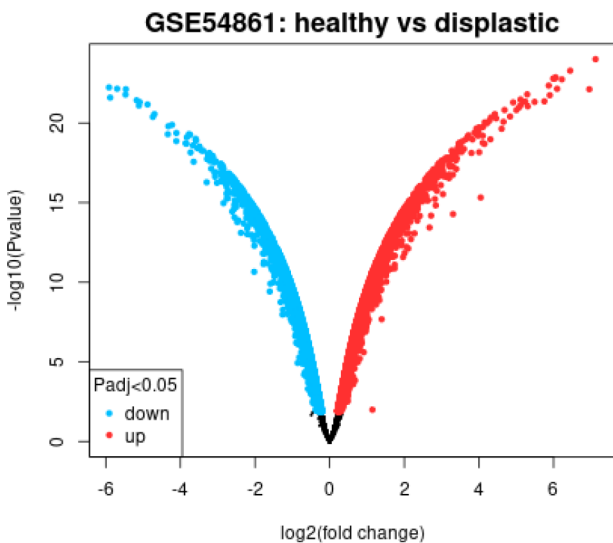


Figure 1: This Volcano plot is created using the limma package. This plot displays statistical significance (-log₁₀ P value) versus magnitude of change (log₂ fold change) and is useful for visualising DEGs.

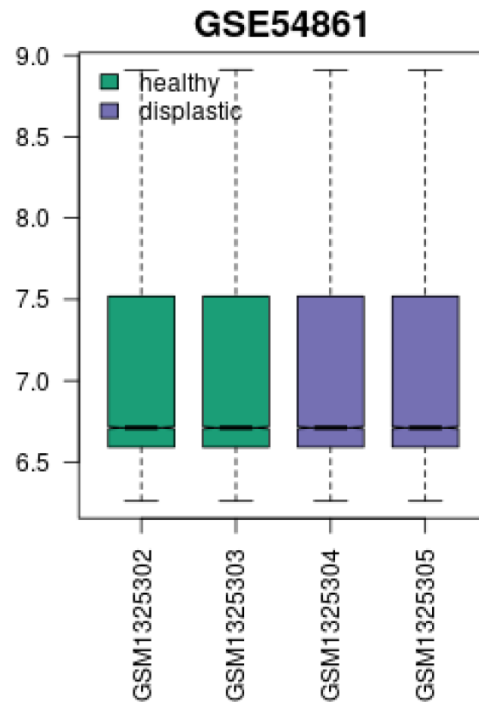


Figure 2: This Boxplot is Generated using the R package. The samples have been defined into two groups: Healthy and dysplastic. The Samples are colored according to groups, usually, median-centred values are indicative that the data are normalised and cross-comparable.

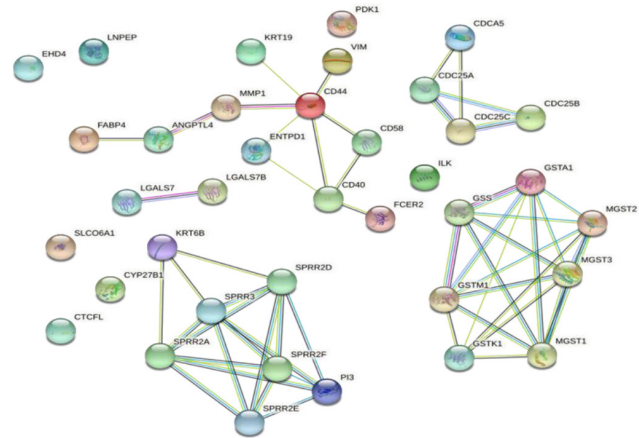


Figure 3: The protein-protein interaction (PPI) network for the differentially expressed genes.

ENTPD1, FCER2) and seven edges, and CD44 was the seed gene in this module.

