Journal of Clinical and Translational Research
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Evaluation of breast cancer stem cells in human primary breast carcinoma and their role in aggressive behavior of the disease

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Handling editor:
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1st Editorial decision
08-Mar-2021

Ref.: Ms. No. JCTRes-D-21-00020
Signature genes from cancer stroma play a critical role in enrichment of cancer stem cells in primary breast tumors
Journal of Clinical and Translational Research

Dear Professor Arora,

Reviewers have now commented on your paper. Reviewer 1 has recommended a reject of the manuscript, while reviewer 2 found the work suitable for publication after minor revision. The editorial board would like to extend the opportunity to revise your work in line with the reviewers' comments. If you are prepared to undertake the work required, I would be pleased to reconsider my decision.

For your guidance, reviewers' comments are appended below. We kindly urge you to fully and comprehensively address the comments of reviewer 1, who posited among other points that the structural make-up and presentation of data do not properly reflect the value and novelty of the work. The editorial board is in agreement with the reviewer's position and requests that the manuscript is restructured accordingly and that the points raised by the reviewer are addressed adequately and to the fullest possible extent.

If you decide to revise the work, please submit a list of changes or a rebuttal against each point which is being raised when you submit the revised manuscript. Also, please ensure that the track changes function is switched on when implementing the revisions. This enables the reviewers to rapidly verify all changes made.

Your revision is due by Apr 07, 2021.

To submit a revision, go to https://www.editorialmanager.com/jctres/ and log in as an Author. You will see a menu item call Submission Needing Revision. You will find your submission record there.

Yours sincerely

Michal Heger
Editor-in-Chief
Journal of Clinical and Translational Research

Reviewers' comments:

Reviewer #1: The correlation of cancer stem cell content and the stromal makeup is interesting but not addressed in sufficient depth and quality to make a convincing case and provide sufficient novelty. The data are presented in a confusing manner, the figures were hard for me to assess in depth.

The title of the manuscript would lead the reader (and this reviewer) to expect a genome wide expression analysis rather than a panel-based method. This makes the identified genes and signalling pathways partly biased.

Also, the use of panels negates the need for GSEA; the authors know exactly which genes were measured so looking for associated biology is a bit odd.

The existence of an ALDH1 positive breast cancer stem cell population is well established. So is the association of such cells with metastatic propensity, and the contributions of for instance IL-6 to this population.

Figure 1 is hard to interpret. For instance, where do we see what is mentioned in the title of this figure's legend?

Figure 1G, second panel (why not make this H?); you cannot do a correlation analysis with 3 samples.

ZEB1 and TAZ are not hypoxia genes per se.

Figure 3A is a circular finding.

I'm not sure the analyses in Figure 4 contribute to the story. Signaling can be shared between tumor and stroma without a biological relevance.

Figure 5; what is the difference exactly between A and B?

Reviewer #2: This is an interesting manuscript adding information on the role of stroma and tumor microenvironment in promoting growth of BCSC. Although the concept is not novel, the manuscript is well organized and the data are sound.
This reviewer would like to address some minor points:
Abstract:
'biologically conducive tumor micro-environment' : the concept is a bit vague
Abstract Conclusions: what is the actual relevance of the study? Just predicting the outcome and providing a risk stratification or providing hints for new treatment strategies? I do not see any association study with the outcome, which would better support the conclusion. What does the stratification into lowBCSC vs highBCSC add with respect to other prognostic variables?

Introduction:
page 2 line 49: 'sorrounding stroma has attracted very little attention'. I think that this cannot be said, many studies have addresses the stroma and the tumor microenvironment.
page 2, line 54 : the list of mechanism for treatment resistance should be supported by references for each of the mentioned mechanisms
page 3 line 14: EMP?
page 3 line 49-52: I do not think that this has been investigated in the present study. This would need functional studies. This is an interesting study, but it limits to correlative data.
Results:
Table 1: this study is enriched in G3 tumors, which may be a bias when interpreting the results and should be acknowledged in the Discussion as possible limitation.
page 10. line 46-57: these are speculative data which should be better moved to the Discussion.
page 11 line 4: why was the CXCR4 gene 'chosen' to address migration of BCSC frpm the tumor to the adjacent tissue?
page 10 line 47-57: These are speculative data which should be moved to the Discussion and supported by literature data.

There is additional documentation related to this decision letter. To access the file(s), please click the link below. You may also login to the system and click the 'View Attachments' link in the Action column.

Authors' response

Comments and Reponses as per the suggestions made for the manuscript titled
"Signature genes from cancer stroma play a critical role in enrichment of cancer stem cells in primary breast tumors"

Pointwise comments and response to the suggestions of Reviewer #1:

Comment 1: The correlation of cancer stem cell content and the stromal makeup is interesting but not addressed in sufficient depth and quality to make a convincing case and provide sufficient novelty.

Response 1: The sequential changes occurring at broader tissue and organ level, beyond localized areas of primary tumor development, are studied under the preview of field cancerization. Still, there are gray areas describing the absence of cellular evidence in this area. Our study revealed that there is a direct correlation between the frequency of BCSCs, not only in the tumor mass but also in the adjacent normal tissue. The present study highlights possible contribution of migratory BCSCs (CXCR4) in field cancerization in adjacent normal tissue. A unique approach to study the contribution of stroma and tumor cells in expansion of BCSCs in High BCSCs vs low BCSCs tumors has been followed.

Comment 2: The data are presented in a confusing manner; the figures were hard for me to assess in depth.
Response 2: Figure 1 is explained in response to Comment 5. Suggested changes have been incorporated in figure 1.

Comment 3: The title of the manuscript would lead the reader (and this reviewer) to expect a genome wide expression analysis rather than a panel-based method. This makes the identified genes and signalling pathways partly biased.
Response 3: Since genome wide expression analysis is out of the scope of the present manuscript, we adopted the expression profiling of most relevant genes. However, we have tried to include all those genes which are expected to make a change using a comprehensive list.

Comment 4: Also, the use of panels negates the need for GSEA; the authors know exactly which genes were measured so looking for associated biology is a bit odd.
Response 4: In continuation to response to Comment No. 3 above, the present study aimed to investigate the differences in well studied genes associated/reported with enrichment of BCSCs, in stromal vs tumor cells compartments in High vs Low BCSCs tumors. It helped us to understand the contribution of stroma and cancer cells towards expansion of BCSCs. It is agreed that the GSEA would be a better approach, but we believe the expression profiling of selected genes has also given a very useful information.

Comment 5: The existence of an ALDH1 positive breast cancer stem cell population is well established. So is the association of such cells with metastatic propensity, and the contributions of for instance IL-6 to this population.
Response 5: Lack of coherent markers to define and characterize breast cancer stem cell population has been a big challenge in field of cancer stem cells. The study emphasizes on one of the isoforms of AlDH1 i.e. AlDH1A1 which is a well-established/studied marker to define BCSCs. It is critically important to validate BCSCs markers (Flow cytometry and IHC analysis), before evaluating the association of these cells with metastatic propensity.
Contribution of IL6 towards BCSCs expansion has been well studied in *in vitro* settings and animal models. But our study re-validated the results in human cancer and the contribution of stroma in self-renewal of BCSCs by IL6 axis in human primary breast tumor samples.

Comment 6: Figure 1 is hard to interpret. For instance, where do we see what is mentioned in the title of this figure's legend?
Response 6: Figure 1's legend has been updated. Now it reads as, "Figure 1: Changes in percentage of BCSCs in tumor and adjacent normal breast tissues of primary breast carcinoma in clinically and pathologically defined aggressive disease setting"
In Figure 1, the identification and characterization of BCSCs with phenotypic markers (flow-cytometry) and AlDH1A1 (IHC) has been quantified. Changes in percentage of BCSCs across clinically and pathologically defined parameters of aggressiveness has been reported.

