Author's response to reviews

Title: Candidate pathways and genes for prostate cancer: a meta-analysis of gene expression data

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Responses to the Reviewer’s comments

We are thankful to the reviewers for their helpful comments and suggestions. Here we provide point by point responses to the reviewer’s critics and comments. All additions to the manuscript are shown in red.

Reviewer: Alvin Liu

“I think Gorlov et al. did a nice job in analyzing a large number of datasets in prostate cancer. With regard to integrin expression in prostate cancer, for example, ITGB4 is expressed by basal cells and not by luminal cells. In tumor, there are no basal cells, hence no ITGB4 expression. Luminal cells are the normal counterpart of cancer epithelial cells, so technically ITGB4 is not downregulated in the transition to cancer when one is looking at the cell level. At the tissue level, that’s true. Same for the basal ITGA2, ITGA3, and ITGA6. Similarly, stromal genes (ITGA1/PELO, CNTN1, CNN1, etc.) may be differentially expressed between tumor-associated stromal cells and their normal counterpart.”

We agree with the Reviewer. We analyzed gene expression at the tissue level. Both the difference in the cell type composition and the difference in the gene expression affect our results. Though the researchers routinely try to choose sites similar in terms of epithelial / stroma composition, the grossly dissected samples are certainly a mix of different cell types and may differ by cell composition. This can be even more complicated because microarray assesses the gene expression on RNA level and its result can not be completely comparable to the results obtained by immunohistochemistry because both posttranslational modification of proteins and multiple splice variants can affect the correlation between the results on mRNA and protein levels. We tried to be cautious with the interpretation of our results in the first version of the paper and we further stress it in the revised manuscript (page 18).
“A study of the available laser-captured datasets was attempted. And the authors mentioned that they will analyze the stromal and epithelial compartments separately (how? Newer or to-be-generated datasets).”

This work is in progress. Currently we have gene expression data from low-grade (sum Gleason score # 6) prostate tumors. We have also gene expression data from 3 samples of bone metastases, and we will obtain the data for 3 more samples. Further, we will assess gene expression from 6 high-grade (sum Gleason score # 7) primary prostate tumors. We use laser captured microdissection to isolate tumor and stromal cells. Our preliminary analysis supports the involvement of cell adhesion in prostate cancer development and also suggests that both epithelia and stromal component may play a role in modulation of cell adhesion.

“Also, many of the earlier datasets were based on in-house cDNA arrays, which were sub-optimal compared to those of Affymetrix (probeset identities). I think these points need to be discussed since the correlation between different datasets is rather poor, and certain genes were not represented in certain datasets.”

We understand this concern. In our analysis only about 20% of the genes were presented in all studies with the majority of them #60% being presented in 3-5 studies. About 5% were assessed in a single study. This results from the fact that different studies use different platforms and that a considerable proportion of the genes in each study (up to 20%) was excluded from the analysis because of low call rate.

It is also true that the correlation between different studies is rather poor: the fact that motivated us to conduct a meta-analysis. We included the used platforms into the Supplementary Table 1.

“With regard to quantitative over qualitative changes in localized-to-extraprostatic transition there are data that showed gene expression (qualitative) differences between the different types of prostate cancer. So I would avoid stating that claim (p.14, second paragraph).”

We agree with this comment and deleted the paragraph.

“I wonder if the authors found a luminal vs. basal signature as in breast cancer.”

Hierarchical cluster analysis is usually used to identify a distinct molecular type of cancer. In our case we were not able to conduct such an analysis because for majority of the datasets the raw expression data were not available. We certainly will consider this analysis for our emerging in-house gene expression data.

Reviewer: Wei D Zhong

“For prostate cancer, there has been no sufficient evidence to "the hypothesis of collagen", which need the authors’ further investigation in order to better explain.”
The Reviewer is concerned by the fact that there is no proof for the collagen hypothesis. It is true and this is why it is only a hypothesis. Though our results allow proposing the hypothesis, further research needs to be conducted to confirm or reject it.

“Microarray technology is an effective way to assess certain genome. The main advantages of it are accuracy and repeatability. I suggest that the control analysis should be combined with Meta analysis.”

We are not sure that we completely comprehend the suggestion. If the reviewer meant that control individuals should be included in the meta-analysis, they are included: all studies used for meta-analysis comprise cases and controls: normal prostate vs. localized for the NP>nMPC transition and localized vs. metastatic – nMPC>MPC transition.

