Author's response to reviews

Title: Epidermal Growth Factor Receptor (EGFR) mutation analysis, gene expression profiling and EGFR protein expression in androgen-dependent prostate cancer

Authors:

Caterina Peraldo-Neia (caterina.peraldoneia@ircc.it)
Giorgia Migliardi (giorgia.migliardi@ircc.it)
Maurizia Mello-Grand (Maurizia.mellogrand@fondoedotempia.it)
Filippo Montemurro (filippo.montemurro@ircc.it)
Raffaella Segir (raffaella.segir@gmail.com)
Ymera Pignochino (ymera.pignochino@ircc.it)
Giuliana Cavalloni (giuliana.cavalloni@ircc.it)
Bruno Torchio (btorchio@mauriziano.it)
Luciano Mosso (lmosso@mauriziano.it)
Giovanna Chiorino (giovanna.chiorino@fondoedotempia.it)
Massimo Aglietta (massimo.aglietta@ircc.it)

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Author's response to reviews: see over
Dear Editor,

We are grateful for the reviewers' careful revision, because their questions and suggestions could increase the clarity and the quality of our paper. We resubmit the manuscript to your attention with the answer to reviewers' comments here below. All the changes in the text and tables are highlighted in gray.

We hope that our answers have addressed the reviewers' suggestions and that our paper will be acceptable for publication on BMC Cancer.
Reviewer: Samantha Larkin
Minor Essential Revisions

1) Sample size quite low, particularly in the gene profiling analysis
   a. Some confusion over sample size in the gene profiling actually. It is mentioned that 13 EGFR mutant samples were analysed using microarrays but in the figure (Fig 2) there are only 8 samples shown. What happened to the other 5? b. Again, qRT-PCR is based on 8 samples – what happened to the n=13 mentioned earlier?

According to the availability of fresh frozen tumour material, different subsets of the initial 100 patients selected for this analysis were studied. Immunohistochemistry and mutational analysis were performed in all the samples from 100 patients. Fifty patients had tumour samples stored at -80°C and were submitted to gene profiling. For one of these patients, two different tumour samples were available. Therefore, the total number of samples submitted to gene profiling was 51. Only 8 of 13 tumours, displaying EGFR mutations, were available for gene profiling. We have added clarifications in the “methods” section, page 4-5, hoping that this clarifies and addresses this point properly.

c. Discussion completely lacks any mention of the low sample size in the mutational analysis of EGFR+ and EGFR- tumours – this was based on a very small number of samples and there needs to be clear critique of this in the discussion and how that might affect the power of the work
   We have added a paragraph in the “discussion” section (page 13) acknowledging the limitations of our study.

3) Biochemical relapse definition is not very clear.
   The definition adopted to identify biochemical relapse and relative reference were added in the “methods” section, page 8 (Cronin et al. 2010).
4) No mention is made of how many histopathologists scored the Gleason of each radical specimen. This is important as the Gleason score can vary hugely between histopathologists. Was the same histo used for all samples? Was the Gleason verified by another histo?

Yes, the same histopathologist has defined the Gleason score.

a. Why were the Gleason groupings 4-6 and 7-9 chosen? No justification given for this division. b. Discussion of this should be included to show the limitations of any Gleason based analysis

The Gleason Score was studied both as a continuous variable and as a categorical variable. To establish categories, we used the classification proposed by Franiel et al (2009), in which Gleason ≤ 6 is considered low grade and Gleason ≥ 7 is considered high grade. This has been specified in the “methods” section, page 5.

While, as a continuous variable Gleason Score showed a weak association with biochemical relapse (HR for PSA progression = 1.37, p = 0.14), the high Gleason Score category showed a large increase in the risk of relapse which fulfilled the criterion for entry in the multivariate model. Therefore, we decided to use this classification.

