Author's response to reviews

Title: Histone demethylase GASC1 - a potential prognostic and predictive marker in invasive breast cancer.

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Author's response to reviews: see over
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Dr Khalil Helou
Editor
BMC Cancer

RE: Review of the manuscript No: 6241375717289647 - version 1

Dear Dr Helou,

We have made corrections and alterations according to the suggestions by the referees (please, see the response to the referees), and additional changes to improve the manuscript.

The abstract was extensively edited to better reflect the reviewers’ comments. Also the language has been revised.

Hereby we are submitting the revised manuscript and our responses regarding the specific points raised by the reviewers. We hope that the manuscript is now acceptable for publication in BMC Cancer.

Yours sincerely,

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I. Response to reviewers’ comments by Piero Tosi

Major compulsory revisions:

1. The question posed in the title is interesting, however in the manuscript GASC1 is considered an useful parameter for classifying breast cancer and predicting its behaviour more than a prognostic indicator;

   **Response:**
   
   In order to adequately reflect the outcome from our study we have changed the title to “Histone demethylase GASC1- a potential prognostic and predictive marker in invasive breast cancer”.

2. Nothing is written on the type of surgical procedure and adjuvant therapy in the cases of the series;

   **Response:**
   
   The following information has been added to the Material and Methods:

   “Sixty eight patients underwent resection, 285 were treated with mastectomy, and two patients did not undergo surgery. Postoperative radiotherapy was given to 206 patients. Altogether 62 patients received adjuvant tamoxifen, and 69 patients were treated with adjuvant chemotherapy, mainly intravenous CMF regimen (500 mg/m², methotrexate 40 mg/m², 5-fluorouracil 500 mg/m).

3. A score system is used for immunohistochemical evaluation, however, it is not specified when a case is defined positive or negative;

   **Response:**
   
   The following information has been added to the Material and Methods:

   “Tumors were designated negative if they scores from both series A and B were 0. Tumors were designated as positive if they score from at least one series was positive.”

4. It is necessary that the Authors specify what is the distribution of immunoreactivity in the various cases of the series; in other words, they have to let us know the number and the distribution of positive cells in order to show the level of omogeneity among neoplastic cells and neoplastic fields, the more so because they use tissue microarray.
Response:

The distribution of immunoreactivity in series A and B (the absolute numbers and the respective percentages) are given in the table below for the reviewer’s reference.

<table>
<thead>
<tr>
<th>Immunoscore</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear number</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Series A, n (%)</td>
<td>198 (55.8)</td>
<td>98 (27.6)</td>
<td>45 (12.7)</td>
<td>14 (3.9)</td>
<td>355 (100)</td>
</tr>
<tr>
<td>Series B, n (%)</td>
<td>220 (62.0)</td>
<td>92 (25.9)</td>
<td>31 (8.7)</td>
<td>12 (3.4)</td>
<td>355 (100)</td>
</tr>
<tr>
<td>Nuclear intensity</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Series A, n (%)</td>
<td>198 (55.8)</td>
<td>89 (25.1)</td>
<td>45 (12.7)</td>
<td>23 (6.5)</td>
<td>355 (100)</td>
</tr>
<tr>
<td>Series B, n (%)</td>
<td>220 (62.0)</td>
<td>93 (26.2)</td>
<td>28 (7.9)</td>
<td>14 (3.9)</td>
<td>355 (100)</td>
</tr>
</tbody>
</table>

To the Material and Methods we included the following informations.

“The results in series A and B were similar (substantial inter-series agreement was achieved; kappa for both nuclear number and intensity was 0.7; p=0.000). The final score was obtained by combining these results in four groups.”

Minor essential revisions:

a. The Authors should comment on the fact that GASC1 is not completely independent of grade;

Response:

The number of GASC1 positive tumors decreases when the histological grade increases. We can imagine two possible explanations: the first that the breast tumors progress through the grades losing GASC1 positivity, what is less possible and the second, which we favor, that the GASC1 positive tumors are derived from different stem or progenitor cells than the GASC1 negative ones. The stem or progenitor cells, which are programmed to give rise to GASC1 positive tumors generate population of better differentiated non-tumorigenic cancer cells[1].

b Numbers and follow-up time of cases should be in Material and Methods and not in Results;

Response:

We moved the information about numbers and follow-up time of cases to the Material and Methods section.
c. Tables are clear, but why S1 instead of 4?

