Research Article

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Methylene tetrahydrofolate dehydrogenase 2 (MTHFD2) is overexpressed in head and neck squamous cell carcinoma (HNSCC) and correlated with patient's poor prognosis

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Abstract

Objective – To investigate methylene tetrahydrofolate dehydrogenase 2 (MTHFD2) expression, biological function, and correlation with head and neck squamous cell carcinoma (HNSCC) patient's prognosis.

Methods – The relative expression levels of MTHFD2 gene mRNA in tumor tissues of HNSCC and adjacent normal tissues were analyzed in the Cancer Genome Atlas and onco-mine database. MTHFD2 protein relative expression in tumor tissue of HNSCC patients was analyzed in human proteome database. Protein–protein interaction (PPI) network of MTHFD2 and correlated genes were constructed in STRING database. Gene ontology and Kyoto Encyclopedia of Genes and Genomes pathway of MTHFD2 and relevant proteins involved in the PPI network was enriched. The Tumor Immune Estimation Resource database was used to analyze the relationship between MTHFD2 expression and immune infiltration. Overall survival (OS) and progression-free survival (PFS) for MTHFD2 high and low expression groups were investigated in the Kaplan–Meier Plotter database.

Results – In HNSCC, MTHFD2 mRNA relative expression level in tumor tissue was significantly higher than the corresponding normal tissue with statistical difference ($p < 0.05$). In the PPI network, 21 protein coding genes were involved in the network with 124 edges, which indicated that the enrichment was significant ($p < 0.05$). MTHFD2 and PPI network involved genes were mainly enriched in tetrahydrofolate metabolic process, one-carbon metabolic process biological process. In KEGG pathway, MTHFD2 and PPI network involved genes were mainly enriched in one-carbon pool by folate, metabolic pathways, glyoxylate, and dicarboxylate metabolism, and carbon metabolism. The relative expression level of MTHFD2 gene was correlated with immune infiltration of macrophage ($r = 0.712$, $p < 0.05$), neutrophil ($r = 0.158$, $p < 0.05$), dendritic cell ($r = 0.1825$, $p < 0.05$), and CD4+ T lymph cell ($r = 0.1825$, $p < 0.05$). HNSCC patients with high expression MTHFD2 had low OS compared to low expression cases (hazard ratio = 1.53, 95% CI: 1.16–2.02, $p < 0.05$).

Conclusion – MTHFD2 is overexpressed in HNSCC and correlated with patient's prognosis. MTHFD2 maybe a potential target for HNSCC target treatment and provides a possible direction for the research and development of related targeted drugs.

Keywords: methylene tetrahydrofolate dehydrogenase 2, head and neck squamous cell carcinoma, bioinformatics analysis, prognosis

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1 Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common malignant tumor globally, with more than 550,000 cases and 300,000 deaths each year [1]. Smoking and drinking are the main risk factors for HNSCC [2]. The treatment of HNSCC is mainly based on TNM staging. Surgery plus comprehensive therapies (radiotherapy,

chemotherapy, and biotherapy) are most applied treatment methods clinically [3]. However, the 5 year survival rate is not improved compared to several decades before. Therefore, new treatment methods are needed to improve the prognosis of HNSCC. With the development of biotechnology, immunology, and high-throughput sequencing technology, more and more studies identified that abnormal spatiotemporal expression of tumor-driving genes played an important role in the development of HNSCC.

Methylene tetrahydrofolate dehydrogenase 2 (MTHFD2) was first found in Ehrlich ascites tumor cells by Scrimgeour and Huennekens [4] in 1960, and encodes a nuclear-encoded mitochondrial bifunctional enzyme with methylene tetrahydro folate dehydrogenase and methenyltetrahydrofolate cyclohydrolase activities. The enzyme functions as a homodimer and is unique in its absolute requirement for magnesium and inorganic phosphate. MTHFD2 has the dual activities of methylene tetrahydrofolate dehydrogenase and cyclohydrolase, and is the key enzyme in the conversion of folate metabolites *in vivo* [5]. Tumor cells have rapid glycolysis and synthesis of amino acids, nucleotides, and lipids to support the rapid proliferation of tumor cells. In particular, the synthesis of single carbon units carried by tetrahydrofolate cofactor is extremely important for the proliferation of cells, which is necessary for nucleotide synthesis and methylation reaction [6].

The overexpression of MTHFD2 has been proved to be related to the occurrence, development, and prognosis of esophageal cancer [7], breast cancer [8] and et al. However, the correlation between MTHFD2 expression and prognosis of HNSCC is rarely reported. Whether MTHFD2 was differently expressed between tumor and corresponding normal tissue of HNSCC and its correlation with patient's prognosis is not clear yet. In this work, we aim to investigate MTHFD2 expression in HNSCC and discuss its biological function through bioinformatics analysis.

