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LETTERS

Deciphering the Role of c-MET in Metabolic Reprogramming of Head and Neck Squamous Cell Carcinoma via *In Silico* Analysis

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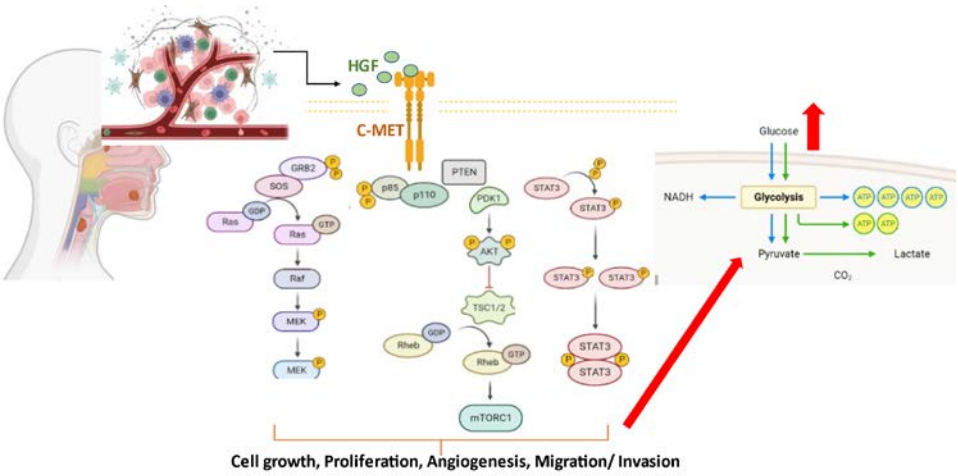
Article

ABSTRACT Targeting of epidermal growth factor receptors (EGFRs) and vascular endothelial growth factor receptors (VEGFRs) has become a major strategy for the control of head and neck cancer. c-MET, a receptor tyrosine kinase is known to be expressed in many cancers including the head and neck squamous cell carcinoma (HNSCC). The c-MET activity has been correlated with many signaling pathways that help the cancer cells to proliferate, migrate and invade into the normal, healthy tissues. The association of c-MET with glycolytic pathway in HNSCC has not been elucidated yet. Since, increased glycolysis has emerged as a major hallmark for cancer cell proliferation, targeting c-MET could bring an impact to inhibit HNSCC progression. In the present study we use various In-silico tools available to identify the association of c-MET with the major metabolic genes such as HK-II (Hexokinase-II), GLUT-1 (Glucose transporter-I), LDH-A (Lactate dehydrogenase-A), PFK-II (Phosphofructokinase-II) and MCT-1 (Monocarboxylate transferase-1) in HNSCC patient datasets available from The Cancer Genome Atlas (TCGA). Protein networking analysis was used to determine the correlation of c-MET with the metabolic genes. Retrieved sequenced data pathway analysis gives the network of genes associated in the activation of glycolytic pathway. Gene ontology and Enrichr studies provide an insight into c-MET activity in metabolism through molecular, functional and pathway basis in HNSCC. Furthermore, we also have shown a negative correlation of c-MET with immune cell infiltration, suggesting c-MET might have a role in immune suppression in HNSCC patients. Further validation on this study could possibly make c-MET as a potential target to inhibit HNSCC.



Keywords: Head and Neck cancer, Glycolysis, In-silico, Warburg effect, c-MET

INTRODUCTION

The Head and Neck Squamous cell cancer (HNSCC) is the 6th most common cancer worldwide with an incidence of more than 500,000 new cases and 380,000 deaths annually.[1] HNSCC originates from the mucosal epithelium in the oral cavity, pharynx and larynx.[2] The burden of HNSCC has been correlated with exposure to tobacco-derived carcinogens, excessive alcohol consumption and Human Papilloma Virus (HPV) derived infections. Most patients with HNSCC are diagnosed at later stages without a clinically evident premalignant lesion.[3] HNSCC patients are generally treated with surgical resection, radiation or chemotherapy based on disease stage. Detailed molecular characterization of HNSCC might suggest incorporation of prognostic or predictive biomarkers for a targeted therapy and enabling prolonged survival.

Malignancy has been correlated with several physiological factors that promotes the cancer proliferation and spread. One such hallmark in cancer progression is cancer metabolism that enables the glycolytic flux in cancer cells to sustain proliferation.[4] This feature enables the cancer cell to acquire necessary nutrients to maintain viability and build new biomass.

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Initial observation by Otto Warburg described an unusual reliance of cancer cells on glycolysis irrespective of the oxygen availability thus reprograming their metabolic profile to meet the cellular energetic demands.[5] The high glucose supply in cancer cells is done with the help of transporters such as GLUT-I.[6] There is elevated glycolysis which allows for the production of intermediates to be channeled into various anabolic pathways of nucleotide biosynthesis, protein synthesis and fatty acid synthesis. Glucose metabolism, as well as oxidative phosphorylation, produce 32 ATPS in normal cells. Whereas, in cancer cells the pyruvate is converted into lactate, which is eliminated outside the cells by lactate carriers such as monocarboxyl transporters. Early production of lactic acid and release into the surrounding environment help cancer cells to protect themselves from attacks by immune cells, and the low pH causes a harmful effect on surrounding healthy cells, which help cancer cells to invade and metastasise.[7]

Metabolic reprogramming involves a set of signaling pathways to support the increased glycolytic activity in cancer cells. The altered metabolism in cancer cells plays a pivotal role in overexpressing various proteins and oncogenes, which can be distinct from normal cells. Primarily, the PI3K/Akt/mTOR pathway act as central regulator of cellular energetics and metabolism and cope up with the demands of increased glycolysis in cancer cells.[8] It is reported that in cancer cells, mutations in c-MET stimulate various downstream signaling pathways, leading to development and progression through migration, invasion, and metabolic reprogramming.[9]

The c-MET (mesenchymal epithelial transition factor) is a Met-family receptor tyrosine kinase, mainly expressed on the surface of epithelial cells.[10] Higher c-MET expression has also been correlated with poor prognosis in cancer patients.[11] The properties of glucose hunger are used in the PET scan of positron emission tomography (PET) in solid tumors.[12] In recent years, tools and techniques of metabolic, proteomics, and *in-silico* analysis have been improved significantly and more sensitive and accurate predictions have been made to determine various drug properties such as pharmacodynamic, pharmacokinetic properties, and metabolic pathways essential in the early drug discovery stages. c-MET regulation and metabolism effects in HNSCC are little known. Therefore, Understanding the association between c-MET and the metabolic pathways is critical and clinically important to develop therapeutic drugs against HNSCC progression. Here we underscore c-MET as a potential biomarker that can be targeted to inhibit the progression of HNSCC.

EXPERIMENTAL

Data Collection.

