Peer Review File

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Reviewer A

The concept of the study is innovative to compare the autophagy related DNA methylation signature with dry lab study, since molecular grading is a critical issue in neuro-oncological research.

However, several issues need to be clarified before this review process can move forward.

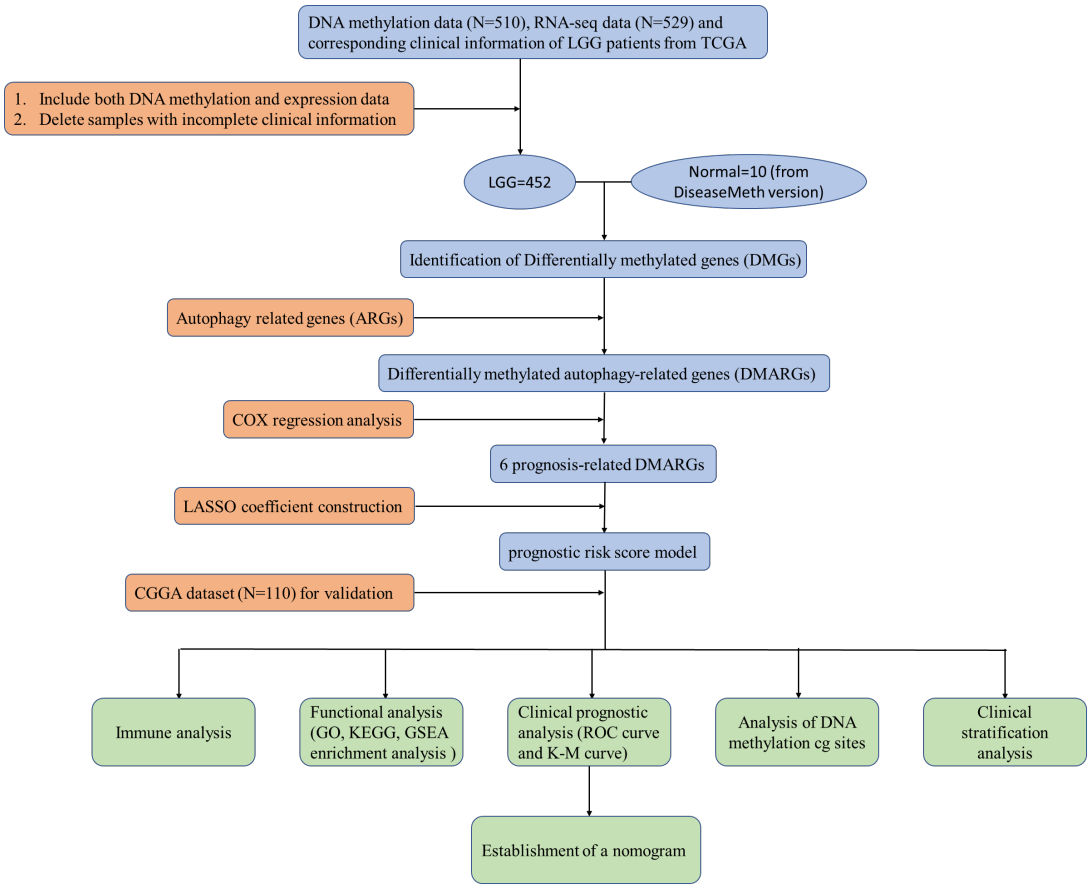
Comment 1: The authors should clearly describe their gene selection, and patient selection process in methodology, not just mention about The Cancer Genome Atlas and MEXPRESS.

Reply 1: Thanks for pointing out this question. A flowchart might help to show gene selection and patient selection process in methodology, so we add a flowchart as a new figure.

Changes in the text: Legends, Page 22, line 19

Figure 1 The workflow chart of this research.

Editable flowchart is uploaded by PPT format



Comment 2: The authors should clearly describe their target gene related to tumor immune microenvironment, since there are at least thousands of transcriptomes related to tumor immune microenvironment that have been published before.

