Author's response to reviews

Title: PIK3R1 underexpression is an independent prognostic marker in breast cancer

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Author's response to reviews: see over
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BMC Cancer
BioMed Central
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Dear members of BMC Cancer Editorial Board,

Please find enclosed revised manuscript entitled "PIK3R1 underexpression is an independent prognostic marker in breast cancer" by Magdalena Cizkova, Sophie Vacher, Didier Meseure, Martine Trassard, Aurélie Susini, Dana Mlcuchova, Celine Callens, Etienne Rouleau, Frederique Spyratos, Rosette Lidereau, Ivan Bièche that we are resubmitting for consideration as a Research Article in BMC Cancer.

In the revised text, we made changes asked for by the two reviewers (marked by yellow background) and also changes following the appropriate article style of BMC Cancer. The detailed replies to the reviewer comments are in the following pages (written in green).

We hope you will find the manuscript of interest and we are looking forward to hearing from you.

Sincerely yours,

Dr. Ivan Bièche
Reviewer's report

Title: PIK3R1 underexpression is an independent prognostic marker in breast cancer

Version: 1

Date: 5 July 2013

Reviewer: Josh Lauring

Reviewer's report:

This is a technically well done study testing the general hypothesis that alterations in the PI3-kinase pathway may have prognostic value in breast cancer. There have been many such studies examining the prognostic impact of PIK3CA mutations, PTEN expression, AKT phosphorylation and other biomarkers of pathway activity. Conflicting results have been demonstrated regarding the prognostic value of PIK3CA mutations, but the largest studies appear to show a favorable prognostic impact, even when adjusting for the fact that the mutations occur most frequently in the more favorable ER+ subset. These investigators have previously published an analysis of the same 450+ breast cancer patients analyzed for PIK3CA exon 9 and 20 hotspot mutation status and prognosis, finding that mutations were a favorable factor on univariate analysis, but not on multivariate analysis.

In this manuscript they re-analyze PIK3CA mutations, adding in rarer mutations in exons 1 and 2, as well as mutations in AKT1 and PIK3R1, which are found at about 3% frequency each (in contrast to the 30-40% PIK3CA mutation frequency. They also analyze RT-PCR based mRNA expression level for a number of PI3-kinase pathway related genes. The study focuses on the degree of association between various mutations and expression levels and then examines the prognostic utility of the analyzed mutations or individual gene expression levels for metastasis free survival in the whole dataset and in individual breast cancer subtypes.

None of the findings regarding PIK3CA, AKT1, or PIK3R1 mutations is especially novel. There are now quite a number of large scale studies showing the relative frequency of these mutations, their mutual exclusivity in breast cancer, and their association with particular breast cancer subtypes (ER+ versus triple negative, for example). These authors have already published their analysis of PIK3CA hotspot mutations in this same cohort. The addition of 7 tumors with exon 1 or 2 mutations (the majority of which already have additional hotspot mutations) adds nothing to their previous findings. It could have been predicted that all of the other mutations analyzed were sufficiently rare that they would not be able to find any statistical association that would add to our current state of knowledge.

The gene expression-based analysis is a bit of a fishing exercise performing multiple comparisons to try to identify various significant associations. However, the focus on PIK3R1 expression adds something to the existing literature since PIK3R1 expression has not previously been examined for its association with prognosis.

The suggestion that PIK3R1 expression could become a clinically useful independent prognostic marker in breast cancer overreaches a bit, although the investigators do note in their discussion that further studies are needed. Many investigators have identified individual genes or gene signatures which are
prognostic in a given dataset of patients. To be a candidate for clinical utility, a marker needs to be replicated in an independent data set and studied prospectively in a population who are untreated or subject to uniform treatment. Furthermore, there is no evidence that PIK3R1 is as good or superior to other widely used clinical parameters with prognostic or predictive value. To be useful as a clinical test from a practical standpoint, IHC would be a better marker than RT-PCR. Although limited data showing correlation of RT-PCR expression and IHC of PIK3R1, the study’s conclusions cannot really be extended to protein IHC. However, these issues are for future research and do not necessarily detract from the relevance of these initial reported findings. If the finding of the prognostic relevance of PIK3R1 expression holds up, it would be of some interest to the breast cancer research community and worthy of publication.