R software was utilized to collect mRNA expression data of c-MET and other metabolic genes of HNSCC patient datasets. The R code used for data collection was as follows

```
install.packages("devtools")
devtools:install_github("mariodeng/FirebrowseR")
require(FirebrowseR)
mRNA.Exp = Samples.mRNASeq(format = "csv", gene = c("CP"), tcga_participant_barcode = c(“TCGA-ABC”, “TCGA-XYZ”))
mRNA.Exp[, c("tcga_participant_barcode", "expression_log2", "z.score")]
write.table(x = mRNA.Exp, file = "mRNA.Exp.csv", sep=",", row.names = FALSE, col.names = TRUE)
write.table(x = mRNA.Exp, file = "mRNA.Exp.txt", sep=" ", row.names = FALSE, col.names = TRUE)
getwd()
```

The log expression values were further analyzed and heat map was generated using GraphPad Prism 8.0 version.

Human Protein Atlas. c-MET protein expression levels in HNSCC tissues and normal tissues were reviewed in the Human Tissue Atlas (http://www.proteinatlas.org/).[17]. Overexpression of c-MET is associated with a poor prognosis and promotes cell proliferation and invasion in HNSCC.

TIMER Analysis. The TIMER database (cistrome.shinyapps.io/timer) is a new website, including 10,897 samples across 39 cancer types from The Cancer Genome Atlas (TCGA). It provides six major analysis modules to estimate immune infiltration and provides estimates of the molecular characteristics of tumor-immune interactions to be systematically excavated including the Gene module, Survival module, Mutation module, SCNA module, Different expression module, and Correlation module. We first analyzed c-MET expression in HNSCC by using the Different expression module.

Expression correlation of c-MET along with metabolic genes in HNSCC. To explore the correlation among the c-MET along with metabolic genes in HNSCC, TCGA datasets were analyzed using the GEPIA2 platform. HK-II, LDH-A, GLUT-1, MCT-1 and PFK-II were observed to be significantly correlated with c-MET expression.

Search Tool for the Retrieval of Interacting Genes (STRING). STRING (version 10.5) contains over 2000 organisms, 9.6 million of proteins and 1380 million of interactions that provides analysis and integration of direct and indirect protein-protein interactions (PPI), and focuses on functional association. c-MET along with the differentially expressed metabolic genes were uploaded, and interactions with at least medium confidence (interaction score > 0.4) were selected.

GO and KEGG pathway analysis of DEGs. To discover the functional roles of DEGs, GO was used for enrichment analysis covering cell components (CCs) biological processes (BP), and molecular function (MF) of the selected genes. KEGG is a database that displays the function and pathways of selected genes. The Database for Annotation, Visualization, and Integrated Discovery (DAVID: https:// david. ncifc rf. gov; last access: 14th February 2021) is a public online bioinformatics database that contains information on functional biological

annotations for genes and proteins. The cut-off criteria were selected based on p<0.01. The enrichment of GO terms and KEGG paths was done for candidates DEGs using DAVID.

UALCAN data analysis. UALCAN (http://ualcan.path.uab.edu) is a web resource for studying cancer transcriptome data. It can analyze gene expression, promoter methylation, correlation, and prognosis across tumors using TCGA data. The gene expression was analyzed in HNSCC patients and normal samples using UALCAN. Subsequently, the correlation between c-MET and metabolic genes at different cancer stages was assessed using UALCAN. A p-value of less than 0.05 (p <0.05) was considered statistically significant for all results.

Heat-map. It is a method to visualize differentially expressed gene lists. Basically, the data is displayed in a grid format where the rows are used to depict the gene names and the column represents the sample. We have generated heat maps for c-MET and the metabolic genes. we observed the following genes c-MET, HK-II, LDH-A, GLUT-1, MCT-1 and PFK2. The data were analysed using GraphPad prism 8.0 version.

Evaluation of the Relationship between c-MET and metabolic gene expression and Patient Survival with HNSCC Tumors. GEPIA2 and Kaplan-Meier survival plotter assessed the relationship between the expression of metabolic genes and the c-MET and the survival rate of HNSCC. We used GEPIA to perform overall survival analysis and assessment of the c-MET as well as metabolic expression levels in HNSCC of the TCGA database. The patient groups of high and low levels were divided by average gene expression. We assessed cancer prognosis, including overall survival (OS), Disease Free Survival (DFS) using the gene chip data sets of the Kaplan-Meier survival plotter with the best cut option, which divides patient groups at the level of gene expression to minimize the log rank P value. These data provide a 95% confidence interval and log rank P value for the Hazard Ration (HR) value.

Functional Gene Annotation. Database for Annotation Visualization and Integrated Discovery (DAVID) were utilized to understand the potential functional amplifications of significant genes in HNSCC.

Statistical Analysis. Statistical analyses were performed using GraphPad Prism 8 Version for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com. Statistical significance was claimed at 95% confidence level (p-value<0.05).

RESULTS AND DISCUSSION

1. *Increased c-MET expression in head and neck tumor patients*

c-MET expression has been correlated with aggressive disease and worst prognosis in many cancers.[13] To check the expression pattern of c-MET in HNSCC patient samples and normal head and neck tissues, we utilized the immunohistochemistry (IHC) analysis from Human Protein Atlas (HPA) database. IHC staining analysis suggested the higher expression of c-MET in head and neck cancer tissue samples of varied age groups when compared to normal tissues (**Figure 1A a-d**). Furthermore, we also investigated the c-MET expression levels in HNSCC patients in tumor as well as normal tissue samples with TIMER database which confirmed the higher expression of c-MET in HNSCC patient samples (**Figure 1B**). Both the analysis confirmed a higher expression of c-MET in HNSCC patients.

