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Reviewer A

In the present manuscript, the authors analyzed the role of sperm-associated antigen 6 (SPAG6) in pan-cancer with particular regard to thyroid carcinoma (THCA). Their study was essentially based on the collection of a number of data from different databases to assess SPAG6 correlation with prognosis, gene heterogeneity, stemness, tumor immunity, and to determine SPAG6 biological function in cancer. They found that SPAG6 expression was differentially expressed in cancers, correlated with prognostic value and was positively linked to immune response in tumor microenvironment. Finally, the authors stated that, as a result of just over a couple of in vitro experiments, SPAG6 overexpression suppressed the malignant phenotypes of THCA cell lines because it slowed down proliferation and decreased migration.

Even so the database analysis of SPAG6 correlation with prognosis, gene heterogeneity, stemness, tumor immunity of different human tumors was barely acceptable (in some cases confusing or unclear), it is surprising to evidence that few data were provided by the authors to assess the role of SPAG6 in thyroid cancer especially if one considers that the manuscript title emphasizes this aspect in particular. Overall, the conclusions drawn by the authors were not supported by the experimental data: proliferation and migration decrease of thyroid cancer cell lines following SPAG6 overexpression does not indicate that cancer cells have lost their malignant phenotype nor that they are unable to invade. It simply indicates that they grow and migrate more slowly. To assess the reduced oncogenic potential in vitro, the authors would have to verify if SPAG6-overexpressing thyroid cancer cells decreased their ability to form foci of transformation in soft agar experiments as well to invade Matrigel in transwell assays.

In addition, it is not clear the entire paragraph “##SPAG6 overexpression suppressed proliferation, migration, and invasion of THCA cell lines”: first of all, papillary thyroid carcinoma arises from follicular thyroid cells, also called thyrocytes, and not from “acinar cells (?) of thyroid gland” as stated by the authors; then, what does it mean the sentence “B-CPAP cells serve as a model for studying Papillary thyroid carcinoma, while KTC-1 cells are utilized in research involving human thyroid cancer”? Papillary thyroid carcinoma is a thyroid cancer!!!!! What is the difference between studying papillary thyroid carcinoma and human thyroid cancer? Maybe would the authors refer to the fact that, since KTC-1 cells derive from poorly differentiated thyroid cancer, this cell line is often used in studies involving undifferentiated thyroid cancer?????

Finally, why do the authors overexpress SPAG6 in a human embryonal kidney-derived cell line, 293T, and not in other thyroid cancer-derived cell lines, besides BCPAP and KTC-1, to substantiate SPAG6 effects on proliferation and migration?

Response： We appreciate it very much for this good suggestion, and we have done it according to your ideas.

1. Our research primarily focuses on the expression and related mechanisms of SPAG6 in pan-cancer analysis, with preliminary experimental validation in thyroid cancer. Based on your suggestion, we acknowledge that our basic experimental research on SPAG6 in thyroid cancer is relatively limited. Therefore, we have revised our paper's title to "A comprehensive pan-cancer analysis revealing SPAG6 as a novel diagnostic, prognostic, and immunological biomarker in the tumor"

2. Our research content and findings mainly focus on the expression and mechanisms of SPAG6 in pan-cancer analysis, with preliminary experimental validation in thyroid cancer. Currently, we have conducted relatively few basic experiments on thyroid cancer. We have carefully considered your suggestions and made changes to the conclusion section of the manuscript. We will continue to investigate the specific molecular mechanisms of SPAG6 in thyroid cancer in future experiments. Thank you for your valuable suggestions.

Changes in the text: In conclusion, this study found that the expression level of SPAG6 was differentially expressed in various cancers at different tumor stages and grades, with gender-associated expression observed in certain cancers. The variation in SPAG6 expression between progression and survival suggests its prognostic value in cancer. The results indicate that SPAG6 affects immune infiltration, regulates the TME, and plays a role in the development of cancers. SPAG6 expression affects the proliferation, growth, and migration of tumor cells, and we conducted some experimental validation specifically in thyroid cancer to affirm these effects. Additionally, SPAG6 was positively correlated with a successful tumor therapy and may be a potential immune checkpoint. SPAG6 expression shows prognostic value in pancancer, particularly THCA, as it influences immune infiltration and TME regulation. Upregulation of SPAG6 inhibits malignant progression in THCA cells through mechanisms related to DNA repair, MYC targets, peroxisome, and G2M checkpoint. This study highlights the inhibitory role of SPAG6 in THCA and suggests that targeting SPAG6 with small molecule drugs may be a novel therapeutic approach.