Major compulsory revisions:

1. A major issue with this clinical dataset is the potential heterogeneity in terms of treatment. The patients in this series were treated over a 30 year period, during which there have been major changes in screening, surgery, radiation, and adjuvant systemic treatment recommendations for breast cancer. The prognostic role of PIK3R1 expression and PIK3CA mutation is assessed in terms of metastasis-free survival of the cohort. However, patients were not necessarily uniformly treated, and no data are presented showing the frequency of hormonal therapy or chemotherapy in the patients with normal expression or underexpression of PIK3R1, for example. Although 344 tumors were ER+, only 268 of them were treated with hormonal therapy, which would be considered sub-standard care by present-day standards. What if there is unequal representation of untreated tumors in the low PIK3R1 subset? The same issue applies to chemotherapy. 183 women received chemotherapy, although 339 were ER-negative. Given that only 26% of the tumors were node-negative, the rate of chemotherapy usage also seems low by today’s standards. Obviously one cannot retrospectively change practice patterns, and one has to work with the data one has, but the authors should explore the possible confounding of their results by differences in treatment and receipt of treatment in the different groups they are analyzing (by PIK3R1 expression level, PIK3CA mutation, etc). A prognostic biomarker cannot be analyzed in the absence of details regarding treatments received.

To describe further the patient cohort, we added information on their diagnosis and treatment: “...where majority of the patients were diagnosed and treated between years 1990 and 2000 (67%).” (page 6, line 4) This ensures majority of patients being treated following similar guidelines. Furthermore, we added information about patient treatment in the Additional Table 5. This shows that proportion of adjuvant non-treated patients was comparable between PIK3R1 underexpressing and non-underexpressing cases (21.2% and 19.3%, respectively). The table also shows that in both adjuvant treated as well as adjuvant non-treated patients the highest proportion was present in PIK3R1 underexpressing cases. Based on this observation we can suggest that despite significant differences in treatment between PIK3R1 underexpressing and non-underexpressing cases, the treatment cannot be accused as the reason for inferior survival in PIK3R1 underexpressing patients.

2. Another possible statistical issue that may apply to this analysis is the lack of correction for multiple comparisons when calculating the significance level of various associations. Many variables (expression level and mutation status of many genes) are analyzed for their associations with survival in the whole cohort and again in individual tumor subtypes. I believe a correction for such multiple
hypothesis testing is needed, but I will defer to a statistical expert. We discussed the issue with statisticians. The result of the discussion is that the performed tests are separate univariate tests that do not require any correction for multiple comparisons. However, to clarify this fact, we have explained further the statistical method used specifically in comments for Table 2: “Chi² test of independency in contingency tables (tumor subtype vs. gene expression). NS: not significant.” And we also specified description of statistical significance assessment: “A level of significance was set at 5%.” (page 9, line 24)

3. There is no discussion of how the expression cut-off of N<0.5 or N>2.0 was chosen. While it might make biological sense to define as important an expression level relative to normal tissue of <0.5 or >2.0 times normal, it does not necessarily make sense for identification of the performance of a prognostic marker. In this study 61% of breast cancers “underexpress” PIK3R1. This is a high proportion of cancers for a putative adverse prognostic marker. While the choice of a cut-off is to some extent arbitrary, one wonders how such a prognostic marker would be useful clinically if it applies to 61% of patients. The utility of a prognostic marker depends on its ability to discriminate between better and worse outcomes. Many prognostic biomarker studies examine expression of the marker in the set of tumors and then separate the tumors into groups based on their relative expression level (highest to lowest quartile, etc.). Such an approach might better define what level of PIK3R1 expression confers the greatest risk of relapse than the arbitrary choice of N<0.5, which places 61% of tumors in the high-risk category. Any future validation of PIK3R1 expression as a prognostic marker would require going forward with the optimal cut-off expression value, so it bears improving upon in this pilot study. The authors could evaluate the performance of different cut-offs, or potentially examine the performance of their marker as a continuous variable.

