Expression profile analysis of head and neck squamous cell carcinomas using data from The Cancer Genome Atlas

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Abstract. Head and neck squamous cell carcinoma (HNSCC) is the major histological type of head and neck cancer and no curative treatments are currently available. Using advanced sequencing technologies, The Cancer Genome Atlas (TCGA) has produced large-scale sequencing data, which provide unprecedented opportunities to reveal molecular mechanisms of cancer. The present study analyzed the mRNA and micro (mi)RNA expression data of HNSCC and normal control tissues released by the TCGA database using a bioinformatics approach to explore underlying molecular mechanisms. The mRNA and miRNA expression data were downloaded from the TCGA database and differentially expressed genes (DEGs) and miRNAs (DEMs) between HNSCC and normal head and neck tissues were identified using TwoClassDif. Subsequently, the gene functions and pathways which are significantly altered in HNSCC were identified using Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. Regulatory networks among DEGs and DEMs were then constructed, and transcription factors (TFs) potentially regulating the DEGs and DEMs were determined and a TF - miRNA - gene network was established. A total of 2,594 significant DEGs (1,087 upregulated and 1,507 downregulated), and 25 DEMs (8 upregulated and 17 downregulated) were identified in HNSCC compared with normal control samples. These DEGs were significantly enriched in GOs and KEGG pathways such as mitosis, cell cycle, Wnt, JAK/STAT and TLR signaling pathway. CPBP, NF-AT1 and miR-1 were situated in the central hub of the TF - miRNA - gene network, underlining their central roles in regulatory processes specific for HNSCC. The present study enhanced the current understanding of the molecular mechanisms underlying HNSCC and may offer novel strategies for its prevention, diagnosis and treatment.

Introduction

Head and neck squamous cell carcinoma (HNSCC), the major histological type of head and neck cancer (90-95%), is the sixth most common cancer type worldwide, with an incidence of <600,000 cases per year (1,2). More than 60% of HNSCC patients are already in the advanced stage at the time-point of first diagnosis (3). Despite the advances in the treatments for HNSCC, including surgical methods, chemoradiotherapy and the introduction of targeted therapies, the overall survival has not significantly improved in the last decades, with a five-year survival rate of only 40-50% (10-30% for patients with stage IVa and IVb tumors) (4,5). Therefore, it is urgently required to thoroughly explore the molecular characteristics of HNSCC to develop novel molecular-based targeted strategies for its treatment.

Numerous recent studies have investigated the biological characteristics of HNSCC. Certain genes, micro (mi)RNAs, transcription factors (TFs) and signaling pathways, including XPA, miR-504, EGR3 and the Notch signaling pathway, respectively, have important roles in the genesis and development of HNSCC, and are correlated to the prognosis of affected patients (6-8). However, to date, a comprehensive and systemic analysis of expression profiles in HNSCC has been lacking. The present study used the high-throughput mRNA and miRNA expression data from hundreds of HNSCC samples released by The Cancer Genome Atlas (TCGA) database to identify differentially expressed genes (DEGs) and miRNAs (DEMs) between human HNSCC and normal head and neck tissues. Significant gene functions and signaling pathways in which those DEGs are enriched were then determined. Finally, the regulatory network among TFs, DEMs and DEGs was mapped. The present study contributed to the current understanding of the molecular basis of HNSCC and may aid in the development of novel means of prevention, diagnosis and treatment.

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Materials and methods

Data sources. Level 3 RNA-sequencing data from 43 normal samples and 498 HNSCC samples, and level 3 miRNA-sequencing data from 45 normal samples and 513 HNSCC samples released by TCGA prior to July 1st, 2014 were all obtained from the TCGA data portal (https://tcga-data. nci.nih.gov/tcga/). As published previously, the mature miRNAs and star miRNAs (3' arms of pre-miRNA) from each pre-miRNA in the miRNA sequencing data were sorted and calculated according to their MIMAT serial number based on miRbase V20.0 (http://www.mirbase.org), whereas stem-loop, precursor or unannotated transcript data were not included in the present analysis (9,10). Reads per kilobase of exon model per million mapped reads (RPKM) and reads per million miRNA mapped (RPM) values were used to represent mRNA and miRNA expression levels, respectively (11). All data were presented as the mean ± standard deviation.