“As expected in the application of clinical medicine, I suggest that the author should add the clinical and pathological information of patients in this study. Although the number of patients are small, but can be used for a trend investigation.”

We agree that the clinical description is important. Because we do not have gene expression data for individual patients we are not sure how individual clinical information can be useful. Nevertheless for all datasets used in the analysis we provide references to the original publication so it is possible to access description of samples provided by the authors.

“In the discussion, the authors should explain the significance of down-regulated genes with the combination of “the hypothesis of collagen”, not only focus on the number of up-regulated or down-regulated genes.”

It is difficult to explain the significance of all down-regulated genes. In this study we focus on the downregulation of the cell adhesion molecules and their ligands because they are involved in the same biological function.

Reviewer: Ajay Singh

“It would be better to discuss in some detail about the Kyoto Encyclopedia of Genes and Genomes (KEGG) and how the analysis of the distribution of genes was performed for the ease of understanding of the readers.”

We included the description of the KEGG pathways. We also outlined statistical approach for pathway clustering (page 8).

“The differentially-expressed genes (21 for the NP-nMPC and 17 for the nMPC-MPC transition) clustered into focal adhesion pathways needs some discussion. Authors should at least show (in a table) how their gene products are involved in adhesion-associated properties.”

We added the schematic of focal adhesion pathway to the supplementary materials and put a reference to it to the text (page 9).
“Furthermore, it can be discussed whether there was a similar trend (as it seems) for the change in expression (up or down-regulation) during both the transitions (NP-nMPC and nMPC-MPC) and how would that affect the progression.”

The discussion of the overlap between NP>nMPC and nMPC>MPC transitions was added to the manuscript (page 10).

“Tables 2 and 3 list the pathways enriched by differentially-expressed genes, but the status of those pathways (induced or repressed) is not clear. While it is clear from the text that the integrin signaling was repressed, it is not apparent for other important pathways (EGF, PDGF, VEGF, Chemokines signaling, etc.) of interest.”

We do not think that it is currently possible to reliably predict the effect of several differentially expressed genes on the whole pathway of function. Indeed if we have several genes differentially expressed in a given pathway with some of them up- and others down-regulated, and those genes are connected by intricate net of interactions, it is practically impossible to predict the final effect on the function without experimental modulation of the gene expressions and assessing the function.

For cell adhesion the situation is different. We found that the expression of the cell adhesion molecules is grossly suppressed in prostate tumorigenesis. Furthermore the expression of the ligands of the cell adhesion molecules was also suppressed. Therefore it seems reasonable to suggest that the cell adhesion function as a whole is suppressed during prostate tumorigenesis.

“The heading ‘the downregulation of integrin ligands” in results section should also include ‘integrins’ (the downregulation of integrins and integrin ligands).”

Done.

“It is not clear how the integrin suppression (by somatic mutation or epigenetic silencing) will avoid apoptosis (as mentioned in page 16, continuing paragraph from page 15 and a basis for the proposed collagen hypothesis). In fact, both the suppression of integrin ligands or integrin itself will abrogate integrin-mediated downstream survival signaling and thus enhance apoptosis. Whereas the suppression of integrin ligands/integrins is obvious and thus the initial hypothesis seems to be valid, it is important to examine and propose some compensatory survival mechanisms (mediated by growth factors etc..) to explain the sustained growth of prostate cancer cells.”

We included a brief description of possible mechanisms by with the integrin induced cell death can be overcome (see page 15).

“A schematic for the “collagen hypothesis” delineating the proposed cellular and molecular events would be an excellent addition in the manuscript.”

We agree that depicting the collagen hypothesis could be useful. However, little is known about possible molecular mechanisms linking cell adhesion, survival
and proliferation. Depiction of the possible molecular mechanism might be, therefore, misleading in this situation.

Reviewer: Ronglai Shen

“The major conclusion of this study is this collagen hypothesis in prostate tumorigenesis based on the meta-analysis findings. However, whether the meta-analysis strategy employed here has sound basis and valid ground is unclear at best in the following aspects: 1) despite the vast literature on meta-analysis in prostate cancer and other types of cancer as well, there was virtually no mention about any one of them in the background. Neither is there discussion on how their results compare to other meta-analysis study findings. “

We agree with this comment and augmented the manuscript with a brief description of the published studies on the meta-analysis of the gene expression data. We also augmented the revised manuscript with a paragraph describing some published approaches to meta-analysis of the gene expression data and results of the meta-analysis (page 3).

We included a discussion of the published meta-analyses on the gene profiling in prostate tumorigenesis (page 15).

