Peer Review File

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Reviewer Comments

Comment 1: 69 cases of oral squamous cell carcinoma (OSCC) were collected. The expression of mthfd1l in tumor tissues was similar to that in healthy tissues? The number of 69 cases is not large. If you can, please increase the sample size.

Reply 1: Many thanks for your suggestions. In this study, we want to explore the potential clinical significance of MTHFD1L in OSCC. We used the data from TCGA database and tissue samples from our hospital, respectively. Although the sample size is not large, the expression level of MTHFD1L in OSCC tissues from these patients was significantly higher than that in normal tissues. We hypothesize that MTHFD1L may play a key role the development of OSCC. In the further, we will expand the samples' quantity and extend the follow-up time to verify the clinical application value of this gene.

Owing to your suggestion, we enlarged the sample size of this study (From June 2007 to July 2016), 96 OSCC cases were finally collected. Moreover, we added some data to evaluate the correlation between MTHFD1L and the clinicopathological variables of OSCC. MTHFD1L levels were correlated with T classification ($p = 0.002$), and local recurrence ($p < 0.001$) in patients with OSCC. No significant difference was observed between the MTHFD1L expression level and any other clinicopathological factor (Table 1).

Changes in the text: To express our opinions as clearly as possible, we have added some content into the **"Material and methods"** and **"Result"** sections as follows. Tumor and matched para-tumor tissues were acquired from 96 OSCC patients who were histologically diagnosed at Bejing Hospital. Surgical specimens of OSCC were obtained from June 2007 to July 2016 (see Page 5, line 100 and 105).

The correlation between MTHFD1L and the clinicopathological variables of OSCC is summarised in Table 1. MTHFD1L levels were correlated with T classification ($p = 0.002$), and local recurrence ($p < 0.001$) in patients with OSCC. No significant difference was observed between the MTHFD1L expression level and any other clinicopathological factor (see Page 12, line 248-252).

Table 1. Correlation between MTHFD1L expression and the clinicopathological characteristics

Characteristic	**No. of patients**	**MTHFD1L**		***P* -value**
		low expression, n (%)	high expression, n (%)	
Gender				0.314
Male	44	19 (19.79)	25 (26.04)	
Female	52	28 (29.17)	24 (25.00)	
Age (years)				0.540
≤60	49	22 (22.92)	27 (28.13)	
≥60	47	25 (26.04)	22 (22.92)	
Clinical stage				0.011
I + II	43	29 (30.21)	14 (14.58)	
III + IV	53	18 (18.75)	35 (36.46)	
T classification				0.002
T_1	10	7 (7.29)	3 (4.35)	
T_2	34	24 (25.00)	10 (10.42)	
T_3	45	14 (14.58)	31 (32.29)	
T_4	7	2 (2.08)	5 (5.21)	
N classification				0.011
Yes	26	7 (7.29)	19 (19.79)	
No	70	40 (41.67)	30 (31.25)	
M classification				0.018
Yes	47	10 (10.42)	37 (38.54)	
No	49	22 (22.92)	27 (28.13)	
Local recurrence				<0.001
Yes	65	29 (30.21)	36 (37.50)	
No	31	18 (18.75)	13 (13.54)	

Comment 2: IHC showed that the expression of mthfd1l in tumor tissues was higher than that in normal tissues. More importantly, Kaplan Meier also analyzed whether mthfd1l expression is clinically significantly associated with overall survival in

HNSCC patients. These results suggest that mthfd1l gene is highly expressed and is associated with survival. Can mthfd1l be used as a biomarker for early diagnosis or prognosis prediction of OSCC?

Reply 2: The present study revealed the expression of MTHFD1L in OSCC specimens and the adjacent normal tissues, and MTHFD1L expression was significantly associated with tumor T classification and local recurrence in OSCC patients. In summary, these findings suggest a novel potential link implicating MTHFD1L with tumor grade or prognosis. Although the study was underpowered for definitive conclusions due to the small sample size, the results were encouraging to search a novel marker for early diagnosis or prognosis prediction of OSCC.

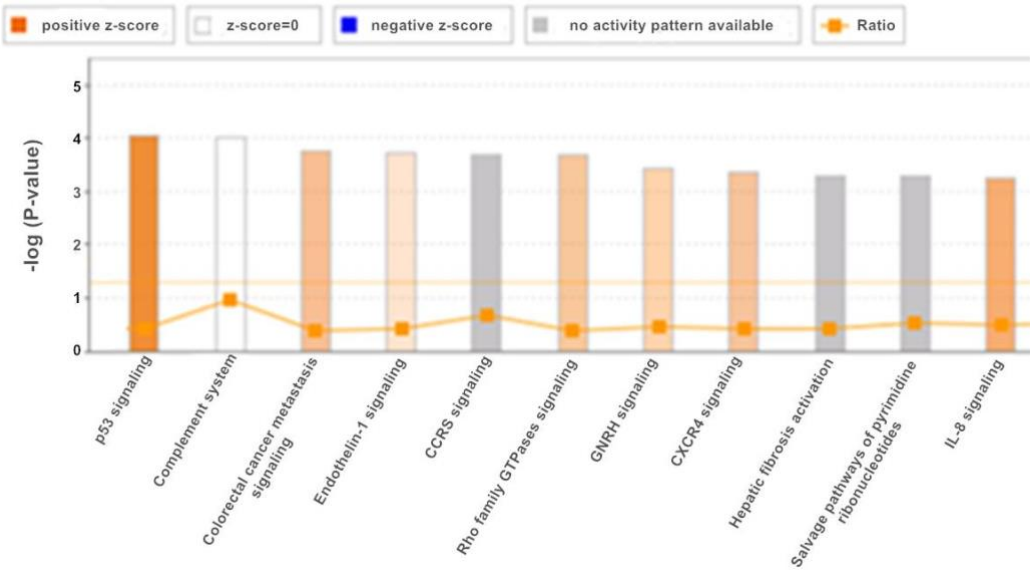
Comment 3: The expression of MTHFD1L in tumor tissue is higher than that in normal tissues and is related to survival. What are the main ways of regulating MTHFD1L?