Identification of DEGs and DEMs. TwoClassDif was used to identify DEGs and DEMs between normal samples and HNSC samples as previously reported (12,13). Briefly, Fold-change (Tumor/Normal) was firstly used to filter DEGs and DEMs. Only genes with a Fold-change (Tumor/Normal) of >2 or <0.5, and miRNAs with a Fold-change (Tumor/Normal) of >2.5 or <0.4 progressed to next stages. Subsequently, the DEGs and DEMs were further confirmed with the t-test and random variance model (RVM)-modified t-test to reduce statistical errors using SPSS for windows, version 20 (International Business Machines, Armonk, NY, USA). In the present study, P<0.05 corrected by the false discovery rate (FDR) was considered to indicate a statistically significant difference.

Gene ontology (GO) analysis. GO analysis was performed according to the GO database (http://geneontology.org/) as previously described (14,15). In brief, the χ^2 test and Fisher's exact test were used to test the significance level of each function, and the FDR was calculated to correct statistical errors derived from multiple tests. The significance threshold was set at P<0.01 and FDR<0.05. The results were then classified in a GO-map to further integrate the functional links between these significant GO functions using Cytoscape v3.2.0 (http://cytoscape.org/).

Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis. KEGG analysis was performed as described previously using the KEGG database (http://www.genome. jp/kegg/) (16-18). The χ^2 test and Fisher's exact test were also performed to screen the significant pathways, and the P-value, FDR and enrichment value were calculated as previously reported (17). Pathways with P<0.01 and FDR<0.05 were considered as significant. Then, based on the pathway associations in the KEGG database, the path network was established to integrate the interactions among these significant pathways using Cytoscape v3.2.0.

TF - miRNA - gene network analysis. First, the target genes of the DEMs were predicted using Targetscan (http://www.targetscan.org/) and Miranda (http://www. microrna.org/) (19,20). The intersections of predictions from the two databases were matched to these DEGs to identify their regulatory targets.

To predict the TFs that regulate these DEGs and DEMs, the DNA sequences of these genes and pre-miRNA near the transcription start site area were acquired, including 1,000-bp upstream and 200-bp downstream regions. Then, the Match™ algorithm in the TRANSFAC database (http://www.gene-regulation.com/index2) was used to search for TF binding sites in these regions. Two score values generated in this algorithm, the core similarity score (CSS) and the matrix similarity score (MSS), were then applied to evaluate the forecast results (21-23). Based on these results, a TF - miRNA - gene network was finally constructed using Cytoscape v3.2.0, to summarize and illustrate the regulatory interactions among TFs, DEMs and DEGs.

Results

Identification of DEGs and DEMs. The present study identified 2,594 significant DEGs (1,087 upregulated and 1,507 down-regulated), and 25 significant DEMs (8 upregulated and 17 downregulated) in HNSCC compared with normal control samples. The DEGs and DEMs with the greatest fold-change are shown in Fig. 1.

GO analysis results. GO analysis was performed to preliminarily summarize the biological functions of the DEGs (Fig. 2).

The upregulated genes were mainly enriched in GO terms including mitosis (GO:0007067), cell proliferation (GO:0008283), cell division (GO 0051301) and the cell cycle (GO:0007049), which are features accountable for the overproliferation of HNSCC cells. They were also enriched in cell migration (GO:0016477), extracellular matrix organization (GO:0030198), cell-cell signaling (GO:0007267), angiogenesis (GO:0001525) and vascular endothelial growth factor receptor signaling pathway (GO:0048010), indicating the metastatic and angiogenic capacities of HNSCC.

Downregulated genes were mainly involved in the G-protein coupled receptor signaling pathway (GO:0007186), epidermal growth factor receptor signaling pathway (GO:0007173), transmembrane transport (GO:0055085) and glucose metabolic processes (GO:0006006), which indicated the potential negative effects of these GO terms in the genesis and development of HNSCC.

Furthermore upregulated, as well as downregulated, genes were significantly enriched in processes including cell differentiation (GO:0030154), cell adhesion (GO:0007155), immune response (GO:0006955) and ion transport (GO:0006811), suggesting that intricate changes were likely to occur in these GOs during the genesis of HNSCC.

