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Original Article

Genomic and transcriptomic alterations in m6A regulatory genes are associated with tumorigenesis and poor prognosis in head and neck squamous cell carcinoma

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Abstract: Genetic alterations in N6-methyladenosine (m6A) regulatory genes are observed in many cancers. Recent studies have shown that newly identified m6A regulatory gene family (IGF2BPs; IGF2BP1, IGF2BP2, and IGF2BP3) were highly expressed in various types of cancer that stabilize and promote translation of multiple oncogenes, resulting in tumor development, survival and drug resistance. However, the oncogenic roles and prognostic values of IGF2BPs in head and neck squamous cell carcinoma (HNSCC) remain largely unknown. In this study, we examined the m6A regulatory genes alteration, their mRNAs expression and the prognostic values in HNSCC. We also analyzed the interaction network and functional enrichment of m6A regulators. Our results showed that m6A regulatory genes were altered in 41% (205/504) of HNSCC patients, of which *IGF2BP2* was amplified in 20% (101/504) of HNSCC patents and positively correlated with its mRNA expression. Importantly, we have validated the expression of *IGF2BP2* in HNSCC and normal tissue samples. Interestingly, we also found that the *IGF2BP2* was frequently co-amplified with the most common oncogenes in HNSCC patients. In addition, this study found that other m6A regulatory genes such as *METTL3, METTL14, WTAP, KIAA1429, ZC3H13, RBM15, ALKBH5, FTO, YTHDF1, YTHDF2, YTHDF3, YTHDC1, IGF2BP1,* and *IGF2BP3* were significantly upregulated in HNSCC samples. Moreover, patients with high expression of *IGF2BP1*, *IGF2BP2*, and *IGF2BP3* had poor overall survival (OS) than those with low expression. Therefore, it is evident that IGF2BP family plays a key role in the oncogenesis of HNSCC and might serve as novel prognostic biomarkers and potential therapeutic targets in HNSCC.

Keywords: m6A regulators, oncogenes, HNSCC, tumorigenesis, poor prognosis

Introduction

HNSCC is the sixth most common malignancy worldwide that usually develops from oral cavity, oropharynx, and larynx [1]. HNSCC is considered to arise with the accumulation of genetic, epigenetic and epitranscriptomic alterations [1-3]. Increasing evidences suggest that copy number variations (CNSs) including oncogenes amplification such as *TP63, PIK3CA, SOX2,* and *ACTL6A* play important roles in HNSCC development [2, 4]. Despite the progress and advancement in diagnosis, and therapeutic strategy during the past decades, the prognosis of HNSCC is still poor. Therefore, the current scenario underscores the need to have a better understanding of the molecular mechanisms underlying HNSCC progression.

N6-Methyladenosine (m6A) is the most abundant internal modification of mRNAs that regulates the gene expression by modulating RNA processing, stability, localization, translation, and decay. The m6A modification is catalyzed by recently discovered group of proteins termed as m6A regulators including "writers", (RNA methyltransferases including METTL3, METTL14, WTAP, KIAA1429 (VIRMA), ZC3H13, RBM15, and RBM15B), "erasers", (RNA demethylases including FTO, and ALKBH5), and "readers" (m6A-binding proteins including YTHDF1, YTHDF2, YTHDF3, YTHDC1, YTHDC2, IGF2BP1,

IGF2BP2, and IGF2BP3) [5-7]. Aberrant m6A modification has been implicated in the development and maintenance of several human diseases including cancers [5-7]. Recent studies have reported that the abnormal expression of m6A regulators affect m6A abundance, thereby disrupting oncogenes expression and promoting tumorigenesis [5, 8].

Insulin-like growth factor 2 mRNA-binding proteins (IGF2BPs; IGF2BP1, IGF2BP2, and IGF2BP3) are new family of m6A readers. IGF2BPs are a conserved family of oncofetal single-stranded RNA-binding proteins (RBPs), which control the localization, translation, and stability of m6A-modified mRNAs. However, the exact molecular mechanisms by which IGF2BP family recognize and regulate the expression of their targets remain elusive. More recent studies have shown that IGF2BPs preferentially recognize m6A-modified mRNAs and promote the stability and translation of thousands of potential mRNA targets in an m6A-dependent manner. Furthermore, IGF2BPs play oncogenic roles by stabilizing m6A-modified mRNAs of oncogenic targets and promote oncogenesis [5, 8].

Deng et al. more recently reported that IGF2BP2 plays a vital role in HNSCC progression [9]. However, the role of IGF2BP1 and IGF2BP3 in the development of HNSCC have not been reported. Importantly, IGF2BP1 and IGF2BP3 are mainly expressed during embryogenesis and absent in adult tissues but recent studies have shown that IGF2BP1 and IGF2BP3 are synthesized *de novo* in cancers, where they act as oncogenes and promote proliferation, differentiation, metastasis, and drug resistance in an m6A-dependent manner [10]. This study was aimed to analyze m6A regulatory genes copy number variations, expression and the prognostic significance in patients with HNSCC.