Comment 7: Figure 1G, second panel (why not make this H?); you cannot do a correlation analysis with 3 samples.
Response 7: Thanks for suggestion, the suggested correction has been incorporated. Figure 1H represents the correlation of mean values of BCSCs in Grade I, II, III tumors with proliferation index. Details has been corrected in the manuscript Figure legends.

Comment 8: ZEB1 and TAZ are not hypoxia genes per se.
Response 8: Agreed, the ZEB1 and TAZ are not hypoxia genes but these are well reported hypoxia associated/affected genes.
References: Zhu J, Huang Z, Zhang M, et al. HIF-1α promotes ZEB1 expression and EMT in a human bladder cancer lung metastasis animal model. Oncol Lett. 2018;15(3):3482-3489. doi:10.3892/ol.2018.7764

Comment 9: Figure 3A is a circular finding.
Response 9: Figure 3A represents the validation of BCSCs percentages in High BCSCs vs Low BCSCs. It represents the cut off value to define High vs Low BCSCs based classification in tumors.

Comment 10: I'm not sure the analyses in Figure 4 contribute to the story. Signaling can be shared between tumor and stroma without a biological relevance.
Response 10: We agree the signaling can be shared between tumor and stroma. The interesting area to focus is the mutually exclusive differentially expressed genes in stromal vs cancer compartment in High vs low BCSCs tumor, which tell the story.

Comment 11: Figure 5 what is the difference exactly between A and B?
Response 11: Figure 5B has been removed.

<u>Pointwise comments and response to the suggestions of Reviewer #1:</u>

Comment 1: Abstract 'biologically conducive tumor micro-environment': the concept is a bit vague
Response 1: Necessary changes have been incorporated. Now it reads as, “Overall, the findings suggest the molecular crosstalk between stromal cells and cancer cells potentially (directly or indirectly) contribute to expansion of cancer stem cells.”

Comment 2: Abstract Conclusions: what is the actual relevance of the study? Just predicting the outcome and providing a risk stratification or providing hints for new treatment strategies?
Response 2: The current study highlights the importance of cancer stem cells as a potential prognostic/predictive marker for aggressive breast cancer. At the same time the findings suggest predicting the potential risk stratification based on percentage of BCSCs in primary breast tumors in addition to existing prognostic factors.

Comment 3: I do not see any association study with the outcome, which would better support the conclusion.
Response 3: Necessary changes have been incorporated. Now it reads as,” The current study highlights the potential importance of cancer stem cells as a potential predictive/prognostic marker for aggressive breast cancer. The findings of the study suggest that it would be possible to predict the potential risk stratification based on percentage of BCSCs in primary breast tumors in addition to existing prognostic factors.”

Comment 4: What does the stratification into low BCSC vs high BCSC add with respect to other prognostic variables?
Response 4: To investigate the differences in potential gene sets associated/reported with enrichment of BCSCs, we have classified the breast tumor samples into High vs Low BCSCs tumors irrespective of pathological grade or other prognostic markers. Differential gene expression profiling has been done in small set of tumor samples (which were classified into High vs Low BCSC tumors). Its association with other prognostic markers has not been evaluated.

Comment 5: Introduction page 2 line 49: 'surrounding stroma has attracted very little attention'. I think that this cannot be said, many studies have addressed the stroma and the tumor microenvironment.
Response 5: The suggested changes based on comment has been incorporated in the manuscript. A recent reference has been added to support the mentioned results. Now it reads as, "It has become interestingly clear that the interactions between tumor cells and stromal cells play a significant role in establishment, progression of tumors as well as expansion and survival of CSCs (8)."

Comment 6: page 2, line 54: the list of mechanism for treatment resistance should be supported by references for each of the mentioned mechanisms
Response 6: The suggested changes based on comment has been incorporated in the manuscript. Recent references have been added to support the mentioned mechanisms.

Comment 7: page 3 line 14: EMP?
Response 7: EMP is abbreviation for Epithelial to Mesenchymal Plasticity. The manuscript is updated with expanded version in introduction. Details on abbreviation can be found in Abbreviation section.

Comment 8: page 3 line 49-52: I do not think that this has been investigated in the present study. This would need functional studies. This is an interesting study, but it limits to correlative data.
Response 8: Thanks for suggestion**.** Necessary changes have been incorporated. Now it reads as, "We hypothesized that the aggressiveness of breast cancer can be directly related to the enrichment of BCSCs in the tumor microenvironment. Further, we investigated the correlation of metastatic disease with percentage of BCSCs."

Comment 9: Results Table 1: this study is enriched in G3 tumors, which may be a bias when interpreting the results and should be acknowledged in the Discussion as possible limitation.
Response 9: One of the major reasons that we received large number of samples in Grade 3 tumor category is the late detection and diagnosis. All possible measures to avoid bias in interpretation of results (random recruitment of patients/samples, non-parametric statistical tests for analysis and others) has been applied. A line acknowledging the bias as a possible limitation has been mentioned in discussion. The line reads as," A possible limitation could be the unintentional bias introduced due to large sample size in grade 3 tumors category."

Comment 10: page 10. line 46-57: these are speculative data which should be better moved to the Discussion. page 10 line 47-57: These are speculative data which should be moved to the Discussion and supported by literature data.

Response 10: The speculative data mentioned in page 10. Line 46-57 has been discussed in detail in discussion section. Line 46-52 has been removed from Page 10. The data in discussion section has been supported by updated references.

Comment 11: page 11 line 4 why was the CXCR4 gene 'chosen' to address migration of BCSC from the tumor to the adjacent tissue?
Response 11: Breast tumors preferentially metastasize to the lung, bones and lymph nodes, all of which represent organs that secrete high levels of CXCL12. CXCL12 acts as a chemoattractant that drives CXCR4-positive primary tumor cells towards secondary metastatic sites leading to the onset of metastatic lesions. With this rationale, we thought to investigate the role of CXCR4+ BCSCs in metastasis to secondary metastatic sites.

2nd Editorial decision
15-Apr-2021

Ref.: Ms. No. JCTRes-D-21-00020R1
Signature genes from cancer stroma play a critical role in enrichment of cancer stem cells in primary breast tumors
Journal of Clinical and Translational Research

Dear Professor Arora,

Reviewers and the editor have now commented on your paper. You will see that they are advising that you revise your manuscript. If you are prepared to undertake the work required, I would be pleased to reconsider my decision.

For your guidance, reviewers' comments are appended below.

If you decide to revise the work, please submit a list of changes or a rebuttal against each point which is being raised when you submit the revised manuscript. Also, please ensure that the track changes function is switched on when implementing the revisions. This enables the reviewers to rapidly verify all changes made.

Your revision is due by May 15, 2021.

To submit a revision, go to https://www.editorialmanager.com/jctres/ and log in as an Author. You will see a menu item call Submission Needing Revision. You will find your submission record there.

Yours sincerely

Michal Heger
Editor-in-Chief
Journal of Clinical and Translational Research

Reviewers' comments:

Editor:
Dear authors, the editorial board has critically evaluated the reviewers' comments and the

depth to which you have implemented the requested changes. At this stage we have decided to side with the reviewers, who are true experts in the field.
Especially reviewer 1 feels that you have not adequately addressed his most fundamental concerns. To prevent us getting into a heated debate, JCTR would like to extend you one more opportunity to revamp the manuscript in line with what has been suggested. We do still see merit in your paper. If you are unable to perform the requested additional work, please let me know (m.heger@jctres.com) and we can withdraw the manuscript, which in such a case will be most fortuitous for all parties involved. Naturally, the board hopes you choose the first option. Thank you, Michal Heger, editor.

Reviewer #1:
The authors have only superficially addressed my concerns.