Aberrant pathways in HNSCC tissues. As displayed in Fig. 3, the upregulated genes in HNSCC were significantly enriched in the cell cycle (Path ID: 4110), Wnt signaling pathway (Path ID: 04310), p53 signaling pathway (Path ID: 04115), Jak/STAT signaling pathway (Path ID: 04630), TGF-β signaling pathway (Path ID: 04350), Toll-like receptor signaling pathway (Path ID: 04620) and extracellular matrix - receptor interaction (Path ID: 04512).
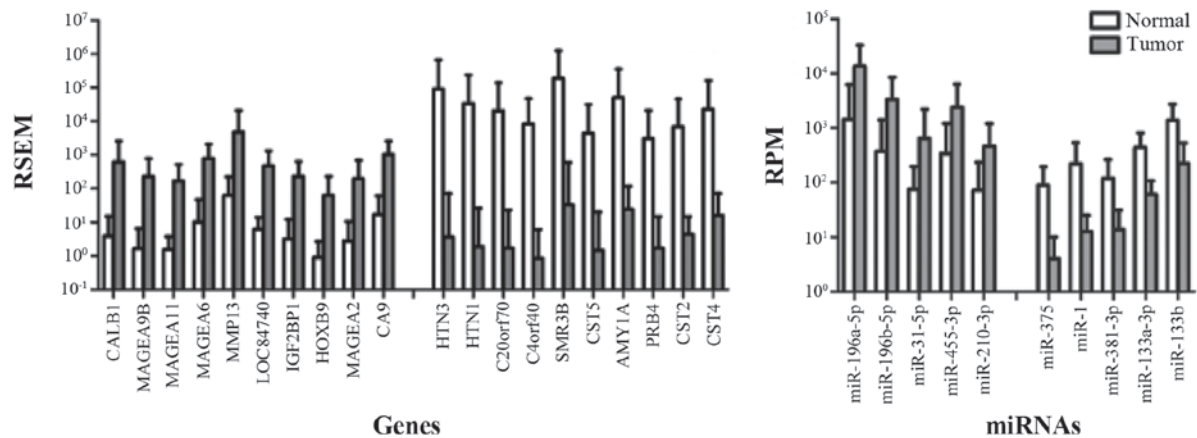
Figure 1. Top-10 differentially expressed genes and top-5 differentially expressed miRNAs in head and neck squamous cell carcinomas ordered by fold-change. miRNA/miR, microRNA; RSEM, RNA-Seq by expectation maximization; RPM, Reads per million miRNAs mapped.

The downregulated genes mostly participated in the calcium signaling pathway (Path ID: 04020), ErbB signaling pathway (Path ID: 04012), GnRH signaling pathway (Path ID: 04912), pyruvate metabolism (Path ID: 00620) and further small molecular metabolism pathways, while the upregulated as well as downregulated genes were enriched in pathways including the MAPK signaling pathway (Path ID: 4010) and cell adhesion molecules (Path ID: 04514).

TF - miRNA - gene network. As shown in Fig. 4, CPBP (also called KLF6) was located in the central hub of the TF - miRNA - gene network and regulated the largest number of DEMs (n=22) and genes (n=97) in HNSCC. This was followed by NF-AT1 (also termed NFATC2), GKLF (also known as KLF4), ZNF333 and Churchill. miR-1, miR-101-3p, miR-486-5p, miR-133a-3p and miR-195-5p were the top-5 DEMs that regulated the highest number of DEGs. These molecular regulators were indicated to have key roles in HNSCC.

Discussion

The present study identified 2,594 DEGs and 25 DEMs in HNSCC based on gene and miRNA expression profiles of HNSCC tissues compared with normal tissues. A large variety of DEMs, including CALB1 and MAGEA9B, DEMs, including miR-196a-5p and miR-196b-5p, in HNSCC were identified. Overexpression of CALB1 has been previously reported to be inversely correlated with apoptosis to be correlated with poor prognosis in other cancer types (24,25). MAGEA9B and three other genes belonging to the melanoma antigen family A (MAGEA) were among the ten most strongly upregulated genes of the present study. This gene family is highly expressed in early embryos and is associated with reduced overall survival in numerous types of cancer (26-28). However, the roles of the CALB1 and MAGEA gene families have been rarely studied in HNSCC and require to be further investigated. As the most strongly upregulated miRNAs in the present study, miR-196a-5p and miR-196b-5p have been frequently reported to be overexpressed in the blood of HNSCC patients and in HNSCC tissues and to be associated

with the prognosis and radio-response, indicating their potential as promising diagnostic and prognostic biomarkers as well as therapeutic targets (29-33). Therefore, the potential of these DEGs and DEMs as biomarkers and therapeutic targets deserve further investigation with regard to their application in the early diagnosis, pathological identification, treatment and monitoring of HNSCC.