**Response:**

The Table S1 was included to the main body of the manuscript as a Table 4 and the numbering of the tables to follow was changed accordingly.

**Discretionary revisions:**

It would be of interest to evaluate whether AR is correlated to GASC1 and to Ki67, since GASC1 is not independent of grade, and grade is dependent on cell proliferation; AR data might suggest alternative therapy in HER2-/GASC1+ cancers as well as in triple negative cancers.

**Response:**

Thank you the Reviewer for this interesting remark. The role of AR in HER2-/GASC1+ and triple negatives is in our interest and we are currently collecting material for this purpose.
II. Response to reviewers’ comments by Jaydutt Vadgama

Major compulsory revisions:

1. Two recent studies have clearly demonstrated the biological functions and clinical relevance of GASC1 in breast cancer (Liu et al; Oncogene 2009; and Wu et al; Oncogene 2012). Liu et al (2009) showed that GASC1 as one of the amplified genes for the 9p23-24 region in breast cancer, particularly in basal-like subtypes. The levels of GASC1 transcript expression were significantly higher in aggressive, basal-like breast cancers compared with nonbasal-like breast cancers. In contrast, the current study by Berdel et al., shows GASC1 downregulation is related to poor outcome. Furthermore, earlier data showed that GASC1 had oncogenic properties and its overexpression was more linked with basal-like phenotypes. In contrast the current study suggests GASC1 negativity is associated with tumors of more aggressive histopathological types (ductal type, grade II and III, ER negative, PR negative). The authors attribute these differences between in vitro cell lines (Liu et al, 2009) and in vivo clinical samples in this study. However, Liu et al had also examined tumor tissues and showed data consistent to their in vitro data.

Response:
We agree that there exist discrepant results concerning the correlation of GASC1 levels and the grade of the breast tumor. We have discussed this in more detail in the discussion section. The following text has been added to the Discussion:

“In addition to cell culture experiments Liu at al. (2009) meta-analyzed data sets concerning gene expression in human breast cancer from ONCOMINE. The results concerning GASC1 mRNA expression from this meta-analysis were consistent with their results obtained from cell culture but did not support our results from IHC analysis of GASC1 expression. The discrepancy might come from differences in material used by us and others. The data set from Fiank at al. concerned gene expression in tumor stroma [2], while we assessed GASC1 expression in epithelial compartment of the tumor. The analysis based on material used by Kreike at al. (2007) encompassed 58% triple negative/basal like carcinomas while in our material triple negative tumors constituted only 11% [3]. The discrepancy might also come from differences in methodology. We performed immunohistochemistry and compared numbers of GASC1 positive and negative tumors in different histopathological and clinical groups, while Liu at al. based their work on GASC1 mRNA and protein expression analysis in different experimental setups. Our sample size available for GASC1 mRNA expression study was too small to do detailed statistical analysis, what makes it impossible to compare our GASC1 expression results with GASC1 expression evaluated by others.”
2. In contrast, others have shown that over expression of GASC1 a histone demethylase acts as an oncogene and induces transformed phenotypes, including growth factor-independent proliferation, anchorage-independent growth, altered morphogenesis in Matrigel, and mammosphere forming ability. Some studies have suggested that GASC1 demethylase activity may be linked to the stem cell phenotypes in breast cancer. The authors in the current study did not adequately explain the contrasting data.

Response:
We agree the obvious role of GASC1 as an oncogene. However, it may also have characteristics of a tumor suppressor through NOTCH1 induction. The following explanation was added to the discussion:
“The above mentioned oncogenic properties of GASC1 the authors linked with induction of NOTCH1 by GASC1. However, there are evidences that NOTCH proteins can act either as oncogenes or as tumor suppressors depending on the cellular context [4, 5]. In case of our study and study by Liu et al. (2009) the cellular context of GASC1 action was different because stromal or physiological interactions are absent in the cell cultures.”