We used the cut off levels for under- and overexpression based on our previous data from other studies. In our opinion, this approach is sufficient to describe primary observation on mRNA level. The presence of PIK3R1 underexpression should be further explored on protein level and in comparison of normal and tumor tissues to determine the best way how to describe expression changes in this protein in tumor cells.

For information, we compared patient survival considering subgroups based on PIK3R1 expression intensity (separation into 4 and 3 subgroups). Furthermore, we searched for expression cut off optimally separating PIK3R1 underexpressing and non-underexpressing cases (identified as 0.45, and so close to our cut off of 0.5). These additional results show that patients with normal expression of PIK3R1 keep superior survival results in comparison to PIK3R1 underexpressing cases. In our article, we prefer to keep previously set cut off to avoid different cut offs in different genes.

4. p.12.-13. The experiments correlating IHC and RT-PCR results for PTEN and PIK3R1 are methodologically flawed. It seems correct that each individual marker must be compared (RT-PCR versus IHC) rather than lumping both proteins together in the analysis. This combined analysis covers over the fact that 2/13 tumors analyzed were discordant for PTEN. I also think it would be better if the authors could show multiple tumors with IHC for PIK3R1 to give the readers a better sense of how PIK3R1 staining intensity was scored and how well the staining correlated with the mRNA level. The criteria for 1+, 2+, and 3+ staining are not explicitly described in the methods. It would also be helpful to see the relative level of PIK3R1 protein expression in the tumor and adjacent normal
tissue from the same patient, rather than a comparison of a tumor from one individual with normal tissue from another. The evidence for PIK3R1 underexpression as a relevant driver event in breast tumorigenesis would be stronger if most tumors showed lower expression than adjacent normal breast ducts.

The IHC analysis was done as additional subanalysis using available formalin-fixed paraffin-embedded samples. Unfortunately, only a limited number of tumor samples were available and none of normal tissue. Since the analysis was done more than one year ago, we unfortunately cannot extend the number of assessed samples. In the Materials section we added description of IHC assessment: “Normal ductal epithelial cells showed a positive cytoplasmic immunostaining, whereas PTEN expression in tumor cells varied with cytoplasmic and/or nuclear staining. A semi-quantitative intensity score was performed (score 0: negative staining, score 1: weak cytoplasmic staining, score 2: moderate cytoplasmic staining, score 3: strong and diffuse cytoplasmic staining). Positive immunohistochemical reactions were defined as a brown cytoplasmic staining for p85. A semi-quantitative intensity scale ranging from 0 for no staining to 3+ for the most intense staining was used by comparing neoplastic cells to adjacent breast cells belonging to normal terminal ductulo-lobular units. p85 underexpression was defined by an IHC score 0, p85 normal expression by an IHC score 1, and p85 overexpression by an IHC score 2+ and 3+.” (page 9, line 12)

5. Much of the results section is descriptive of associations that are not very meaningful without further analysis. For example, page 12 paragraph 3: “PIK3R1 underexpression was also associated with AKT3 and WEE1 underexpression.” Was there any hypothesis being tested here? Is this interesting in any way? Does this tell us anything about the biology of PIK3R1 underexpressing tumors? If it is worth discussing in the results at such length, there should be some discussion of these particular results in the Discussion section, and there is not, in most cases. Otherwise, the tables can show this less interesting data sufficiently well.

The length of detailed description of some results (including relationship between PIK3R1 underexpression and AKT3 and WEE1 expression status) was reduced in the text and in the abstract. (pages 12 and 13)

6. p. 13 “Survival analysis” paragraph. It is strange that mutation of either PIK3CA, PIK3R1, or AKT1 is associated with a worse prognosis, yet PIK3CA mutation considered alone is associated with a better prognosis. Given that there were 151 PIK3CA mutations and a combined 26 PIK3R1 or AKT1 mutation, this contradiction seems strange, particularly since AKT1 mutations are almost exclusively found in better prognosis ER+ tumors.