To explore the main functional enrichment and signaling pathways of these DEGs at the cellular level, GO and KEGG pathway analyses were performed, respectively. GO functions and pathways including mitosis and cell cycle as well as Wnt, JAK/STAT and Toll-like receptor signaling pathways were markedly altered in HNSCC compared with normal controls. The mutation, abnormal expression and modification of these GOs and pathways have been frequently reported in HNSCC and other cancer types (34-38). In tumor cells, the normal energy metabolism of aerobic respiration is replaced by glycolysis, which is harnessed for accumulating intermediates of macromolecule biosynthesis, known as the 'Warburg effect' (39-41). Along with this, the present study revealed that the downregulated DEGs were significantly enriched in gene functions and pathways associated with the metabolism of glucose, pyruvate and numerous other small molecules. These results suggested that these significant GOs and pathways are critical drivers in the carcinogenesis and development of HNSCC, and so are the DEGs that participate in these GOs and pathways. As shown in the interaction networks, considerable cross-links exist among these gene functions and pathways, and therapeutic targeting of one of them may modulate others, indicating that several parts of the network may be affected by targeting one component for the treatment of HNSCC. The hub genes involved in the significant GO and pathway networks might be applied as novel targets in HNSCC therapeutic strategies.

There are two major ways in which cells generally regulate gene expression; one is to regulate the transcription from DNA to RNA via elements, such as TFs, while the other is to regulate the stability of RNA via factors, such as miRNAs (42). Using the mRNA and corresponding miRNA expression data released by TCGA, the present study constructed the TF - miRNA - gene network in HNSCC. In this network,
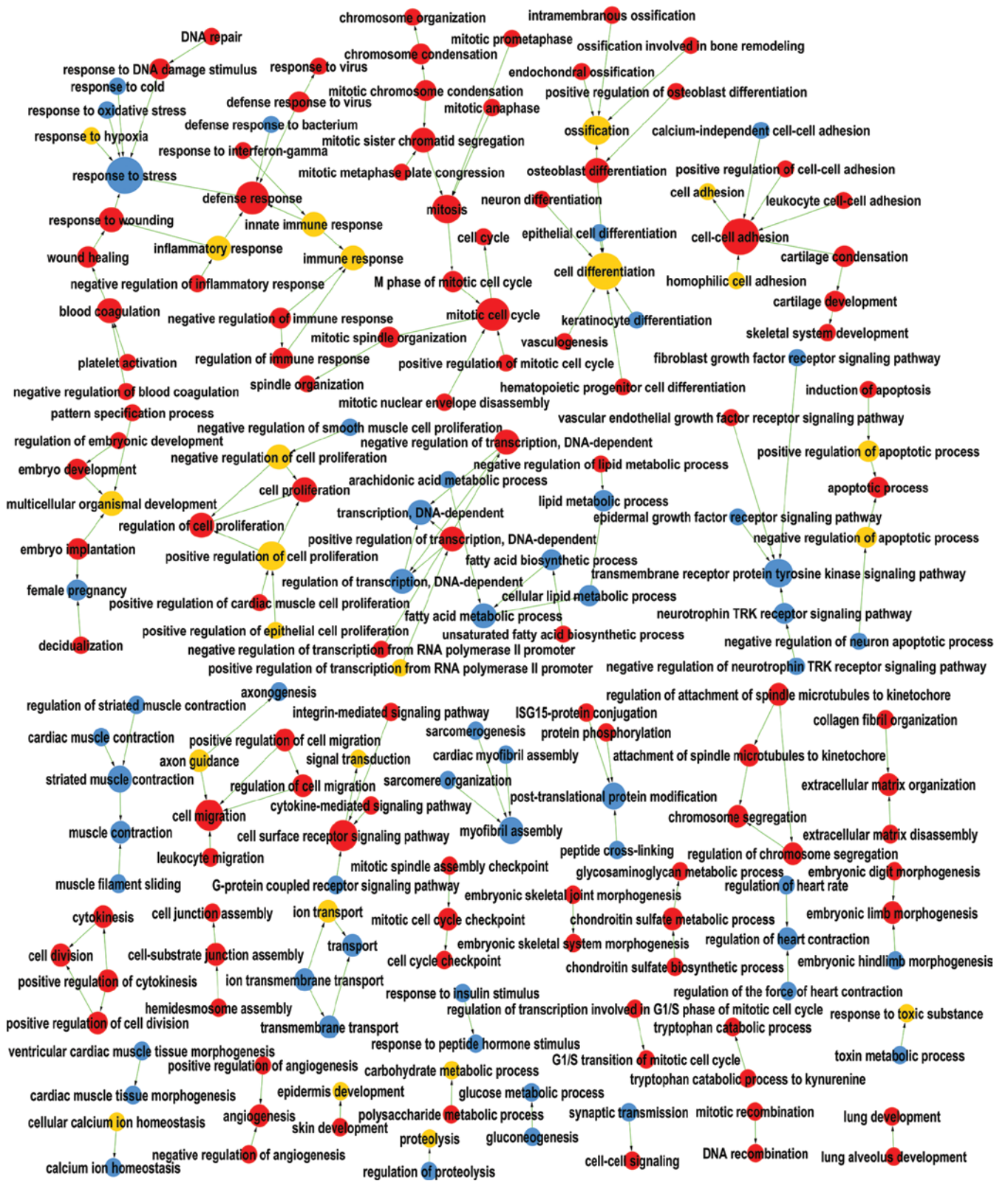
Figure 2. Gene ontology map for head and neck squamous cell carcinomas. Circles represent genetic functions of differentially expressed genes, with their size being proportional to the importance of their function. Red represents functions encoded by upregulated genes, blue represents functions encoded by downregulated genes and yellow represents functions encoded by up- as well as downregulated genes. Straight lines represent links between genetic functions.