5) It’s a shame that there is such limited follow up on the T3 staged tumours –only 9/26 have follow up – it would have been interesting to see how tumour stage correlated with some of your findings. Why did so many patients not complete follow up? It would be worth expanding on the reason behind the lack of follow up in those 32 patients

We acknowledge that lack of follow-up information for a substantial number of patients is a limitation of our study. However, the patients with complete information were all treated and followed-up at the same institution, which ensures completeness and consistency of the available information. We have retrieved additional follow-up information for 4 patients and now the data are complete for 59 patients in total.

We decided not to pursue additional follow-up information retrieval for patients treated at other Institutions to preserve the solidity of the data and to avoid inconsistencies.

Causes for missing follow-up data have been detailed in the results section, page 8.
6) Summary of clinical pathological characteristics needs amending – 26 were T3.
**Thank you, we have corrected this mistake.**

7) All figure legends need expanding to detail the experiments more precisely.
   a. Figure 1 should have some annotations – point out normal glandular staining and over expression in the tumour areas.
   **Thank you for your advice; we have expanded figure legends and added new images in figure 1 to point out the different pattern of EGFR expression in normal and tumour tissues.**

8) On the immunostaining, how was the 1% of tumour staining (was this the basal level?) quantified? And by whom?
   **We have re-scored the EGFR protein expression as described in the “methods” section, page 6.**
   The pathologists who reviewed the EGFR staining were Luciano Mosso and Bruno Torchio (LM and BT) and we have added this information in the “methods”, section page 6.

9) Mutational analysis – why is the association between EGFR protein expression and mutation status given a p-value of >0.05 whereas the other p-values in that sentence have a specific p-value? Be consistent.
   **Thank you, we have changed it.**

10) How were the Ct values analysed in the qRT-PCR gene profiling experiment. I feel that a brief summary of how the Ct values were manipulated and analysed should be included rather than there just being a column in the table stating ‘Log10Ratio’ and a reference.
    **The ‘Log10Ratio’ is defined in table 4 legend and is referred to microarray data.**
    The evaluation of amount of mRNA target by comparative cycle threshold (Ct) methods was now described in the “methods” section, page 7.

11) Some references missing (‘a PSA follow-up of 4 years is considered sufficient to declare a patient disease free’ should be referenced)
    **We acknowledge that “a follow up of 4 years” could be considered a somewhat arbitrary cut-off. We therefore agree that it should be erased. Instead, by**
considering the overall follow-up information in months, we could treat biochemical relapse as a time-dependent outcome and run Cox and Kaplan Meier analysis. Follow-up has been described by median and range values.

Reviewer: Kathleen Ignatoski

This paper would be strengthened if the following were included:

1) A citation and discussion on the paper by Shah, et al. (2006) Prostate 66(13):1437-44. This paper used TMA analysis to show that EGFR over expression correlated with androgen independence. 
We have added this citation in the “background” (page 3 reference 7) and in the “discussion” (page 11) section as suggested.

2) A pathologist's review of the staining for EGFR (or it cited that a pathologist did review and score the IHCs).
The pathologists who reviewed the EGFR staining were Luciano Mosso (LM) and Bruno Torchio (BT) we have added this information in the “methods” section, page 6.

3) Western blot analysis of the genes that were deregulated in the microarray analysis. Quantitative RT-PCR analysis was performed in order to confirm microarray data. In our case, it is not possible to validate the expression of selected genes by Western Blot because we do not have frozen tissue from which extract proteins.
Reviewer: Srikala Sridhar

Major Revisions.
1. The paper should include references to the trials that have been done exploring EGFR inhibitor in prostate cancer. (Gefitinib, Omnitarg, Lapatinib), as this highlights the disconnect between preclinical and clinical role of EGFR inhibitors, and the need to determine if a subset of pts are more likely to respond.

We have added in the “discussion” section, page 11, some references (16, 26-29) of clinical trials that explore EGFR inhibitors in PCs.

- Minor Essential Revisions

Introduction:
Second paragraph, Line 1.should be changed to ...molecular mechanisms responsible ‘for’ PC

Thank you, we have corrected it.

Line 2, Line 14: “Several evidences” and "clinical evidences" should be changed
Line 9: should be changed to ....types and to its role "as a” drug target

We have re-edited some sentences in the “background” section.

