Title: Cyclin D1, Id1 and EMT in Breast cancer

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

Please find attached the revised manuscript “Cyclin D1, Id1 and EMT in breast cancer” (MS: 2115140737536846). Below is the reviewer’s report along with a point-by-point response to each of the concerns raised. Thank you kindly for the opportunity to resubmit our manuscript to BMC Cancer.

Kind regards,

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Reviewer's report
Title: Cyclin D1, Id1 and EMT in Breast cancer

Version: 1 Date: 23 May 2011

Reviewer: Ingeborg Tinhofer

Reviewer's report:
In the study of Tobin et al the authors focused their experiments on the crosstalk between cyclin D1 and Id1 and their role in the acquisition of an EMT phenotype with increased migratory potential by breast cancer cells. The authors used two cell lines models in which they overexpressed or silenced Cyclin D1 and Id1 and subsequently performed migration assays. In addition, they performed a meta-analysis of six Affymetrix gene expression data sets including a total of 1107 primary breast cancers. Two further gene expression datasets were used for correlation of Cyclin D1, Id1 and EMT markers with breast cancer subtypes. Overall, there are several major concerns regarding the experimental settings and interpretation of data of this manuscript:

Major compulsory revisions:

1) When presenting cell migration the authors should give the absolute numbers of migrated cells instead of the fold-change values in order to get an idea on the extent of Cyclin D1 siRNA-induced migration.

This change has been made, all migration figures now show total numbers of migrated cells rather than fold change levels. All relevant text in main body of the manuscript has been adjusted to reflect this.

2) The authors mention but do not show that the basal ID1 expression levels are very low in ZR75-2 cells. If Id1 expression is suppressed by high Cyclin
D1 expression one would expect low basal levels in MDA-MB-231 cells as well. The authors should discuss why such correlation was not observed in their cell models.

In addition to the interaction we have demonstrated between cyclin D1 and Id1, other regulators of Id1 have been previously identified. TGF-beta [1], KLF17 [2] and Src [3] are all known to interact and influence Id1 expression. Thus, levels of Id1 in ZR75-1 and MDA-MB-231 cells may reflect interactions with transcriptional regulators other than cyclin D1. To directly address this hypothesis, we examined TGF-beta (a known inducer of Id1 [1]) gene expression in a range of breast cancer cell lines and noted high levels in MDA-MB-231 cells relative to ZR75-1 cells. However, none of the aforementioned transcripts (TGF-beta, KLF17 or Src) were altered in our expression array data in response to cyclin D1 silencing and are hence unlikely to contribute to our cyclin D1-Id1-EMT data.

This discussion point had been added to the manuscript along with a new supplemental figure displaying the TGF-beta data, with MDA-MB-231s and ZR75-1 cell lines highlighted for clarity.
3) In order to be able to generalize from their experimental data on the interference of Cyclin D1/Id1 and cell migration the authors should include a larger number of breast cancer cell lines. Overall, they present only results from two cell lines models which seem to significantly differ in the regulatory circuit between Cyclin D1 and Id1.

In our experimental model we have shown that silencing cyclin D1 in MDA-MB-231 and ZR75-1 cells increases their migration, and in the case of the former, does so not only through enhanced Id1 expression, but also by increasing EMT markers. Indeed cyclin D1 was unable to influence migration in the absence of Slug (Supplemental Fig. 2).

These data demonstrate a complex relationship between cyclin D1, Id1, EMT and cell migration in MDA-MB-231 cells, and as such, generalizing our data to be based solely on cyclin D1/Id1 and cell migration would both ignore our experimental paradigm and omit the vital role of an enhanced EMT phenotype in promoting this migration. As such, we have not applied our results in a broad sense to many breast cancer cells lines and state in our discussion- “It is important to note that here we are only proposing this mechanism in MDA-MB-231 cells and in a distinct subset of representative breast tumours.”.