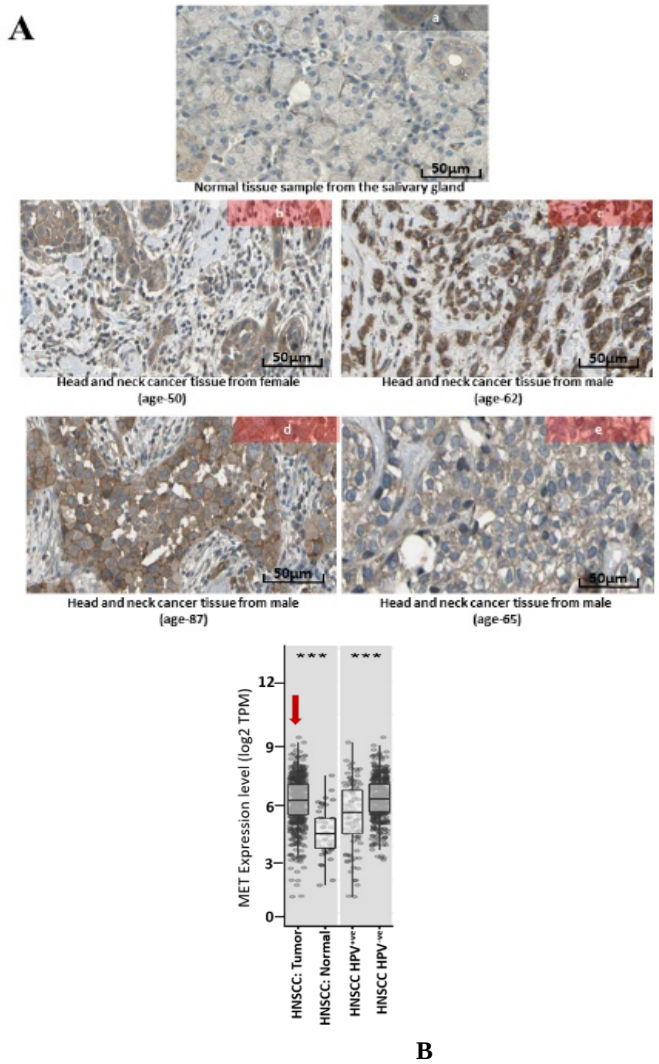


Figure 1: A. Immunohistochemical analysis of protein expression levels of c-MET genes on normal and cancerous HNSCC tissues based on The Human Protein Atlas (THPA) database. (a) Normal tissue from the salivary gland. (b) Head and neck cancer tissue from female (age-50). (c) Head and neck cancer tissue from male (age-62). (d) Head and neck cancer tissue from male (age-87). (e) Head and neck cancer tissue from male (age-65). Scale bar 50 µm B. c-MET expression levels in HNSCC tumor with HPV^{-ve} and HPV^{+ve} tissues and normal tissues were analyzed in TIMER database (*P < 0.05, **P < 0.01, ***P < 0.001).

2. *Expression correlation of c-MET and metabolic genes in HNSCC patients*

HNSCCs are reported to be highly glycolytic and increased glycolysis has been associated with tumor progression and metastasis.[14] To explore the correlation between the c-MET and metabolic genes in HNSCC, The TCGA data sets were analyzed with the GEPIA2 platform. HK-II, GLUT-1, PFK-II, LDH-A,

and MCT-1 were observed to be significantly correlated with c-MET (**Figure. 2A**). The PPI network of DEGs consisted of 9 nodes and 18 edges (**Figure. 2B**). The interaction showed a PPI enrichment p-score of 9.17e-11 which signifies strong protein interactions at biological function as a group. c-MET demonstrate significantly higher correlation with key glycolytic genes.

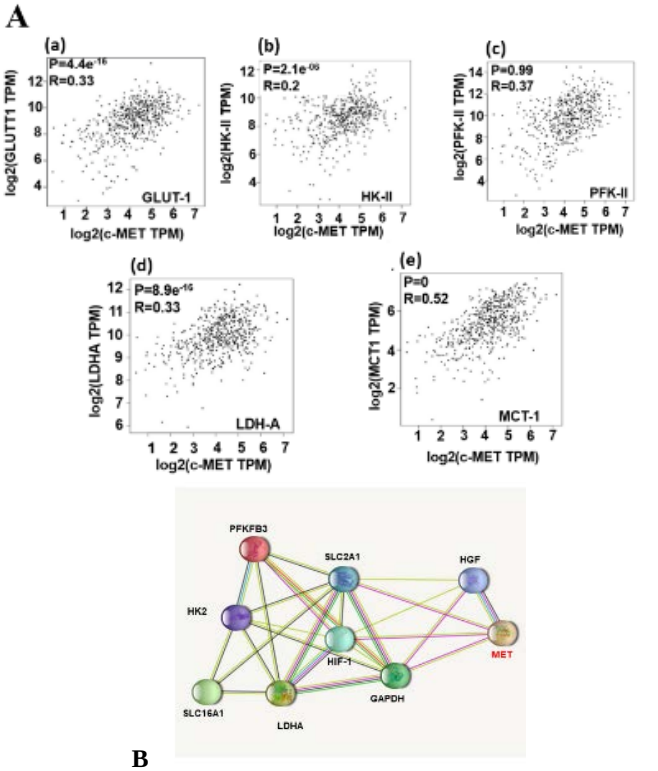
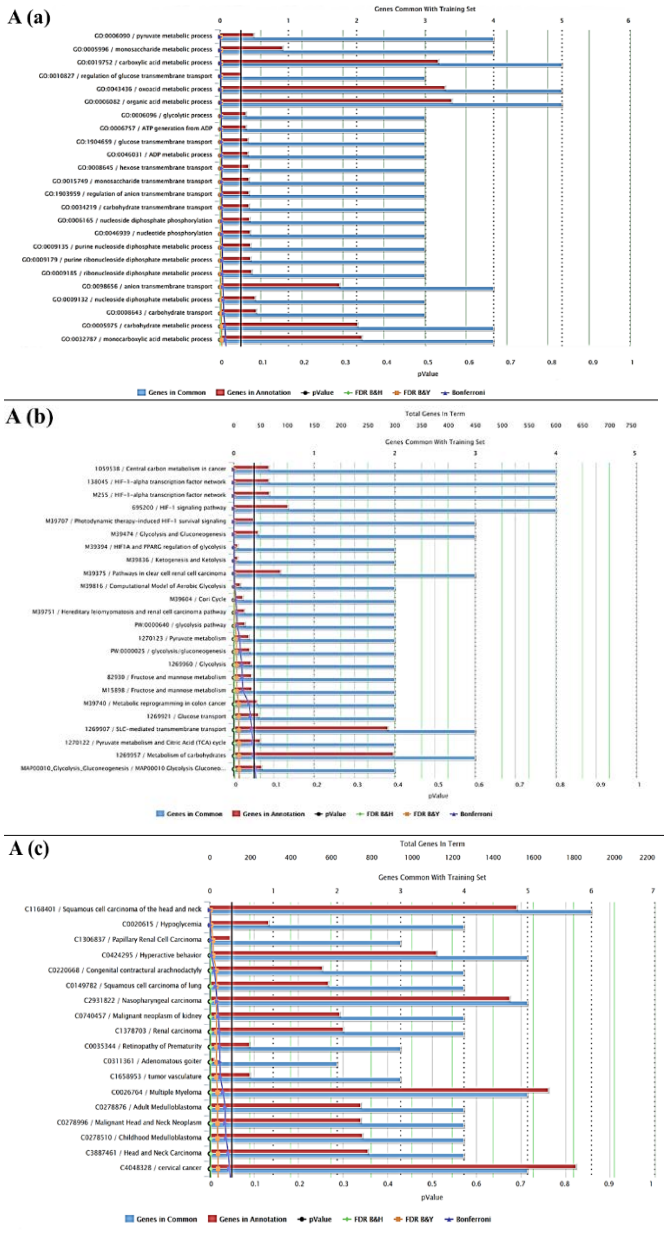


Figure 2: A. Correlation analysis of the c-MET and metabolic genes was conducted using the GEPIA 2 tool. (a) c-MET and GLUT-1 (SLC2A1) were positively correlated (P<0.01; R=0.33). (b) c-MET and HK-II were positively correlated (P<0.01; R=0.2). (c) c-MET and PFK-II were positively correlated (P=0.99; R=0.37). (d) c-MET and LDH-A were positively correlated (P<0.01; R=0.33). (e) c-MET and MCT-1 (SLC16A1) were positively correlated (P=0.00; R=0.52. c-MET, mesenchymal epithelial transition factor; GLUT-1, glucose transporter-1; HK-II, hexokinase-II; PFK-II, Phosphofructokinase-II; LDH-A, lactate dehydrogenase-A; MCT-1, Monocarboxylase transferase-1; TPM; transcripts per million reads. B. String analysis reveals a high degree of protein: protein interaction (PPI) among the members of the c-MET and metabolic network containing HK-II, GLUT-1 (SLC2A1), LDH-A, PFK-II (PFKFB3), MCT-1 (SLC16A1), GAPDH. Circles represent genes, lines represent the interaction of proteins between genes, and the results within the circle represent the structure of proteins. Line color represent evidence of the interaction between the proteins**.**