“Despite much advancement in meta-analysis methods in gene expression data setting, the authors used an outdated approach (Stouffer’s method) where its applicability to microarray data is basically unclear.”

We used a method of meta-analysis that was suggested about thirty years ago. Since then other more effective methods including those developed by the Reviewer were proposed. Our choice was dictated by the data available for meta-analysis. For the majority of the datasets raw gene expression data were available. However, t-statistics and P-values were available for each probe in all studies. Therefore, Z-score-based approach was a natural choice for the meta-analysis in this situation. We hope that the reviewer will agree that the latest published does not necessarily mean the most appropriate in any situation. As for the date of publication, just a month ago a paper by Ochsner et al. (2009) was published in Cancer Research, where the authors used a very similar Z-score based approach to meta-analysis.

We agree that the justification of the choice of the meta-analysis method was not presented. We corrected this in the revised version (page 7).

Reviewer: Phillip G Febbo

“1) The authors refer to statistical ‘true discoveries” on page 6 but perform no validation to demonstrate that their statistical method actually is valid. I.e., there is no quantitative PCR or immunohistochemistry in an independent dataset that confirms the consistent finding and demonstrates that the observed genes that represent “true discoveries” are differentially expressed.”

The Reviewer correctly noted that we used “true discoveries” in pure statistical
sense. Nevertheless, the usage of the term “true discovery” was probably misleading. We decided to change true and false discoveries to the true and false positives.

It is practically impossible to experimentally validate thousands of statistically significant genes detected by the meta-analysis. However it certainly should and will be done when we narrow the list to the few candidate genes.

“2) After ranking genes based upon the P value, the authors used “top-ranked genes to analyze their clustering, pathway, molecular, and cellular functions”. If the authors are confident that their meta-analysis identifies “true discoveries” then why is it not best to use all genes?”

Not all genes differentially expressed in prostate tumorigenesis are directly related to cancer development. Indeed, the genes in the human genome are connected in the complex net of interactions, so the modulation of the expression of the limited number of causal genes is likely to cause a ripple effect – modulation of the expression of multiple downstream targets. It is reasonable to suggest that the statistical evidence for causal genes on average will be stronger compared to the ripple effect genes. Therefore we limit our analysis to the top genes with strongest statistical evidence for their differential expression during prostate tumorigenesis.

“3) In the supplemental materials, they tested multiple cut offs and used their functional annotation to determine the number of genes resulting in the highest number of discovered functions. They then go on to discuss the relevance of the functions and pathways identified. While their attempt to avoid arbitrary cut-offs is to be lauded, by testing the same data to identify the number of genes implicating the largest number of functions they detract from the validity of their findings. Ideally, the number of genes would be established in a training analysis the optimized parameters of which are used in an independent analysis.”

We agree that it would be better to have a more formal approach to decide on the number genes that should be included in functional annotation analysis. However, subdividing available data in training and validation sets may lead to lose of statistical power in validation set. We understand that our approach for deciding how many genes to include in functional annotation is not perfect but it is the best of what we can do now.

“4) The decreased expression of the focal adhesion pathway in the transition from, normal prostate to local prostate cancer can be explained by the decreased, percentage of stroma seen in tumor samples compared to local prostate cancer., This has been well described and as only one of the datasets analyzed was, microdissected, the findings can all be attributed to tissue composition rather, than biology. In fact, the authors state that the observed changes are not, observed when differences in microdissected samples are looked at specifically, on page 11. They propose that the observed changes are occurring within the, fibroblasts. Without confirmation in microdissected samples or independent, analysis that identifies the same changes while accounting for tissue composition, differences, the value of these findings is unclear. The same
is true for the transition from local to metastatic as there is often less stromal tissue with the metastatic samples that have been analyzed by microarray when compared to the local cancers. This is my major concern with the paper.”

We agree with the reviewer that this is one of the major concerns of the study. We tried to further address this concern by comparing available gene expression profiling from 4 samples of the benign prostatic epithelium hyperplasia and benign prostatic hyperplasia stroma from the same individuals (Tomlins, 2007 #8). We compared the expression of 69 cell adhesion molecules: cadherins, integrins, selectins, ICAMs and the genes involved in the formation of tight cell junctions. The list of the cell adhesion genes is included in supplementary materials. We used the adhesion genes from two cell adhesion databases {Sadanandam, 2008 #274; Li, 2009 #273}.