Analysis on these candidate hub genes to check out their effects on oral cancer survival

We Perform the Recurrence free survival (RFS) analysis and overall survival (OS) analysis based on expression data by Kaplan Meier plotter (kmplot.com/). Dynamically Kalpan- Meier plots generate curated survival data which includes all TCGA cohorts. Groups of hallmark genes and seed genes are colored and

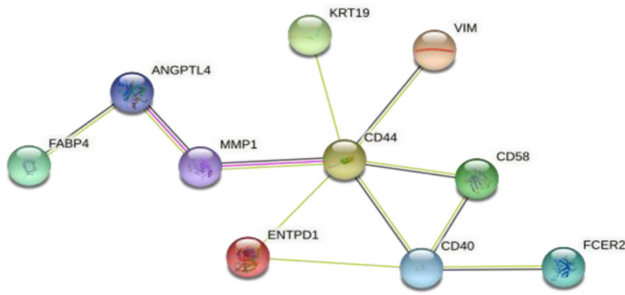


Figure 4: The only significant module.

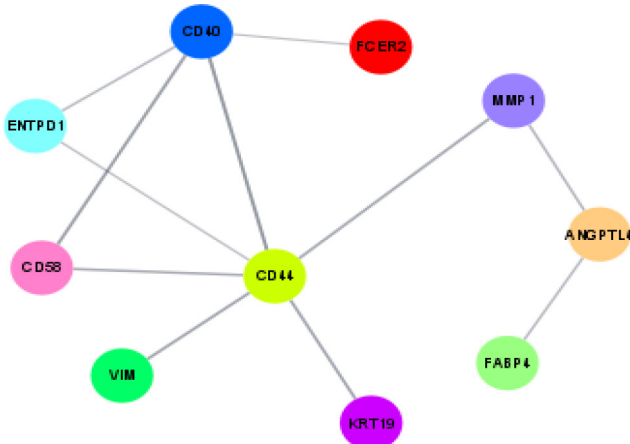
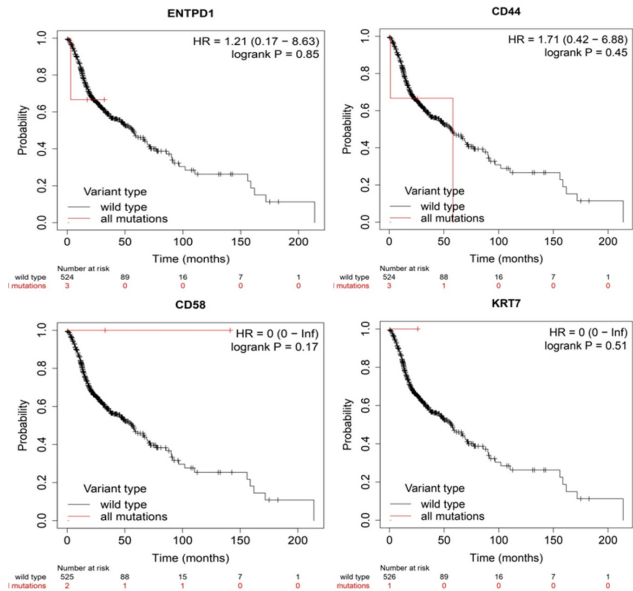


Figure 5: The only network is showing the 1st Cluster with the seed gene CD44 and CD58, ENTPD1, CD10, FCER2, and SECOND CLUSTER with MMP1, ANGPTL4, FABP4, KRT19: which are responsible for keratinisation by using cytoscape.

labelled according to the leftmost annotation column so that users can quickly assess if any annotation field has an impact on survival. In KM PLOTS advanced options allow investigators to select other survival parameters by analysing Figure 5. We have chosen ENTPD1 for head and neck squamous cell carcinoma to show the probable mutations with the wild types in respect to seed gene CD44 and some others related genes CD58, and CD40. We have tried to analyse the plot of KRT19, but we didn't get any result of the same so we have chosen KRT7 which is directly linked with KRT19 and attached that data too.