3. c-MET *Gene ontology and Enrichr analysis*

To discover the potential functional roles of DEGs, the GO was used to perform enrichment analysis, which covers the cellular component (CC), biological process (BP), and molecular function (MF) of the selected genes. In the enrichment analysis of metabolic genes along with c-MET, the DEGs weresignificantly enriched in ‘pyruvate metabolic pathway’,‘carboxylic acid metabolic pathway’, ‘glucose transport pathway’, ‘oxoacid metabolic pathway’ and ‘glycolytic pathway’ (**Fig. 3A-a**). In the molecular function analysis, the DEGs were significantly enriched in‘central carbon metabolism pathway’, ‘HIF-1a transcription factor pathway’, ‘glycolysis and gluconeogenesis’, ketogenesis and ketolysis’ and several other metabolic pathways (**Fig. 3A-b**).In the pathway function analysis, the DEGs were predominantly enriched in ‘squamous cell of head and neck cancer’,(**Figure. 3A-c**). KEGG pathway analysis revealed that DEG was significantly enriched in metabolic pathways, and other significant signaling pathways with the highest gene numbers (P<0.05). The majority of these pathways are closely associated with the metabolic signaling pathways in HNSCC. KEGG is a database that describes a specific gene function and pathway. The cut-off criteria were selected based on p < 0.01. (**Figure 3B**).



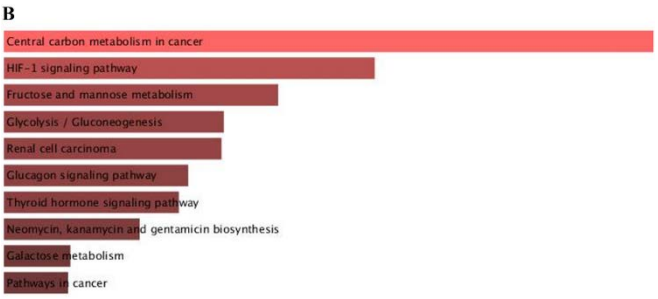
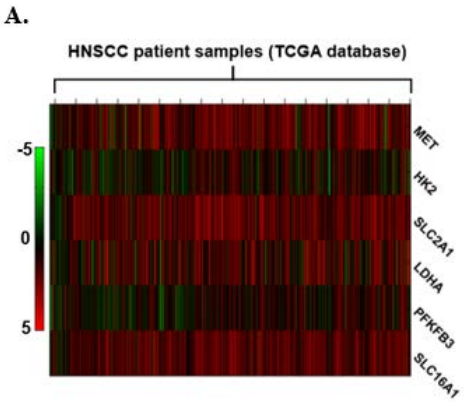


Figure 3: A. Gene ontology analysis of c-MET and metabolic genes in HNSCC patients. (a-c) The numbers of enriched genes according to the (a) biological process, (b) molecular function (c) pathway function. B. GO term and KEGG pathway enrichment analyses performed using Enrichr on c-MET and metabolic genes identified from HNSCC samples. The top 10 enriched KEGG pathway for c-MET and metabolic genes. The horizontal axis represents the number of genes, and the y-axis represents KEGG pathway names. (Enlarged view of figure provided in Supplementary file).

4. Increased c-MET and metabolic gene expression in Head and Neck cancer

The differential expression of metabolic genes along with c-MET expression was analyzed using the mRNA expression levels obtained from TCGA database. The log expression values of metabolic genes along with c-MET from 523 HNSCC patient data sets from TCGA were collected using R studio software using specific code to retrieve the expression data. The differential expression pattern was analyzed using GraphPad prism software. The analysis revealed increased expression of c-MET along with metabolic genes in HNSCC patient samples with a p-value <0.0001 (**Figure 4A)**. Since, the expression of c-MET and metabolic genes were upregulated in HNSCC patient samples we validated the results using ULCAN database (**Figure 4B)**. c-MET expression was observed to be significantly upregulated in HNSCC patient samples. Similarly, a significant increase in expression of metabolic genes were also observed in HNSCC patient data sets. These observations gave us a clear picture of c-MET correlation with the metabolic genes in HNSCC patients.



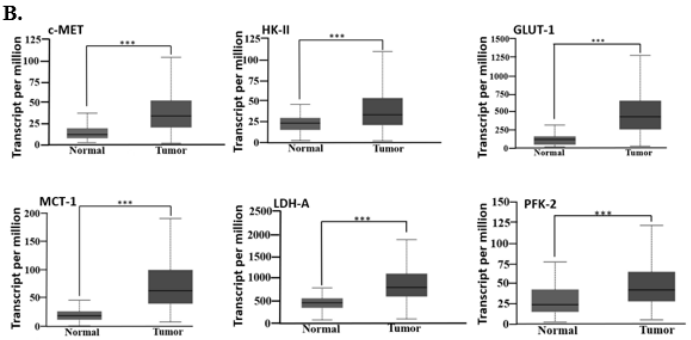


Figure 4: A. Heat map of log 2 gene expression for the most differentially expressed c-MET and metabolic genes in the selected 523 HNSCC patients via TCGA database. Data are represented in a matrix format: each row representing a single gene, and each column representing a specific patient sample. Red represents the overexpressed genes (expression levels over the median) and green represents the underexpressed genes (expression levels under the median). **B.** c-MET and metabolic gene expression analysis using different variables of TCGA HNSCC patients with ULCAN web. Box-whisker plots showing the expression of genes in sub groups of HNSCC samples. (a) Boxplot showing relative expression of c-MET in normal and HNSCC samples. (b) Boxplot showing relative expression of HK-II in normal and HNSCC samples patients. (c) Boxplot showing relative expression of GLUT-1 in normal and HNSCC samples (d) Boxplot showing relative expression of MCT-1 in normal and HNSCC samples, (e) Boxplot showing relative expression of LDH-A in normal and HNSCC samples. (f) Boxplot showing relative expression of LDH-A in normal and HNSCC samples. All data are presented as the mean ± standard deviation (SD). Expression level of gene is represented as log2(TPM).

5. Relationship between c-MET and metabolic genes at different stages of HNSCC progression

Next, we examined c-MET and metabolic gene expression based on pathological stages of HNSCC patients. Although there was no significant expression changes at initial stages of HNSCC but as the tumor progressed to later stages there was an significant increase observed in the HNSCC patient samples (**Figure 5 a-f**). Clearly, stating at later stages of HNSCC, c-MET along with metabolic gene expression significantly increases and might be contributing towards the progression of the disease.