2 Databases and methods

2.1 MTHFD2 expression analysis

The relative expression levels of MTHFD2 gene mRNA in cancer tissues and adjacent normal tissues of patients with HNSCC and other malignancies were analyzed in the Cancer Genome Atlas (TCGA; <https://portal.gdc.cancer.gov/>) and oncomine (<https://portal.gdc.cancer.gov/>) databases with the searching words/variables of HNSCC and expression. MTHFD2 protein relative expression was investigated in the human protein atlas database (<https://www.proteinatlas.org/>).

TCGA, a landmark cancer genomics program, molecularly characterized over 20,000 primary cancer and matched normal samples spanning 33 cancer types. The human protein atlas database, the Human Protein Atlas, maps most the human proteins in cells, tissues, and organs using an integration of various omics technologies, including antibody-based imaging, mass spectrometry-based proteomics, transcriptomics, and systems biology.

2.2 Protein–protein interaction (PPI) network construction

The PPI network relevant to MTHFD2 and correlated genes was constructed in functional protein association network database (STRING, <http://string-db.org/cgi/input.pl>). The PPI network was constructed under the following conditions: (1) proteins involved in the PPI no more than 20 and (2) the confidence of interaction was more than 0.7.

2.3 Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis

The GO enrichment and KEGG signal pathway analysis of MTHFD2 and network interacting protein coding genes were performed by using David database (<https://david.ncifcrf.gov/>). GO enrichment includes biological process (BP), cellular component (CC), and molecular function (MF). David database can provide annotation, visualization, and integrated discovery of interest genes.

2.4 MTHFD2 expression and immune infiltration analysis

The correlation between MTHFD2 gene expression and immune infiltration such as macrophage, neutrophil, dendritic cell, B lymph cell, CD8+ T lymph cell, and CD4+ T lymph cell was analyzed in the Tumor Immune Estimation Resource (TIMER) database (<https://cistrome.shinyapps.io/timer/>). TIMER database is a comprehensive resource for systematical analysis of immune infiltrates across diverse cancer types. The abundances of six immune infiltrates (B cells, CD4+ T cells, CD8+ T cells, neutrophils, macrophages, and dendritic cells) are estimated by TIMER algorithm.

2.5 Survival analysis

According to the median expression level of MTHFD2 gene in tumor tissue of HNSCC cases, the patients were divided into MTHFD2 high ($\geq$ MTHFD2 median expression) and low expression ($<$ MTHFD2 median expression) groups. The OS and progression-free survival (PFS) were compared between high and low expression groups by log-rank test and demonstrated by survival curve.

2.6 Statistics analysis

The data analysis was partly based on R software (<https://www.r-project.org/>) and relevant databases online data analysis tool. Two tails $p < 0.05$ was considered as statistically significant.

3 Results

3.1 MTHFD2 overexpressed in cancer tissue of HNSCC

MTHFD2 mRNA was up-regulated in most malignant carcinomas in tumor tissue compared to corresponding normal tissue (Figure 1a). In oncomine database, MTHFD2 mRNA was also overexpressed in tumor tissue compared to normal tissue except for leukemia (Figure 1b). In HNSCC, MTHFD2 mRNA relative expression level in cancer tissue was significantly higher than the corresponding normal tissue with statistical difference ($p < 0.05$; Figure 1c). However, MTHFD2 mRNA expression level was not different between different stages of HNSCC patients (Figure 1d). MTHFD2 protein was mainly positive expressed intracellular of brown staining in HNSCC tissue (Figure 2).

3.2 21 proteins were included in the PPI network

In the PPI network, 21 protein coding genes were involved with 124 edges. The average node degree was 11.8 with the average local clustering confidence of 0.835, which indicated that the enrichment was significant ($p < 0.05$; Figure 3).

3.3 Enrichment of GO and KEGG pathway

In the aspects of GO, MTHFD2 and PPI network involved genes were mainly enriched in tetrahydrofolate metabolic

process, one-carbon metabolic process, dicarboxylic acid metabolic process, and folic acid metabolic process for BP (Table 1). For CC, MTHFD2 and involved genes were mainly enriched in mitochondrial matrix, mitochondrion, and cytoplasm (Table 2). For MF, MTHFD2 and related genes were enriched in hydroxymethyl-, formyl-, and related transferase activity, methenyltetrahydrofolate cyclohydrolase activity, methylenetetrahydrofolate dehydrogenase (NADP+) activity, and so on (Table 3). In terms of KEGG pathway, MTHFD2 and PPI network involved genes were mainly enriched in one-carbon pool by folate, metabolic pathways, glyoxylate and dicarboxylate metabolism, carbon metabolism, and so on (Figure 4).