DISCUSSION

The identified seed gene CD44 is a GP90 lymphocyte homing/adhesion receptor, that is Receptor for HA (hyaluronic acid). Mediates cell-cell and cell-matrix interactions through its affinity for HA, and possibly also through its affinity for other ligands such as osteopontin, collagens, and matrix metalloproteinases (MMPs). Adhesion with hyaluronic acid plays an important role in cell migration, tumour growth and



progression. In cancer cells, it may play an important role in invadopodia formation. Also involved in lymphocyte activation, recirculation and homing, and in hematopoiesis. Network showing that it is connected with CD40 which is Tumour necrosis factor receptor superfamily member 5 and Receptor for TNFSF5/CD40LG that Transduces TRAF6- and MAP3K8-mediated signals that activate ERK in macrophages and B cells, leading to induction of immunoglobulin secretion. The GP90 lymphocyte homing/adhesion receptor is connected with CD58, Lymphocyte function-associated antigen 3 which is the Ligand of the T-lymphocyte CD2 glycoprotein. This interaction is important in mediating thymocyte interactions with outer layer of thymus (as a primary lymphoid organ) epithelial cells, antigen-independent and -dependent interactions of T-lymphocytes with target cells and antigen-presenting cells and the T-lymphocyte rosetting with erythrocytes. In addition, the LFA-3/CD2 interaction may be a prime response by both the CD²⁺ and LFA-³⁺ cells. Vimentin (VIM) is attached to the nucleus, ER (endoplasmic reticulum), and mitochondria, either laterally or terminally and found in oral epithelial cells as class III intermediate filaments also connected with KRT8; type II cytoskeletal 8, together with KRT19, helps to link the contractile apparatus to dystrophin at the costameres of striated muscle. Altered expression or dysfunction causes numerous pathogenic phenotypes. Great protein heterogeneity due to numerous alternative splicing and post-translational modification events. Receptor for LGALS9; the interaction enhances binding of SMAD3 to the FOXP3 promoter, leading to up-regulation of FOXP3 expression and increased induced regulatory

T (iTreg) cell stability and suppressive function of CD44 is connected with the MMP1, Matrix metalloproteinase 1 interstitial collagenase Cleaves collagens of types I, II, and III at one site in the helical domain which Also cleaves collagens of types VII and X. MMP4 is connected with the node Hepatic fibrinogen/angiopoietin-related protein ANGPTL4, the Protein with hypoxia-induced expression in endothelial cells. It can act as a regulator of angiogenesis and modulate tumorigenesis and Inhibits proliferation, migration, and tubule formation of endothelial cells and reduces vascular leakage. In response to hypoxia, the unprocessed form of the protein accumulates in the sub-endothelial extracellular matrix (ECM). The matrix-associated and immobilised unprocessed form limits the formation of actin stress fibres and focal contacts in the adhering endothelial cells and inhibits their adhesion. That may exert a protective function on endothelial cells through an endocrine action. It is directly involved in regulating glucose homeostasis, lipid metabolism, and insulin sensitivity. Lipid transport protein in adipocytes from the Adipocyte-type fatty acid-binding protein, binds both long chain fatty acids and retinoic acid and delivers long-chain fatty acids and retinoic acid to their cognate receptors in the nucleus is FAB4 connected with the ANGPTL4. ALL THESE INFORMATIONS TAKEN FROM <https://string-db.org>.^[32] Keratinisation of oral squamous cells and connective tissues makes the differences in their morphology and function in respect to non keratinized cells of the oral cavity.^[33-35] Keratin 17 expression occurred in approximately half of the gastric cancer patients in a previous study, which was positively correlated with tumour progression and poor prognosis.^[36]