Out of 69 cell adhesion genes analyzed in this study 63 showed no difference in the expression level between epithelial and stromal cells (P-value of 0.05 and no correction for multiple testing). Out of 6 differentially expressed genes 3 genes: CDH1, ITGB5 and TJP3 were down- and 3: ICAM1, ITGA8 and ITGB5 were up-regulated in stroma compared to epithelial.

When we looked at the expression of the same 69 genes in the transition from normal prostate to localized prostate cancer we found 30 differentially expressed genes: 23 were down and 7 up-regulated. Four genes differentially expressed between stromal and epithelial cells were also differentially expressed in NP>nMPC transition: CDH1 was up- and TJP3, ICAM1 and ITGA8 were down-regulated. Therefore we found that for majority of cell adhesion genes there were no significant differences in the expression between stromal and epithelial cells. Furthermore, differentially expressed genes were not biased in terms direction: the same number of genes were up and down-regulated. These results suggest that epithelium vs. stroma differences in the gene expression are unlikely to explain the observed regular suppression of cell adhesion genes in prostate tumorigenesis.

We appended the revised version of the manuscript by the stroma versus epithelium analysis (page 18).

“Minor Essential Revisions

1) The authors do not state the statistic used to generate a P value for their initial assessment of overlap. Because they refer to this as a “liberal P value of 0.05” it is not clear if their statistical measure takes into account multiple testings and is consistent with methods proposed for microarray data by Tibshirani et al and/or if the measure takes into account gene variation as well as fold change. It is important to use measures that account for variation as without such accounting analysis can be significantly impacted by spikes and/or outlying expression of genes in single samples.”

The reason to select this threshold was that different studies used different platforms. In this situation the number of the genes assessed in both studies can be relatively low, especially after excluding probes with absent call and ambiguous hybridization. Therefore we chose this threshold to ensure that we
would have enough number of common genes for statistical analysis.

“2) The authors rank genes based upon a P value that represents effectively a sum of a gene’s Z score across all datasets divided by square root of the number of tests (k). It is not clear from the methods what “tests” were used for k.”

We clarified that (page 8).

“3) There seems to be little difference between 500, 700, 1000, and 2000 genes with respect to the number of functions and pathways implicated. Is there significant overlap in the implicated functions and pathways? The impact of number of genes included on analysis on the results has to be described in detail.”

We included an additional description of the results of the functional annotation in supplementary materials. In brief, the results were remarkably stable for the top functions and pathways. As for the functions and pathways located down the list there was a significant motility in terms of rank, with some of them going up and others going down, as the number of number of the genes included in functional annotation increases.

“4) In the first part of the results – the authors describe more methods under “proof of principle”. The analysis should be presented in the methods and the results here. In addition, they report only a single “null” testing of the lowest 500 genes. The more appropriate and more common approach is to perform multiple tests of randomly chosen genes and to use the distribution of results from usually 1000 randomly aggregated genes to the experimental group of 500 genes. It is insufficient to test to a single group.”

The functional annotation is based on the idea that when genes are randomly selected from the human genome the probability that the gene will be assigned to a given pathway is proportional to the number of the genes in the pathway. If the genes fall to a specific pathway more often than one can expect it suggests that the pathway (biological function) is associated with the phenotype under study. Based on this consideration we believe that we do not need the “proof of the principle”, so we deleted it.

“5) The analysis of the overlap between NP-nMPC and those between nMPC and MPC assumes that all 17,859 genes are equal with independent expression and it is clear from the corpus of microarray work this is not the case. The authors should only use genes from the 17,859 matched for expression value and expression variation before they can argue that the overlap is greater than expected by chance alone.”

The reviewer suggests that because the genes are not independent in terms of the expression we can not treat individual genes as independent statistical event. In other words because of the internal structure of the gene expression data the real number of independently expressed units is less than 17,859. We completely agree with this in principle. However, it is really difficult to provide a reasonable estimate of the degree of freedom in this case. To do this we need to identify the
correlation matrix. It is not obvious whether we should use the correlation matrix for normal tissues only or tumors. It is not obvious either how to define the threshold for correlated genes.

Because the estimates of the expected number of overlapping genes is not really crucial for the study we decided not to change this part but simply indicate that the approach we used is an idealization and the real number of independent units is likely to be lower. We believe, however, that it is reasonable to suggest that after correction for internal correlations both 500 top genes and 17,859 will be reduced by approximately the same factor, and in this case the results of the analysis may be similar to that based on the used idealization.

“Minor:
1. Typo in Supplemental materials page second page “expecte””

Corrected.