Reply 3: In our study, we have discussed the known ways of regulating MTHFD1L.The regulate effects of MTHFD1L were attributed to NADPH reduction and ROS accumulation in the biological behaviour of OSCC. To illustrate this point clearly, we added some into the "**Discussion**" sections as follows.

Changes in the text: MTHFD1L contributes to the production of NADPH that are sufficient to combat oxidative stress in OSCC. They determined that the expression of MTHFD1L was significantly affected by the mTORC1-4EBP1-eIF4E axis (see Page 16, line 338-341).

Comment 4: Nad dependent methylenetetrahydrofolate dehydrogenase inhibits proliferation and promotes apoptosis of OSCC cells by regulating c-myc gene and p53 dependent pathway. Does a nad-dependent methylenetetrahydrofolate dehydrogenase inhibit proliferation and promote apoptosis of OSCC cells by regulating other genes or signaling pathways?

Reply 4: In this study, some differently expressed genes and fundamental biological signaling pathways were identified by Ingenuity Pathway Analysis (IPA). Including p53 and IL-8 signaling pathway were potentially activated (Z-score≥2) (see Page 14, line 301) . Among these signaling pathways, we chose the p53 signaling pathway to explore the possible mechanism of shMTHFD1L on OSCC.



Comment 5: OSCC is a highly complex multi-step process involving genetic and epigenetic changes and dynamic genomic changes. The current methods of molecular detection are still insufficient in the choice of oral treatment for cancer. What is the progress of OSCC molecular detection?

Reply 5: Over the past few decades, the molecular detection techniques of OSCC have been made great progress. Standard methods used to detect OSCC remain comprehensive clinical examination, biochemical investigations, and invasive biopsy. In proteomics, the technology of micro-array and bio-informatics carved out a new way to effectively seek tumor markers.

Salivary microRNAs were increasingly studied as non-invasive molecular biomarkers which could aid in early diagnosis, monitoring, and prognosis of oral cancers.Saliva's direct contact with oral cancer lesions makes it more specific and potentially sensitive screening tool, whereas more than 100 salivary biomarkers (DNA, RNA, mRNA, protein markers) have already been identified, including cytokines (IL-8, IL-1b,

TNF-α), defensin-1, P53, Cyfra 21-1, tissue polypeptide-specific antigen, dual specificity phosphatase, spermidine/spermineN1-acetyltransferase , profilin, cofilin-1, transferrin, and many more.

Surface enhanced laser desorption/ionization-time of flight-mass spectrometry is such a new path to provide high-throughput protein profiling. Protein mass peaks were screened out to build a serum diagnosis model with a significant P value, respectively, and the sensitivity, specificity, and total accuracy were 93.75%, 92.86%, and 93.33%.

Imaging mass spectrometry (IMS) is a powerful approach allowing unique combination of molecular and morphological information. The particular advantage of IMS in cancer research is allocation of molecular profiles to specific cell types, such as cancerous, preneoplastic or inflammatory. Moreover, IMS can be used in studies aimed at interfacing tumor and normal tissue (tumor niche) and intra-tumor heterogeneity. It is noteworthy that automated (unsupervised) methods of clustering of IMS data, particularly based on component analysis and spatial segmentation, appeared to be a particularly suitable approach in studies of intra-tumor heterogeneity and classification of tumor sub-regions. Hence, IMS proved its role as a powerful tool in clinical proteomics, with obvious applicability in biomarker research and molecular tissue classification. This approach revealed its exceptional value in studies of complex heterogeneous systems exemplified by many tumors. About 4% of the detected peptides showed significantly different abundances between normal epithelium and tumor, and could be considered as a molecular signature of OSCC.

Comment 6: Interestingly, enzymes in the key mthfd1l folate cycle not only support tumor growth, but also increase the sensitivity of targeted therapy. Can the enzymes in the folate cycle of mthfd1l be used as a sensitizer for targeted therapy?

Reply 6: Thank you for your instructively reminding. In our study, we found that an enzyme in the folate cycle of MTHFD1L, plays an essential role in support of OSCC

cancer growth. And we examined the literature to seek evidence of it as a sensitizer for targeted therapy. Lee et al. revealed that MTHFD1L knockdown could increase the sensitivity to a targeted drug of hepatocellular carcinoma by inducing oxidative stress injury (**Reference 1**). Exciting, MTHFD1L silencing also increased reactive oxygen species and accelerated cell death under oxidative stress of OSCC (**Reference 2**). So we hypothesized that enzymes in the MTHFD1L folate cycle may be a sensitizer for therapeutic targeting in oral cancer. Your suggestion is of great theoretical significance in guiding our subsequent research.

1. Lee D, Xu IM, Chiu DK, Lai RK, Tse AP, Lan Li L, Law CT, Tsang FH, Wei LL, Chan CY, Wong CM, Ng IO, Wong CC. Folate cycle enzyme MTHFD1L confers metabolic advantages in hepatocellular carcinoma. J Clin Invest. 2017, 127(5):1856-1872.

2. Li H, Fu X, Yao F, Tian T, Wang C, Yang A. MTHFD1L-Mediated Redox Homeostasis Promotes Tumor Progression in Tongue Squamous Cell Carcinoma. Front Oncol. 2019, 9:1278.