Materials and methods

Copy number variations (CNVs) analysis of m6A regulatory genes in HNSCC patients

Recent studies have shown that m6A regulatory genes play important oncogenic roles in various types of cancer. In the current study, genetic alteration in m6A regulatory genes such as amplification, and deep deletions analysis were done from TCGA (The Cancer Genome Atlas) dataset for 504 HNSCC patients using the cBioPortal tool (www.cbioportal.org/) [11].

The m6A regulatory genes and their targets expression analysis

Furthermore, the expression of m6A regulatory genes including IGF2BP family and their targets in 520 primary HNSCC and 44 normal tissues were analyzed using TCGA dataset through UALCAN database (http://ualcan.path.uab.edu/) [12]. The protein expression of IGF2BP2 was analyzed in HNSCC and normal tissues by immuno-histochemistry images [13].

The validation of IGF2BP2 and its target expression

The expression of IGF2BP2 and its target HMGA2 were validated in 48 oral squamous cell carcinoma (OSCC) and 16 adjacent normal tissues using the CFX96 Real-Time PCR detection system (Bio-Rad Lab-oratories Inc., Hercules, CA, USA) and primer sequences that were published elsewhere [14, 15]. All the patients were pathologically confirmed. Written informed consent was obtained from all the patients, and the study protocol was approved by the Ethic Committee of Saveetha Medical College.

Survival analysis by Kaplan-Meier plotter

The prognostic values of IGF2BPs in HNSCC patients were analyzed using Kaplan-Meier plotter (http://kmplot.com/analysis/) is an online database containing gene expression profiles and survival information of cancer patients [16]. In addition, multivariate Cox regression analyses also performed to identify prognostic factors for HNSCC.

Protein-protein interaction (PPI) and functional enrichment analysis

The functional protein association network of IGF2BPs and their targets were analyzed using GeneMANIA (https://genemania.org/) [17] and STRING (https://string-db.org/) [18] databases. Metascape (http://metascape.org) [19], is a web-based portal used for comprehensive functional analysis of IGF2BPs. Cytoscape was applied to generate the visualized gene co-expression network result.

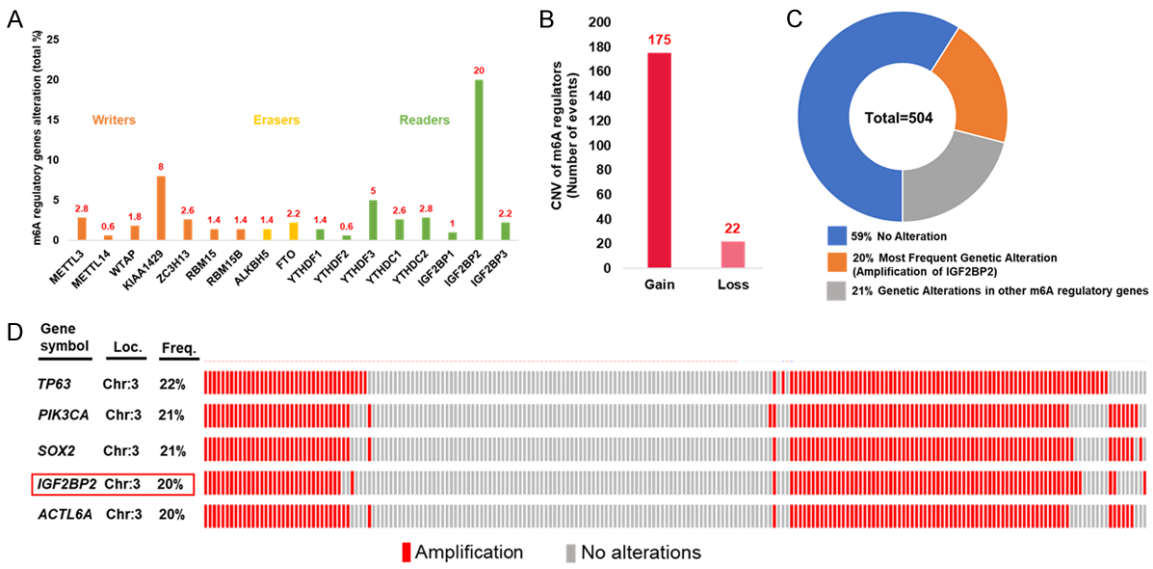

Figure 1. Genetic alterations of the m6A regulatory genes in HNSCC. Percentage of HNSCC samples with CNVs of the m6A regulatory genes based on the data from TCGA (A). Events of copy number gain or loss of m6A regulatory genes in HNSCC samples (B). The most common patterns of CNVs in m6A regulatory genes in HNSCC samples (C). Oncoprint in cBioPortal database exhibited the most common oncogenes alterations (D).