We are sorry for the mistake in survival analysis description. As is shown in additional table 4, the survival analysis of all mutations brings similar results to PIK3CA mutations only – mutations in all three genes are also associated with better survival. “Patients presenting any of the mutations assessed in this study (PIK3CA, PIK3R1 or AKT1) had a significantly better MFS (p=0.024).” (page 14, line 7)

Minor essential revisions:
1. Page 3 Abstract. The conclusion of the abstract is misstated. Alterations in PIK3CA but not PIK3R1 were shown to be prognostic. Lower expression of PIK3R1 was shown to be prognostic in this study, but low expression is not an “alteration.”
The conclusion of the abstract was changed according to the comment: “PIK3CA mutations and PIK3R1 underexpression show opposite effects…” (page 3, line 16).

2. Page 7, Mutation screening paragraph. In one line it says PIK3R1 exons 11-15 were screened and in the next line it says exons 10-14. The mistake in exon numbering was fixed: “…PIK3R1 exons 11 to 15…” (page 8, line 2).

3. p.10-paragraph 2, the synonymous nucleotide change in PIK3R1 is discussed as a potential polymorphism, yet it is counted as one of 11 “mutations.” This “mutation” should be verified as a somatic change in the tumor by sequencing normal tissue from the same patient. This change is most likely a sequencing artifact or a germline polymorphism. If it is a somatic change, it is still of doubtful significance, as it does not change the amino acid sequence of the protein. I think it would be best to report the frequency of non-synonymous changes. Unfortunately, we don’t have any sample of adjacent normal tissue of the patient. The actual significance of the described change can however be highlighted by other authors who might have samples of both normal and tumor tissue. The change can be a polymorphism but it can also be a silent mutation with a distinct impact on the protein structure. However, as suggested by the reviewer, in the revised manuscript, we did not retain the non-synonymous nucleotide change and further therefore counted only 10 PIK3R1 mutations: “PIK3R1 mutations were screened in exons 11 - 15 and were present in 10 (2.2%) of the 454 available samples (Additional Table 3). Seven cases of deletions of 3-nucleotide multiples were observed in exons 11 and 13 (in the area between nucleotides 1345-1368 and 1701-1743, respectively), 2 cases of duplications of 3-nucleotide multiples were observed in exon 13 (in the area between nucleotides 1650-1723) and 1 case of point mutations were observed in exon 15 (c.1925G>T). It is noteworthy that we found also c.1590G>A giving the AAG-->AAA (Lys) nucleotide substitution located in exon 13 that is probably a polymorphism with no amino acid change. PIK3R1 mutations were found in only 1 of the 151 PIK3CA-mutated cases and in 10 of the 297 PIK3CA wild-type cases.” And “Altogether, we observed PIK3CA and/or PIK3R1 and/or AKT1 mutations in 174/454 (38.3%) breast cancer tumors. Breast cancer subgroup analysis demonstrated mutation of at least one of the three genes with the highest frequency in HR+/ERBB2- tumors (133/289; 46.0%).” (page 11 and 12, line 16 and 4, respectively) The corresponding sentences in the Discussion were also changed: “PIK3CA mutations were detected in 33.0% of cases (exons 1, 2, 9, 20) and PIK3R1 mutations were detected in 2.2% of cases (exons 11, 12, 13, 15).” and “The highest mutational frequency for all of the genes assessed in this study (PIK3CA and/or PIK3R1 and/or AKT1) was observed in HR+/ERBB2- tumors (133/289; 46.0%).” (page 16 and 17, line 6 and 8, respectively) We also changed results concerning PIK3R1 mutations in the Tables 3 and in the Additional Tables 3 and 4.