CPBP, NF-AT1 and miR-1 were situated in the central hub, indicating their marked importance in the regulatory net of HNSCC. CPBP is a member of the Kruppel-like family of TFs, some of which are implicated in carcinogenesis, acting in processes ranging from cell proliferation and apoptosis to differentiation, migration and pluripotency (43). NF-AT1 has a central role in gene transcription during the immune response and is associated with several tumor types, including glio-
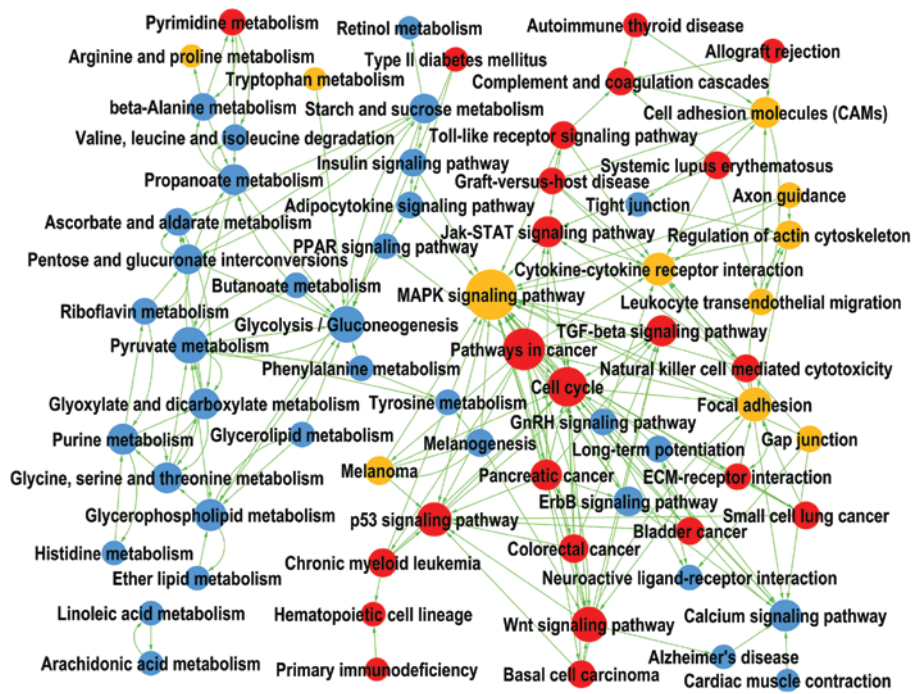
Figure 3. Kyoto Encyclopedia of Genes and Genomes path network in head and neck squamous cell carcinomas. Circles represent pathways, and their size is relative to the importance of the respective pathway. Red represents pathways of upregulated genes, blue represents pathways of downregulated genes and yellow represents pathways of up- as well as downregulated genes. Straight lines represent interactions between pathways.
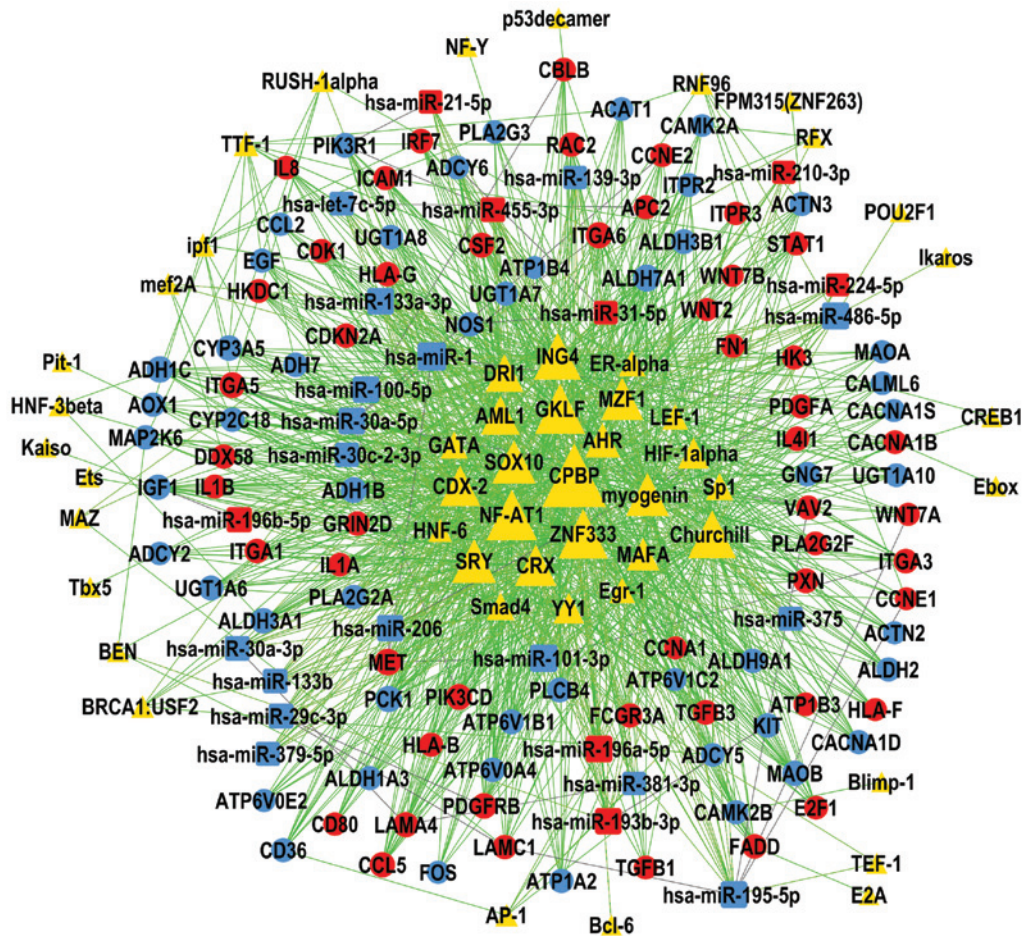