Results

CNVs of m6A regulatory genes in HNSCC patients

CNVs of m6A regulatory genes analyzed in 504 HNSCC samples using the cBioPortal TCGA dataset, found that m6A regulatory genes had different levels of CNV events, ranging from 0.6% to 20%. Importantly, the m6A "reader" *IGF2BP2* had the highest frequency of CNV events (20%, 101/504) (**Figure 1A**). Moreover, it was observed that most of the CNV events led to gain of copy number (**Figure 1B**). Amplification of *IGF2BP2* was the most frequent alteration in all the CNVs of m6A regulatory genes (**Figure 1C**). Gene amplification is a relatively frequent event in various types of cancer genomes including in HNSCC. Therefore, the present study analyzed the most common oncogenes and *IGF2BP2* amplifications in HNSCC samples. The results showed that *TP63* (22%), *PIK3CA* (21%), *SOX2* (21%), *IGF2BP2* (20%), and *ACTL6A* (20%) amplified in HNSCC samples. Interestingly, *IGF2BP2* was co-amplified along with these oncogenes in 99% (100/101) of HNSCC patients (**Figure 1D**).

Association between IGF2BP2 expression and HNSCC patients survival

Recent studies have provided substantial evidence on the CNV patterns and m6A regulatory gene expression. The results of the present study demonstrate that the copy number status of *IGF2BP2* gene positively correlated with its mRNA expression in HNSCC patients (**Figure 2A**). In addition, UALCAN database and the Human Protein Atlas analysis revealed that IGF2BP2 mRNA (P = 1.642e-12, **Figure 2B**) and protein (**Figure 2C**) were highly expressed in HNSCC tissues. Moreover, high expression of IGF2BP2 correlated with the poor prognosis of HNSCC patients (P = 5.1e-05, **Figure 2D**).

Association between IGF2BP1 and IGF2BP3 expression and HNSCC patients survival

Studies have shown that IGF2BP1 and IGF2BP3 high expression were associated with poor prognosis in patients with various types of cancer. The results of the current study reveal that *IGF2BP1* (P < 1e-12, **Figure 3A**) and *IGF2BP3* (P = 1.642e-12, **Figure 3B**) were highly expressed in HNSCC tissues compared to control tissues. Moreover, high expression of *IGF2BP1* (P = 0.0091, **Figure 3C**) and *IGF2BP3* (P = 0.03, **Figure 3D**) were associated with poor prognosis of HNSCC patients.

PPI and functional enrichment analysis

The protein-protein interaction network revealed a correlation among genes for IGF2BPs. The relationship of IGF2BPs were analyzed by using GeneMANIA (**Figure 4A**). The results showed all