Authors' response

May 15, 2021
Comments and Reponses as per the suggestions made for the manuscript titled
"Enrichment of breast cancer stem cells in human primary breast carcinoma play a critical role in aggressive behavior of the disease"

RE: Revisions to Journal of Clinical and Translational Research Manuscript ID: JCTRes-D-21-00020R1

We would like to thank the editor and the reviewers for the time and effort put into the review of this manuscript. The comments/suggested revisions have significantly improved our paper. In the attached, revised version of the paper, all changes have been done following track changes and the updated figures attached. The detail responses to individual comments are below and hope they adequately address all concerns. Please let us know if you require any additional information.

Pointwise comments and response to the suggestions of Reviewer #1:

Comment 1: The correlation of cancer stem cell content and the stromal makeup is interesting but not addressed in sufficient depth and quality to make a convincing case and provide sufficient novelty.

Response 1: Major focus of the manuscript was to assess if frequency of intra-tumoral BCSCs is associated with aggressive behavior of the disease (high pathological grade, molecularly aggressive tumors, metastatic lymph nodes) and factors leading to expansion of BCSCs in these aggressive cancers. Our study reveals that there is a direct correlation between the frequency of BCSCs, not only in the tumor mass but also in the adjacent normal tissue. The present study highlights possible contribution of migratory BCSCs (CXCR4) in field cancerization in adjacent normal tissue. We have tried to address this issue with a unique approach to study the contribution of stroma and tumor cells in expansion of BCSCs in High BCSCs vs low BCSCs tumors, which revealed certain pathways originating from the factors released by the stromal cells seem to promote the expansion of BCSCs in the aggressive cancer phenotypes.

Comment 2: The data are presented in a confusing manner; the figures were hard for me to assess in depth.

Response 2: Substantial modification in data presentation has been done. Please refer to following changes:

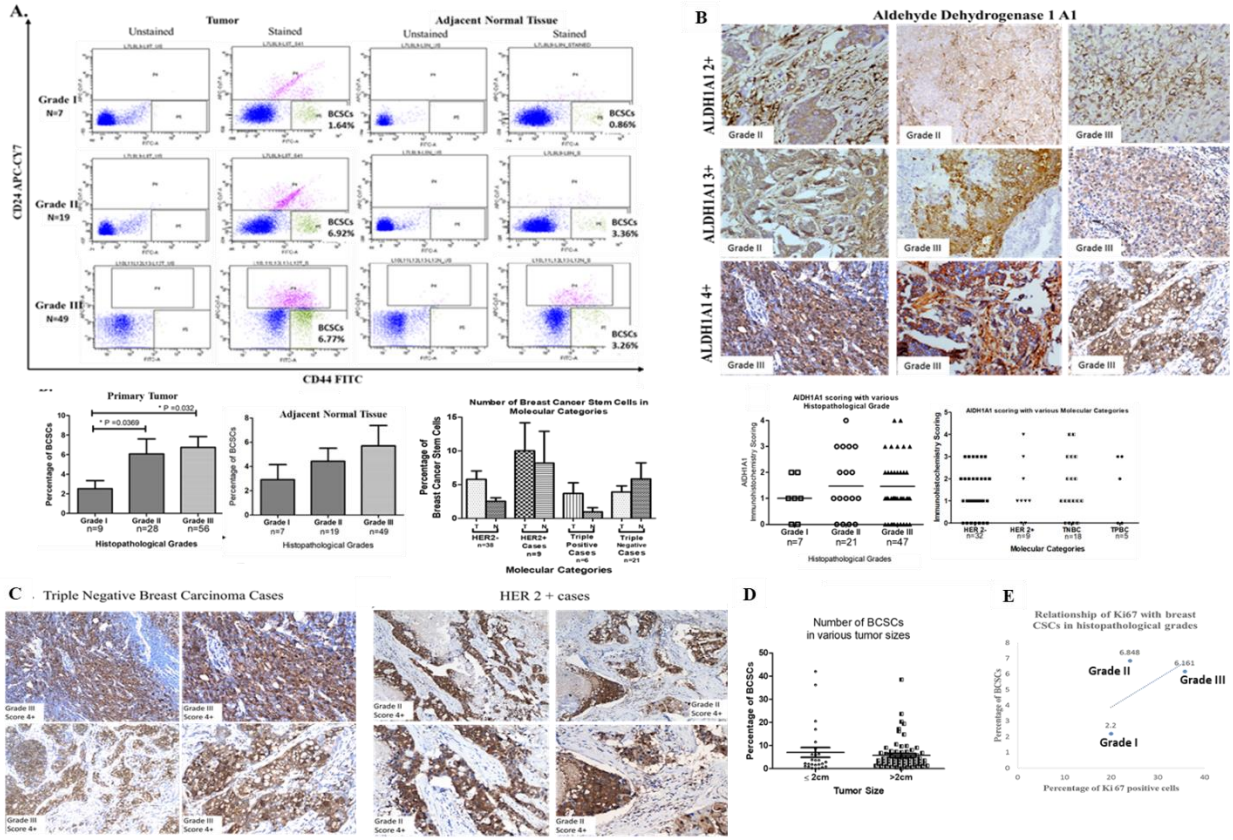

Figure 1: Identification and distribution profile of BCSCs in primary breast tumor specimens in clinically and pathologically defined aggressive breast tumors (A). Representative flow cytograms of identification of BCSCs of tumor and adjacent normal tissues in various histological grades of breast cancer(Grade I n=7; Grade II n=19; Grade III n=49) Significant increase in percentages of BCSCs in tumor tissues of Grade II (p=0.0369) and Grade III (p=0.032) were found as compared to Grade I. (Grade I n=9; Grade II n=28; Grade III n=56). No difference in percentage of BCSCs were found in adjacent normal tissue of various histological grades (Grade I n=7; Grade II n=19; Grade III n=49). Bar graphs presenting Mean ± SEM were plotted. Quantification of percentage of BCSCs (Mean ± SEM) in various molecular categories in tumor and normal tissue suggest potential trend of increase of BCSCs percentage in molecularly aggressive breast carcinoma (ER/PR+ HER2- n=38; ER/PR- HER2+ n=9; ER/PR+ HER2+ n=6; ER/PR- HER2– n=21). (B) Representative micrographs of immunohistochemical staining of ALDH1A1 and its scoring in tumor sections of various histological grades (40X). Comparison of IHC scores (Mean±SEM) of ALDH1A1 in various histological grades (Grade I n=7; Grade II n=21; Grade III n=47) and molecular categories (ER/PR+ HER2- n=32; ER/PR- HER2+ n=9; ER/PR- HER2- n=18; ER/PR+ HER2 + n=5). (C) Representative micrographs of ALDH1A1staining in molecularly aggressive breast carcinoma Triple Negative Breast Carcinoma category, Her 2 category (D) No correlation was

observed between percentages of BCSCs and tumor size (≤2cm and >2cm). (E) A positive trend line of correlation between histopathological grades and proliferation index was observed. Data points represent the correlation of mean values of BCSCs in Grade I, II, III tumors with proliferation index.