Reply 2: Thank you so much for your valuable suggestions. The prognostic risk model constructed in this paper is consisted by six target genes. We add a description of the relationship between target genes and immune microenvironment in the Discussion part.

Changes in the text: Discussion, Page 16, line 22- Page 18, line 2

Autophagy was an important factor in regulating the tumor immune microenvironment, the six autophagy-related genes in this model had also been reported to be related to the immune response of tumors. The innate immune response was regulated by toll-like receptors (TLRs), and dysregulation of TLRs' function could occur in multiple malignant tumors. A C et al. found that the expression of TLR2 was significantly increased in gastric cancer, and the DNA expression matrix showed that the growth response of human gastric cancer cells induced by TLR2 was associated with the up-regulation of the anti-apoptotic gene CFLAR(53). Meanwhile, Carlos et al.

showed that the low expression of CFLAR would contribute to high apoptosis rate of regulatory T cell, which was important for regulating immune responses and might be a target for tumor immunotherapy(54). A study had shown that dysregulation of RAB24 is closely related to oncogenic genetic alterations of MXD3, which mediate immune cell dysfunction in a variety of tumors(55). Guo et al. demonstrated cocaine-induced autophagy could reduce microglia activation and the release of inflammatory factors, while the activation of ERN1-dependent endoplasmic reticulum stress pathway was involved in this induction of autophagy(56). Liao et al. reported rVP1 increased the formation of LC3-associated autophagosomes via WIPI1 and WIPI2 and promotes the migration of macrophages, which was important for regulating immune responses and antitumor activity. Knockdown of WIPI1 and WIPI2 could inhibited rVP1-mediated autophagy and reduce MAPK1/3 phosphorylation and activation of MMP9(57). A recent study showed that SNX5 initiated autophagy during viral infection via recruitment of WIPI2 to regulate intracellular immune and inflammatory responses(58). Regrettably, the impact of these six autophagy-related genes on the immune microenvironment remained to be studied in low-grade glioma because there were few related studies in LGG.

References, Page 22, line 3-16:

53. West AC, Tang K, Tye H, et al. Identification of a TLR2-regulated gene signature associated with tumor cell growth in gastric cancer. Oncogene 2017;36:5134-44.

54. Plaza-Sirvent C, Schuster M, Neumann Y, et al. c-FLIP Expression in Foxp3-Expressing Cells Is Essential for Survival of Regulatory T Cells and Prevention of Autoimmunity. Cell Rep 2017;18:12-22.

55. Wu SY, Lin KC, Lawal B, et al. MXD3 as an onco-immunological biomarker encompassing the tumor microenvironment, disease staging, prognoses, and therapeutic responses in multiple cancer types. Comput Struct Biotechnol J 2021;19:4970-83.

56. Guo ML, Liao K, Periyasamy P, et al. Cocaine-mediated microglial activation involves the ER stress-autophagy axis. Autophagy 2015;11:995-1009.

57. Liao CC, Ho MY, Liang SM, et al. Recombinant protein rVP1 upregulates BECN1-independent autophagy, MAPK1/3 phosphorylation and MMP9 activity via WIPI1/WIPI2 to promote macrophage migration. Autophagy 2013;9:5-19.

58. Dong X, Yang Y, Zou Z, et al. Sorting nexin 5 mediates virus-induced autophagy and immunity. Nature 2021;589:456-61.

Comment 3: The quality of English writing should be improved.

Reply3: Thanks for your kind suggestion. We carefully checked the grammar of the full text, and the manuscript has been revised by a native speaker.

Reviewer B

The authors wrote a well-designed bio-informatics study on the importance of autofagy-related genes on clinical prognosis and immune micro-environments in low-grade gliomas. The underlying hypothesis is clear and relevant, the description of the state-of-art key knowledge on these topics well summarized. The methodology mainly used a training set from publicly available TCGA database and a validation set of CGGA dataset. The methods used are sound. There are a few remarks to be made:

Comment 1: LGG (typically involving grade II and not really grade III like stated in the introduction) are to be considered immunologically cold tumors. In that regard, it is important to know how exactly the 22 different types of immune cells in the micro-environments have been identified in the TCGA and CGGA databases. In other words, which metagene-signatures were used to identify these (rare) cells in the LGG?

