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Reviewer A:

- Comment 1: Domen et al. investigated "Prognostic implications of cellular senescence in resected non-small cell lung cancer," but the study's design does not support this hypothesis well.
  Reply 1: We are convinced that the findings of our observational study make a significant contribution to the limited knowledge of cellular senescence in NSCLC. We have applied a conventional study design to study the prognostic implications of cellular senescence according to the definition of a prognostic biomarker, i.e., a prognostic biomarker informs about a likely cancer outcome (e.g., disease recurrence, disease progression, death) independent of treatment received [1]. However, we are aware of the limitations of the retrospective nature of the study. Therefore, these limitations are explicitly described in the Discussion (See page 20, line 502).

- Comment 2: To assess cellular senescence, Senescence-associated beta-galactosidase (SA-β-gal) is considered an excellent marker.
  Reply 2: SA-β-Gal is only applicable in fresh snap-frozen tissue samples [2]. In order to detect cellular senescence in formalin-fixed paraffin-embedded (FFPE) samples, a combination of immunohistochemical senescence markers, such as lipofuscin aggregates, cell cycle inhibitors $p16^{INK4A}$ and $p21^{WAF1/Cip1}$ and proliferation marker Ki67 is recommended. This methodology is a validated technique of detecting cellular senescence described in multiple high-impact journals, i.e., *Nature Protocols* [3] and *Cell* [4].
  Changes in the text: We added and rephrased the following sentences to the Introduction:

  "*Also, the detection of senescence-associated beta-galactosidase (SA-β-Gal) activity - often considered as the gold standard for identifying cells - is only applicable in fresh snap-frozen tissue samples [2]. Therefore, detection of cellular senescence in archival formalin-fixed paraffin-embedded (FFPE) samples should be achieved by combining the measurements of different markers for cellular senescence, as previously reported [3,4].* (See page 5, line 150).

  We also added the following sentence to the Material & methods:

  "*as the detection of SA-β-Gal activity is only applicable in fresh snap-frozen tissue samples [2].*" (See page 6, line 187).

Reviewer B:

- Comment 1: Prepare a deposited electronic dataset of the images from the various H & E and IHC stains coupled with the main clinical demographic data (e.g. age, gender, histology, stage, treatment, smoking history, survival) and oncogenotype.
  Reply 1: We agree with the reviewer that a deposited electronic dataset of the images from the various H&E and IHC stains coupled with main clinical demographic data and oncogenotypes would be very helpful for other investigators for independent image analyses and validation of our results. We are aware of the https://bigpicture.eu initiative, but this repository is still under development. In the meantime, we are willing to share our images and clinical data with other researchers upon reasonable request.
  Changes in the text: We added:

  "*The images and clinical data that support the findings of this study are available from the corresponding author, A.D., upon reasonable request.*" (See page 23, line 567).

Reviewer C:

- Comment 1: Figure 1 shows several markers from a single sample that confirms the senescent signature. It would be helpful to give additional examples that illustrate the different patterns of gene expression. Especially interesting for the 9 SS adenocarcinoma patients treated with neoadjuvant therapy. Perhaps this could be presented as supplemental data.
  Reply 1: We added the immunohistochemical stainings from a patient treated with neoadjuvant therapy, showing the senescent signature, in Figure 1. Additional images of other patients can be requested from the corresponding author, A.D., upon reasonable request.
  Changes in the text: We adapted Figure 1. (See page 12, line 307).

- Comment 2: Have the authors attempted double staining, ex., p16 + Ki67?
  Reply 2: We did not opt for a double staining for p16 + Ki67 in order to prevent false positive interpretation of such a double staining. p16 can result in a mixed cytoplasmic/nuclear staining and Ki67 in a nuclear staining. Therefore, we opted to perform our four stainings on sequential and adjacent FFPE sections (5 µm thick).

- Comment 3: In previous studies, p53 was examined and gives information for only a subset of patients in the current study, revealing an association between p53 mutation and low p21 levels. Similarly, it would be interesting to show the status of p16 (deleted, methylated, etc) in non-SS samples in order to better understand the mechanism for the non-SS status.
  Reply 3: Unfortunately, for this retrospective study, we do not have access to

additional FFPE patient samples to determine the status of p16, as we already have used 4 sequential sections of the primary tumor for the senescence stainings. However, in samples with a non-SS status, 36/107 (33.7%) samples showed a negative p16 expression, suggesting a potential p16 gene deletion or functional p16 silencing through methylation (adapted Table 2).

