Peer Review File

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Reviewer A

Comment 1: The authors present a comprehensive set of analysis to propose NSUN1 as a potential biomarker for prognosis of different cancers. All data is based on various online resources. Nevertheless, the depth of analysis is so comprehensive that it merits publication. The authors could consider to make it clearer to the readers in the abstract that the study is based on online resources and not on in-house tumor material.
Reply 1: We have modified our text as advised.
Changes in the text: The study was based on online resources and public databases. (see Page 2, line 25-26)

Reviewer B

This article describes the analysis made on public databases of cancer gene expression about the possible significance of RNA m5C methyltransferase NSUN1 on cancer diagnosis, prognosis and therapy. The authors analyzed data from bulk RNA-sequencing, single-cell RNA sequencing, protein expression and protein phosphorylation. This study extends previous studies on NSUN1 expression in six cancer types. In agreement with these previous results, the authors found that NSUN1 mRNA and protein expression are higher in most cancer types analyzed than in normal tissue. Single-cell RNA sequencing data indicated that NSUN1 expression was high specifically in immune cells, such as T cells, B cells and DC cells. In addition, high NSUN1 expression correlated with poor overall survival and disease-free survival. Expression of NSUN1 also correlated with tumor-infiltrating immune cells and immune checkpoints. The authors conclude from these studies that NSUN1 may be and effective biomarker for early cancer diagnosis, prognosis and therapy.

This work is of interest since it describes a potential new cancer biomarker. The possible role of NSUN1 in cancer immune cells is also very relevant in this field of research. The article is well presented and the results clear and sound. The results obtained would require validation by experimental studies as the authors indicate at the end of the Discussion Section.

Comment 1: One of the more interesting results reported is the expression of NSUN1 in tumor immune cells and the possible role in immune surveillance. The article would be improved if the authors could provide additional data about the importance of this finding. For example, it would be important to determine if NSUN1 is expressed to similar levels in tumor immune cells and in non-tumor-associated immune cells in control tissues or in the blood. In other words, is high NSUN1 expression in T cells, B cells and DC cells a specific characteristic of these immune cells when they are

associated with tumors that could be used as a tumor biomarker and even in liquid biopsy assays?

Reply 1: Thank you for your comments. You point out directions for future research. In this study, we found that NSUN1 is associated with immune cell infiltration. The results of single-cell data analysis indicated that the expression of NSUN1 was increased in immune cells. Therefore, we speculate that NSUN1 may regulate the tumor immune microenvironment in cancer. To confirm this finding, basic research is needed. The significance of this article is to report a good finding: NUSN1 may be a promising biomarker. In the future, more research needs to be carried out.

Comment 2: According to the hypothesis indicated by the authors in the Discussion (lines 217-218), NSUN1 is associated with immune cell infiltration in the tumor which would be in agreement with the observed increased expression of the gene. However, in line 134-135 the authors indicate that NSUN1 expression is negatively associated with the number of infiltrating immune cells in some cancers. Also, in the Discussion section (lines 198-202) the authors indicate that NSUN1 significantly negatively regulates the infiltration of immune cells in most tumors. Later on, in the same paragraph, the authors indicate that NSUN1 inhibits the function of immune cells in tumor tissues. However, the authors do not provide any indication of the possible role of NSUN1 in the immune cells, activating or repressing them. I consider that the possible role of NSUN1 in the immune cells should be discussed in more detail by the authors since there seems to be some contradictions along the text of the manuscript at the present time. In this respect, the authors observe a good correlation between NSUN1 expression and T cell and B cell markers in most cancers (Figure S3) that should be indicated and discussed in further detail.

Reply 2: We have modified our text as advised. This study found that NSUN1 may inhibit immune cell infiltration. Therefore, we have revised the expression in the manuscript. The specific role of NSUN1 in immune cells remains unclear. This requires fundamental research for in-depth exploration. Based on your comments, we have further discussed related content in the Discussion section.

Changes in the text:

The immune-related research results in this study found that NSUN1 significantly negatively regulates the infiltration of immune cells in most tumors and is involved in regulating the tumor immune microenvironment. NSUN1 inhibited the infiltration of dendritic cells, monocytes/macrophages and CD8+ T cells, especially in SKCM, STAD, TGCT. This result preliminarily confirmed our hypothesis: NSUN1 promotes the proliferation, invasion and metastasis of cancer cells by inhibiting the infiltration of immune cells in tumor tissues. (see Page 11, line 197-202)

Minor points.

Comment 3: 1. A list of abbreviations should be presented as a table or in the figure legends to facilitated understanding of the data to non-specialized readers. In particular, the meaning of TMB and MSI should be indicated in the Abstract Section.

Reply 3: We have modified our text as advised.