4. p.10 paragraph 2. The sentence beginning “PIK3R1 mutations were found in only 1 of the 151…” should state that PIK3R1 mutations are mutually exclusive with PIK3CA mutations, rather than AKT1 mutations. The sentence was changed: “PIK3R1 mutations were found in only 1 of the 151 PIK3CA-mutated cases and in 10 of the 297 PIK3CA wild-type cases.” (page 11, line 22)

5. p.12 The statement beginning “On the other hand. The authors speculate about what altered gene expression of pathway components “might” mean for pathway activity, but they should explicitly note that their data does not in fact provide any actual evidence about the relative level of PI3-kinase pathway activity in these tumors.
As suggested by the reviewer, we have moderated the sentence concerning the relationship between “gene expression of pathway components” and “pathway activity”. We explained the pointed statement: “The 4 molecular subgroups of breast cancer therefore appeared to undergo distinct changes at the levels of mRNA expression of the genes involved in the PI3K/AKT pathway. These data would benefit from confirmation at protein level (both quantity and activity).” (page 13, line 9)

6. Table 1 and Table 2 duplicate a lot of data. One column in the table 2 was erased to make the table simpler and avoid data duplication.

7. In p.13 “survival analysis” paragraph and Additional Figure 1, it is not clear what comparison the p value refers to, since there are four lines on the plot. The p-value refers to general comparison of all 4 curves. We added a sentence to clarify this point: “Comparison of all four survival curves showed statistical differences with p=0.00046 (log-rank test for 4-level factor, Figure 2).” To clarify further the statistical comparison made, we changed the title of the Figure 2: “Figure 2. Survival curves of four patient groups according to PIK3R1 expression status and PIK3CA mutations.” (page 14, line 19)

8. On page 17, In the sentence “PIK3R1 underexpressing tumors were also prone to...” the word “cumulate” should be “accumulate.” In the following sentence, the claim that PIK3R1 underexpression is associated with increased signaling activation is unsupported and hypothetical. The authors should state that it “could be associated with increased signaling activation.” We wrote “accumulate” in the first sentence and we changed the following one as: “PIK3R1 underexpression is therefore associated with additional pathway deregulation and possibly also with increased signaling activation.” (page 18, line 23)

9. On page 18, paragraph 2, the authors should suggest that PIK3R1 should or could be explored as a predictive marker for trastuzumab resistance, etc., since there are currently no data to suggest that it can be used as such. Trastuzumab was mentioned in the paragraph as one of drugs that might show resistance in the presence of PIK3R1 decreased expression: “Finally, PIK3R1 underexpression (and PIK3CA mutation) could be used as predictors of resistance to treatment with ERBB2 inhibitors such as trastuzumab” (page 19, line 11)

10. On page 18, paragraph 3. The sentence beginning “The present study...” Again, the authors provide no evidence supporting an effect of PIK3R1 underexpression on PI3-kinase pathway activation and should simply state that they found an effect on survival. We adjusted the statement to follow the reviewer’s suggestion: “The present study showed that alterations in these two genes have a complementary impact on breast cancer patient survival.” (page 19, line 12)

Discretionary revisions:
1. p. 11 last paragraph. “Marked overexpression” of several genes is discussed, but the numbers reported only show the percentage of tumors with N>2.0 overexpression. The range of values indicates that some tumors have very high expression, which might be termed “marked.” It would be more interesting to see what proportion of tumors have such “outlier” very high expression. Such tumors are more likely to have chromosomal amplification of the genes in question.
We added a colon in the Table 1 to show percentage of tumors with very high expression \( N \geq 5 \) and we also commented on this results in the manuscript: “Markedly high expression that might be caused by gene amplification was observed only in low frequency (<4%) of tumors as shows the last colon in the Table 1.” (page 12, line26)

2. The data is all presented in tabular format. For ease of comprehension of the major findings, it might be good to also present the Table 2 data in graphical format, as box-and-whisker plots.

We tried graphical format, but it is less informative than a table in our study.

Level of interest:
An article whose findings are important to those with closely related research interests

Quality of written English:
Acceptable

Statistical review:
Yes, but I do not feel adequately qualified to assess the statistics.