CONCLUSION

The genes responsible for Keratinisation of mucous cells are related to the genes which are responsible for ORAL carcinoma. KRT19 has been reported as active factor of oral dysplasia is associated with CD40 which is a prognostic marker and transduces TRAF6- and MAP3K8-mediated signals that activate ERK in macrophages and B cells. Therefore we hypothesized that the differentially expressed genes regulate keratinisation in the oral cavity. Dynamic changes during cell cycle and apoptosis of a cell is related to the CD40, CD44 and CD58. (Figure 4) The annotation cluster is showing that ENTPD1 is connected to the DEGs which are related to Tumour necrosis factor receptor, lymphocyte homing/adhesion receptor; Receptor for HA (hyaluronic acid); Lymphocyte function-associated

antigen 3 resp. AND also to the gene KRT19 which together acts with KRT8causes numerous alternative splicing and post-translational modification events. In cancer cells, may play an important role in invadopodia formation also involved in lymphocyte activation, recirculation and homing, and in hematopoiesis. All these detailed analyses on the validation set from GEO showed consistent results. The prognostic values of the genes responsible for the keratinisation process in cancers have been widely studied. Hence we can conclude that ENTPD1 can be a hallmark gene for oral squamous cell carcinoma with the seed gene CD44and connected with CD40, CD58which are responsible for the mutations of head and neck cancer too.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

GO: Gene ontology; **CC:** cellular component; **MF:** molecular function; **BP:** biological process; **KEGG:** Kyoto Encyclopedia of Genes and genomes; **DRG:** down regulated genes; **URG:** Up regulated genes; **PPIN:** Protein-protein interaction network; **DEGs:** differentially expressed genes; **OSC:** oral squamous cells; **SC:** squamous cell; **OSCC:** oral squamous cell carcinoma; **EC:** extracellular components; **IIR:** inflammatory immune response; **VP:** volcano plot; **RFS:** Recurrence free survival; **OS:** overall survival; **HA:** hyaluronic acid; **MMPs:** matrix metalloproteinases; **TNF:** tumor necrosis factor; **SM:** super family member; **LFA:** Lymphocyte function-associated antigen; Antigen: Ag; **APCs-antigen:** presenting cells; **RBCs:** Red Blood cells; **VIM:** Vimentin; **ER:** endoplasmic reticulum; **iTreg:** Induced regulatory T; **MMP1:** Matrix metalloproteinase 1 interstitial collagenase; **ANGPTL4:** angiopoietin-related protein; **ECM:** sub-endothelial extracellular matrix; **RA:** retinoic acid.

SUMMARY

Keratinisation of oral squamous cells and connective tissues makes the differences in their morphology and function in respect to non keratinized cells of the oral cavity.^[33-35] The genes responsible for Keratinisation of

mucous cells are related to the genes which are responsible for ORAL carcinoma. KRT19 has been reported as active factor of oral dysplasia is associated with CD40 which is a prognostic marker and transduces TRAF6- and MAP3K8-mediated signals that activate ERK in macrophages and B cells. Therefore we hypothesized that the differentially expressed genes regulate keratinisation in the oral cavity. Dynamic changes during cell cycle and apoptosis of a cell is related to the CD40, CD44 and CD58. (Figure 4) The annotation cluster is showing that ENTPD1 is connected to the DEGs which are related to Tumour necrosis factor receptor, lymphocyte homing/adhesion receptor; Receptor for HA (hyaluronic acid); Lymphocyte function-associated antigen 3 resp. AND also to the gene KRT19 which together acts with KRT8 causes numerous alternative splicing and post-translational modification events. In cancer cells, may play an important role in invadopodia formation also involved in lymphocyte activation, recirculation and homing, and in hematopoiesis. All these detailed analyses on the validation set from GEO showed consistent results. The prognostic values of the genes responsible for the keratinisation process in cancers have been widely studied. Keratin 17 expression occurred in approximately half of the gastric cancer patients in a previous study, which was positively correlated with tumour progression and poor prognosis.^[36] Hence we can conclude that ENTPD1 can be a hallmark gene for oral squamous cell carcinoma with the seed gene CD44 and connected with CD40, CD58 which are responsible for the mutations of head and neck cancer too.

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