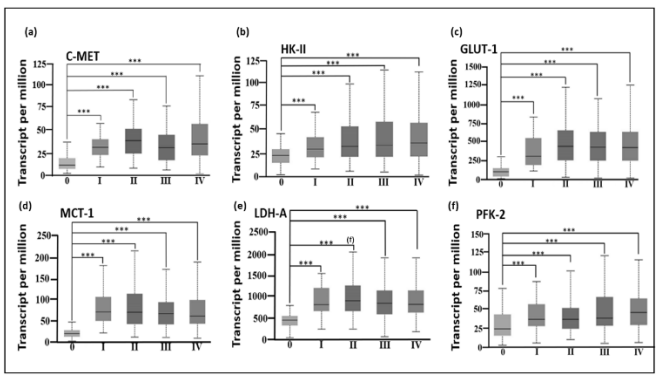


Figure 5: c-MET and metabolic gene expression for stage wise analysis using different variables of TCGA HNSCC patients with ULCAN web. Box-whisker plots showing the expression of genes in sub groups of HNSCC samples at different stages of HNSCC cancer patients. (a) Boxplot indicates the relative expression of c-MET from

stage 1 to stage 4 of HNSCC progression. (b) Boxplot indicates the relative expression of HK-II from stage 1 to stage 4 of HNSCC progression. (c) Boxplot indicates the relative expression of GLUT-1 from stage 1 to stage 4 of HNSCC progression (d) Boxplot indicates the relative expression of MCT-1 from stage 1 to stage 4 of HNSCC progression (e) Boxplot indicates the relative expression of LDH-A from stage 1 to stage 4 of HNSCC progression. (f) Boxplot indicates the relative expression of PFK-2 from stage 1 to stage 4 of HNSCC progression. Expression level of gene is represented as log2(TPM).

6. *Relationship between c-MET along with metabolic gene expression in over all survival and prognosis in HNSCC patients*

Kaplan-Meier survival plots showed that c-MET expression is inversely correlated with patient survival in HNSCC patients. similar observations were done with metabolic genes which stated an inverse correlation with the patient survival of HNSCC patients (**Figure 6A**). In order to assess the potential effects of glycolytic genes on the prognosis of HNSCC, we examined these genes as associated with the survival of patients without disease in clinical samples. Data from GEPIA2 were analyzed to construct box plots and perform Kaplan–Meier analysis [35]. Among the enriched genes, elevated expression of c-MET, HK-II, GLUT-1, LDH-A, MCT-1 and PFK-II were significantly associated (log-rank $p < 0.05$) with worse prognosis (**Figure 6B**).

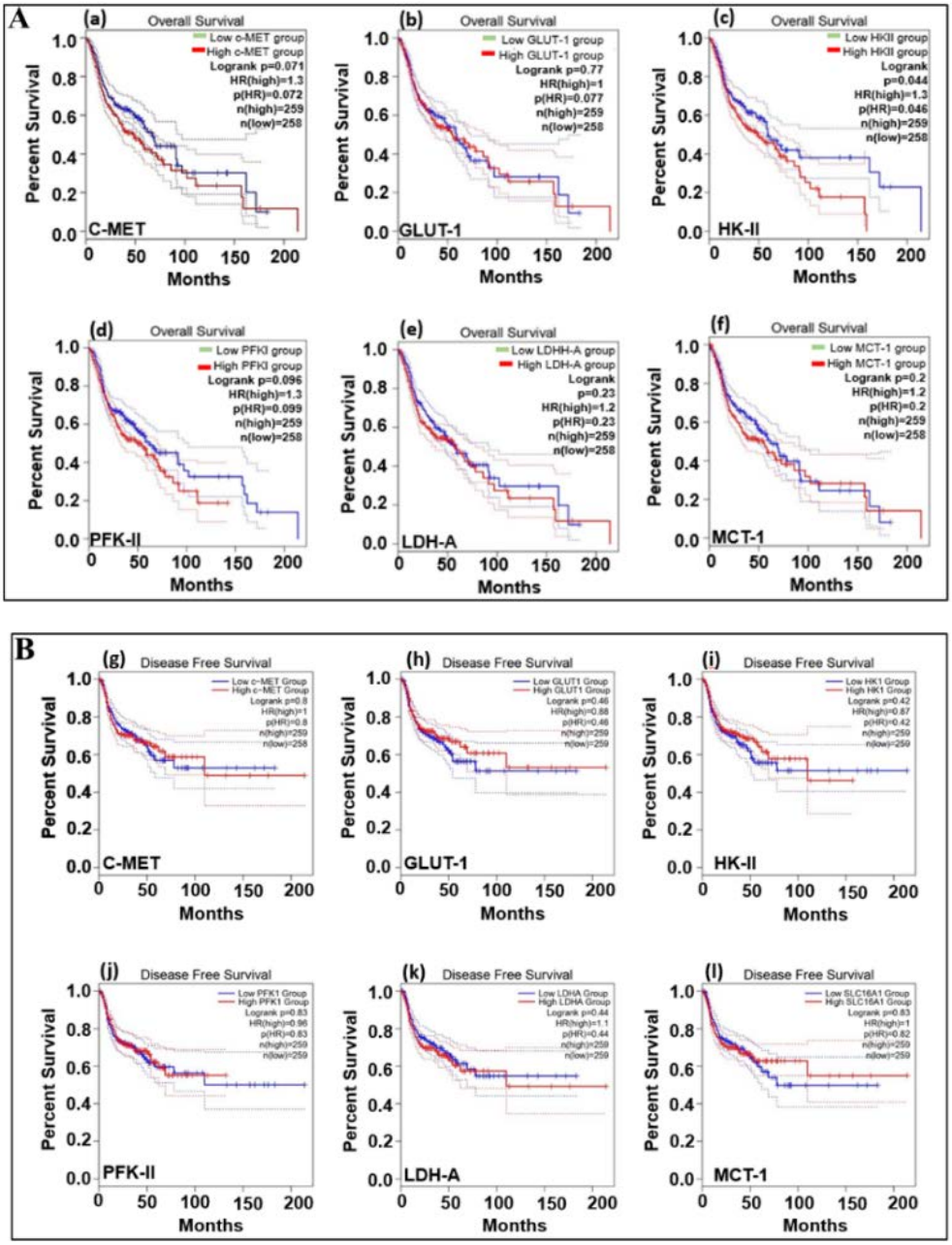


Figure 6: Correlation between c-MET, HK-II, LDHA, GLUT-1, MCT-1, PFKFB expression and patient survival in HNSCC patients. We used the GEPIA2 tool to analyze the overall survival (A) and disease-free survival (B) analyses of HNSCC tumors in TCGA by c-MET and metabolic gene expression. The survival map and Kaplan-Meier curves with positive results are given. Patient's survivals were compared between two groups divided at median value of gene expression as higher (red) and lower (green) in TCGA data. The *P*-value with log-rank analysis.

7. *Pathway analysis of c-MET and its association with metabolic pathway in HNSCC.*

The correlation between c-MET and metabolic genes were evident from the above analysis performed. To further validate the significant connection of c-MET with metabolic genes in HNSCC we performed DAVID pathway analysis. The analysis gave a significant association of c-MET with the glycolytic pahway through PI3K/AKT/mTOR pathway (**Figure 7**).