However, we have aimed to take on board reviewers comment and to provide more information regarding possible general inferences that could be drawn from our manuscript. Our final figure (Fig. 5 A) points towards the cyclin D1, Id1 and EMT interaction occurring in the subset of breast cancer cell lines which fall into the basal B subgroup. If we wanted to infer that this group of cell lines respond to cyclin D1 silencing with increased Id1, an enhanced EMT phenotype and increased migration we would compare them to the luminal and basal A subgroups. The number of samples required to have 90% statistical power of observing even a 1.3 fold change in migration (between these groups) with a standard deviation of 0.2, and 5% error is 9 cell lines in each group i.e. 9 basal B cell lines vs 9 luminal or basal A. As this would entail a huge financial outlay not currently possible (each cell line would require validation, migration assays, microarray, qPCR and protein analysis), we made use of the Fig. 5A cell line data to draw tentative conclusions.

Fig. 5 A shows the ZR75-1 cell line clustering as part of the luminal subgroup. As Id1 expression was not present in ZR75-1 cells we would not expect it to cluster with MDA-MB-231s, and thus is in keeping with our hypothesis. Also present in the same luminal subgroup are the MCF7 and T47D cell lines. We examined the effect of cyclin D1 silencing on the migratory properties of both MCF7 and T47D cell lines by boyden chamber assay (n=3 for both cell lines) and found no significant migratory increase despite effective cyclin D1 silencing (see new figure attached in this document). Conversely, we have previously published data showing an increase in MDA-MB-435 cell migration following cyclin D1 silencing [4]. This cell line is part of the basal B subgroup and clusters with MDA-MB-231 cells. Taken together these data show that two additional cell lines of a luminal subtype (MCF7 and T47D) do not behave similarly to two cell lines of a basal
B subtype with respect to cyclin D1 silencing and induction of cell migration, when chosen based on ID1 and EMT gene expression.

Whilst these results are in keeping with our central cyclin D1-Id1-EMT hypothesis, no statistical inferences can be drawn and as noted previously, extensive analysis of at least 18 cell lines would be required to do so. We therefore respectfully request not to include this data in the main manuscript, and keep our analysis to the fully characterised MDA-MB-231 cell line. The new MCF7 and T47D migration figures and western blots have been attached at the end of this document.

4) The differences in the mRNA expression levels of EMT markers SNAI2, CDH11 and TWIST1 after Cyclin D1 downregulation are very small.

We have addressed this point in the discussion,

“We note that these figures are more meaningful when taken in the context of the most increased gene in our expression array, which was only up-regulated 1.8 fold (Lehn et. al). As may be expected from treatment with siRNA, many more genes were down-regulated in the array analysis than up-regulated, again highlighting the importance of the increases in our mesenchymal markers. It is likely that all of these factors work in concert to promote a migratory and EMT-like phenotype, and that small gains in expression of a number of EMT genes can contribute to a greater overall effect.”

Only one time point after transfection has been analyzed. What is the kinetic of their upregulation? Is the selected time point the maximum of upregulation which was observed?

In our previous publication on this subject where we made the original migratory observation (i.e. that migration of MDA-MB-231 cells increases following cyclin D1 silencing [4]), we extensively analysed how migration changes over time following cyclin D1 silencing. We have thus identified the optimum timepoint at which these cells displayed the greatest levels of migration. This timepoint has been employed throughout all of our experimental protocols for this paper, including western blots, migration assays, microarray, qPCR and ChIP analysis.

As such, it is only relevant in the context of this manuscript to examine expression of EMT gene mRNA expression at this timepoint. To alter it would invalidate both our previous publication and the numerous aspects of this manuscript.

Although stated otherwise, only results from one cell line is presented in Figure 2A. Which one? What about the other cell line?

We apologise for an unclear legend to Fig. 2, it was not our intention to give the impression we have run a microarray on more than one cell line. Thank
you for drawing this to our attention, we have made appropriate changes to the figure legend. The data refer only the MDA-MB-231 cell line.

5) Although significant differences in recurrence-free survival of tumor patients with different Cyclin D1 and Id1 expression levels could be observed, these differences did not depend on the expression levels. For examples, the survival curves from patients the lowest and highest Cyclin D1 expression (figure 3 A) overlap during the first 5-years of follow-up and only split thereafter.