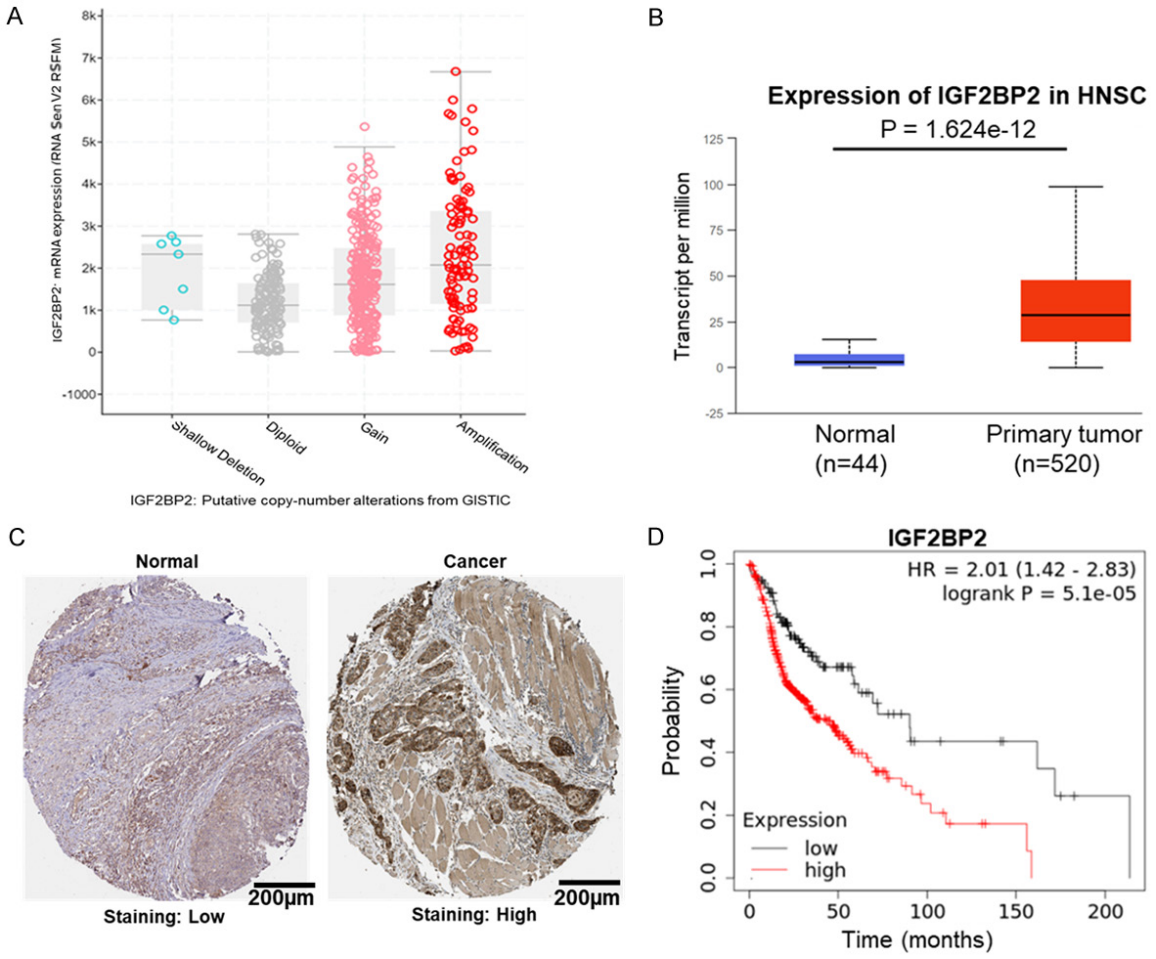

Figure 2. IGF2BP2 expression was associated with HNSCC patients survival. Correlation between the genetic alterations of *IGF2BP2* and its mRNA level in HNSCC tissues (A). *IGF2BP2* mRNA expression was analyzed using UALCAN database (P = 1.624e-12, B). GF2BP2 proteins expression was analyzed in HNSCC tissue using the Human Protein Atlas (https://www.proteinatlas.org/) (C). The prognostic value of *IGF2BP2* in HNSCC patients, analyzed by Kaplan-Meier plotter (P = 5.1e-05, D).

IGF2BPs have shared protein domains. Physical interactions were found between m6A "readers" IGF2BP1, IGF2BP2, IGF2BP3, and HNRNPA1. Further, relationships were noted between IGF2BP1 and IGF2BP3 in co-expression. The present study, identified interactions of IGF2BPs at the protein expression level by using STRING (**Figure 4B**). IGF2BP1 was shown to interact with IGF2BP3 as assessed by gene coexpression, text-mining, and protein homology data.

Further, kyoto encyclopedia of genes and genomes (KEGG) pathway and process enrichment analysis were performed. The top 8 clusters with their representative enriched terms are shown in **Figure 4C**. Enriched terms across these candidate genes were identified for pathways involved in regulation of mRNA metabolic process, nucleobase-containing compound transport, metabolism of RNA, regulation of insulin secretion, regulation of viral transcription, transcriptional misregulation in cancer, adherence junction, RNA modification, and a network plot was constructed by Metascape (**Figure 4D**). As demonstrated in the KEGG pathway, RNA modification, regulation of mRNA metabolic process and transcriptional mis-regulation were more enriched, suggesting that these pathways may be closely related to HNSCC development.

IGF2BP family target oncogenes expression in HNSCC patients

IGF2BP family plays an oncogenic role by stabilizing and translating m6A-modified oncogenic mRNAs including *HMGA2*, *TK1*, *HDGF*, *FSCN1*,