Declaration of competing interests:
I declare that I have no competing interests.
Reviewer's report
Title:
PIK3R1 underexpression is an independent prognostic marker in breast cancer
Version: 1
Date: 7 August 2013
Reviewer: Katarina Ejeskär
Reviewer's report:
General
In this paper the authors have studied mutational status and expression levels of genes in the PI3K-pathway, with emphasis on PIK3CA and PIK3R1 in 458 well defined breast tumor samples. They can confirm the common mutations of PIK3CA, AKT1 and PIK3R1 from previous studies, and conclude that underexpression of PIK3R1 is common in metastatic tumors, contrary to PIK3CA-mutation that is a good prognostic marker. The finding is interesting; the sample size is good, methods used are good, and the English is acceptable:

-Minor Essential Revisions
- In Abstract, Results and Discussion: PIK3R1 and AKT1-mutations were more common in non-PIK3CA-mutated cases, however mutually exclusive is an incorrect term, since it did occur in PIK3CA-mutated cases, even if it was rare. Change in the text were this term was used.

As suggested by the reviewer, we have moderated the sentences concerning mutual exclusivity. We changed description of results: in abstract we wrote “PIK3R1 underexpression tended to mutual exclusivity with PIK3CA mutations (p=0.00097)” instead of “PIK3R1 underexpression was mutually exclusive with PIK3CA mutations (p=0.00097).”

In the results section: “PIK3R1 mutations were found in only 1 of the 151 PIK3CA-mutated cases and in 10 of the 297 PIK3CA wild-type cases.” instead of “PIK3R1 mutations were found in only 1 of the 151 PIK3CA-mutated cases and in 10 of the 297 PIK3CA wild-type cases and were therefore mutually exclusive with AKT1 mutations.” And “AKT1 mutations were found in only 1 of the 161 PIK3CA/PIK3R1-mutated cases and 14 of the 297 PIK3CA/PIK3R1 wild-type cases and tended therefore to mutual exclusivity with PI3K mutations (p=0.019).” instead of “AKT1 mutations were found in only 1 of the 161 PIK3CA/PIK3R1-mutated cases and 14 of the 297 PIK3CA/PIK3R1 wild-type cases and were therefore mutually exclusive with PI3K mutations (p=0.019).”

(page 3, 11 and 12, line 9, 26 and 3, respectively) Finally we changed in the discussion section: “We also found that PIK3R1 mutations tended to mutual exclusivity with PIK3CA and AKT1 mutations.” instead of “We also found that PIK3R1 mutations were mutually exclusive with PIK3CA and AKT1 mutations.”

(page 17, line 1)

-In Methods: Explanation is needed for the choice of the exons chosen to sequence, why not all exones were examined.

In the methods section we added a sentence: “Exons to be screened in the three genes were chosen following mutational frequency described at COSMIC: Catalogue Of Somatic
Mutations In Cancer (cancer.sanger.ac.uk/).” (page 7, line 24) The selected exons also show clear activating mutations confirmed by functional studies in vitro or in vivo.

- In Results: PIK3R1-mutation: AAG-AAA gives no amino acid change for sure, in the text it says probably. We changed the marked sentence. (page 11, line 21) (see also Reviewer 1; item “minor essential revisions” n°3)

- In Table 2, 3 and 4: It is not clear to what differences the p-value is indicating significance, change to make it clear. The p-values marked in the tables describe general comparison between all the subgroups in each group so the p-values cannot be more precisely associated to the data (NS when p>0.05).

- Table 3 and 4 would benefit to be fused into one single table, for less repetition of data. We prefer to keep the two tables apart because we tried a fused table and this obtained table was lengthy and less clear.

- Additional file 1: Figure 1: Important figure! It would be better as an original figure in the paper. We renamed the figure as Figure 2.

Discretionary Revisions
- In Introduction: Information is missing regarding other isoforms of catalytic subunit p110, p110beta and p110delta, that also could influence the PI3K activity. We added information on other PI3K encoding genes: “It is noteworthy that other PI3K subunit encoding genes (PIK3CB, PIK3CD, PIK3CG, PIK3R2, PIK3R3...) are altered with much lower frequency than PIK3CA and PIK3R1 [17].” (page 5, line 2)

- In Methods: 170 patients developed metastases. Write in numbers.
Level of interest:
An article of importance in its field
Quality of written English:
Acceptable
Statistical review:
No, the manuscript does not need to be seen by a statistician.
Declaration of competing interests:
'I declare that I have no competing interests'