In order to gain a greater understanding of our data we examined all of our genes of interest individually in a Kaplan-meier analysis. This data is presented in Fig. 3 and Supplemental Fig. 1. It is not always clear the most appropriate way to split data for high expression vs low expression analysis, however, given the vast range of expression values and large numbers of patients, we separated gene expression into quartiles. The results we noted for this figure were that high expression of cyclin D1 (Fig. 3 A) and low expression of Id1 (Fig. 3 B) were associated with reduced recurrence free survival in our cohort. We make no claim regarding a concentration-dependent response to expression of these genes and RFS regarding this figure, and use a log-rank test as a statistical measure of this conclusion.

Moreover, it is in fact the CCND1 low vs. high curve separation (and not the intermediate groups) that makes Kaplan-meier significant and removal of the intermediate groups improves the p-value of CCND1 in all patients to 0.045 and further strengthens the statistical significance of ID1. However, for the sake of transparency in our results we wanted to present all data and not just the highest and lowest quartiles, even though we believe these to be the important groups.

On a more general note, the data we present has at least 20 years patient follow-up. This length of follow-up allows for improved analysis and provides far greater information than a dataset with only 5 years follow-up information. In our dataset, a difference exists in RFS for patients with high CCND1 and low ID1 expression, the fact that this difference only becomes apparent after 5 years, only serves to highlight the importance of employing datasets with long-term follow-up. These curves are very typical of Kaplan-meier analysis. Survival curves cannot always separate immediately from time of cancer diagnosis, it can take years for gene expression levels to impact on cancer recurrences. Take for example the data from Trere et al. [5]: in Figure 1 of their manuscript it is clear that from the Kaplan-meier that the difference in disease-free survival between triple negative and Her2+ breast cancer only become apparent after 40 months.

We would urge caution in drawing conclusions about the effect of genes on RFS at early timepoints, but greatly thank the reviewer for bringing any potential ambiguity of these statements to our attention. Changes have been made to the language of both the results and discussion sections of the manuscript to ensure only appropriate conclusions are drawn.
In addition, the curves presented in Figure 4 B and C show also no concentration-dependent influence of Id1 and Cyclin D1 expression on recurrence-free survival. Do the authors have any explanation for this? If not, any conclusion on the prognostic value of these two markers seems premature.

As with Fig 3, we do not make any claims regarding a concentration-dependent influence of cyclin D1 and Id1 on recurrence free survival, we have interpreted these Kaplan-meier graphs in the results section as follows:

“…high ID1 expression was associated with the shortest RFS (Fig. 4B, left and right panels) in CCND1^low ER-positive tumours. In addition, both low and high CCND1 expression was associated with the shortest RFS in ID1^high ER-positive tumours…(Fig 4C)”

In Fig. 4 B (ER-positive patients), the highest quartile of ID1 is associated with the shortest RFS and the lowest quartile with the longest RFS, however the two intermediate quartiles do not linearly increase in RFS, thus as the reviewer correctly notes there is no clear concentration-dependent influence of ID1 on RFS. For this reason we have not stated this conclusion in our manuscript.

However, we do believe that the interpretation of high ID1 expression being associated with the shortest RFS in CCND1^low ER-positive tumours, is a valid conclusion to draw regarding prognosis based on our results. The same is true of for both low and high CCND1 expression in ID1^high ER-positive tumours. We hope that the way in which we have presented this data is clear, relevant and logical.

6) If upregulation of Id1 upon downregulation of Cyclin D1 leads to the transition to a mesenchymal phenotype with enhanced migratory potential of cells, how do the authors explain the lack of a prognostic role for the EMT markers in the patient cohorts?

The reviewer raises an important and interesting point here. There has been an explosion of EMT related data in recent years in the breast cancer field. Central to many of these publications has been the ability of EMT to putatively enhance stem cell-related features and promote the metastatic process [6, 7]. Of particular note, the idea of cells that have undergone EMT residing at the leading edge of an invasive tumour and promoting metastasis at the tumour-stroma interface has garnered much attention [8]. This hypothesis may be one explanation as to why EMT markers such as SNAI1, SNAI2, TWIST1 and VIM do not show any prognostic significance in our model- if the cells that have undergone EMT reside at the leading edge of the tumour, strong expression of their genes could easily be lost amongst the entirety of the tumour body. In these circumstances, any strong links to prognosis would also be diluted.