Figure 2: Presence of BCSCs in metastatic lymph node and adjacent normal tissue indicate invasive behavior of BCSCs in breast cancer metastasis (A) Increased BCSCs (Mean±SEM) in Metastatic LN (n=43) tumor samples as compared to non-metastatic LN category (n=48) (flow cytometry). Presence of ALDH1A1+ BCSCs validates in lymph node sections in metastatic(n=10) tumor cases as compared to non-metastatic(n=3) lymph node category (B). Micrographs of ALDH1A1 staining in metastatic (n=10)/non-metastatic (n=3) lymph nodes. (C) Immunohistochemical staining of ALDH1A1 on shortlisted cases showing high percentages of BCSCs on flow cytometry showed ALDH1A1 positivity in all eight cases suggesting a role played by cancer stem cell component in field cancerization. (D) Presence of ALDH1A1+ cells within well-arranged histologically normal mammary ducts in tumor vicinity (n=3) (E) Immunohistochemical staining for vimentin (n=3) on tumor sections showed ALDH1A1+ normal cells in tumor vicinity indicating occurrence of EMT mechanism for conversion of NSCC to BCSCs. (F) Representative flow cytographs showing presence of BCSCs at different distances from primary tumor tissue in histopathological grade II (n=6) and grade III (n=11). (G) Line plot representing mean distribution of BCSCs (upper left) (Grade II n= 6; Grade III n=11) and BCSCs showing CXCR4 expression (upper right) at distant site from primary tumor. CXCR4 MFI (upper left) (Grade II n= 6; Grade III n=11) of BCSCs at distant tissue distance from primary tumor tissue.

Figure 3: Increased relative expression of genes associated with growth factors, cytokines, hypoxia, EMT observed in tumors with High BCSCs containing tumors (A) Percentage of BCSCs in Hi-BCSCs and Lo-BCSCs breast tumors. It represents the differences in mean values of BCSCs percentage of primary tumors (High BCSCs vs Low BCSCs tumors) used for panel-based gene expression profile. It represents the cut off value to define High vs Low BCSCs based classification in tumors. Significant difference in percentage of BCSCs in Hi-BCSCs (16.68%) vs. Lo-BCSCs (2.52%) (p=0.0002) breast tumors. (B) Differential gene expression profile between Hi-BCSCs_SC group (Test) vs. Lo-BCSCs_SC group (Control). Heat map showing the differential gene expression (fold change) in Hi-BCSCs_SC group (Test group) as compared to Lo-BCSCs_SC group (Control group). Low gene expression is represented by green color and high gene expression is represented by red color in the test vs. control. (C) Cluster diagram showing differential gene expression in stromal cells of Hi-BCSCs tumors (Test) vs. Lo-BCSCs tumors (Control). Low gene expression is represented by green color and high gene expression is represented by red color in the test vs. control. (D) Scatterplot showing the differential gene expression in Hi-BCSCs_SC group (Test) as compared to Lo-BCSCs_SC group (control). Scatterplot showing the differential gene expression of up-regulated and down-regulated genes in stromal cells of Hi-BCSCs (Hi-BCSCs _SC) as compared to Lo-BCSCs (Lo-BCSCs_SC) tumors. (E) Differential gene expression profile between Hi-BCSCs_CCgroup (Test) vs. Lo-BCSCs_CC group (Control). Heat map showing the differential gene expression (fold change) in Hi-BCSCs_CC group (Test group) as compared to Lo-BCSCs_CC group (Control group).Low

gene expression is represented by green color and high gene expression is represented by red color in the test vs. control. (F) Cluster gram showing differential gene expression in cancer cells of Hi-BCSCs tumors (Test) vs. Lo-BCSCs tumors (Control). Low gene expression is represented by green color and high gene expression is represented by red color in the test vs. control. (G) Scatterplot showing the differential gene expression in Hi-BCSCs_CC group (Test) as compared to Lo-BCSCs_CC group (Control). Scatterplot showing the differential gene expression of up-regulated and down-regulated genes in Hi-BCSCs_CC group as compared to Lo-BCSCs_CC group. (H) Line diagram showing down-regulated genes in Hi-BCSCs tumors (Test) as compared to Lo-BCSCs_tumors (Control). Line diagram showing down-regulation in genes (fold change) involved in EMT mechanism, Extracellular matrix protein contributing to aggressive behavior of disease and signaling molecules in stromal cells of Hi-BCSCs tumors as compared to Lo-BCSCs tumors. (I) Fold change in genes involved in EMT mechanism, chemokine receptors, Extracellular matrix protein contributing to aggressive behavior of disease and signaling molecules in cancer cells of Hi-BCSCs tumors as compared to Lo-BCSCs tumors.

Figure 4: Compartment specific gene expression profile associated with BCSCs expansion (A). A total of 19 genes were found to be commonly over-expressed by cancer cells as well as stromal cells, whereas 7 genes were exclusively over-expressed by either stromal cells or cancer cells. Only one gene, SNAI1 was found to be significantly under-expressed in stromal cell compartment of Hi-BCSCs tumors as compared to Lo-BCSCs tumors. (B) Change in relative gene expression profile of hypoxia related genes HIF1α, ARNT, EPAS1, TAZ and SIAH1 in stromal cells and cancer cells of Hi-BCSCs tumors and Lo-BCSCs tumors as compared to adjacent normal tissue levels of (C) Correlation of ECM genes (Lumican and Periostin) and BCSCs percentage in BCSC expansion. (D) Correlation of BCSCs percentage with fold change in gene expression of VEGFA and CXCL12 (E) Relative changes in gene expression levels of IL-6, IL-8, TNF-α and TGF-β1 in stromal cells and cancer cells of Hi-BCSCs tumors and Lo-BCSCs tumors as compared to adjacent normal tissue gene expression levels. (F) Correlation of BCSCs percentage with fold change in gene expression of IL-6, IL-8 and TNF-α

Comment 3: The title of the manuscript would lead the reader (and this reviewer) to expect a genome wide expression analysis rather than a panel-based method. This makes the identified genes and signaling pathways partly biased.
Response 3: Although we do agree that in general the perception would be the GWA in such studies, but there are numerous published studies where a select panel of genes have been used in form of custom arrays to evaluate the differentially expressed genes. We have made an effort to select a very comprehensive list of genes which may have influence on the expansion of stem cells. This study has given a very useful information, although not as elaborate as it could be with GWA, yet interesting enough to make few logical conclusions, that too within limited budget. Moreover, the study has been done in the human clinical tissue and not in the cell-lines. But, keeping in mind the concern raised by the worthy reviewer for this particular study, we have now changed the title to, "**Enrichment of breast cancer stem cells in human primary breast carcinoma play a critical role in aggressive behavior of the disease**".

Comment 4: Also, the use of panels negates the need for GSEA; the authors know exactly which genes were measured so looking for associated biology is a bit odd.

Response 4: As mentioned in Response 3 above, the present study aimed to investigate the differences in well studied genes associated/reported with enrichment of BCSCs, in stromal vs tumor cells compartments in High vs Low BCSCs tumors. It helped us to understand the contribution of stroma and cancer cells towards expansion of BCSCs. The present study validates upregulation of genes associated with stromal component and cancer component.

Comment 5: The existence of an ALDH1 positive breast cancer stem cell population is well established. So is the association of such cells with metastatic propensity, and the contributions of for instance IL-6 to this population.

Response 5: Lack of coherent markers to define and characterize breast cancer stem cell population has been a big challenge in field of cancer stem cells. The study emphases on one of the isoforms of AlDH1 i.e. AlDH1A1 which is a well-established/studied marker to define BCSCs. It is critically important to validate BCSCs markers (Flow cytometry and IHC analysis), before evaluating the association of these cells with metastatic propensity. The present study conducted on clinical samples validates the reports, mostly using only breast cancer cell-lines, with AlDH1A1 as specific BCSCs marker.

Contribution of IL 6 towards BCSCs expansion has been well studied in *in vitro* settings and animal models. Our study re-validated the results; the contribution of stroma in self-renewal of BCSCs by IL6 axis in human primary breast tumor samples.

Comment 6: Figure 1 is hard to interpret. For instance, where do we see what is mentioned in the title of this figure's legend?

Response 6: Figure 1 legend has been changed, which now reads as, “Figure 1: Distribution profile of BCSCs in primary breast tumor specimens in clinically and pathologically defined aggressive breast tumors”

In Figure 1, the identification and characterization of BCSCs with phenotypic markers (flow-cytometry) and AlDH1A1 (IHC) have been quantified. Changes in percentage of BCSCs across clinically and pathologically defined parameters of aggressiveness have been reported.