3.4 MTHFD2 expression was partly correlated with the immune infiltration

The relative expression level of MTHFD2 gene was correlated with immune infiltration of macrophage ($r = 0.712$, $p < 0.05$), neutrophil ($r = 0.158$, $p < 0.05$), dendritic cell ($r = 0.1825$, $p < 0.05$), and CD4+ T lymph cell ($r = 0.1825$, $p < 0.05$). However, it was not correlated with CD8+ T lymph cell ($r = 0.049$, $p > 0.05$) and B lymph cell ($r = 0.004$, $p > 0.05$) (Figure 5).

3.5 MTHFD2 expression was correlated with patient's overall survival

HNSCC patients with high expression MTHFD2 had low OS compared to low expression cases [hazard ratio (HR) = 1.53, 95% CI: 1.16–2.02, $p < 0.05$]. However, the PFS was not statistically different between the high and low expression groups (HR = 2.50, 95% CI: 0.87–7.21, $p > 0.05$) (Figure 6).

4 Discussion

In the present analysis, we found that MTHFD2 mRNA was up-regulated in most of the malignant carcinomas including HNSCC. MTHFD2 and PPI network involved genes were mainly enriched in tetrahydrofolate metabolic process, hydroxymethyl-, formyl-, and related transferase activity, and carbon metabolism pathway. Further analysis indicted that MTHFD2 gene was correlated with immune infiltration and HNSCC patient's prognosis. The OS was

Figure 1: Expression analysis of MTHFD2 gene in HNSCC (a) relative expression level of MTHFD2 in various human solid tumors; (b) MTHFD2 expression pattern in major malignant carcinoma in oncomine database; (c) box plot of MTHFD2 expression in HNSCC; (d) MTHFD2 expression between different stages of HNSCC.

Figure 2: MTHFD2 protein relative expression analysis detected by immunohistochemistry assay (a) MTHFD2 protein high expression; (b) MTHFD2 protein median expression; (c) MTHFD2 low expression.

Figure 3: The protein–protein network for MTHFD2 and relevant gens.

significantly decreased in MTHFD2 high expression groups. MTHFD2 and PPI network involved genes were mainly enriched in one-carbon pool by folate, metabolic pathways, glyoxylate, and dicarboxylate metabolism, carbon metabolism and so on. One-carbon pool by folate, metabolic pathways, glyoxylate, and dicarboxylate metabolism pathway

was closely related with folic acid and can further cause apoptosis of cancer cells.

Studies have shown that MTHFD2 is expressed in fetal cells and transformed cell lines, but it is low or absent in most adult tissues and cell types [9,10]. In recent years, MTHFD2 has been proved to be important for rapidly growing cells (such as embryonic cells or tumor cells), mainly by supporting the high level of purine synthesis [11]. Nilsson *et al.* found that MTHFD2 mRNA and protein expression in mitochondrial folate pathway were increased in highly proliferative cancer [12]. Furthermore, several studies have shown that MTHFD2 is highly expressed in breast cancer [8] and renal cell carcinoma [13], and is associated with patient's poor prognosis.

Several experimental studies have confirmed that MTHFD2 was up-regulated specifically in various types of cancer and is related to the poor prognosis of patients [7,8]. Wang *et al.* [7] performed a bioinformatics study and found that MTHFD2 was a novel prognosis biomarker in esophageal carcinoma patients. The authors identified that MTHFD2 was up-regulated in esophageal cancer and can be applied as potential biomarker for patient's prognosis. Liu *et al.* [8] also identified that increased MTHFD2 expression is associated with poor prognosis in breast cancer. MTHFD2 has been identified as a new drug target [14–16]. Noguchi *et al.* [17] carried out studies on the expression of single carbon metabolizing enzymes including MTHFD2 in 103 patients who underwent surgical resection of pancreatic ductal adenocarcinoma. The results showed that the OS and PFS of patients with high