Of note, cellular senescence can also be induced by activation of p21, independent of p16 [5], where p21 seems to be more involved in the initiation of senescence, while p16 seems to be more crucial for maintaining the senescence-associated arrest [6]. Hence, samples with a non-SS status showing a negative p16 staining do not necessarily exclusively harbor non-senescent proliferating cancer cells, but nevertheless can also harbor few senescent cancer cells (e.g., in case of initiation of early senescence based on p21). Since senescence is considered antagonistically pleiotropic [7], it could be suggested that the quantity of senescent cancer cells in these samples is not sufficient to exhibit similar protumorigenic effects as in samples with a high tumoral senescence burden, and the occasional senescent cells primarily act tumorsuppressive through the senescent-associated cell cycle arrest.

Changes in the text: We adapted Table 2 presenting the expression of senescence markers in the total patient cohort and according to the tumoral SS status (See page 11, line 301), and added the following additional correlation of expression of senescence markers according to the tumoral SS status to the text:

"*In patients with a tumoral SS (n = 48), immunohistochemical p16^{INK4A} expression was positively correlated with high-level lipofuscin accumulation (< 30% NSCLC cells positive) (correlation coefficient = 0.26, p-value = 0.071) and significantly inversely correlated with p21$^{WAF1/Cip1}$ expression (correlation coefficient = -0.34, p-value = 0.018). Conversely, in patients with no tumoral SS (n = 107), immunohistochemical p16^{INK4A} expression was significantly inversely correlated with lipofuscin accumulation (correlation coefficient = -0.24, p-value = 0.014) and significantly positively correlated with p21$^{WAF1/Cip1}$ expression (correlation coefficient = 0.22, p-value = 0.024). Also, in patients with no tumoral SS, immunohistochemical Ki67 expression was significantly positively correlated with lipofuscin accumulation (correlation coefficient = 0.33, p-value = <0.001) and significantly inversely correlated with p16^{INK4A} expression (correlation coefficient = -0.22, p-value = 0.023).*" See page 12, line 326.

We also added the following sentence:

"*Of note, samples without a tumoral SS do not necessarily exclusively harbor non-senescent proliferating cancer cells but nevertheless can also harbor few senescent cancer cells. Since senescence is considered antagonistically pleiotropic [7], it could be suggested that the quantity of senescent cancer cells in these*

samples is not sufficient to exhibit similar protumorigenic effects as in samples with a high tumoral senescence burden, and the occasional senescent cells act primarily tumor suppressive through the senescent-associated cell cycle arrest." (See page 19, line 462).

- Comment 4: Was the SS ever seen in adjacent tissues? And if this were to happen, how would it impact the interpretation of the SS in the tumor tissue?
  Reply 4: For our study, unfortunately, we only had the tumoral tissue at our disposal. Therefore, we are not able to provide any information about the presence of a SS in adjacent tissues. However, senescent cells can paracrinally spread the senescent phenotype to adjacent cells through the SASP [4,8]. Also, evidence suggests that cellular senescence occurring in normal non-malignant tissue can promote cancer relapse [9]. Hence, adjacent non-malignant senescent cells can contribute to the protumorigenic effects exhibited by the tumoral SS.
  Changes in the text: We adapted the following sentence:

  "*In later stages, the SASP can reinforce the senescent phenotype in an autocrine way, and paracrinally spread the senescent phenotype to adjacent malignant and non-malignant cells. With an increasing tumoral senescence burden, the accumulation of SASP can subsequently stimulate growth and proliferation of neighboring benign, premalignant and malignant cells.*" (See page 18, line 453).

  We also added the following sentence:

  "*The senescent phenotype spread by the tumoral SS to the adjacent non-tumoral tissue can contribute to the protumorigenic effects exhibited by the tumoral SS, ultimately promoting cancer relapse [9]*". (See page 19, line 460).

  Finally, we also adapted Figure 5 and added the residual adjacent non-tumoral tissue containing cellular senescence. (See page 21, line 521).

