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Reviewer Comments

In this study, the authors evaluated NHLRC2 expression in lung cell and tissue samples from patients with ADC and SCC by immunohistochemistry and mRNA in situ hybridization. They concluded that immunohistochemical NHLRC2 expression was higher in ADC to SCC clinical samples and high NHLRC2 expression was associated with poor survival in lung adenocarcinoma patients. This immunohistochemical study has certain value in addition to the previous studies on total RNA expression. Notably, it is important to show evidence of the specificity of the methods and reagents (antibodies and probes) used in this study. Unfortunately, there is a lack of evidence to confirm the specificity in some parts.

Major points:

Comment 1: The S/N ratio in immunohistochemical study (Fig 1 and Fig2) does not appear to be very high, so each negative control should be shown. Only one figure (F in Fig2) is shown but the tissue image is much different from others. Authors should show each negative control using serial sections in Fig 1 and Fig 2, which is most important data in this study.

Reply 1: Thank you for the comment. We have now prepared novel images of immunohistochemical staining on serial sections using NHLRC2 antibody and rabbit isotype control. We have now replaced the Figure 1 and Figure 2 with new NHLRC2 images with negative controls (rabbit isotype controls) for each case. Original Figure 1 (showing immunohistochemical NHLRC2 expression in lung ADC) was divided into two new figures (Figure 1 and Figure 2) and original Figure 2 (showing immunohistochemical NHLRC2 expression in lung SCC) was renamed to Figure 3. Due to addition of new negative control stainings, we removed the original Figure 2F showing negative control staining. We also removed the original Figure 2E representing NHLRC2 staining in bronchus since it is not relevant to this study on NHLRC2 expression in lung cancer.

Changes in the text: Legends of Figures 1-3 were updated to correspond the new figures (see Page 20, lines 469–492) as follows:

Figure 1. Immunohistochemical NHLRC2 expression in lung adenocarcinoma. (A and B) Lepidic adenocarcinoma. (C and D) Acinar adenocarcinoma (cribriform pattern). (E and F) Papillary adenocarcinoma. (A, C and E) Immunohistochemical stain for NHLRC2. (B, D and F) Negative control in which the primary antibody was substituted with rabbit isotype control.

Cytoplasmic NHLRC2 expression was observed mainly in the tumor cells (arrows) and inflammatory cells (black arrowheads) within tumor stroma of all histologic subtypes. Some macrophages (white arrowheads) within tumors were also positive for NHLRC2. Scale bar 50 μm.

Figure 2. Immunohistochemical NHLRC2 expression in lung adenocarcinoma. (A and B) Micropapillary adenocarcinoma. (C and D) Solid adenocarcinoma. (E and F) Invasive mucinous adenocarcinoma. (A, C and E) Immunohistochemical stain for NHLRC2. (B, D and F) Negative control in which the primary antibody was substituted with rabbit isotype control. Cytoplasmic NHLRC2 expression was observed mainly in the tumor cells (arrows) and inflammatory cells (arrowheads) within tumor stroma. Scale bar 50 μm.

Figure 3. Immunohistochemical NHLRC2 expression in lung squamous cell carcinoma. (A, C, E and G) Immunohistochemical stain for NHLRC2. (B, D, F and H) Negative control in which the primary antibody was substituted with rabbit isotype control. Cytoplasmic NHLRC2 expression was detected in some of the tumor cells (black arrows) and inflammatory cells (arrowhead) within tumor stroma. (A) Negative expression of NHLRC2 in tumor cells of non-keratinizing squamous cell carcinoma. (C) Positive NHLRC2 expression in tumor cells of non-keratinizing squamous cell carcinoma. (D) Keratinizing squamous cell carcinoma with NHLRC2-positive tumor cells. (E) Basaloid squamous cell carcinoma. Scale bar 50 μm.

Comment 2: Similarly, Fig S2 should also show a negative control using sense probe.

Reply 2: Thank you for the relevant comment. Instead of sense probe for NHLRC2 we performed the mRNA *in situ* hybridization with negative control probe (DapB 310043, Advanced Cell Diagnostics) targeting bacterial gene dapB, recommended by the manufacturer of the RNAscope® 2.5 HD Detection kit (Advanced Cell Diagnostics) (Wang F, et al. J Mol Diagn. 2012;14:22–9). The same probe has been used as a negative control also in other publications using RNAscope reagents (Wang S, et al. J Immunother Cancer. 2021;9:e002926, Ren X, et al. J Clin Invest. 2022;132:e163620, Zhang X, et al. Transl Cancer Res. 2020;9:3573–3585).

Originally, we have prepared RNAscope mRNA *in situ* hybridization on the lung tissue materials consisting of both fibrotic and malignant diseases with negative controls. The results of mRNA *in situ* hybridization of idiopathic pulmonary fibrosis with a negative control have been published previously (Kreus M, et al. Respir Res. 2022;23:206). Due to given timeline for this revision, we could perform RNAscope using probes for NHLRC2 and negative control only for one lung cancer case. If more cases for mRNA *in situ* hybridization are required, a new reagent kit is needed to be purchased, which will cause a considerable delay in the publication

process.