This analysis demonstrates a clear interaction of c-MET with glycolytic pathway in HNSCC. This suggests the activation of c-MET triggers phosphorylaton of PI3K/AKT pathway which in turn regulates the glycolysis in HNSCCs.

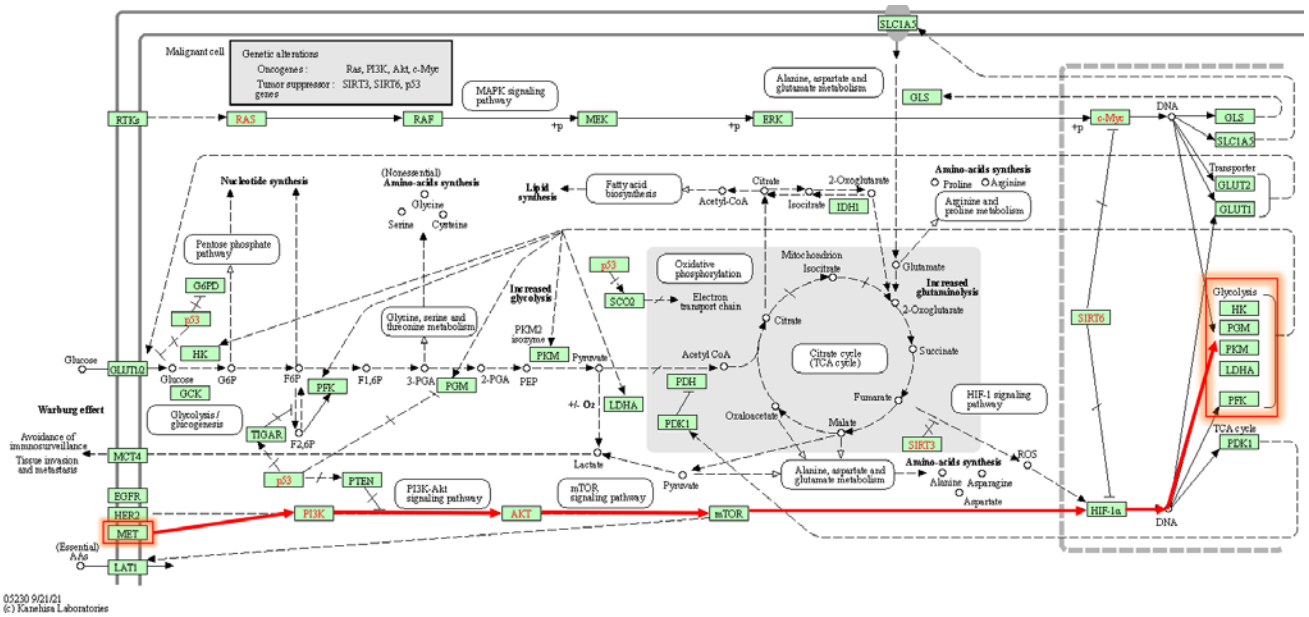


Figure 7: DAVID analysis of the linkages of c-MET. Diagram from the KEGG pathway analysis database produced by DAVID 61. The diagram shows the involvement of c-MET in metabolic alteration in HNSCC progression.

8. *Correlation between c-MET Expression and Gene Expression of Immune-Suppressive Cytokines.*

Immunosuppression is a well-known mechanism for the progression of tumors, resulting in tumor growth and metastasis.[15] In addition to cancer cells, tumors also include many different types of stromal components, which include tumor infiltrating immune cells, endothelial cells, neuronal cells, lymphatic cells, cancer associated fibroblasts (CAFs) and the extracellular matrix (ECM).[16] These inhibitory cytokines have anticancer effects through immune suppressive functions in effector cells, including NK cells and CD8+ T cells. In this study, a negative correlation was showed between c-MET expression and gene expression of Treg cell, B-cell, NK cell and $CD8^+$ cells low infiltration of the immune-suppressive cells. Therefore, here we investigate whether immune-suppressive cytokines derived from cells are also reduced by expression of c-MET in HNSCC, we analyzed the correlation between c-MET expression and immune cytokine gene markers (Treg cell, B-cell, NK cell and $CD8^+$ cells) using the TIMER database **(Figure 8)**. CAF infiltration was highly correlated with c-MET expression in HNSCC patients. In HNSCC, the surrounding stroma contains an abundance of CAFs.[17] CAF has been reported to release growth factors that stimulate the activity of c-MET in various cancers.

Immunotherapy with PDL-1 inhibitors has recently been explored in the treatment of multiple cancers. Studies show nearly 60% of HNSCC tumor cells expressing high levels of PDL-1, creating an immunosuppressive micro-environment around the tumor.[18,19,20] Data obtained from ULCAN database shows a higher expression of CD247 (PDL-1) in HNSCC patients as compared to the normal samples in the dataset **(Figure S1a)**. However, c-MET expression had no significant correlation with CD247 in HNSCC patient samples **(Figure S1b).**

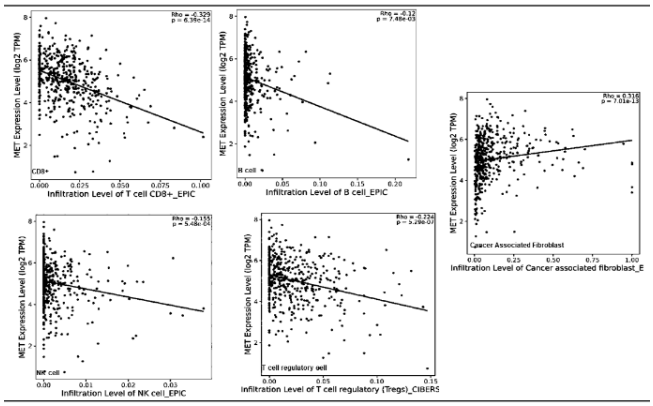


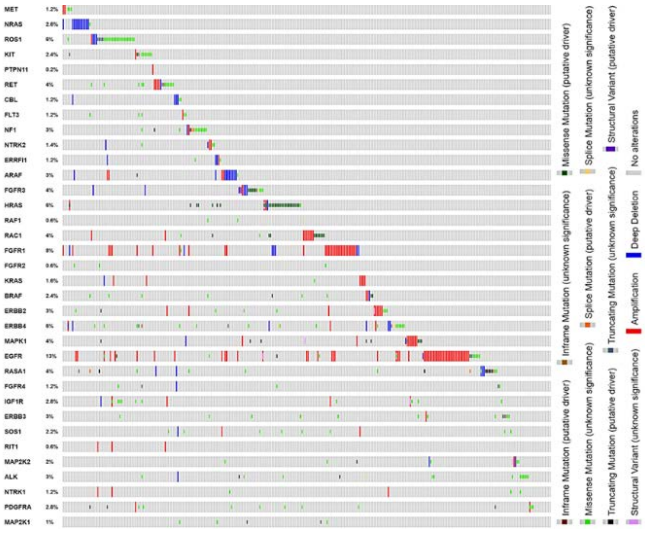
Figure 8: Correlation of c-MET expression and immune cell infiltration in HNSCC. Correlation of c-MET gene with immune cells was examined using TIMER. A significant negative correlation between c-MET expression and such as B cells, $CD8^+$ cells, NK cells and T regulatory cells were observed in HNSCC. However, correlation of c-MET expression with cancer associated fibroblast cells were positively correlated.