- Comment 5: In line 341, would it not be clearer to state 'low Ki67' as an individual marker of senescence?
  Reply 5: In the referred study [10], patients with high $p21^{WAF1/Cip1}$ and high Ki67 had a worse overall survival.
  Changes in the text: We added "*high*" to the sentence in question. (See page 18, line 442).

Reviewer D:

- Comment 1: Abstract: Method part was lengthy.
  Reply 1: We have shortened the methods part of the abstract.
  Changes in the text: We shortened the methods part of the abstract by deleting the

following sentence:

"*A tumoral senescence signature was defined by the presence of high-level lipofuscin and high p16^{INK4A} and/or p21$^{WAF1/Cip1}$ expression (≥ 30% NSCLC cells positive) in combination with low Ki67 expression (< 30% NSCLC cells positive).*" (See page 2, line 66).

- Comment 2: Introduction: The goal of this study should be emphasized. And possible results or hypothesis should be presented.
  Reply 2: As this was an observational study, provisional results could not be provided in the introduction. Regarding the goal and rationale, we added another sentence to the Introduction to emphasize the goal and rationale of the study.
  Changes in the text: We added the following sentence:

  "*As a result, in vivo evidence of cellular senescence in cancer patient samples is sparse and is only now catching up [4].*" (See page 5, line 154).

- Comment 3: Method: Too long period of cohort of patient! It make some differences of patient's survival result.
  Reply 3: Several referred papers in the manuscript provide survival data comprising a similar period of follow-up of 132 months (11 years) [10] or even a longer period of follow-up up to 15 years [11]. Of note, we have provided the 5-year overall survival rate.
  Changes in the text: We added:

  "*with a 5-year OS rate of 44.9% versus 66.9%, respectively*". (See page 14, line 369).

- Comment 4: TCGA data analysis or cell line study for molecular mechanism should be added if possible.
  Reply 4: Apart from the mounting *in vitro* evidence that senescent cells can exhibit protumorigenic effects, to which we refer in the Introduction and Discussion, the goal of our study was to evaluate the clinical impact and the significance of the presence of cellular senescence in NSCLC patients, rather than to provide a mechanistic molecular explanation on how senescent cells exhibit their protumorigenic effects.

  Regarding TCGA data analysis, single high mRNA expression of CDKN2A (p16^{INK4a}) (https://www.proteinatlas.org/ENSG00000147889CDKN2A/pathology/lung+cancer/LUAD), CDKN1A (p21$^{WAF1/Cip1}$) (https://www.proteinatlas.org/ENSG00000124762-CDKN1A/pathology/lung+cancer/LUAD) and MKI67 (Ki67) (https://www.proteinatlas.org/ENSG00000148773-MKI67/pathology/lung+cancer/LUAD)

shows a significant worse overall survival for NSCLC adenocarcinoma patients with Kaplan-Meier survival analysis. Since lipofuscin, another hallmark of senescence [4], results from non-degradable aggregates of oxidized lipids and proteins that accumulate in lysosomes of senescent cells due to senescence-related lysosomal malfunction [4], there is no corresponding coding gene for lipofuscin. In addition, there is currently no specific and universal gene set or single protein marker for the detection of cellular senescence [3]. Hence, the use of multiple markers remains the sole option for accurate validation of senescence in cultured cells and in vivo [3]. Therefore, in our study, we opted to combine four immunohistochemical senescence markers on protein level. As such, this research strategy is, in our opinion, more reliable to determine senescence than determination of senescence by mRNA expression on translational level. Also, CDKN2A ($p16^{INK4a}$), CDKN1A ($p21^{WAF1/Cip1}$) and MKI67 (Ki67) are not exclusively related to cellular senescence but are involved in a myriad of cellular processes. Hence, presenting the single prognostic value of mRNA expression of single senescence related genes is not in line with our research strategy and therefore, in our opinion, will not have considerable added value. Also, Kaplan-Meier survival analysis does not correct for other independent variables (e.g., age and TNM-stage) as we did in our Cox PH model analysis. Therefore, these survival data should be interpreted with caution.

ID: TLCR-22-192
Title: Prognostic implications of cellular senescence in resected non-small cell lung cancer.

Reviewer A

Domen et al. investigated "Prognostic implications of cellular senescence in resected non-small cell lung cancer," but the study's design does not support this hypothesis well.