We have replaced original Figure S2 with two figures; the first one (Figure S2) contains the original Figure S2A (mRNA *in situ* hybridization performed on lung adenocarcinoma using probe for NHLRC2), and the second one (Figure S3) represents mRNA *in situ* hybridization performed on serial sections of lung squamous cell carcinoma using probes for NHLRC2 and negative control (DapB ACD_310043). The figures were renamed to Figure S2 and Figure S3.

Changes in the text: We added information of the control probes in the materials and methods section (see Pages 7-8, lines 140–143) as follows: "Probe for the bacterial gene 4-hydroxy-tetrahydrodipicolinate reductase (DapB, 310043, Advanced cell diagnostics) was used as negative control to assess background signals. Probe for the endogenous housekeeping gene ubiquitin C (Hs-UBC, 310041, Advanced cell diagnostics) was used as positive control to assess assay procedure." We also added a citation to a publication where RNAscope method is described (see Page 7, line 140).

We also updated the legends of supplementary figures to correspond the new figures (see Page 22, lines 522–527) as follows:

Figure S2. *NHLRC2* expression in lung adenocarcinoma by mRNA *in situ* hybridization. *NHLRC2* expression is detected mainly in tumor cells (arrows). Scale bar 50 μm.

Figure S3. *NHLRC2* expression in lung squamous cell carcinoma by mRNA *in situ* hybridization. (A) *NHLRC2* expression is observed mainly in tumor cells (arrows). (B) Negative control probe for bacterial gene DapB shows no signal. Scale bar 50 μm.

Minor points:

Comment 3: In Fig 3A, what is the meaning of the control value? Is it negative controls on both ADC and ACC? Please describe it in the legend.

Reply 3: Thank you for the comment. The control in Figure 3A represents NHLRC2 expression in ten control samples which were derived from histologically normal-looking peripheral lung tissue outside tumor from non-smoking ADC patients. Normal-looking lung was not obtained from lung SCC patients since majority of the patients with SCC included in this study were ex- or current smokers who often have smoking associated changes in their lung.

Changes in the text: We have added the following description of controls in the legend of original Figure 3 (now Figure 4) as advised (see Page 21, lines 495–496): "Control lung tissue

samples were derived from tumor-free peripheral lung of ten non-smoking lung ADC patients".

Comment 4: As shown in Fig 3F, NHLRC2 protein expression levels were nearly identical in all culture cell types including ADC and SCC cell lines, meaning that NHLRC2 protein are ubiquitously expressed. Authors described higher NHLRC2 expression in lung ADC tissue is associated with mitotic activity. Taken together, is it possible that the NHLRC2 positive cell counts in lung ADC and SCC tumor tissues reflect the number of proliferating cells? If possible, please show more evidence or discuss about it.

Reply 4: Thank you for the interesting point of view. We assume that several facts may affect this finding. Each cell line was cultured from one patient, and the cells may undergo changes during culture, when gene expression might be altered during culture as shown previously (Rodriguez LR, et al. Sci Rep. 2018;8:3983). On the other hand, the results of immunohistochemical stainings assessed by image analysis software were presented as median NHLRC2 expression in tumors with low and moderate/high mitotic activity. Furthermore, the immunohistochemical NHLRC2 expression was determined in all cell types within tumors, not only in individual cell type as it has been done in cell lines. We think that the Reviewer's question regarding to whether NHLRC2 positive cell count in lung ADC and SCC reflect the number of proliferating cells is very interesting and possible, but we don't have more evidence on that.

Changes in the text: We added the following sentences in the discussion section (see Pages 14–15, lines 317–324): "We observed that the NHLRC2 protein levels in different cell lines (including cancer cells from ADC and SCC) were nearly equal. Additionally, high immunohistochemical NHLRC2 expression was associated with high mitotic activity in ADC lung tissue samples, and thus it can be speculated whether NHLRC2 expression reflected the number of proliferating cells. When interpreting the results of cell line experiments, however, it is notable that each cell line was isolated and cultured from a tissue sample of one patient, and moreover, the gene expression may be altered during culture. Furthermore, the immunohistochemical NHLRC2 expression was determined in all cell types within tumors, not only in individual cell type as it has been done in cell lines."

Comment 5: In Fig S3(Immunoblot of NHLRC2 expression in lung tissue samples), several bands of smaller molecular weights are detected, in addition to the main bands of 75kD. What does this mean?

Reply 5: Thank you for the relevant comment. The bands smaller than 75 kDa detected with NHLRC2 antibody may be non-specific binding of antibody to another protein with almost similar peptide area to the antigen or they may originate from the NHLRC2 protein. It has been shown that certain caspases can cleave NHLRC2 from several different sites resulting in smaller

fragments of the protein (Nishi K, et al. Cell Death Dis 2017;8:3218). Thus, it is possible that bands of smaller molecular weight in original Figure S3 (now Figure S4) are cleaved forms of NHLRC2. However, we do not have currently enough information on NHLRC2 turnover and cleavage to identify the bands with certainty.

Changes in the text: -