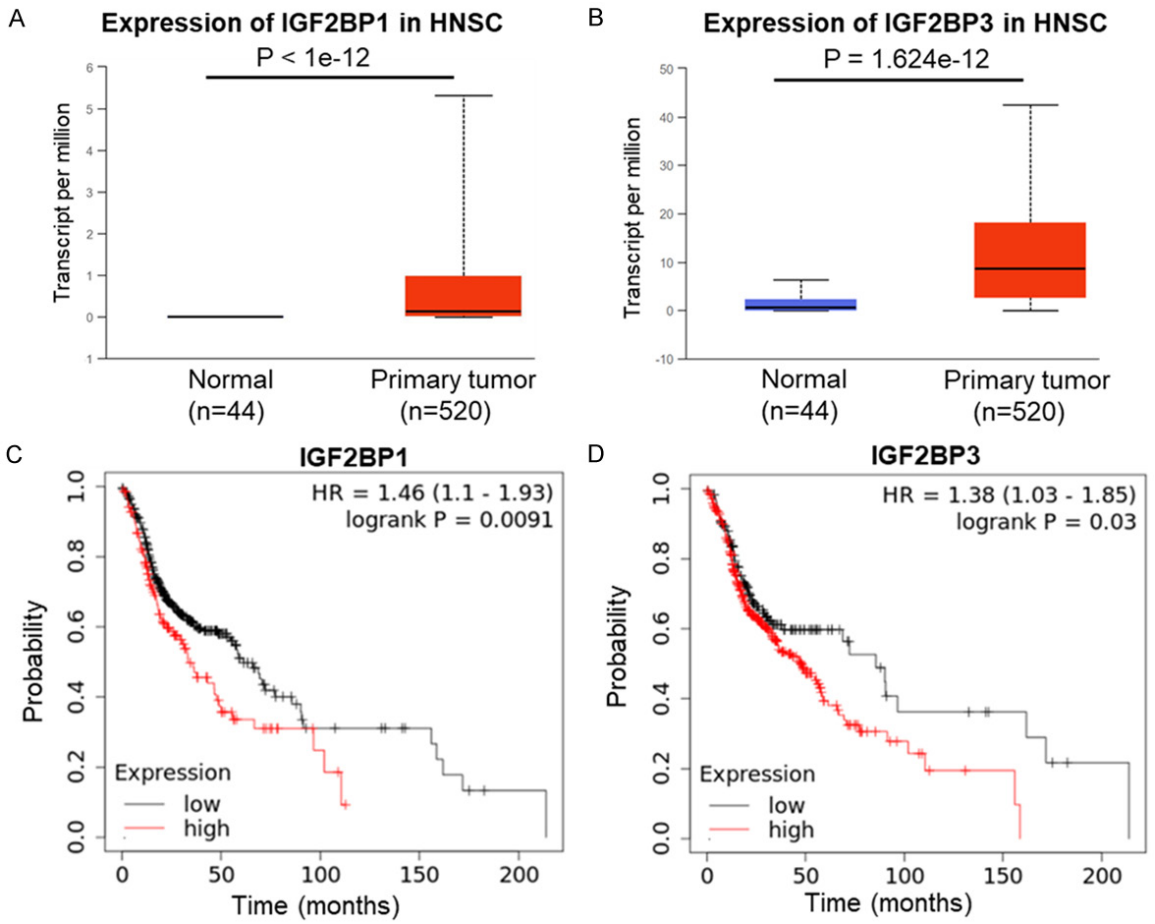

Figure 3. IG*F2BP1* and *IGF2BP3* expression were associated with HNSCC patients survival. *IGF2BP1* (P < 1e-12, A) and *IGF2BP3* (P < 1.624e-12, B) mRNAs expression were analyzed using UALCAN database. The prognostic value of *IGF2BP1* (P = 0.0091, C) and *IGF2BP3* (P = 0.03, D) in HNSCC patients, analyzed by Kaplan-Meier plotter.

MKI67, and *CD44* thereby promoting tumorigenesis. Results of the present study showed that IGF2BP family targets oncogenes including *HMGA2* (1.624-12, **Figure 5A**), *TK1* (P < 1e-12, **Figure 5B**), *HDGF* (P < 1e-12, **Figure 5C**), *FSCN1* (P = 1.624-12, **Figure 5D**), *MKI67* (P = 1.624-12, **Figure 5E**), and *CD44* (P < 1e-12, **Figure 5F**) which were highly expressed in HNSCC. Importantaly, we also found that *HMGA2* was highly expressed in OSCC patients with high expression of IGF2BP2. Our results also revealed that *IGF2BP2* gene amplification frequently co-occurred with oncogenic *TP53* mutations (**Figure 6A**) and *IGF2BP2* mRNA highly expressed in HNSCC patients with *TP53* mutations (**Figure 6B**).

Discussion

The functional role of m6A modification on cancer epitranscriptomics is an active area of research. Emerging evidence implies that dysregulation of m6A modification is tightly associated with proto-oncogene activation and cancers development [8, 20-22]. m6A modification is dynamic and reversible that plays a crucial role in post-transcriptional regulation. The enzyme systems involved in the regulation of m6A modification mainly include a series of proteins (m6A regulators) [23, 24]. Recent studies have shown that dysfunction of m6A regulators are associated with tumorigenesis and poor prognosis in various types of cancer [25-27]. However, the regulation of m6A modification in HNSCC is largely unknown.

Zhao et al. recently investigated the function and mechanism of m6A "writer" METTL3 in oral squamous cell carcinoma (OSCC) tumorigenesis and they found METTL3 promotes c-Myc mRNA m6A modification and translation via m6A "reader" YTHDF1, thereby leading to OSCC