Cell cycle arrest resulting from activation of p16INK4a/pRB and p53/p21 pathways is associated with cellular senescence. Additionally, oxidative DNA damage can lead to cellular senescence mediated by ROS generation. Cellular senescence may have a beneficial or harmful effect depending on the type of cancer cell. During chemotherapy, the removal of cellular senescence sometimes benefits patients.

To assess cellular senescence, Senescence-associated beta-galactosidase (SA-β-gal) is considered an excellent marker.

Reviewer B

The authors perform immunohistochemical studies for p16, p21, lipofuscin, and Ki67 in 155 clinically annotated non-small cell lung cancer (NSCLC) resected tumor samples the vast majority of which were lung adenocarcinoma (LUAD). They find that 48 of these had a “senescence signature” (SS) and that this was associated with worse prognosis. They performed a variety of other analyses of patient subset that found that the 4 SS markers were expressed independently of one another with the exception of p16 inversely correlating with Ki67, and that the combination of p16 and p21 after adjusting for many factors were the best predictors of adverse prognosis. With a limited oncogenotyping they found more SS in TP53 wildtype tumors and more SS in LUAD that had received neoadjuvant chemotherapy. Their main findings were also seen in the largest group of patients which were LUADs without EGFR mutations. Finally, the best adverse predictor involved use of all 4 markers. They conclude: “ The presence of a tumoral senescence signature particularly based on high p16INK4A expression significantly affects overall survival in non-small cell lung cancer adenocarcinoma. “ In their discussion they raise the possibility of “senolytic therapy” (therapy targeting senescent cells). They also discuss the role of senescent cells which initially could act as a tumor suppression mechanism, but then by providing secreted factors and other mechanisms to help tumor cells with more malignant potential or immune suppression to arise.

Comments to the Authors:
This is a technically well-done study that is clearly presented in the Results section. All of the methods and reagents used in the study are clearly described. The Discussion section is evenly balanced. I have only one suggestion for the authors. As they describe in the Methods section – after IHC staining the images were scanned and then an Image Management System used for analyses. Given the recent advances

in image analyses combined with artificial intelligence it would be very helpful for them to prepare a deposited electronic dataset of the images from the various H & E and IHC stains coupled with the main clinical demographic data (e.g. age, gender, histology, stage, treatment, smoking history, survival), oncogenotype. These data could then be used by other investigators (alone or in collaboration with the authors) to facilitate state of the art approaches to image analyses for prognostic information. This would be very similar to depositing molecular datasets (such as mRNA expression or mutational analyses) coupled with clinical data which are now the standard for such publication reporting. This would, in the long run, greatly enhance the value of this manuscript and the authors' work and also allow independent validation and analyses of their findings.

Reviewer C

The work by Domen et al examined more than 150 lung cancer samples for the senescence signature exposed by histopathologic examination. Not surprisingly, high expression of p16 was associated with poor prognosis. In general the work was porperly performed and interpreted. Even so, a few improvements would strengthen this study.

1) Figure 1 shows several markers from a single sample that confirms the senescent signature. It would be helpful to give additional examples that illustrate the different patterns of gene expression. Especially interesting for the 9 SS adenocarcinoma patients treated with neoadjuvant therapy. Perhaps this could be presented as supplemental data.

2) Have the authors attempted double staining, ex., p16 + Ki67?

3) In previous studies, p53 was examined and gives information for only a subset of patients in the current study, revealing an association between p53 mutation and low p21 levels. Similarly, it would be interesting to show the status of p16 (deleted, methylated, etc) in non-SS samples in order to better understand the mechanism for the non-SS status.

4) Was the SS ever seen in adjacent tissues? And if this were to happen, how would it impact the interpretation of the SS in the tumor tissue?

5) In line 341, would it not be clearer to state 'low Ki67' as an individual marker of senescence?

Reviewer D

This article studied senescence signature expression in 155 NSCLC by IHC. Its approach is frequent, and detail mechanism study was not performed. Some major point should be revised and the specific comments are as following:

ABSTRACT: Method part was lengthy.

INTRODUCTION: The goal of this study should be emphasized. And, possible results or hypothesis should be presented.

Method: Too long period of cohort of patient! It make some differences of patient's survival result. TCGA data analysis or cell line study for molecular mechanism should be added if possible.

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