

## Peer Review File

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### Reviewer A

The paper is well written and logically organized. In our lab we have more or less 60 variables that can affect the results and when considering those with predictive value this can have a paramount importance. Would recommend to provide images at higher magnification to better appreciate (10x or 20x).

Furthermore a lot of evidence has been published on this topic so my recommendation is to expand some parts of your discussion quoting PMID: 35926433, PMID: 34530257, PMID: 34157159

Re: We have added 200× field images to both Fig1 and Fig2. We cited references and discussed them.

Changes in the text: (see Page 11, line 224-233)

### Reviewer B

This study aimed to analyze the effects of specific preanalytical variables, specifically decalcification and depigmentation of various durations, on PD-L1 immunohistochemistry. While investigating the impact of different preanalytical variables on PD-L1 expression holds significant value and deserves attention, this study's design has some issues. Additionally, the manuscript suffers from a lack of attention to detail and language-related concerns.

In the section 'Study Subjects and Methods' (Lines 89-97), the statement, 'The samples were fixed in 10% neutral formalin, routinely dehydrated, and paraffin-embedded... Placental tissues were made into tissue chips with a diameter of 3.5mm, and each section was made into tissue microarrays at 3\*3 and decalcified using the Rapidcal. Immuno,' suggests that decalcification was conducted on paraffin-embedded tissue. This raises questions about the rationale behind testing it on paraffin-embedded tissue. Typically, decalcification is performed before tissue processing and paraffin embedding, which aligns more closely with clinical practice.

Re: The reviewers gave very professional opinions. We must recognize that some deficiencies of this research, the fresh tissue is closer to the clinical, but it is a pity that our institutions (tumor specialized subject hospital) has been difficult to obtain fresh placental tissue.

Concerning the esophageal squamous cell carcinoma specimens, the description mentions, 'In addition, ten PD-L1 positive esophageal squamous cell carcinoma samples were treated with decalcification.' It's unclear whether these were whole tissue sections or also microarray samples, and whether they were fresh or also paraffin-embedded tissues. Further clarification on these specifics is needed.

Re: In order to avoid interpretation, result difference caused by tumor heterogeneity, we chose the complete esophageal paraffin embedding tissue section instead of microarray samples.

Changes in the text: (see Page 5, line 99-103)

In addition, there are multiple grammatical and stylistic issues, along with several errors:

Line 60: The sentence “Currently, immunohistochemistry is used most common,…” is grammatically incorrect.

Re: Has been revised

Changes in the text: (see Page 3, line 60)

Lines 71 and 72: “decalcification and decalcification”... It was likely intended to be ‘decalcification and depigmentation’.

Re: Has been revised

Changes in the text: (see Page 4, line 75)

Line 73: This last sentence lacks coherence and clarity.

Re: Has been revised

Changes in the text: (see Page 4, line 76)

Lines 105-107: “immunocyte Proportion Score (IPS) = Total number of tumor-related immune cells/tumor-related immune cells with positive PD-L1 membrane and plasma of any strength \*100%” doesn’t make sense. This equation seems to contain an error, possibly a reversal of the nominator and denominator. Additionally, “plasma” in this sentence probably refers to ‘cytoplasm’.

Re: Has been revised

Changes in the text: (see Page 6, line 111)

Lines 107-108: The latter part of the sentence should be a separate sentence.

Re: Has been revised

Changes in the text: (see Page 6, line 111)

Lines 123-127: This sentence suffers from poor grammar and would benefit from being split into two sentences.

Re: Has been revised

Changes in the text: (see Page 6, line 131)

Lines 181-183: This sentence contain grammatical errors and that need correction.

Re: Has been revised

Changes in the text: (see Page 9, line 194)

Additional concerns include:

Lines 187-189: If including this hypothesis, this sentence lacks clarity and would benefit from further explanation regarding the differences between binding to tumor cells and immune cells.

Re: Has been revised

Changes in the text: (see Page 10, line 201)

Figure 1D: 'Decalcification' should likely be 'depigmentation'.

Re: Has been revised

There are also other language-related issues. This paper would benefit from better English language editing.

Re: Has been revised