Figure 4. TF - miRNA - gene regulation network in head and neck squamous cell carcinomas. Triangles represent TFs, circles represent differentially expressed genes and rounded rectangles represent differentially expressed miRNAs, with their area being relative to the significance. Red represents upregulation, blue represents downregulation and yellow represents up- as well as downregulation. Straight lines represent regulatory interactions. TF, transcription factor; miR, microRNA.

blastoma and human melanoma (44,45). However, the roles of these two TFs in have remained elusive in HNSCC. As the second most downregulated DEM, miR-1 has been reported to be a tumor suppressor miRNA targeting transgelin 2, purine nucleoside phosphorylase, fibronectin 1 and prothymosin alpha, and to accelerate apoptosis and inhibit proliferation in HNSCC (46-49). Further investigation on these TFs and miRNAs will enhance the current understanding of the molecular mechanisms of HNSCC and help to identify potential therapeutic targets for the treatment of HNSCC.

Based on the mRNA and corresponding miRNA expression data for hundreds of HNSCC samples, the present study identified the DEGs and DEMs, and then investigated the GOs and pathways in which the DEGs were significantly enriched. The regulatory links among the DEMs and DEGs were determined and potential TFs, which regulate these DEMs and DEGs were predicted to finally construct the TF - miRNA - gene network. Additionally, the hub genes, TFs and miRNAs may potentially be targeted by novel therapeutic strategies in the future. To the best of our knowledge, the present study was the first systematic bioinformatics analysis in HNSCC, and for the first time, significantly altered GOs and pathways, as well as DEMs and TFs were identified in HNSCC. The results of the present study enhanced the current understanding of the molecular mechanisms underlying HNSCC, and may provide a source for developing novel strategies for its prevention, diagnosis and treatment.

Acknowledgements

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