3. In addition, the current study did not comment on GASC1 overexpression and demethylation of H3K9me3.

Response:
This is now commented in the text in the Discussion section.

“GASC1 possesses enzymatic activity which specifically demethylates tri- and dimethylated (me3 and me2) lysine (K) in residues 9 and 36 on histone H3 (H3K9me3/2 and H3K36me3/2) [6-8] and together with histone methyltransferases dynamically modulates the methylation status of H3. The biological meaning of H3 methylation depends on the modified residue (K9 or K36), the degree of methylation (me1, me2, me3) and the genomic position of H3 (promoter region or coding region) [9, 10]. In general, H3K9me3/2 is found at promoter regions of inactive genes and demethylation in this site causes promoter activation, whereas H3K36me3/2 is enriched within the body of active genes and demetylation in this site is related to the ending of transcription [11, 12]. However, the increase of H3K9me3 inactive mark in coding region of the gene has also been associated with active gene expression [13].

4. GASC1 protein demethylates tri- and dimethylated H3K9 and H3K36 marks (Cloos et al.,2006; Klose and Zhang, 2007; Shi and Whetstine, 2007; Whetstine et al., 2006). Demethylation of H3K9 activates transcription and loss of H3K9 methyltransferase activity is likely associated with many types of tumors. The current study fails to discuss these findings.
Response:

We thank the reviewer for pointing out the roles of H3K9 and H3k36. The following information was added to the discussion:

“The transcriptional repressive effect of the H3K9 methylation can be attributed to the association with the repressive protein HP1 [14]. Cloos at al. [6] showed that GASC1 can effectively remove H3K9me3 and H3K9me2 marks what releases the repressive protein HP1 (heterochromatin protein 1) and reduces heterochromatin in vivo. We can assume that in the absence of GASC1 HP1 is recruited to H3K9me3/2 and stabilizes heterochromatin. Recent data have shown that HP1α is over-expressed in numerous cancers and that HP1α over-expression is associated with increased cell proliferation most likely through silencing of genes inhibiting cell proliferation. Moreover the same authors demonstrated that HP1α overexpression in breast cancer patient samples correlates with an increased risk of death [15]. These data could partly explain why GASC1 negativity is associated with more aggressive tumors and worse breast cancer specific survival.

H3K9me3 status is also mediated by different histone methyltransferases. Suv39H1 (suppressor of variegation 3-9 homolog 1) is a major methyltransferase responsible for H3K9me3 that intimately links to DNA methylation. Dong at al. (2012) has found that H3K9me3 and DNA methylation on the E-cadherin promoter were higher in basal-like breast cancer cell lines. Further they showed that knockdown of Suv39H1 restored E-cadherin expression by blocking H3K9me3 and DNA methylation and resulted in the inhibition of cell migration, invasion and metastasis of in basal-like breast cancer [16]. GASC1 activity eliminates H3K9me3 what with consideration of the abovementioned data might partly explain the protective role of GASC1 expression our results suggest.”

Minor essential revisions:

The questions posed by the authors are well defined, and the methods are well described. The title and abstract clearly and accurately conveys their findings, and the writing is acceptable with exception to a few typo/grammatical errors made in the paper (noted below). The data is sound, with exception to comments listed below. The conclusions are solid, but the discussion was found to be lacking and does not adequately justify contrasting data.

With regards to data, discussion of limitations and discussion:

1. Figure 7: The error bar for relative GASC1 mRNA expression in Grade III samples is large; are results truly statistically significant?
Response:
We have reanalyzed our results and confirmed that the difference was statistically significant. The Figure 7 shows the box and whisker plots. The appropriate explanation and the mean values with standard errors were added to the figure legend as follows:

“Figure 7. Graph showing GASC1 mRNA expression in tumors of different histological grades. Boxes represent the 25–75th percentile; whiskers: range; black line: median; black dots: outliers. The highest GASC1 mRNA expression is detected in tumors of grade I (GR I; 0.761±0.0986). Tumors of grade II (GR II; 0.510±0.070) and III (GR III; 0.510±0.069) show lower GASC1 mRNA expression than tumors of grade I. There is no difference in GASC1 mRNA expression between tumors of grade II and III. Kruskal-Wallis test, p=0.02. Mann-Whitney test: grade I versus II - p=0.006; grade I versus III - p=0.019, grade II versus III - p=0.821.”