Comment 7: Figure 1G, second panel (why not make this H?); you cannot do a correlation analysis with 3 samples.

Response 7: Suggested correction has been incorporated. A second panel of Figure 1H has been created. Figure 1H represents the correlation of mean values of BCSCs in Grade I, II, III tumors with proliferation index. Details have been corrected in the manuscript Figure legends.

Comment 8: ZEB1 and TAZ are not hypoxia genes per se.

Response 8: Suggested corrections have been made. Now it reads as,” These genes are associated with hypoxia and its affected genes (HIF1A, ARNT, EPAS1, SIAH1, ZEB1, TAZ), inflammatory cytokines (IL-6, IL-8, TGF-β1, TNF-α), growth factors (VEGFA, FGF2,

PDGFD, HGF), epithelial to mesenchymal transition (TWIST1, SOX9, CDH1, CDH2, VIM), and matricellular proteins (LUM, COL6A3, HAS2, POSTN, TNC, SPP1, SPARC) ."

Comment 9: Figure 3A is a circular finding.

Response 9: Figure 3A represents the differences in mean values of BCSCs percentage of primary tumors (High BCSCs vs Low BCSCs tumors) used for panel-based gene expression profile. It represents the cut off value to define High vs Low BCSCs based classification in tumors.

Comment 10: I'm not sure the analyses in Figure 4 contribute to the story. Signaling can be shared between tumor and stroma without a biological relevance.

Response 10: We agree the signaling can be shared between tumor and stroma. The interesting area to focus is the mutually exclusive differentially expressed genes in stromal vs cancer compartment in High vs low BCSCs tumor. It would be interesting to investigate these genes further for their contribution in BCSCs expansion/ origin/self-renewal based on host specific response/ cancer cell specific response.

Comment 11: Figure 5 what is the difference exactly between A and B?

Response 11: Figure 5A represents the gene interaction of different growth factors, cytokines, ECM component genes with EMT regulatory genes involved in BCSCs origin/expansion. Figure 5B highlights the importance of hypoxic microenvironment in origin/expansion of BCSCs phenotype. Induction of hypoxia and hypoxia related genes leads to activation of some commonly activated pathways by growth factors leading in origin/expansion of BCSC phenotype.

Figure legends for figure 5A and figure 5B have been updated and now reads as, "**Proposed model of BCSCs origin/expansion.** (A) Gene Interactions involved in BCSCs origin/expansion. Interactions of different growth factors, cytokines, ECM component genes with EMT regulatory genes, thereby helping in acquisition of BCSC phenotype. (B) Proposed mechanism suggesting role of hypoxia/hypoxia related genes in origin/expansion of BCSC phenotype. Induction of hypoxia and hypoxia related genes leads to activation of some commonly activated pathways by growth factors leading in origin/expansion of BCSC phenotype. (C) Cross talk between stromal cells and cancer cells initiate a cascade of events leading to increase in various intra-tumoral factors contributing to intra-tumoral expansion of CSCs. Based on our findings, we propose that the growth factors along with inflammatory cytokines through Notch and SMAD signaling induce EMT-transcription factors which in turn initiate epithelial to mesenchymal transition. EMT might be one of the possible mechanisms responsible for acquisition of BCSCs phenotype."

3rd Editorial decision
02-Jun-2021

Ref.: Ms. No. JCTRes-D-21-00020R2
Enrichment of breast cancer stem cells in human primary breast carcinoma play a critical role

in aggressive behavior of the disease
Journal of Clinical and Translational Research

Dear Professor Arora,

Reviewers have now commented on your paper. You will see that they are advising that you revise your manuscript. If you are prepared to undertake the work required, I would be pleased to reconsider my decision.

For your guidance, reviewers' comments are appended below.

If you decide to revise the work, please submit a list of changes or a rebuttal against each point which is being raised when you submit the revised manuscript. Also, please ensure that the track changes function is switched on when implementing the revisions. This enables the reviewers to rapidly verify all changes made.

Your revision is due by Jul 02, 2021.

To submit a revision, go to https://www.editorialmanager.com/jctres/ and log in as an Author. You will see a menu item call Submission Needing Revision. You will find your submission record there.

Yours sincerely

Michal Heger
Editor-in-Chief
Journal of Clinical and Translational Research

Reviewers' comments:

Reviewer #1: The authors have improved the manuscript, but several points of concern remain:

Many of the figures are still very hard to read, with very small font and cluttered makeup. Many panels do not have a label. This makes it very hard to do an in-depth assessment.

Can the authors prove VIM is an EMT marker and not a stromal marker (Figure 2). Likewise, the authors should indicate much more clearly in section 3.3 that these are sorted cells, thus informing on whether the genes identified are truly stromal or not. Took me a while to figure that out.

Why are Lin-CD44+CD24- cells considered BCSCs? This needs a bit more explanation.

Figure 1E; it's hard to tell (see my first comment) but are the authors basing a linear

regression from three data points?

I have concerns with specificity in Figure 1: "Surprisingly the adjoining normal tissue to tumor also showed comparable numbers of BCSCs" this likely means that the tissue was not normal. Likewise, the staining in panel B looks unspecific, with mostly stromal staining. Why is the histology so different between samples?

Figure 5 is very complex and upon closer scrutiny, has some strange annotations. For instance, why are CDH1 and VIM both considered EMT genes (panel A)? And why do regulators or pluripotent/stemness not included in the network? What is the rationale for panel B? Hypoxia is already included in panel A. Panel C does not really help to condense the findings in an intuitive manner. Everything signals to everything else?

Minor comments:
Does the (new) Title really reflect what's reported in the paper?

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Figure 3B and E, how are these samples and genes clustered? Figure 3C and F, how are these genes ranked (why not by expression in stroma), and why is the heat map only two colors?

Authors' response

July 02, 2021
Comments and Reponses as per the suggestions made for the manuscript titled
"Evaluation of breast cancer stem cells in human primary breast carcinoma and its role in aggressive behavior of the disease"

RE: Revisions to Journal of Clinical and Translational Research Manuscript ID: JCTRes-D-21-00020R1

We would like to thank the editor and the reviewers for the time and effort put into the review of this manuscript. The comments/suggested revisions have significantly improved our paper. In the attached, revised version of the paper, all changes have been done following track changes and the updated figures attached. The detail responses to individual comments are below and hope they adequately address all concerns. Please let us know if you require any additional information.

We thank the reviewer for his/her time and kindly request that the reviewer see the below mentioned detailed response to each comment/concern/suggestion provided in the critique above. Thank you.

Pointwise comments and response to the suggestions of Reviewer #1:

Reviewer #1: The authors have improved the manuscript, but several points of concern remain:

Comment 1: Many of the figures are still very hard to read, with very small font and cluttered makeup. Many panels do not have a label. This makes it very hard to do an in-depth assessment.

Response 1: Suggested changes have been incorporated in all figures. The font has been changed to a readable format and other details have been added to all figures. Accordingly, the figure legends have been modified. Please find the changes incorporated.