3. B-CPAP and KTC-1 are two commonly used cell lines for studying thyroid cancer. Upon reviewing relevant literature, we found that KTC-1 is frequently used in thyroid cancer research, while B-CPAP is also used in thyroid cancer research, particularly in studies of papillary thyroid carcinoma. The initial lack of clarity in

our expression may have caused some confusion for readers. To address this, we have revised the phrasing in the text.

Changes in the text: Papillary thyroid carcinoma arises from the follicular thyroid cells, representing the predominant subtype, constituting over 80% of thyroid carcinoma cases. B-CPAP cells and KTC-1 cells are two common types of cell lines for studying thyroid carcinoma. Notably, KTC-1 cells are utilized in research involving human thyroid cancer, while B-CPAP cells also serve as a model for studying human thyroid cancer, especially for papillary thyroid carcinoma.

4. The content of the paper primarily focuses on the role of SPAG6 in pan-cancer analysis, with preliminary experimental validation in thyroid cancer. Since 293T is a renal cancer cell line with stable expression and is widely used in tumor research, we initially used this cell line to verify the transfection of SPAG6 and the effectiveness of our experimental techniques. However, as you pointed out, this might cause confusion for readers. Including this exploratory research in the paper could lead to misunderstandings about the content. Therefore, based on your suggestion, we have removed the part about 293T from the paper. Our research primarily focuses on the expression and related mechanisms of SPAG6 in pan-cancer analysis, with preliminary experimental validation in thyroid cancer.

   Changes in the text: We have removed the part about 293T from the paper(including related figure).

Reviewer B

The paper titled “The pancancer landscape of SPAG6 and the mechanism underlying its effect in thyroid carcinoma” is interesting. SPAG6 plays a significant role as an oncogene and can be used as a marker to predict the prognosis of cancers, particularly in THCA. SPAG6 influences both the tumor immune infiltration and microenvironment, making it a promising immunotherapeutic target for tumor therapy. However, there are several minor issues that if addressed would significantly improve the manuscript.

1)What are the correlations between SPAG6 and THCA staging, degree of differentiation, neural invasion and lymphatic metastasis? It is recommended to add relevant content.

2)The description of some methods in this study is too simplistic, please describe in detail.

3)The abstract is not sufficient and needs further modification. The research background did not indicate the clinical needs of the research focus.

4)There are many detection methods for cell proliferation and migration. Why this

study only uses one method? If multiple methods are used, the results may be more reliable. It is suggested to add test results of other methods.
5)Figure 7 has no scale bars. Please add relevant information.
6)Some fonts in figures need to be enlarged.
7)What is the impact of this study on the further treatment and prognosis of THCA? It is recommended to include relevant content in the discussion.

Response： Your opinion is highly professional and important. Our team shares your perspective, and we have made changes based on your suggestions

1. Thank you for your valuable suggestion. Our research primarily focuses on the expression and related mechanisms of SPAG6 in pan-cancer analysis, with preliminary experimental validation in thyroid cancer.
   Changes in the text: We have revised our paper's title to "A comprehensive pan-cancer analysis revealing SPAG6 as a novel diagnostic, prognostic, and immunological biomarker in the tumor"

2. Thank you for your valuable suggestion. Previously, due to word count limitations, the Methods section was overly concise. Within the allowable range, we have now enriched certain parts of the original text and made the necessary annotations.

   Changes in the text:
   Pearson's correlation method was used to calculate the association between spag6 expression and other factors.
   Formalin-fixed tissue (the tissues were got from hospital and the study was approved by the Ethical Committee) sections from various cancers were fully embedded and cut into 10 μm sections for immunohistochemical staining. After dewaxing and antigen retrieval, the sections were blocked with PBT-1 for 60 minutes at room temperature. The sections were then incubated overnight with a primary antibody against spag6 (anti-SPAG6, 1:200, ab155653, Abcam, Cambridge, MA) at 4°C. Subsequently, a secondary antibody conjugated with Alexa Fluor 546 (1:1000, Invitrogen, Carlsbad, CA) and DAPI (1:1000, D9542, Sigma-Aldrich, St. Louis, MO) were incubated for 1 hour at room temperature. The sections were washed with buffer at each step. The final staining was visualized using a laser scanning confocal microscope (Leica SP8, Leica, Wetzlar, Germany).
   PEIpro (Polyplus) was applied for conducting cell transfection, which lasted 48 hours as per its standard guide.
   B-CPAP, and KTC-1 cells were processed as required and seeded into 96-well plates at a density of 3-5 × 103 per well. After treatment for 0, 24, 48, 72 and 96 h, 10 μL Cell Counting Kit 8 (CCK8; BioSharp, Anhui, China) was added to each well, and

absorbance was measured at 450 nm with a spectrophotometer after incubation for 2 h.