9. *C-MET functions*

Genetic alterations of c-MET, its co-occurrence with tumor suppressor and oncogene in HNSCC tissue from the TCGA database was analyzed. The genetic data on tumor suppressor genes and tumor upregulating genes were extracted through cBioPortal from the TCGA HNSCC project, and presented as OncoPrint for 279 HNSCC cases. Samples (n = 279) are categorized in columns and was designed to emphasize mutual exclusivity between mutations. The color code indicates the type of mutation: red, homogeneous amplification; blue, homogeneous deletion; green, mutation. Left, mutation percentage. Top, HPV status. The analysis showed the complex

alteration of genes having role in complex process in HNSCC **(Figure 9A).** To explore the correlation between the c-MET and genes analyzed through oncoprint in HNSCC, TCGA datasets were analyzed using the STRING platform. All the genes were shown to be significantly correlated with c-MET signifying a complex functioning of c-MET in HNSCC (**Figure. 9B**). The PPI network of DEGs consisted of 35 nodes and 349 edges. The interaction showed a PPI enrichment p-score of 10e-16 which signifies strong protein interactions at biological function as a group. Further, gene ontology assay with selected genes from the TCGA oncoprint revealed a high association of c-MET and related genes in complex cellular pathways in HNSCC (**Figure. 9C)**. These findings confirmed the involvement of c-MET in complex pathways of HNSCC progression and is highly corelated with major genes involved in different survival signaling pathways in HNSCC.

A



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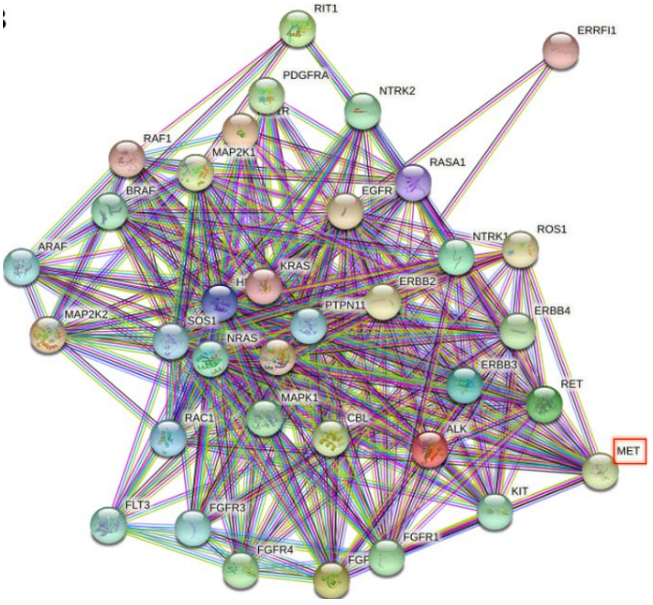

C

Molecular Function (Gene Ontology)

GO-term	description	count in network	strength	false discovery rate
GO:0005007	fibroblast growth factor-activated receptor activity	4 of 5	2.65	1.74e-07
GO:0005030	neurotrophin receptor activity	2 of 4	2.45	0.0048
GO:0043125	ErbB-3 class receptor binding	2 of 5	2.35	0.0066
GO:0004714	transmembrane receptor protein tyrosine kinase activity	18 of 63	2.2	3.64e-31
GO:0005021	vascular endothelial growth factor-activated receptor activity	2 of 7	2.2	0.0107
GO:0043121	neurotrophin binding	2 of 9	2.09	0.0155
GO:0043560	insulin receptor substrate binding	2 of 10	2.05	0.0176
GO:0017134	fibroblast growth factor binding	4 of 21	2.03	1.61e-05
GO:0005078	MAP-kinase scaffold activity	2 of 11	2.01	0.0204
GO:0004708	MAP kinase kinase activity	3 of 18	1.97	0.00089
GO:0004713	protein tyrosine kinase activity	20 of 137	1.91	2.59e-30
GO:0001784	phosphotyrosine residue binding	4 of 42	1.73	0.00019
GO:0019838	growth factor binding	12 of 136	1.69	3.90e-15
GO:0019003	GDP binding	4 of 74	1.48	0.0014
GO:0097110	scaffold protein binding	3 of 58	1.46	0.0155
GO:0035591	signaling adaptor activity	3 of 65	1.41	0.0204
GO:0004672	protein kinase activity	24 of 568	1.37	8.38e-26
GO:0030295	protein kinase activator activity	3 of 87	1.29	0.0443
GO:0019903	protein phosphatase binding	4 of 149	1.18	0.0144
GO:0019902	phosphatase binding	5 of 194	1.16	0.0029
GO:0003924	GTPase activity	6 of 318	1.02	0.0024
GO:0005524	ATP binding	24 of 1464	0.96	6.39e-17
GO:0035639	purine ribonucleoside triphosphate binding	29 of 1799	0.95	7.49e-22
GO:0004674	protein serine/threonine kinase activity	7 of 437	0.95	0.0014
GO:0032555	purine ribonucleotide binding	29 of 1864	0.94	1.58e-21
GO:0008047	enzyme activator activity	8 of 520	0.93	0.00044
GO:0019900	kinase binding	10 of 742	0.88	7.26e-05
GO:0005525	GTP binding	5 of 370	0.88	0.0419
GO:0140096	catalytic activity, acting on a protein	26 of 2116	0.84	6.02e-16
GO:0016740	transferase activity	25 of 2170	0.81	2.12e-14
GO:0019901	protein kinase binding	7 of 653	0.78	0.0133
GO:0042803	protein homodimerization activity	7 of 673	0.76	0.0155
GO:0046983	protein dimerization activity	10 of 1037	0.73	0.0012
GO:0042802	identical protein binding	18 of 1896	0.72	9.06e-08
GO:0030234	enzyme regulator activity	9 of 1044	0.68	0.0072
GO:0044877	protein-containing complex binding	10 of 1216	0.66	0.0041
GO:0019899	enzyme binding	18 of 2239	0.65	1.23e-06
GO:0005102	signaling receptor binding	12 of 1581	0.63	0.0014
GO:0003824	catalytic activity	32 of 5486	0.51	1.30e-12
GO:1901363	heterocyclic compound binding	30 of 5831	0.46	1.96e-09
GO:0043167	ion binding	31 of 6188	0.45	8.45e-10
GO:0097159	organic cyclic compound binding	30 of 5916	0.45	2.83e-09
GO:0005515	protein binding	31 of 7026	0.39	2.80e-08
GO:0005488	binding	35 of 12516	0.19	2.46e-05

Figure 9: A. Differential gene mutation analysis in HNSCC. Oncoprint represents the distribution and percentage of different type of changes to samples consistent with the c-MET gene. The right part of the figure without alterations was not included. **B.** String analysis reveals a high degree of protein: protein interaction (PPI) among the members of the c-MET and several other genes involved in cellular function and growth. The circle represents the gene, the line represents the protein interaction between the gene and the results within the circle represents the protein structure. Line color represent evidence of the interaction between the proteins. **C.** Gene ontology analysis was done to uncover the molecular function of c-MET and related genes in HNSCC. c-MET and selected genes showed an association with complex cellular pathways in HNSCC.