Changes in the text:

Tumor mutation burden (TMB), microsatellite instability (MSI), and immune

checkpoints. (Abstract, see Page 2, line 22-26)
Abbreviations TCGA, The Cancer Genome Atlas; TMB, Tumor Mutation Burden ; MSI, Microsatellite Instability; ACC, Adrenocortical carcinoma; BLCA, Bladder Urothelial Carcinoma; BRCA, Breast invasive carcinoma; CESC, Cervical squamous cell carcinoma and endocervical adenocarcinoma; CHOL, Cholangiocarcinoma; COAD, Colon adenocarcinoma; DLBC, Lymphoid Neoplasm Diffuse Large B-cell Lymphoma; ESCA, Esophageal carcinoma; GBM, Glioblastoma multiforme; HNSC, Head and Neck squamous cell carcinoma; KICH, Kidney Chromophobe; KIRC, Kidney renal clear cell carcinoma; KIRP, Kidney renal papillary cell carcinoma; LGG, Brain Lower Grade Glioma; LIHC, Liver hepatocellular carcinoma; LUAD, Lung adenocarcinoma; LUSC, Lung squamous cell carcinoma; MESO, Mesothelioma; OV, Ovarian serous cystadenocarcinoma; PAAD, Pancreatic adenocarcinoma; PCPG, Pheochromocytoma and Paraganglioma; PRAD, Prostate adenocarcinoma; READ, Rectum adenocarcinoma; SARC, Sarcoma; SKCM, Skin Cutaneous Melanoma; STAD, Stomach adenocarcinoma; TGCT, Testicular Germ Cell Tumours; THCA, Thyroid carcinoma; THYM, Thymoma; UCEC, Uterine Corpus Endometrial Carcinoma; UCS, Uterine Carcinosarcoma; UVM, Uveal Melanoma. (see Page 13, line 233-246)

Comment 4: 2. Abstract, line 24 and Results section. The authors determine the localization of NSUN1 in tumor cells (A-431, U-2, U-251) but, since they show that this gene is mainly expressed in immune cells, the possible localization it these last cells should be considered.
Reply 4: Thank you very much for your question. This study shows that NSUN1 is highly expressed in immune cells. For example, in breast, the expression of NSUN1 was higher in T cells and DC cells. However, this does not mean that NSUN1 is not expressed in other types of cells. This was confirmed by the results of cellular immunofluorescence. Therefore, this result is plausible and there are no contradictions.

Comment 5: 3. Introduction, line 47. The authors state that NSUN1 expression has been reported in seven human cancers. However, they only indicate six cancer in lines 49-50. This discrepancy should be solved.
Reply 5: The discrepancy has been solved.
Changes in the text: NSUN1 has been reported in six human cancers. (see Page 4, line 47-49)

Comment 6: 4. Methods, lines 82-83 “Used to analysis” probably would better be “used to analyze”
Reply 6: We have modified our text as advised.
Changes in the text: was used to analyze the alteration frequency and mutation type. (see Page 6, line 82)

Comment 7: 5. Results, lines 113-114. “NSUN1 was significantly high expressed” is not very clear. It might be changed, for example by “NSUN1 expression was significantly high”
Reply 7: We have modified our text as advised.
Changes in the text: we found that NSUN1 expression was significantly high in immune cells than in other cell types in… (see Page 8, line 134-135)

Reviewer C

The study focuses on the role of NSUN1, an RNA m5C methylase, in various types of cancer. The authors analyze bulk RNA-seq and single-cell sequencing data from public databases to evaluate the expression and potential clinical value of NSUN1 in predicting cancer. Their findings suggest that NSUN1 is highly expressed in most tumors, has significant correlations with TMB, MSI, and immune checkpoints, and is involved in immune regulation. They also found that high expression of NSUN1 predicts poor prognosis in various cancers. Additionally, the study explores the location, variation, methylation, and phosphorylation characteristics of NSUN1, which might be critical factors in cancer development.

Comment 1: However, the authors should further discuss some of their limitations. The specific molecular mechanisms by which NSUN1 affects the function of immune cells are not well understood, limiting the potential clinical application of the findings. The study does not provide direct experimental evidence for the role of NSUN1 in cancer development, relying mainly on correlative data. The study does not discuss any potential off-target effects or complications that might arise if NSUN1 is targeted for cancer therapy.

Reply 1: Your comment is very important. We have modified our text as advised. We have further discussed the limitations of this study. A limitation of this study is that all research findings are based on data analysis. The specific molecular mechanism still needs to be explored and discovered through basic research. The significance of this study is that it provides a new direction for future basic research. NSUN1 is a tumor marker with clinical application prospects, but there is still a long way to go before NSUN1 can be applied clinically. We've improved the Discussion section based on your comments.

Changes in the text: A limitation of this study is that all research findings are based on data analysis. The specific molecular mechanism still needs to be explored and discovered through basic research. The significance of this study is that it provides a new direction for future basic research. (see Page 12, line 211-214)