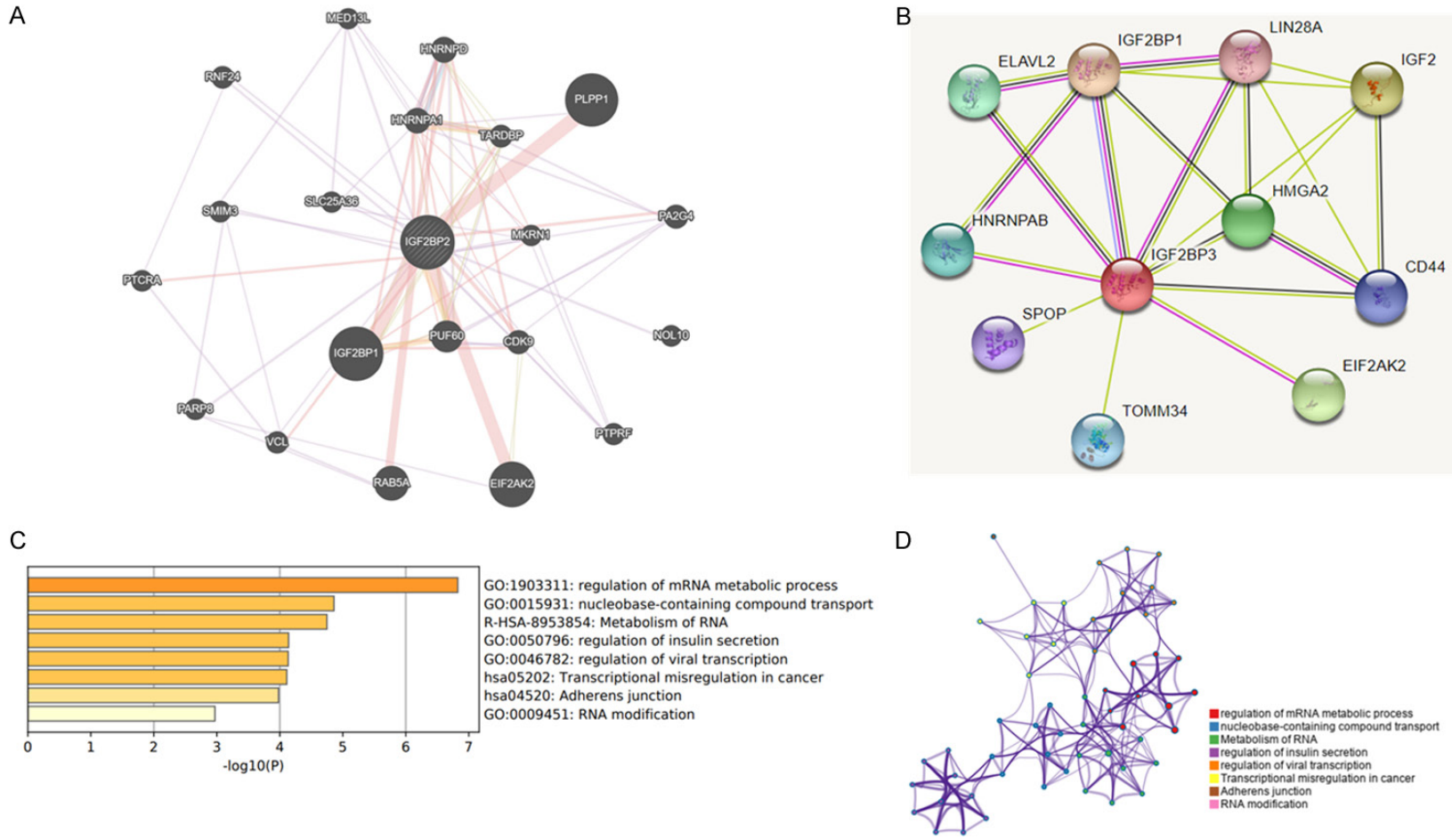

Figure 4. Protein-protein interactions and functional analysis of IGF2BPs in HNSCC. Gene-gene interaction network among IGF2BPs and genes whose expression was closely associated with IGF2BPs expression in the GeneMANIA dataset (A). Protein-protein interaction network among IGF2BPs and proteins whose expression was closely associated with IGF2BPs expression in the STRING dataset (B). Kyoto Encyclopedia of Genes and Genomes (KEGG) functional analysis of 8 key genes in HNSCC (C). Detailed net structure of key genes in HNSCC (D).