2. Table 1: More number of patients with GASC1 overexpression was associated with higher tumor size and the difference was significant. The authors do not provide adequate explanation.

Response:
In Table 1 we provided only the p-values for the survival analysis, where we analyzed the difference in survival between GASC1 positive and negative tumors in groups of the same clinical characteristics. Even though among GASC1 positive patients there were more cases with bigger tumors (81 cases) than with smaller ones (76 cases) the difference in the proportion of cases was not statistically significant (Fisher’s exact test, p=0.749). We supplemented the Table 1 with adequate p-values and corresponding explanations.

3. Table 2: Tumor size (T2, T3 and T4) and Clinical Stage (II, III and IV) has no effect on breast cancer specific survival and time to relapse – counter intuitive? Is this possibly due to the small number of patients with T3 and T4 tumors and at Stage III and Stage IV? If so, this limitation should be addressed.

Response:
We thank the Reviewer for this important notice. Indeed there were only 34 patients (9.6%) with T3 and T4 tumors and 42 patients (12%) at Stage III and Stage IV in our material. Probably this number of cases was not sufficient to show significant influence on survival in multivariate analysis. However in univariate analysis the patients with T1 and at Stage I survived significantly better and had significantly longer time to relapse than the patients with more advanced disease. Similarly in bivariate analysis, where as the second variable in addition to tumor size or Stage GASC1 status was entered, tumor size or stage and GASC1 status had significant effect on the breast cancer specific survival and time to relapse. This explanation has been added to the Results.
4. In the discussion section, it was mentioned that GASC1 demethylase activity has been possibly linked to the stem cell phenotype in breast cancer, and that this function (of GASC1) might be responsible for a lower recurrence rate in GASC1 positive cases compared with GASC1 negative and better outcome of GASC1 positive patients treated with radiotherapy. My question is if GASC1 functions to maintain a stem cell phenotype in breast cancer, why would you expect the GASC1 positive cases to have a better outcome than the negative cases? Cancer stem cells are thought to be responsible for recurrence of cancer. Statement seems counterintuitive.

**Response:**

Breast tumors arise from normal stem cells or early progenitor cells through deregulation of normal self-renewal leading to appearance of cancer stem or progenitor cells. We think that GASC1 protects the normal stem cells population through maintaining right levels of genes inhibiting cell proliferation by removing inactive mark H3K9me3/2 from their promoters. This mechanism we described in more details in the Discussion section pages: 17-18.

**Discretionary Revisions**

1. Figure 6: Could possibly increase the number of patient samples analyzed for GASC1 mRNA expression levels, and analyze breast cancer specific survival. Too few samples (20 for high GASC1 mRNA expression levels & 37 for low GASC1 mRNA expression levels) were utilized in that analysis.

**Response:**

We agree that the results would be more reliable if we would analyze more cases but that was the sample set available when the work was done. Currently we do not have samples which could be analyzed for GASC1 mRNA expression levels but we are gradually collecting them and we are planning to analyze them in the future. However, we emphasize that the results were obtained predominantly on immunohistochemical analysis of the GASC1 in the breast tumor sections and we consider the RNA expression results as supportive.

**Minor Essential Revisions**

1. Figure 1. The images of positive and negative stains for epithelial carcinoma cells should be at the same magnification (either 100x or 200x magnification)
Response:
We made a new photo for Figure 1 B under magnification 200x.

2. Page 9 under segment GASC1 mRNA expression is in line with immunohistochemical data – Change statement, “In contrast, HER2 negative cases showed significantly higher GASC1 mRNA expression than HER2 positive ones, which was in line with the protein staining results”.