Reply 1: Thank you very much for your constructive comments. CIBERSORT is an analytical tool from the Alizadeh Lab and Newman Lab to impute gene expression profiles and provide an estimation of the abundances of member cell types in a mixed cell population, using gene expression data. Comparing with previous approaches using cell type–specific marker genes, CIBERSORT does not require cell type–specific expression for every gene, suggesting applicability to diverse cell phenotypes. Many researches about immune cell infiltration in LGG also used this method (for example: PMID: 35252165, PMID: 34805164), so our article also used this method for the

comparison of immune cell infiltration in the high-risk group and the low-risk group. In fact, the true difference between the two groups still needs further verification. As pointed out in the comments, LGG (typically involving grade II and not really grade III) sometimes were to be considered immunologically cold tumors, calculation of immune cell infiltration using CIBERSORT might be biased, so we add a statement about this part in Discussion.

Changes in the text: Discussion, Page 16, line 15-18

The immune infiltration scores and immune cell infiltration were also significantly different between high- and low-risk groups, providing a theoretical basis for the clinical application of immunotherapy. However, LGG sometimes were considered to be immunologically cold tumors, calculation of immune cell infiltration using CIBERSORT might be biased. The true differences between the two groups still needs further verification.

Comment 2: for many bio-informatics methods used in this manuscript, a reference-link is provided. For some others however, it would be good to include the most relevant references e.g.LASSO coefficient construction, ESITMATE algorithm, Cibersort algorithm.

Reply 2: Thank you for your correction and we apologize for our carelessness. We have added the relevant citations

Changes in the text: Methods, Page 6, line 20; Methods, Page 7, line 3; Methods, Page 7, line 18; Methods, Page 7, line 20;

Selected differentially methylated autophagy-related genes associated with prognosis by COX regression analysis(23).

The prognostic risk score model was constructed by LASSO coefficient and the corresponding relative methylation level by formula: risk score = sum (the relative methylation level × corresponding coefficient)(24).

To explore the potential differences of immune microenvironment in two groups, the ESITMATE algorithm was used to calculate the immune scores, stromal scores of the high and low-risk groups(25).

The Cibersort algorithm which calculated enrichment of different immune cells by deconvolution algorithm, was applied to examine distribution of 22 types of immune infiltrating cells(26).

References, Page 20, line 26-33:

23. Linden A, Yarnold PR. Modeling time-to-event (survival) data using classification tree analysis. J Eval Clin Pract 2017;23:1299-308.

24. Friedman J, Hastie T, Tibshirani R. Regularization Paths for Generalized Linear Models via Coordinate Descent. J Stat Softw 2010;33:1-22.

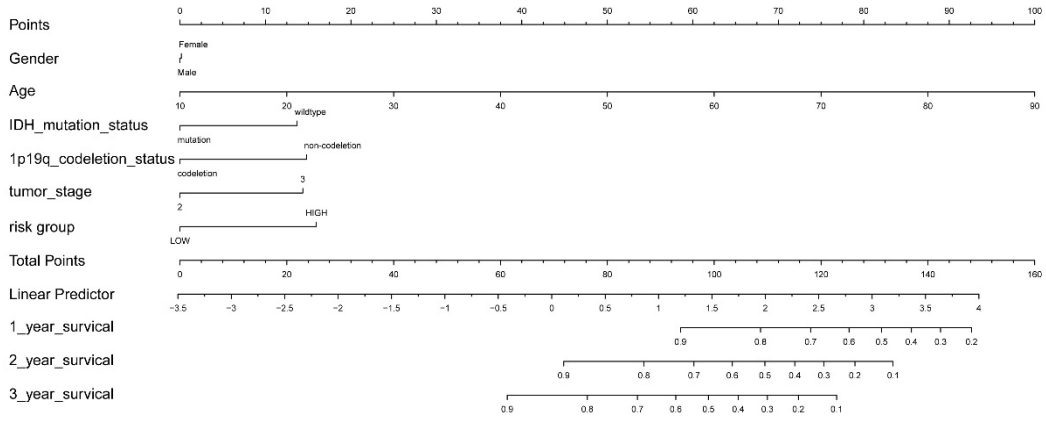
25. Yoshihara K, Shahmoradgoli M, Martínez E, et al. Inferring tumour purity and stromal and immune cell admixture from expression data. Nat Commun 2013;4:2612.