Cell migration was assessed using an 8-μm Transwell chamber (BioSharp, Anhui, China). Briefly, Serum-free 1640 medium (200 μl) containing 4×104 cells were seeded in the upper chamber. 500 μL 1640 medium containing 20% FBS was added to the lower chamber. After incubation for 24 h, the cells were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. Migrated cells were photographed in four random fields using an inverted light microscope (Olympus, Tokyo, Japan). The experiments were repeated three times independently. Data were quantified via the ImageJ software.

3. We strongly agree with your viewpoint. We have made changes to the abstract and background sections of the original text, marking the revisions in red.

Changes in the text:

Study background: The aim of this study was to clarify the role of sperm-associated antigen 6 (SPAG6) in pancancer, with some findings about thyroid carcinoma (THCA) validated through experiments particularly thyroid carcinoma (THCA).

Study objective: To explore the role of SPAG6 in pancancer.

Methods: We employed an observational study in Shandong Provincial ENT Hospital to examine the role of SPAG6 in pancancer, with the data being collected from databases. Further analysis was conducted to assess its correlations with prognosis, gene heterogeneity, stemness, and tumor immunity. The interacting proteins of SPAG6 were also identified, and gene ontology enrichment analysis was performed to determine its biological function. Overexpression function assays were performed to identify the functions of SPAG6 on the malignant behavior and tumor growth of THCA via in vitro experiments.

Results: This study found that SPAG6 expression was differentially expressed in cancers and at various tumor stages and grades.

Previous research has linked SPAG6 to immunodeficiency, which included reduced CD8 cytotoxicity, decreased CD8 T-cell interferon-γ (IFNγ) secretion, and impaired antibody production (19). However, the role of SPAG6 in thyroid carcinoma (THCA) and other cancers remains unclear.

In this study, we aimed to clarify the relationship between SPAG6 and multiple cancers by integrating data from multiple databases using polyomics methods. We analyzed the expression, prognosis, gene heterogeneity, and tumor

microenvironment (TME) of SPAG6 and further examined the association between SPAG6 and immunotherapy across different types of cancers (Figure 1). The results of this study provide a comprehensive understanding of the role of SPAG6 in various types of cancer, and serve as a valuable reference for further research.

4. Our research is a preliminary investigation into thyroid cancer. Building on this foundation, we will further explore the role and mechanisms of SPAG6 in thyroid cancer in future studies.

   Changes in the text:None

5. We have made some adjustments to the research content in Figure 7 of the article and have uploaded a new version of Figure 7.

6. Thank you for your valuable suggestions. We have adjusted the font size for some figures. Due to formatting constraints, we couldn't further increase the font size for certain figures. However, we have ensured that the resolution is sufficient to allow for clear text visibility when zooming in on the images.

7. In the original text, we conducted some analysis on SPAG6 expression in thyroid cancer. Based on your suggestion, we agree that further analysis and discussion on the diagnosis and treatment of thyroid cancer should be included. We have marked the original content in the article and added new discussions on SPAG6 and thyroid cancer.

   Changes in the text: In this study, we focused on the high incidence of thyroid cancer and conducted in vitro experiments to preliminarily confirm the possible role of spag6 in cancer. The expression of SPAG6 was downregulated in THCA and was associated with prognosis. The differential expression of SPAG6 regulates especially proliferation and metastasis in THCA. In thyroid cancer with SPAG6 expression, its expression is associated with immune cell infiltration. Additionally, the expression of immune checkpoints shows a strong correlation with SPAG6. When SPAG6 expression is low, the risk of THCA tumor recurrence is lower, but the risk of death from non-tumor causes is relatively higher. Therefore, for the treatment and prognosis evaluation of THCA, SPAG6 expression may be an important target.