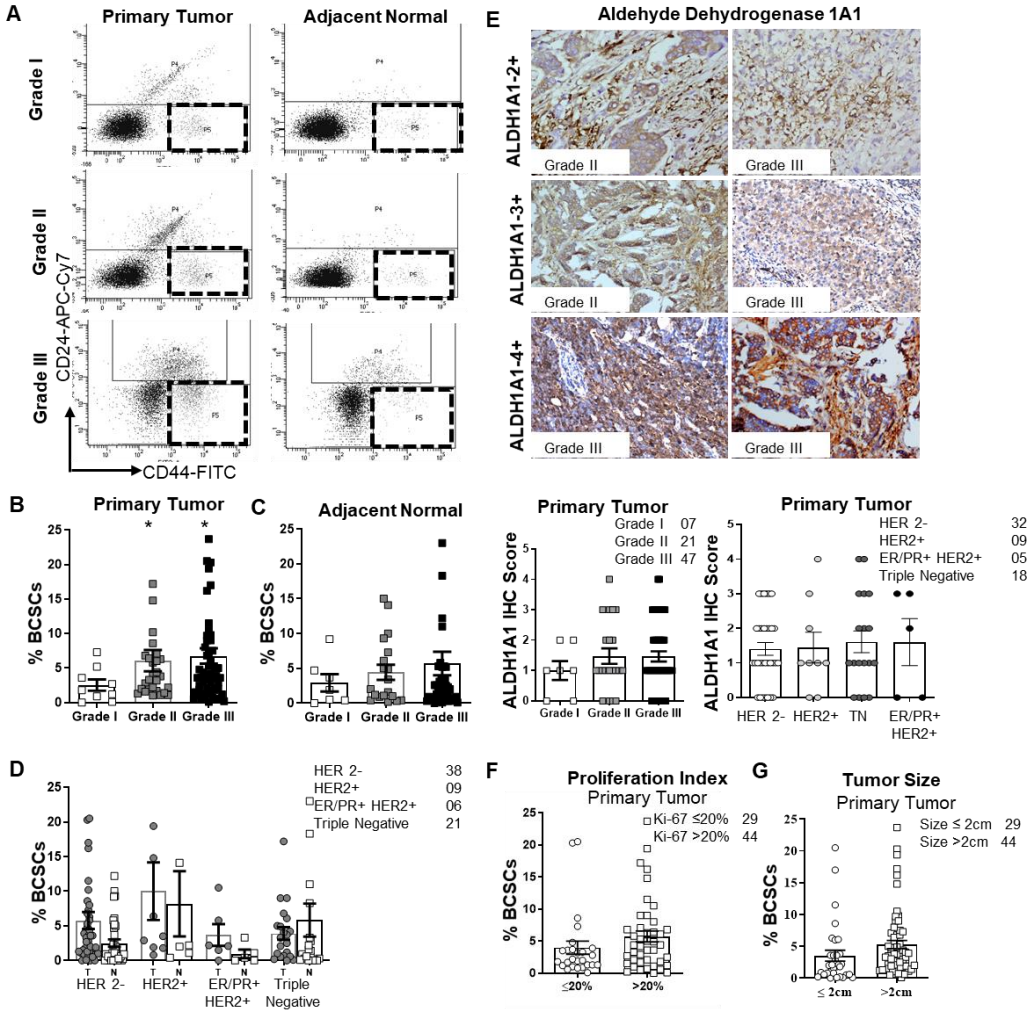


Figure 1: Frequency of BCSCs in tumor and adjacent normal breast tissues of primary breast carcinoma in clinically and pathologically defined aggressive disease setting (A). Flow cytograms representing comparative percentages of BCSCs in various histological grades in tumor (Grade I n=9; Grade II n=28; Grade III n=56) and adjacent normal tissues (Grade I n=7; Grade II n=19; Grade III n=49). Quantification of percentage of BCSCs by flow cytometry (Lin^- $CD44^+$ $CD24^-$) in various histopathological grades (B). Primary Tumors (Grade II (p=0.0369), Grade III (p=0.032) vs Grade I) (C) Adjacent Normal tissues. (D) Quantification of percentage of BCSCs in various molecular categories in tumor and normal tissue (ER/PR+ HER2- n=38; ER/PR- HER2+ n=9; ER/PR+ HER2+ n=6; ER/PR- HER2– n=21). (E) Quantification of IHC stained microphotographs of tumor cells with ALDH1A1 antibody (40

X) in different histological grades. Comparison of IHC scores of ALDH1A1 in various histological grades (bottom panel-left) (Grade I n=7; Grade II n=21; Grade III n=47) and molecular categories (ER/PR+ HER2- n=32; ER/PR- HER2+ n=9; ER/PR- HER2- n=18; ER/PR+ HER2 + n=5). (bottom panel-right) (F) Differences in percentage of BCSCs in tumors sized ≤2cm and >2cm (G) Differences in percentage of BCSCs in Ki-67 ≤20% and >20% tumors. Bars represent Mean and error bars represent ± SEM. Statistical comparisons between groups were performed, using Kruskal–Wallis and Dunn's multiple comparisons tests unless until mentioned. *p<0.05, **p<0.01, ***p<0.001.