Response: We changed the statement as follows:
“In contrast, HER2 negative cases showed significantly higher GASC1 mRNA expression than HER2 positive ones (Mann-Whitney: p=0.004) which was in line with the protein staining results (Fig. 8).

3. Figure 7 and Figure 8: Should include mean expression value for each category in figure legend (histological grade, PR weak/neg, PR strong, HER2-, HER2+).

Response: We included mean expression value for each category in the figure legend as follows:

Figure 7. Graph showing GASC1 mRNA expression in tumors of different histological grades represented by boxplots with whiskers from minimum to maximum. The highest GASC1 mRNA expression is detected in tumors of grade I (GR I; 0.761±0.099). Tumors of grade II (GR II; 0.510±0.070) and III (GR III; 0.510±0.069) show lower GASC1 mRNA expression than tumors of grade I. There is no difference in GASC1 mRNA expression between tumors of grade II and III. Kruskal-Wallis test, p=0.02. Mann-Whitney test: grade I versus II - p=0.006; grade I versus III - p=0.019, grade II versus III - p=0.821.

Figure 8. Graphs showing GASC1 mRNA expression according to progesterone receptor (PR) and HER2 status. (A) GASC1 mRNA relative level is lower in tumors showing negative or weak expression of PR (0.483±0.051) compared with tumors showing high PR expression (0.691±0.092; Mann-Whitney: p=0.016). (B) GASC1 mRNA relative level is higher in HER2 negative tumors (0.599±0.055) than in HER2 positive tumors (0.326±0.054; Mann-Whitney: p=0.004).

4. Page 11 – correct spelling error – demetylation to demethylation

Response: “Word has been written as suggested by reviewer.
III. Response to reviewers’ comments by Anna Sapino

**Major revisions**

1. **Background** This introduction does not emphasize enough the significance of the marker presented in the study. We suggest the authors to better underline the study purpose and the hypothetical central role of GASC1.

   **Response:**
   
   We rewrote the introduction adding important information to better show the context of our study.

2. **Material and Methods** The criteria used for the selection of the cases are not shown. In the Immunohistochemistry section the authors say: “After initial standard procedures the sections were incubated overnight at 4°C…” but the procedures are not described. Both positive and negative controls are lacking. The cut-off value for GASC1 positivity is absent.

   **Response:**
   
   Information about the initial procedures has been added to the Material and Method section and the cut-off value for GASC1 positivity has been stated. The section regarding immunohistochemistry has been modified as follows:

   “. After deparafinisation and rehydration, the sections were heated in a microwave oven for 3 × 5 min in citrate buffer (pH 6.0). Then they were treated for 5 min with 5% hydrogen peroxide to block endogenous peroxidase. Next the sections were incubated for 35 min at room temperature in 1.5% normal serum diluted in PBS to block non-specific binding. After that the sections were incubated overnight at 4°C with the mouse monoclonal anti – GASC-1 antibody (Origene, TA 500587) at dilution 1:100. The slides were then incubated with a biotinylated secondary antibody (35 min) and avidin-biotin-peroxidase complex (45 min) (ABC Vectastain Mouse Elite Kit, Vector Laboratories, Burlingame, CA, USA). After each step of the immunostaining procedure slides were rinsed with PBS. The color was developed with diaminobenzidine tetrahydrochloride (DAB) (Sigma, St. Louis, MO, USA). The slides were counterstained with Mayer's haematoxylin, washed, dehydrated, cleared and mounted with Depex (BDH, Poole, UK). In the negative controls, the primary antibody was omitted.”

   and

   “The results in series A and B were similar (substantial inter-series agreement was achieved; kappa for both nuclear number and intensity was 0.7; p=0.000; Table 1). The final score was obtained by combining these results in four groups. Tumors were designated negative if they scores from both series A and B were 0. Tumors were designated as positive if they score from at least one series was positive.”
3. The authors illustrate the kit for mRNA analysis but they do not specify if the reaction has been performed following the manufacturer’s instruction or a specific protocol. Reference (endogenous control) genes should be more than one.