10. Enrichment analysis of all HNSCC-associated genes correlated with c-MET function

After compiling our list of c-MET associated genes using TCGA database, we undertook a gene ontology enrichment analysis of this gene set, processing genes in terms of their associated molecular function. The top ranked biological process was in cell junction organization followed by the involvement in motility, adhesion, metabolic process and many other such complex processes necessary for cancer progression. The entire gene set was ranked by fold enrichment as shown in **figure 10.**

Despite the vast advancement in cancer research, current therapies for HNSCC are associated with poor survival and high morbidity. There are only few targeted therapies associated with HNSCC treatment mainly the EGFR targeted therapy with cetuximab monoclonal antibody.[21-25] The efficacy of EGFR targeted treatment has not been much due to the chemo resistant activity taking place within cancer cells. The integration of tumor cell metabolism is a great opportunity to design new therapies, which are being very much studied in the cancer research community.

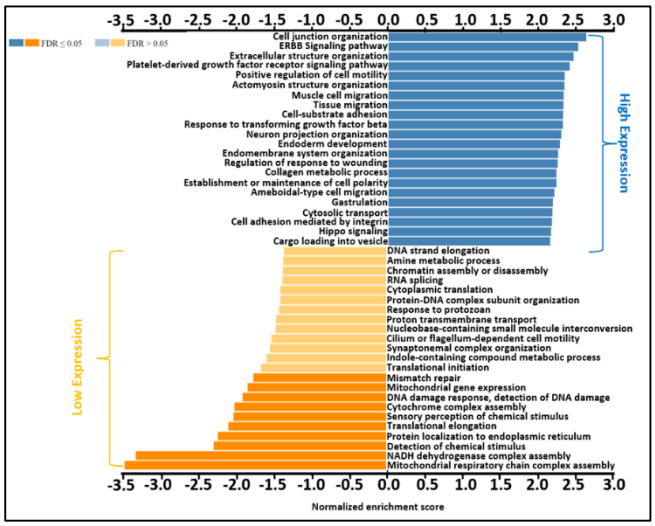

Figure 10: Gene Ontology enrichment analysis of the differentially expressed genes. The number of genes enriched according to the biological process. The entire gene set was ranked by fold enrichment.

The effect of the suppression of endogenous proto-oncogenes of c-MET is shown in the suppression of tumor growth, regression of established metastases, and decreased generation of new metastases, indicating the importance of persistent c-MET expression in the early phase of cancer progression.[22] HNSCC tumors are highly glycolic and are usually diagnosed with PET (18F-FDG-PET) fluorodeoxyglucose. The absorption of ^{18}F-FDG in HNSCC tumors is associated with high levels of lactate, poor prognosis and low survival rates.[23] In addition to glycolysis, glutaminolysis produces lactate in cancerous cells.[24] However, in a panel of 15 HNSCC cell lines, glucose and not glutamine have been shown to be the main sources of energy.[25] Clinical data emphasizes the overexpression of c-MET results in shorter overall survival of HNSCC patients. Here, we have focused on the c-MET kinase pathway, which has been identified to be correlated with the increased glycolysis in HNSCC patients. This approach can be utilized to target c-MET along with major metabolic genes to inhibit the progression of HNSCC. Since, similar studies have reposted the association of c -Met with the central signaling pathways such as mTOR, EGFR, FGFR, STAT 3 and COX2. Thus, c-MET can be considered as a potential target against HNSCC. The c-MET expression was significantly higher in HNSCC patient samples obtained from the TCGA database. The IHC analysis confirms the increased expression of c-MET in the patient tissue samples.

Importantly, the increased expression of c-MET was corelated with the increased expression of metabolic genes such as HK-II, GLUT-I, LDH-A, MCT-1 and PFK-II in HNSCC patients. Network analysis further confirms the association of c-MET with major metabolic genes that participate in glycolysis events in cancer progression. The changes in these key enzymes of glycolysis seem to be enough for an effect on glycolysis. Taking together, co-Inhibition of c-MET with major metabolic genes are also a possible therapeutic strategy against HNSCC. The enrichment analysis shows the activation of signal pathways and the characteristics of a set of genes for metabolic stimulation. Furthermore, in cancer, the central carbon metabolism (hsa05230) was observed to be significantly increased. This gene set assembles genes associated with the Warburg effect. Also, the gene sets were quite evidently over-expressed in HNSCC patient samples.

Next, to suggest the possibility c-MET as a marker to predict the prognosis of HNSCC, we analyzed the correlation between c-MET and metabolic gene expression with patient survival using the Kaplan-Meier curve. c-MET as well as metabolic gene expression, as based on Kaplan-Meier survival curves, was significantly correlated with overall survival and disease free survival showing that these gene expressions were negatively correlated with the HNSCC patient survival. This study also demonstrates that c-MET expression correlates with immune cell infiltration in HNSCC. Infiltration levels of $CD8^+$, B cell, NK cell and T regulatory cells negatively correlated with c-MET expression. Interestingly, cancer associated fibroblasts (CAF), which has mesenchymal properties in the tumor microenvironment was seen to moderately corelated to c-MET expression in HNSCC. Recently, CAFs have shown that they can also suppress immune responses in tumor microorganisms by recruiting M2 macrophages or directly suppressing immune cells. The study however has demonstrated the clinical importance of c-MET, further in vitro and in vivo studies are needed to demonstrate the role of c-MET in the glycolysis mechanism of HNSCC.

Conclusion

This study underscore a significant upregulation of c-MET gene in HNSCC patient data obtained from TCGA database. The expression of c-MET is significantly correlated with the key metabolic genes such as HK-II, GLUT-1, LDH-A, PFK-2 and MCT-1 in HNSCC patients. c-MET function has been highly correlated in HNSCC patients making it as a possible biomarker for HNSCC prognosis. High c-MET expression has also been negatively correlated with immune infiltration suggesting it to suppress the immune function in HNSCC patients. Interestingly, CAF expression has been highly correlated with c-MET expression in HNSCC. Thus, this systematic analysis provides evidence suggesting the potential use of c-MET as well as metabolic genes as an effective target to discover and develop therapeutics against HNSCC.

Future Aspects

Due to the heterogeneity in head and neck cancer, it is very important to study in detail the molecular aspects for head and neck cancer to develop novel targeted therapies. Targeting a single receptor like in the case of EGFR consequently activates other receptors in the loop such as c-MET and leads to chemoresistance. Correlation of metabolic activation with c-MET receptor can be a major breakthrough for treating HNSCC. Although the study confirms the association of c-MET with metabolic genes in HNSCC datasets, the c-MET high expression rate has to be validated among HNSCC population as to narrow

it down to a personalized therapy concept. The in-vitro and in-vivo studies can provide a clear picture on this co-relation and can help to develop novel therapeutic agents to target HNSCC progression. Since, combination therapy is now in the lead to target different cancers and in this regard targeting c-MET along with metabolism can provide effective treatment against HNSCC.

CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

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