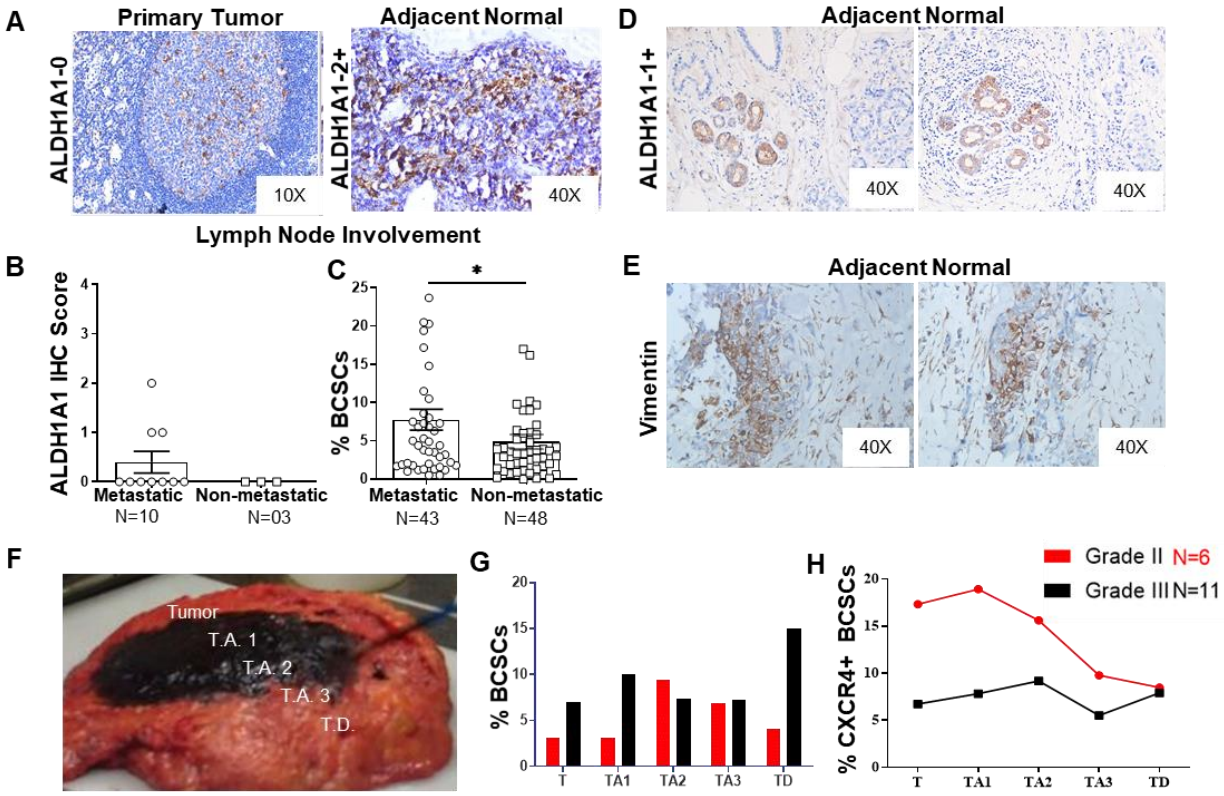


Figure 2: Invasive behavior of BCSCs and its involvement in breast cancer metastasis (A). Representative IHC stained micrographs of ALDH1A1 staining in metastatic and non-metastatic lymph nodes; (B) Bar graph representing percentage of BCSCs in Metastatic LN (n=43) and non-metastatic LN tumors (n=48) by flow cytometry; (C) ALDH1A1 staining on lymph node sections in metastatic (n=10) and non-metastatic (n=3) lymph nodes; (D) Presence of ALDH1A1 positive cells within well-arranged histologically normal mammary ducts in tumor vicinity (n=3); (E) Immunohistochemical staining for vimentin (n=3) on adjacent normal sections near tumor vicinity; (F) Representative mastectomy specimen serially dissected by histologist to obtain following tissues: primary tumor (T), T.A. 1 (Tumor Adjacent 1; 3mm from T); T.A. 2 (Tumor Adjacent 2; 1cm from T); T.A. 3 (Tumor Adjacent 3; 2cm from T); T.D. (Tumor Distant; 4cm from T); (G) Histogram showing percentage of BCSCs at primary tumor site compared with different tissue intervals in grade II (n=6) and grade III (n=11); (H) Line plot representing distribution of CXCR4 expressing BCSCs at different tissue levels (Grade II n= 6; Grade III n=11). For all data, bars indicate means and error bars indicate ±SEMs. **p<0.01, ****p<0.0001

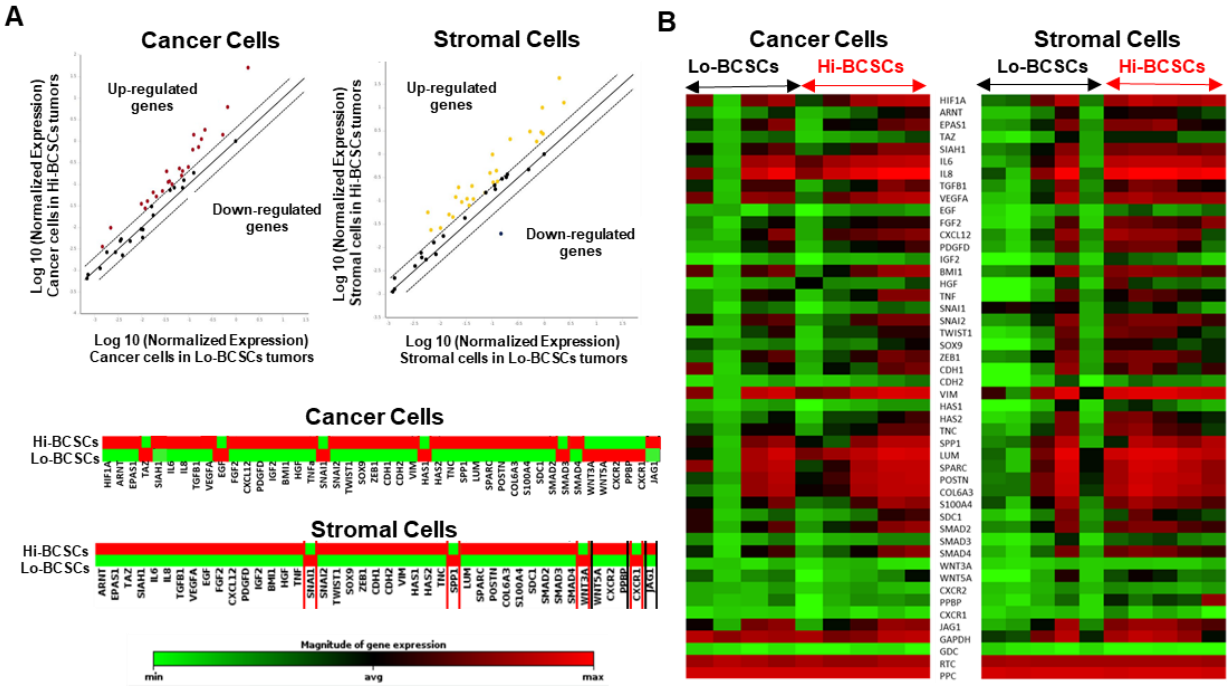


Figure 3: Differential gene expression profile of cancer cells and stromal cells in tumors with High BCSCs vs Low BCSCs tumors (A) Scatterplot showing the differentially expressed genes in cancer cells and stromal cells in Hi-BCSCs tumors (16.68%) vs Lo-BCSCs tumors (2.52%). Cluster diagram showing average gene expression for individual genes in cancer cells (upper) and stromal cells (lower) in Hi-BCSCs tumors vs. Lo-BCSCs tumors; (B) Heat map showing differential gene expression of selected gene sets in cancer cells and stromal cells isolated from Hi-BCSCs tumors and Lo-BCSCs tumors. Low gene expression is represented by green color and high gene expression is represented by red color in the test vs. control. Statistical comparisons between groups were performed, using Kruskal–Wallis and Dunn's multiple comparisons tests.