26. Newman AM, Liu CL, Green MR, et al. Robust enumeration of cell subsets from tissue expression profiles. Nat Methods 2015;12:453-7.

Comment 3: the nomogram built in FIG 2 could be a nice tool, but from the figure it is quite unclear what exactly (if any) would be the relative contribution of factors like IDH mutation or 1p19q codeletion to the nomogram? It appears to be irrelevant for the final sumscore: is that correct?

Reply 3: We appreciate your comments and apologize for this mistake. We correct the division of risk group and build a new nomogram. IDH mutation status and 1p19q codeletion status were associated with prognosis in LGG and relevant for the final sumscore.

Changes in the text: (see FIG3 H)



Comment 4: the authors refer to the WHO 2016 classification to define LGG: in the mean time, we have a WHO 2021 classification up and running. Which one was finally used in this work? This might be highly relevant since the ' IDHwt LGG' from the 2016 classification have become 'glioblastomas' in the 2021 classification. The authors should clarify this point in the manuscript.

Reply 4: We sincerely appreciate your significant comments, this point should be clarified in the manuscript. According to your comments, we make specific explanations for the parts that were not clearly stated. In this paper, we still use the 2016 WHO classification of central nervous system tumors. In the 2021 WHO classification of central nervous system tumors, adult-type diffuse gliomas are divided into astrocytoma (IDH-mutated), oligodendroglioma (IDH-mutated and 1p/19q co-deletion), glioblastoma (IDH wild-type), and for adult diffuse gliomas which are unsatisfactory with the above diagnostic conditions, use NOS or NEC including histological diagnosis, WHO grading, and molecular diagnosis as the comprehensive diagnostic results. Gliomas are still classified according to the LGG and GBM in the TCGA and TGGA databases and it is inappropriate for this paper to use the new classification.

Changes in the text: Methods, Page 6, line 6-7

The DNA methylation data (Illumina HumanMethylation450 BeadChip), gene expression profile and corresponding clinical information of LGG patients were originated from The Cancer Genome Atlas (TCGA, https://portal.gdc.cancer.gov/) and MEXPRESS(21,22). The collection criteria for low-grade glioma were still in accordance with the 2016 WHO classification of central nervous system tumors.

Comment 5: language and grammar:

-please replace the word 'judging/judgement' rather by 'predicting/prediction' e.g. in p.1, line 28; p.4, line 19; p.11; line 14;

-p.4, line 3: autophage is AN important direction

-p.6, line 14 Analysis instead of analyze

-p.6, line 17, cg sites AND the relative expression

-p.14, line 15: a total of six DIFFERENTIALLY METHYLATED ARG ASSOCIATED WITH PROGNOSIS were screened

Reply 5: Thank you for your correction and pointing out the problem. we have modified the incorrect words in our text as advised.

Changes in the text: ① Abstract, Page 2, line 9; Introduction, Page 5, line 21; Discussion, Page 13, line 16; ②Introduction, Page 5, line 4 ③Methods, Page 8, line 2 ④ Methods, Page 8, line 6 ⑤Conclusion, Page 18, line 12

① The receiver operating curve and KM survival curve were used to verify the model's effectiveness in predicting prognosis.

and provides a new research target for the prognosis prediction and treatment of low-grade glioma.

Therefore, the methylation level of various genes in glioma cells is a standard biomarker for predicting tumor prognosis

② Epigenetic modification of autophagy is an important direction in cancer and cancer therapeutics.

③ Analysis of DNA methylation cg sites

④ The correlation between DNA methylation cg sites and the relative expression of the corresponding genes were examined by Pearson correlation analysis.

⑤ In this study, a total of six differentially methylated ARG associated with prognosis were screened.