**Response:**
The following information has been added to the Material and Method section concerning mRNA expression analysis:

“We used High Capacity c, DNA Reverse Transcription Kit to synthesize cDNA (Applied Biosystems, Foster City, USA) following the manufacturer’s instructions. The endogenous control gene was chosen by the investigation made by McNeill et al., where they found PPIA to be the best choice for breast cancer mRNA analysis [17].

4. Results “From the total material (392 tumors) we excluded 37 benign and in situ cases”. This means that the study has been conducted on 355 specimens.

**Response:**
Yes, to statistical analysis we included only invasive cancers without distant metastases, which is 355 cases, even though the tissue microarrays comprised 392 tumors.

5. Section: “GASC1 negativity is an independent prognostic factor of worse breast cancer specific survival.”
Section: “GASC1 negative cases are more likely to have a relapse of breast cancer and to suffer from more aggressive tumors than the GASC1 positive cases” It is not clear which is the difference in the two paragraphs. The term “MORE” has to be cancelled and substituted with numbers.

**Response:**
We canceled the term “MORE” and commented the significant difference between GASC1 positives and negatives.

In the breast cancer specific survival analysis in the first section there was no difference in proportion of GASC1 negative and positive cases in analyzed clinical groups (Table 1 in the revised manuscript) while in the second section the proportion of GASC1 positive and negative cases was significantly different in analyzed histopathological and molecular groups (Table 4 and 5 in the revised manuscript).

In the first section we are evaluating time from the diagnosis of breast cancer to the death from breast cancer (breast cancer specific survival), which means that the end point for survival analysis is death from breast cancer and death from other causes are censored. Based on the analysis in this section we can conclude that GASC1 positive patients survive approximately 2 years longer than the patients without GASC1 expression overall and 2.4 to 3.4 years longer when they are in advanced stages of the disease (Kaplan Meier analysis).
From the multivariate analysis in this section we can say that GASC1 negative patients have 2 times higher risk of dying from breast cancer than GASC1 positive ones (Cox regression).

In the second section we are describing our group of patients at the end of follow up period giving figures regarding how many patients with or without relapse were GASC1 positive or negative. Based on that, we concluded that among patients with relapse there are 1.7 times more GASC1 negative patients than GASC1 positive. Furthermore, we are giving evidence that GASC1 negative tumors are more likely ductal type, histological grade II or III, more often estrogen or progesterone receptor negative and HER2 positive. This result raises the question of whether GASC1 is really a prognostic factor for breast cancer specific survival or is only a marker of aggressive tumor subtype. We did the survival analysis in histopathological and molecular groups and showed that the ductal GASC1 negative cases and HER2 negative GASC1 negative cases had worse breast cancer specific survival than ductal GASC1 positive and HER2 negative GASC1 positive cases confirming that GASC1 negativity is prognostic factor of worse breast cancer specific survival.

6. Section: “GASC1 mRNA expression is in line with the immunohistochemical data.” Please state the value used to define low/high expression of GASC1 mRNA.

Response:
GASC1 mRNA expression was divided to low/high expression by the mean value of the expression (0.545).

7. “In contrast, HER2 negative cases showed significantly higher GASC1 mRNA expression than HER2 negative ones (Mann-Whitney: p=0.004) which was in line with the protein staining results”: “negative” is repeated two times and the sentence has no meanings.

Response:
The sentence has been corrected as follows: “In contrast, HER2 negative cases showed significantly higher GASC1 mRNA expression than HER2 positive ones (Mann-Whitney: p=0.004) which was in line with the protein staining results (Fig. 8).”

8. Discussion “An interesting finding from the survival analysis was that HER2 negative patients survived significantly better when they had GASC1 positive tumors regardless of their clinical stage. This observation might open new therapeutic possibilities, especially for the patients with triple negative tumors”. Why? The relationship with the AR is not clear.
Response:
Here we intended to suggest that restoring or inducing GASC1 function in GASC1 negative tumors can prolong the survival of patients with most aggressive tumors. This can be done by targeting directly GASC1 or its downstream molecules, but first the function of GASC1 signaling in breast cancer has to be clarified. These sentences were modified as follows:

“This observation might direct the search for new therapeutic possibilities towards GASC1 signaling pathway. Identification of molecules involved in this pathway and elucidating their role in the breast cancer patophysiology can be beneficial especially for the patients with triple negative tumors.”