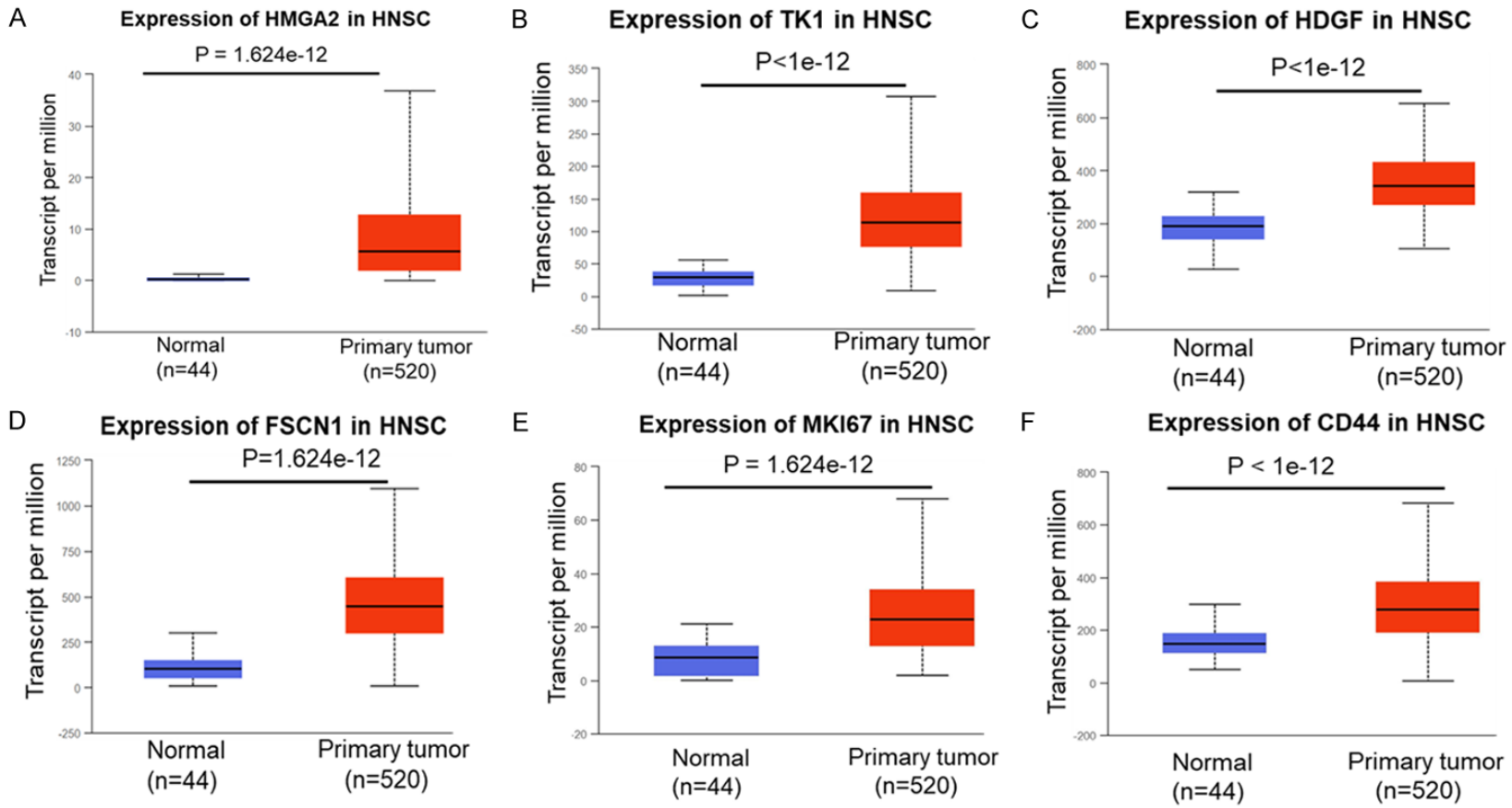

Figure 5. IGF2BPs target oncogenes expression in HNSCC. IGF2BPs target oncogenes such as *HMGA2* (A), *TK1* (B), *HDGF* (C), *FSCN1* (D), *MKI67* (E), and *CD44* (F) mRNAs expression were analyzed using UALCAN database.

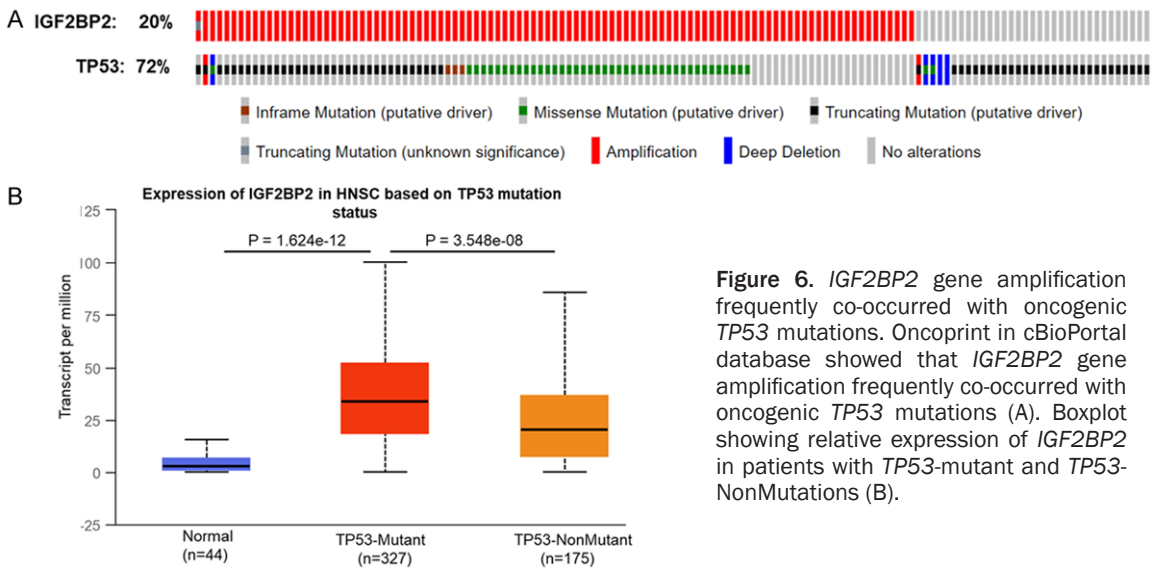

Figure 6. *IGF2BP2* gene amplification frequently co-occurred with oncogenic *TP53* mutations. Oncoprint in cBioPortal database showed that *IGF2BP2* gene amplification frequently co-occurred with oncogenic *TP53* mutations (A). Boxplot showing relative expression of *IGF2BP2* in patients with *TP53*-mutant and *TP53*-NonMutations (B).

