Peer Review File

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Reviewer A

The authors explored the prognostic value of the expressions of CB1 and CB2 receptors in early-stage non-small cell lung cancers. By checking the protein expression and gene expression levels, they concluded their hypothesis that expression of these markers is helpful in predicting NSCLC. The article is detailed, results are backed by enough data, and the conclusion is supported by the results. I'd give the article a pass with minor English and grammar checking.

Reply: In order to ensure highest possible language quality, the manuscript has been re-checked by a professional editing agency (Sees-editing, Ltd, Great Britain).

Reviewer B

The article entitled 'Cannabinoid receptor 2 expression in early-stage non-small cell lung cancers identifies good prognosis patients with longer survival' concerns an interesting topic. The authors examined the expression of CB1 and CB2 at the RNA and protein levels. Below are comments on the article.

1. Throughout the manuscript: gene names and abbreviations should be in italics
   Reply: Agreed. Abbreviations has been put into the italics where appropriate.
   See: Due to the large amount of these relatively minor changes the lines are not specified.

2. In the methods section, line 125: '...according to relevant national and international guidelines...' imprecise wording. If the authors are already writing about the guidelines according to which the clinical procedure was implemented, it would be appropriate to write what the guidelines were.
   Reply: The patients were treated according NCCN Lung cancer guidelines 2010. In detail, stage IA-IIIA NSCLC patients underwent radical surgical resection based on the consensus of a multidisciplinary tumor board (surgery, oncology, radiology, etc.). Stage IB-IIIA NSCLC patients received platinum-based adjuvant chemotherapy and patients with microscopically positive resection margins (R1) underwent adjuvant radiotherapy. The manuscript has been modified accordingly and new reference (Ettinger et al., 2010) was added.
   See: Methods – Patients. Lines 149 – 154, 529 - 530.

3. The authors determined gene expression by qPCR and used the ΔCT calculation formula. The question arises as to why the authors chose these and not 2-ΔCT or 2-ΔΔCT. If the forms mentioned here had been used, taking into account reaction efficiency, would the results have been the same?

   Reply: The ΔΔCT method compares the differences of normalized target gene CT values ($CT_{target\ gene}$- $CT_{reference\ gene}$=ΔCT) between treated and control group. In our case, we couldn't use ΔΔCT method, because there were no such a group of patients (treated, non-treated, control, healthy) to be compared to. Thus, the qPCR reaction efficiency doesn't matter in our study, because we analyzed one gene expression in one group of patients in one experiment.

Reviewer C

Cannabis use/interest in the cancer population is growing rapidly and the impact of the cannabinoid system on cancer growth and survival is not well known. Overall, the study design was constructed well. I felt the authors did a nice job with the methods section, clearly describing the sample population and experimental techniques used. The results reported were comprehensive.

I have a few specific comments/questions for the authors:

Intro: Very well written and gives a good overview of what is known with the cannabinoid receptors and cancer outcomes.
Methods: Generally clear and easy to follow

1. In statistical analysis section, it would be nice to list the variables used in multivariable analysis and why they were chosen (age, stage, CB expression, etc.).
   Reply: All the tested variables are currently listed in the Supplementary Tab. 1, 2. In the multivariate models, the age and gender were used as a standard adjusting variables and disease stage was used as a stratification variable. The CB1 and CB2 expressions were the variables of our interest. The body weight, BMI and chemotherapy administration were used as an adjusting variable for survival. Based on reviewer comments the manuscript and table legends have been modified accordingly.
   See: Statistical analysis and supplementary table legends. Lines 205 - 209.

Results:

2. Correction needed? Lines 183-184 – you list HR on 0.274, but it appears from table this is 0.166. please clarify.
   Reply: Agreed and mistyping error in the table was corrected. The HR is 0.274.
   See: Supplementary Tab. 1.

3. Line 188 – I believe this is from table 2, not figure 2.
   Reply: Agreed and mistyping error corrected. The results are summarized in Supplementary Tab. 2.
   See: Supplementary Tab. 2.

4. It might be nice if you could explain somewhere in results what you mean by "clinically managed according to relevant national and international guidelines". Specifically, while supplemental figure 2 shows adjuvant vs no adjuvant chemotherapy a brief listing of what regimens were used (if known) might be nice. Based on time frame of 2009-2013, one would presume this is a platinum-doublet backbone of some type and that no immunotherapy was utilized as those trials weren't conducted until 2014.
   Reply: Agreed. The patients were treated according NCCN Lung cancer guidelines 2010. In detail, stage IA-IIIA NSCLC patients underwent radical surgical resection based on the consensus of a multidisciplinary tumor board (surgery, oncology, radiology etc.). Stage IB-IIIA NSCLC patients received platinum-based adjuvant chemotherapy and patients with microscopically positive resection margins (R1) underwent adjuvant radiotherapy. The manuscript has been modified accordingly and a new reference (Ettinger et al., 2010) was added.
   See: Methods – Patients. Lines 149 – 154, 529 - 530.