Materials and Methods
Line 4: forzen should be changed to frozen

Thank you, we have corrected it.

Results;
Second paragraph: Line 2...should be changed from cytoplasmatic to Cytoplasmic

Thank you, we have corrected it.

Gene Profiling
Paragraph 1: should be changed to: "analysis on frozen tissue was performed"

Thank you, we have changed it.

Discussion
paragraph 1: line 3: should be changed to: leading to the search for...
Line 3/4: suggest reword: The role of EGFR in PC progression......progression of PC
Line 5: arising should be rising
Paragraph 4: sustains should be changed to sustains

Since other reviewers asked to edit the “discussion”, we have changed this section.

- Discretionary Revisions
These are recommendations for improvement which the author can choose to ignore. For example clarifications, data that would be useful but not essential.
A table showing the commonly recognized EGFR mutations may help for clarify.

We have prepared a summarizing table of the thirteen mutations found, specifying which were already described in literature (Table 2).
Reviewer: Rosita Winkler

Methods
Immunohistochemistry was used to detect EGFR protein, but the positive and negative controls are not mentioned.

We have provided this information in the “results” section (page 8). A new figure 1 was inserted.

The method used to amplify the mRNA for the microarray should be clarified – do they obtain 5 µg of mRNA from 5 µg of total RNA but how this was verified is not mentioned.

We have clarified this point in the “methods” section, page 6.

As mentioned below the discussion section is too long and some results are too preliminary to allow a definitive conclusion.

Thank you for the advice; we have re-edited the “discussion” section.

The conclusion of the abstract should be more moderate:

Thank you for the suggestion; we have modified the conclusions.

The discussion section writing needs some editing.

We have re-edited the “discussion” section.

Major compulsory revision
The manuscript contains weaknesses that should be corrected before publication.

1. Immunohistochemistry results are not convincing as presented. Figure 1 presents two magnifications of the same section. Several sections should be shown displaying different levels of positivity, and the cancerous and healthy cells should be pointed out. The positive and negative controls are not stated.
Thank you for the suggestion; we have provided a new figure 1 in which a negative control (lobular breast carcinoma) a positive control (colon cancer), and different scores of EGFR expression in normal and tumour prostate tissues were shown.

Considering a tumour as “overexpressing” if only 1% of the cancerous cells are EGFR positive leads to over estimation of EGFR+ tumours. The literature reports different levels of EGFR expression in prostate cancers, but the lower threshold for overexpression is around 15% positive cells. What the authors mean by “positivity” or “overexpression” should be clarified.

Indeed, healthy basal cells of the adult prostate express EGFR, so what is the cellular population to which the tumour is compared?

How the number or proportion of positive cells is estimated. How many regions of the section have been examined?

Does “overexpression” mean that the protein is detected or that the quantity is increased when compared with the receptor expressed in healthy cells?

*We have re-scored the EGFR expression as described in the “methods” section, page 6. About 100 cells were counted in three different fields.*

2. It is surprising that there was no difference between the genes expressed in EGFR positive and negative tumours. This could result from the over estimation of overexpressing tumours. Another possibility would be that the receptor is inactive. The authors should include an experiment showing the activation status of the receptor such as for instance phosphorylated EGFR or an activated target.

*Unfortunately, we have performed p-MAPK and p-Akt evaluation by IHC on Glyo-fixx fixed tissues, but we are not able to detect them, maybe because the phosphorylation of these proteins was not preserved with this kind of fixative.*

The number of tumours is too small to allow for a valid statistically significant comparison between the genes expressed in mutated EGFR positive and negative tumours. To investigate the importance of the mutation the genes expressed differentially between EGFR positive tumours with or without the mutation should be compared.