9. Conclusion “Evaluation of the GASC1 status could enable more accurate qualification of patients for adjuvant therapy”. Why?

Response:
The following text was added to the discussion:

“The patients with ER-positive, HER2-negative disease are the group in whom decisions about adjuvant chemotherapy are most difficult. The relative indications for chemoendocrine therapy and endocrine therapy alone are given by the St Gallen International Expert Consensus [18]. However, there are no guidelines for those ER-positive, HER2-negative patients who are node positive with grade 2 tumors of size 2.1 – 5 cm (T2-T3). Our results indicate that the patients with HER2 negative, grade II tumors of ductal type have better prognosis when they are GASC1 positive (Table 5). Moreover GASC1 positive patients respond better to hormonal treatment. The results suggest that GASC1 positivity in these patients might be an indication for endocrine therapy alone, however this will need to be confirmed in a larger group of patients.”

Minor revisions
1. Abstract is not organized properly. The aim of the work is missing. The Background section contains some Material and Methods. In the sentence “In our material 56% cases were GASC1 negative and 44% positive” the authors have to state the method used to obtain these results. Please substitute the expression “more” (i.e. Among GASC1 negative tumors there were more estrogen and progesterone receptor negative cases and more HER2 positive cases than among GASC1 positive one) with an appropriate scientific terminology.

Response:

We modified abstract as suggested by the reviewer. The section “Methods” has been added and appropriate scientific terminology replaced vague terms. The Abstract has been modified as follows:
“**Background:** Histone demethylase GASC1 (JMJD2C) is an epigenetic factor implicated in development of different cancers including breast cancer. It is thought to be overexpressed in more aggressive breast cancer types based on mRNA expression studies on cell lines and meta analysis of human breast cancer sets. The aim of this study was to evaluate the prognostic and predictive value of GASC1 for women with invasive breast cancer.

**Methods:** All the 355 cases were selected from a cohort enrolled to the Kuopio Breast Cancer Project between April 1990 and December 1995. The expression of GASC1 was studied by immunohistochemistry (IHC) on tissue microarrays. Additionally relative GASC1 mRNA expression was measured of available 57 cases.

**Results:** In our material 56% cases were GASC1 negative and 44% positive in IHC staining. Women with GASC1 negative tumors had two years shorter breast cancer specific survival and time to relapse than the women with GASC1 positive tumors (p=0.017 and p=0.034 respectively). The majority of GASC1 negative tumors were ductal cases (72%) of higher histological grade (84% of grade II and III altogether). Considering estrogen receptor negative and progesterone receptor negative cases separately there was 2 times more GASC1 negative tumors than GASC1 positive ones in each group (chi2, p= 0.033 and 0.001 respectively). Among HER2 positive cases there was 3 times more GASC1 negative cases than GASC1 positive ones (chi2, p= 0.029).

2. Figure 1: The IHC figure shows an intense background. Where should the IHC-staining be located? “Positive immunostaining in nuclei of epithelial cells (immunoscores: 3 for the nuclear number and 3 for intensity of nuclear staining), positive staining also visible in cytoplasm.”

**Response:**

The positive staining is located in both nuclei and cytoplasm. In this paper we evaluated only nuclear staining because GASC1 function in the nucleus is known whereas GASC1 function in the cytoplasm is unknown. It might be that in the cytoplasm GASC1 is only synthesized and the positive staining there has no biological meaning. The background staining is impossible to avoid in some sections but, in our case, it does not affect the results because the staining intensity in the nuclei was evaluated by pathologists regardless of the background.
IV. Additional revisions to the manuscript

1. The list of Abbreviations and References had been updated.
2. The Conclusions have been modified according to the extended Discussion.
3. The language has been checked by an English speaking researcher.
References