5. Figure 1 – listing number of patients at risk below the time(months) would be helpful.
   Reply: Agreed. The number of patients at risk were added to the Figure 1.
   See: Figure 1

6. Figure 2 – it is stated in results (line 208-209) that lower cb2 gene expression is seen in tumor tissue samples vs unaffected lung. However, this figure (figure 2B) looks very similar to me. It would be helpful to explain this more in terms of degree of difference, significant of p-value/analysis.
   Reply: The Figure 2 describes the results of paired ΔCT data (*CB1* and *CB2* gene expression) analyzed by Paired t-test. In case of *CB2*, the difference between tumor and tumor-free lung

tissue didn't reach statistical significance (p= 0.056). The manuscript text has been changed for better understanding**.**
See: Results. Lines 253 - 257.

7. Supplementary Table 1 and table 2- what was MV analysis not done for overall survival as well? I'm having difficulty understanding the two tables as they have same title and this issue was not well described in methods section. In addition, please explain why age, gender, and CB positivity were the only variable selected. What about stage? Adjuvant chemotherapy?
Reply: In the multivariate models, age and gender were used as a standard adjusting variables and the disease stage was used as a stratification variable. The CB1 and CB2 expressions were the variables of our interest (Supplementary Tab. 1). Body weight, BMI and chemotherapy were used as adjusting variables for survival. (Supplementary Tab. 2). The tested multivariate model was non-convergent for the overall survival and thus could not be used. Based on reviewer's comments the manuscript and table legends were modified accordingly.
See: Statistical analysis and supplementary table legends. Lines 205 - 209.

8. For supplementary table 2 – it is not visually well laid out. I think you are doing 3 different analysis where you add a variable to each. Table should be better formatted and described in title…..table 1a, 1b, 1c.
Reply: Agreed. The Supplementary Tab. 2 and the corresponding legend has been modified for ease of understanding.
See: The Supplementary Tab. 2 and the legend.

Discussion: Overall it was a nice review of data presented and compared to existing literature. I though authors did a nice job explaining some of the potential reasons for the results.

Reviewer D

The family of NSCLC is comprised of adenocarcinoma, squamous cell carcinoma and large cell carcinoma. Of these, adenocarcinoma has been shown to be inhibited by Gi-coupled receptors via reduction in cAMP. By contrast, there is evidence that squamous cell carcinoma is inhibited by cAMP. The authors therefore need to break down their data to include the influence of histological subtype on their data. Unfortunately, it is often overlooked that NSCLC is not one type of cancer but instead a family of histological subtypes of cancer with different regulatory mechanisms. The generalizations made by the authors conclusions are unjustified and can potentially lead to therapeutic strategies that would selectively stimulate the growth and progression of squamous cell carcinoma

Reply: In general, we agreed. Therefore, we have categorized our cohort according to histology into adenocarcinoma (n = 40), large-cell carcinoma (n = 7) and squamous-cell carcinoma (n = 51) and adjusted the Table 1A accordingly. We found no significant difference in CB1 and CB2 expression across histological subtypes (Tab. 1A). Also, univariate analysis detected no influence of histology subtypes on survival parameters (Supplementary Fig. 2A).
The multivariate analysis, stratified on stage and adjusted on age and gender, identified the following effects of histology subtypes on CSS and DFS:
Squamous-cell carcinoma patients have more than three times lower risk of CSS event than patients with adenocarcinoma (CSS: HR=0.26, CI=(0.07, 0.93), p= 0.04).
Large-cell carcinoma patients have more than seven times higher risk of CSS event than patients with adenocarcinoma (CSS: HR = 7.5, CI = (1.2, 47.0), p= 0.03) (see the table below).
However, these findings did not affect prognostic significance of *CB2* gene expression on survival in respective histological subtypes of NSCLC. Due to the small number of large-cell

carcinoma patients (n = 7), resulting HR confidence interval is very broad, which renders multivariate model with histology subtypes unreliable. Therefore, we did not include these results into the manuscript/supplementary files.

Since the data related to histological NSCLC subtypes did not affect CB1/CB2 influence on survival, we do not discuss these findings in the Discussion section. Otherwise, the manuscript has been modified accordingly.

See: Table 1A, Supplementary Fig. 2A, Results

	CSS			DFS		
A	**HR**	**95% CI**	**p-value**	**HR**	**95% CI**	**p-value**
Age	1,189	1,064-1,328	**0,002**	1,048	0,998-1,101	0,060
Gender M	0,767	0,251-2,347	0,642	0,753	0,316-1,796	0,522
Histology large-cell	7,574	1,220-47,000	**0,030**	3,493	1,016-12,004	**0,047**
Histology squamous-cell	0,263	0,074-0,934	**0,039**	0,590	0,237-1,472	0,258
***CB2* gene qRT-PCR positivity (<= 1.0)**	0,209	0,068-0,641	**0,006**	0,265	0,107-0,655	**0,004**