*We have 13 mutated samples, for only 8 gene expression analysis is available. This number is too small to give a strong message. We acknowledge that these are preliminary data based on a small number of cases.*
Thank you for your advice; we have performed this kind of analysis, a 79 gene signature separated the $\text{EGFR}_{\text{high}}$/mutated from $\text{EGFR}_{\text{high}}$/Wt samples. We have added this information in the “results” section, page 10. The functional significance of mutation in tumours not expressing the receptor is not clear. All tumour samples expressed basal level of EGFR ($\text{EGFR}_{\text{low}}$).

3. Results in Figure 3 are difficult to understand. The legend does not indicate what the number on the Y axis mean nor to what P51, G65 etc are referring to….

These signs are referring to patients for which microarray analysis was available. In order to clarify this concept, we have added this information in additional file 1, table S1.

The LogRatio definition is the logarithm (base 10) of the ratio between prostate cancer sample and prostate reference expression values, as added in table 4 legend.

I think that the number of tumours is too small for a valid comparison. The authors should include additional samples to be able to obtain conclusive results. If this is not possible, I suggest to keep these results for another publication. Indeed, what conclusions can be drawn from the expression of FOXC1 – negative in EGFR and in 2/3 EGFR+ cases? And from PSA negative in 3/5 EGFR- and in all 3 EGFR + cases?

We agree with you, since the small number of mutated samples, no conclusive results could be given from our findings. At this moment, we do not have the possibility to increase the cohort of patients. Nonetheless, EGFR overexpression correlated with an androgen-independent status (Shah et al), FOXC1 is expressed in androgen independent xenografts (van der Heul-Nieuwenhuijzen et al), and KLK3 (PSA) is higher expressed in androgen dependent PCs (Zhao et al). On these bases, we could suppose that FOXC1 will be expressed only in $\text{EGFR}_{\text{high}}$ samples while PSA in $\text{EGFR}_{\text{low}}$ respectively, as seems to emerge from the trend towards observed in our preliminary data.

4. qRT-PCR results are not significantly different among EGFR+ and EGFR- cases. This experiment must be repeated on a higher number of tumours.

We agree with you, but now we are unable to recruit new patients.
Minor essential revision

1. The ground on which the tumours are considered as androgen dependent has to be indicated.

We have changed the title from “androgen-dependent” to “primary”.

2. The description of the mechanism of action of drugs targeting EGFR in the 2nd paragraph of the introduction should be included in the discussion section. The discussion is too long. The language in this section should be corrected.

Thank you for the advice; we have corrected the language in discussion section and modified several paragraphs.

3. Has the DNA from healthy cells of the same patients been sequenced?

Yes, we have sequenced the EGFR TK domain of the normal counterpart of mutated samples; all normal tissues are WT, so we can conclude that mutations found are somatic. We have added this information in the “results” section, page 9.

4. Since the extracellular domain of the EGFR was not sequenced paragraph 4 of the discussion section should be omitted or re-written.

We have decided to omit this paragraph.

Eight of the 13 tumours bearing EGFR mutations were negative for EGFR expression. The significance of this is not discussed

All tumour samples expressed basal level of EGFR (EGFR$_{\text{low}}$).

5. The novel mutations are the real interesting part of the manuscript. I would suggest to include in the discussion section a comment on the predictable consequences of the novel mutations on EGFR activity.

Thank you for the suggestion; it could be very interesting to know how these mutations affect the EGFR pathway activation. A great amount of work is requested
to answer to this question. We think that we could keep these results for another publication.

6. Discussion paragraph 6 – reporting the results of Cai et al on the functional consequence of the E804G mutation “most active and significant somatic missense mutation…” but in comparison with the other tested mutations. It should be clearly indicated

We have clarified this point in the “discussion” section, page 12.

7. Discussion end of paragraph 7: “An evaluation of EGFR…” should be deleted since this claim is not supported by enough data.

8. The end of the discussion section (paragraph 8 and following) is too long. It would be more correct to say that the expression data are too preliminary are the significance of the results needs additional data.

We have re-edited the “discussion” section.

Discretionary revisions

1. Could the region coding for the extracellular domain, which could identify the EGFRvIII be sequenced?

Thank you for your suggestion but a great amount of work is requested to answer to this question.