A second, more straight-forward explanation as to why we have not observed
prognostic significance of EMT-related genes centers upon a keystone principal. Upregulation of one EMT gene, e.g. SNAI1, is not enough to induce a transition to mesenchymal phenotype. This fact is clear from the board range of expression values of EMT genes across all breast cancer tumours and subtypes in our study (Fig. 5 D). Induction of EMT requires a reduction in CDH1 expression and upregulation of the potent SNAI1, 2 and TWIST1 genes (amongst others). In order to examine the effect of EMT in our cohort, we would have to combine all tumours with these gene properties- essentially giving us the ‘claudin-low’ subgroup. Unfortunately, we only have 14 such cases- not enough to give any relevant prognostic information. In order to explore this further one would need to analyse a cohort consisting of a large representation of claudin-low tumours, and preferably with micro-dissection of the tumour-stroma interface.

We have added these comments to the discussion section of the manuscript.

7) Statistical analyses: In survival curves the patient numbers in subgroups and the numbers of patients at risk for tumor recurrence should be given.

This has been amended, the relevant numbers have been added to the survival curves.

Do the authors have corrected for multiple testing?

We are aware of and have considered multiple testing in our analysis of clinical data (Table 1) where applicable. However, we decided against its use (and on the Bonferroni method in particular) based on two main observations. Firstly, the parameters that display statistical significance in our analysis have very low p-values (between 0.005 and <0.001), correcting for multiple testing would reduce these p-values, but still render them highly statistically significant. As such we deemed it unnecessary, especially in light of opinions on this kind of adjustment (see point 2).

Secondly, correcting for multiple testing has drawn much criticism and is thought to make sense in only a few situations [9]. We do not believe our data fall into the categories that are deemed appropriate for its use.

Do the authors have corrected for other important clinical parameters like LN status, grading, tumor stage in multivariate models?

Yes, we have examined both CCND1 and ID1 in a Cox Multivariate analysis adjusting for size, lymph node status, grade and ER positivity. Parameters not included in the Cox model were left out due to insufficient numbers (e.g. Age- was missing for 60% of cases). Neither CCND1 nor ID1 were significant as independent prognostic indicators and as such, we omitted the analysis from the manuscript. However, this has now been rectified and added to the results section.

8) It is impossible to understand the data in Table 1.
The table has been altered to make it clearer. R values have been placed in brackets directly below the p-value.

Which values are given in the column entitled P?

The column consists of p-values with R values underneath in each section. E.g. ER-α p-value: <0.001, R value 0.306.

Why are some of these values negative?

These are the R values, a negative value indicates an inverse correlation.

To which comparison the P-values are referring to?

They are referring to a comparison between the parameter to which they are horizontal from, we have added a separating line to make this easier to interpret.

Why do the numbers of cases differ in the subgroups: for example, the association between Id1 and ER status has been assessed for 939 cases, but there were only 879 cases for which Cyclin D1 expression has been correlated with the ER status?

We thank the reviewer for bringing this to our attention, it was down to a typing error, and has been correctly edited.

9) Given an important role of EMT for cell migration and metastasis and a potential involvement of CyclinD1 and Id1 in the EMT process, it would be interesting to know the role of Cyclin D1 and Id1 for metastasis-free survival rather than recurrence-free survival. Do the authors have access to this sort of clinical data? If yes, these data should be presented.

Unfortunately we do not have access to this type of data, but readily agree with the reviewer that metastasis-free survival would make an interesting endpoint.

Unused Fig. 1A- Western blot showing effective cyclin D1 silencing with siRNA in MCF7 and T47D cells lines.
Unused Fig. 1B- Boyden chamber migration assay showing no significant change in migration relative to control of MCF7 and T47D cells lines following cyclin D1 silencing.

References