Table 1: BP enrichment for MTHFD2 and PPI network involved genes

Term description	Observed gene count	Background gene count	Strength	False discovery rate
Tetrahydrofolate metabolic process	14	18	2.86	2.09×10^{-33}
One-carbon metabolic process	12	24	2.67	7.37×10^{-27}
Dicarboxylic acid metabolic process	13	97	2.1	5.59×10^{-23}
Folic acid metabolic process	10	18	2.71	1.80×10^{-22}
Tetrahydrofolate interconversion	9	9	2.97	8.18×10^{-22}
Carboxylic acid metabolic process	18	854	1.29	2.79×10^{-20}
Coenzyme metabolic process	14	297	1.64	2.28×10^{-19}
Drug metabolic process	16	622	1.38	9.65×10^{-19}
Alpha-amino acid metabolic process	12	209	1.73	3.25×10^{-17}
Cofactor metabolic process	14	467	1.45	8.03×10^{-17}
Cellular amide metabolic process	15	732	1.28	6.34×10^{-16}
10-Formyltetrahydrofolate metabolic process	5	5	2.97	5.44×10^{-12}
Folic acid-containing compound biosynthetic process	5	9	2.71	4.12×10^{-11}
Serine family amino acid metabolic process	6	40	2.15	1.47×10^{-10}
Cellular modified amino acid biosynthetic process	6	41	2.13	1.62×10^{-10}
Glycine metabolic process	5	16	2.46	3.52×10^{-10}
Carboxylic acid biosynthetic process	9	311	1.43	3.65×10^{-10}

MTHFD2 expression were significantly lower than those with low expression level. It is confirmed that MTHFD2 is an independent prognostic factor and potential therapeutic target for pancreatic cancer [17]. Consistent with the role of MTHFD2 in supporting cancer cell proliferation, MTHFD2 was found to be co-expressed with proteins involved in cell

Table 2: CC enrichment for MTHFD2 and PPI network involved genes

Term description	Observed gene count	Background gene count	Strength	False discovery rate
Mitochondrial matrix	9	463	1.26	3.64×10^{-8}
Mitochondrion	12	1531	0.86	2.59×10^{-7}
Cytoplasm	20	11,238	0.22	0.0031
Mitochondrial inner membrane	4	456	0.91	0.0206
Cytosol	12	4958	0.35	0.0243

Table 3: MF enrichment for MTHFD2 and PPI network involved genes

Term description	Count	Background gene	Strength	p-value
Hydroxymethyl-, formyl-, and related transferase activity	8	8	2.97	5.73×10^{-19}
Oxidoreductase activity, acting on the CH-NH group of donors, NAD or NADP as acceptor	7	17	2.58	1.07×10^{-14}
Transferase activity, transferring one-carbon groups	10	213	1.64	2.79×10^{-13}
Cyclohydrolase activity	5	6	2.89	9.20×10^{-12}
Methenyltetrahydrofolate cyclohydrolase activity	4	4	2.97	1.34×10^{-9}
Methylenetetrahydrofolate dehydrogenase (NADP+) activity	4	4	2.97	1.34×10^{-9}
Ligase activity, forming carbon-nitrogen bonds	5	50	1.97	3.74×10^{-8}
Catalytic activity	19	5,592	0.5	6.22×10^{-8}
Vitamin binding	6	135	1.62	6.66×10^{-8}
Methylenetetrahydrofolate dehydrogenase (NAD+) activity	3	3	2.97	2.08×10^{-7}
Oxidoreductase activity	9	716	1.07	2.22×10^{-7}
Carboxylic acid binding	6	187	1.48	3.42×10^{-7}
Drug binding	11	1,710	0.78	2.65×10^{-6}
Folic acid binding	3	11	2.41	2.66×10^{-6}

Figure 4: Bubble plot of KEGG pathway enrichment for MTHFD2 gene.

Figure 5: Scatter plot between MTHFD2 expression and immune infiltration.

cycle progression, especially in S, G2, and M stages, and often overexpressed in human tumors [18].

Animal study demonstrated that MTHFD2 promotes rapid cell growth in mice during embryonic development [19]. In addition, knocking down MTHFD2 expression can reduce the population of CD44+ cancer stem cell marker-

positive cells, indicating that loss of MTHFD2 expression reduces cancer stem cell properties. Study also identified that MTHFD2 can be silenced by miR-9, and hence, followed by reduced cell migration [20].

In conclusion, we performed a comprehensive bioinformatics analysis by applying TCGA, PPI, Oncomine,

Figure 6: Survival curve of between MTHFD2 low and high expression groups (a) overall survival; (b) progression free survival.

David, and TIMER databases. The above databases can provide expression, enrichment, survival, and immune infiltration analysis, and obtain credible results. We found that MTHFD2 was over expressed in malignant carcinomas including HNSCC and correlated with patient's prognosis. MTHFD2 maybe a potential target for HNSCC target treatment and provide a possible direction for the research and development of related targeted drugs. However, the present work also had limitations: (i) most of the findings are based on bioinformatics analysis, which need verification by cell experiments; and (ii) the conclusion of the present work especially on survival needs further confirmation by clinical data.

Conflict of interest: Authors state no conflict of interest.

Data availability statement: The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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