tumorigenesis [28]. Another study reported that dysregulation of m6A regulators are associated with tumorigenesis and poor prognosis in HNSCC patients [29]. However, the availability of research reports directly focuses on the role of m6A regulatory genes in HNSCC, the specific function of m6A regulatory genes in tumorigenesis and their underlying mechanisms remain elusive.

The present study investigated the m6A regulatory genes aberrations in HNSCC to determine their frequency and prognostic impact, and also to gain further insights into HNSCC related carcinogenesis. The results of this study showed that m6A regulatory genes were altered in 41% (205/504) of HNSCC patients. Importantly, *IGF2BP2* was amplified in 20% (101/504) of HNSCC patients. Recent studies have shown the relationships between CNV patterns and m6A regulatory genes expression in acute myeloid leukemia (AML) [27], and clear cell renal cell carcinoma (ccRCC) [30]. They found that m6A "readers" YTHDC2 and YTHDC3 had the highest frequency of CNV events than other m6A regulatory genes, suggesting that m6A regulatory genes dysfunction may play crucial roles in the tumorigenesis of ccRCC, AML and HNSCC. Interestingly, the present study results revealed that the newly identified m6A "reader" *IGF2BP2* exhibited the highest frequency of CNV events than other m6A regulatory genes in HNSCC patients. In addition, *IGF2BP2* CNV correlated with its mRNA expression and poor prognosis, and the results of multivariate regression analysis showed that the risk score of the IGF2BP family might be an independent prognostic indicator in HNSCC. Furthermore, other m6A regulatory genes such as *METTL3, METTL14, WTAP, KIAA1429, ZC3-H13, RBM15, ALKBH5, FTO, YTHDF1, YTHDF2, YTHDF3, YTHDC1, IGF2BP1,* and *IGF2BP3* were significantly upregulated, while *RBM15B*, and *YTHDC2* expressions were not significantly altered in HNSCC samples. Therefore, these findings suggest that dysfunction of m6A regulators play important roles in HNSCC carcinogenesis.

The *TP53* gene is the most commonly mutated gene in human cancers including HNSCC. Further, many studies reported the co-occurrence oncogenes amplification and oncogenic TP53 mutations [31]. The results of this study showed that *IGF2BP2* gene amplification frequently co-occurred with oncogenic TP53 mutations and that *IGF2BP2* mRNA was highly expressed in HNSCC patients with oncogenic *TP53* mutations. Moreover, studies reported that amplification of the long arm of chromosome 3 (3q) in cancer is a major signature of neoplastic transformation and it was found in early stages of cancer development [2, 4, 32, 33]. Saladi et al. recently reported that the *ACTL6A* gene on 3q was co-amplified with TP53 family oncogene *TP63* on 3q, promotes proliferation and poor prognosis of HNSCC [34]. The results of this study showed that *IGF2BP2* gene on 3q was frequently co-amplified with *TP63* and other oncogenes such as *PIK3CA, SOX2,*

IGF2BP2, and *ACTL6A* on 3q. Therefore, the co-occurrence of *IGF2BP2* amplification with oncogenic *TP53* mutations and *IGF2BP2* co-amplification with TP53 family and other oncogenes may contribute to HNSCC development and progression.

More recent studies have shown that IGF2BP family members were highly expressed in several cancers and involved in the translation of many known oncogenes. The results of this study show that IGF2BPs target oncogenes including *HMGA2*, *MKI67*, *CD44*, *TK1*, *HDGF*, and *FSCN1* were highly expressed in HNSCC samples. Importantaly, our result also showed that *HMGA2* was highly expressed in OSCC patients with high expression of IGF2BP2. This study therefore indicates that IGF2BP2 plays an oncogenic role in HNSCC development by promoting *HMGA2* oncogene translation in an m6A-dependent manner, however, further functional studies are needed to verify this observation.

Further, the protein functional enrichment and the mechanism of the IGF2BPs studied by Metascape. The results revealed that the pathways involved in IGF2BPs might include RNA modification, regulation of mRNA metabolic process, and transcriptional misregulation. Many studies have reported that these pathways are involved in the tumorigenesis. Therefore, these finding help to study the role of IGF2BPs and relevant signaling pathways in HNSCC development and progression.

In conclusion, this data demonstrates that IGF2BP family plays a key role in the oncogenesis of HNSCC. These findings suggest that IGF2BP family might serves as novel prognostic biomarkers and potential therapeutic targets in HNSCC.

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Disclosure of conflict of interest

None.

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