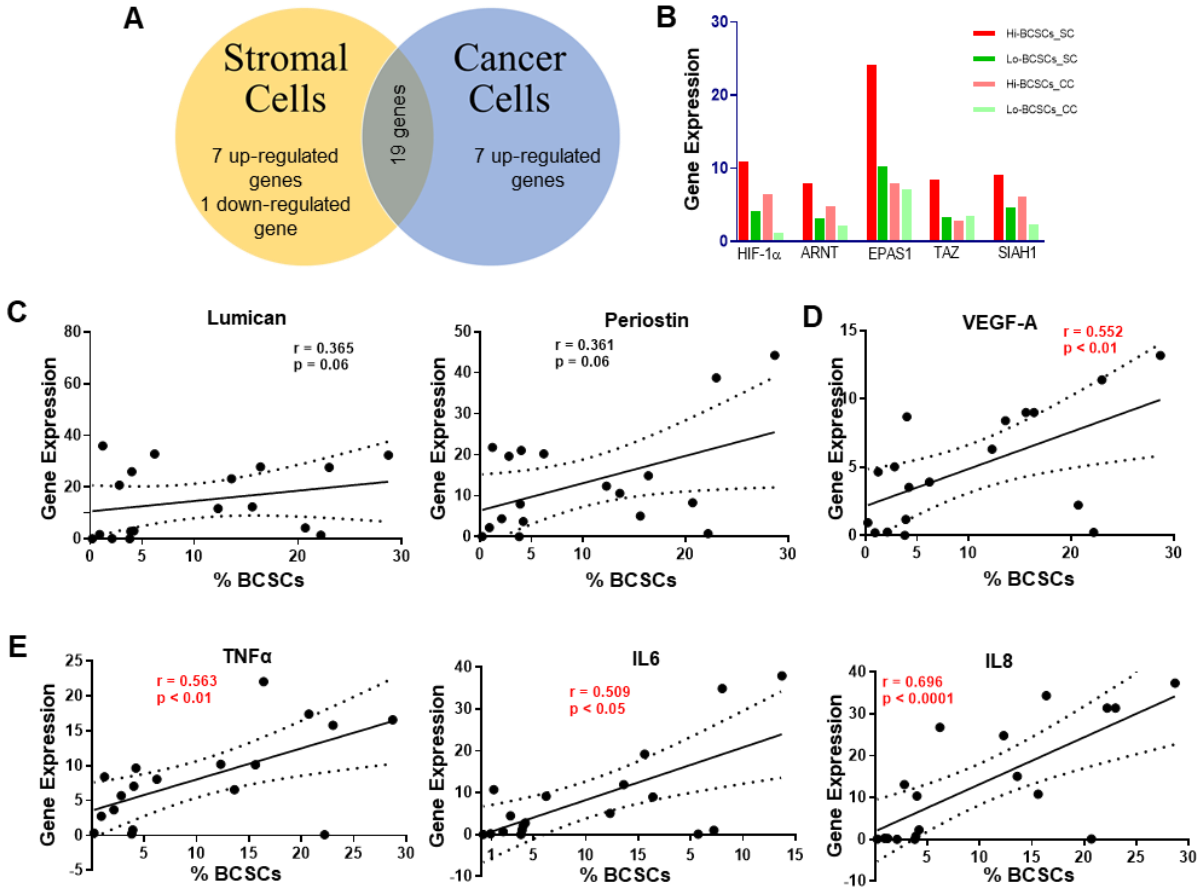


Figure 4: High BCSCs in primary tumor correlates with inflammatory tumor microenvironment (A). Differential gene expression indicating a total of 19 genes to be commonly over-expressed in cancer cells as well as stromal cells, whereas 7 genes were exclusively over-expressed in stromal cells or in cancer cells. Only one gene, SNAI1 was found to be significantly under-expressed in stromal cell compartment; (B) Histogram showing expression profiles of hypoxia related genes in cancer cells and stromal cells in Hi-BCSC tumors vs Lo-BCSC tumors. (C) Correlation of BCSCs percentage with gene expression of ECM genes: Lumican and Periostin; (D) Correlation of BCSCs percentage with gene expression of VEGFA; (E) Correlation of inflammatory cytokines (TNFα, IL6, IL8) with BCSC expansion. To evaluate the correlation between different variables, Spearman correlation test was applied. P value <0.05 was considered statistically significant.

Comment 2: Can the authors prove VIM is an EMT marker and not a stromal marker (Figure 2).

Response 2: Suggested changes have been incorporated in figure legends, discussion and result section. Vimentin is not an EMT marker, but a mesenchymal marker.

Now it reads as,

Result Section 3.2 “Additionally, vimentin expression in 5-10% tumor cells suggests that possibly these cells have been transformed to mesenchymal cell type (Figure 2E).”

Figure 2 legend: Immunohistochemical staining for vimentin (n=3) on adjacent normal sections near tumor vicinity

Discussion: Our gene-expression data reveals that the factors known to regulate the EMT transcription factors such as TWIST1, SOX9, SNAI1, SNAI2 and mesenchymal markers like Vimentin and N-cadherin, were all found to be significantly highly expressed in Hi-BCSC tumors as compared to Lo-BCSC tumors, which suggests the significant role being played by EMT induction in expansion of CSCs in the breast cancer.

Comment 3: Likewise, the authors should indicate much more clearly in section 3.3 that these are sorted cells, thus informing on whether the genes identified are truly stromal or not. Took me a while to figure that out.

Response 3: Suggested changes have been incorporated.

Now it reads as, "Transcriptomic analysis using shortlisted gene panel was performed in flowcytometrically sorted cancer cells and stromal cells in 20 samples of different histopathological grades and different BCSCs frequency. The tumors were divided into High BCSCs (Hi-BCSCs: Tumors with >5% BCSCs) and Low BCSCs (Lo-BCSCs: Tumors with <5% BCSCs) category. The mean values of BCSCs in Hi-BCSC and Lo-BCSC tumors were 16.68% and 2.52% respectively. Further, stromal cells and cancer cells were sorted from these tumors for RNA analysis."

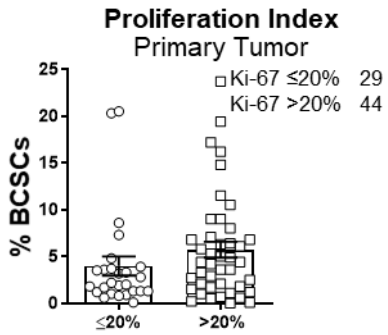
Comment 4: Why are $Lin^-CD44^+CD24^-$ cells considered BCSCs? This needs a bit more explanation.

Response 4: Necessary changes have been incorporated. A line describing the phenotypic identification of BCSCs along with reference has been added to the result section 3.1.

Now it reads as, "In 2003, Al-Hajj *et al*. identified BCSCs in breast tumor with a phenotype of Lin^- $CD44^+$ $CD24^-$ using flowcytometry (15), which is well excepted now."

Comment 5: Figure 1E; it's hard to tell (see my first comment) but are the authors basing a linear regression from three data points?

Response 5: Linear regression graph has been removed. A new scatter graph (Figure 1 F) has been added, which gives a better picture. Please find below the modified graph.

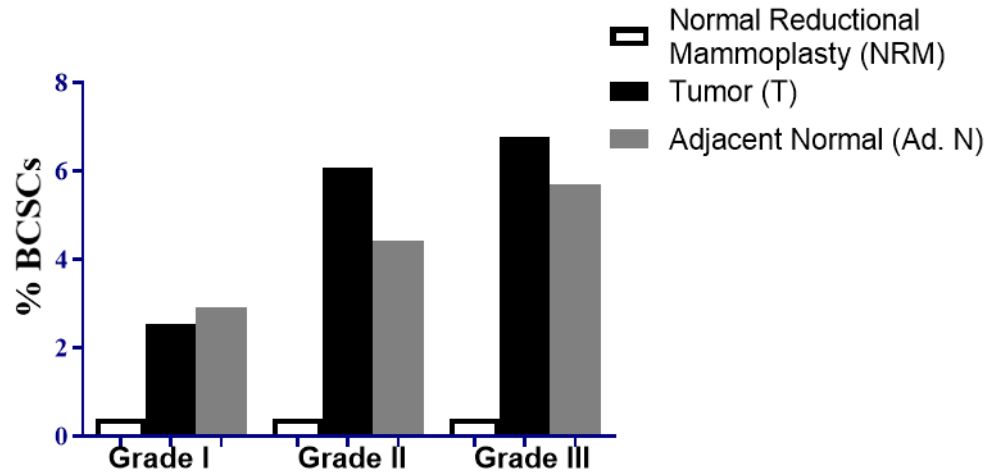


Comment 6: I have concerns with specificity in Figure 1: "Surprisingly the adjoining normal tissue to tumor also showed comparable numbers of BCSCs" this likely means that the tissue was not normal. Likewise, the staining in panel B looks unspecific, with mostly stromal staining. Why is the histology so different between samples?

Response 6: Staining for BCSCs is specific and similar cells have been seen in normal mammary tissue also (Normal reductional mammoplasty). A supplementary Figure 1 (Appendix 2: Supplementary Figure 1) has been added to describe the BCSCs (%) in normal mammoplasty sample as compared to tumor samples and adjacent normal samples. As previously indicated (ref) a very small percentage of cells in normal mammary tissue also express Lin- CD44+ CD24- profile but numbers are negligible as compared to tumor and adjacent normal tissue.

Appalaraju Jaggupilli, Eyad Elkord, "Significance of CD44 and CD24 as Cancer Stem Cell Markers: An Enduring Ambiguity", Journal of Immunology Research, vol. 2012, Article ID 708036, 11 pages, 2012. https://doi.org/10.1155/2012/708036

The surrounding tissue to tumor is generally taken as a paired control sample. As adjacent tissue to tumor tissue, it is only under diseased conditions that the histology as well as stroma could also have been affected/ diseased (as per the concept of field cancerizartion).



Now, it reads as, Result section 3.1 “We found that the adjoining normal tissue also contain BCSCs-like cells (Figure 1C and Figure 1D). The adjacent normal tissues to tumor are taken as

paired control samples in mastectomy samples but it does not represent/resemble normal mammary tissue (Supplementary Figure 1)."

Comment 7: Figure 5 is very complex and upon closer scrutiny, has some strange annotations. For instance, why are CDH1 and VIM both considered EMT genes (panel A)? And why do regulators or pluripotent/stemness not included in the network? What is the rationale for panel B? Hypoxia is already included in panel A. Panel C does not really help to condense the findings in an intuitive manner. Everything signals to everything else?

Response 7: Suggested changes have been incorporated. Figure 5 has been removed.

Minor comments

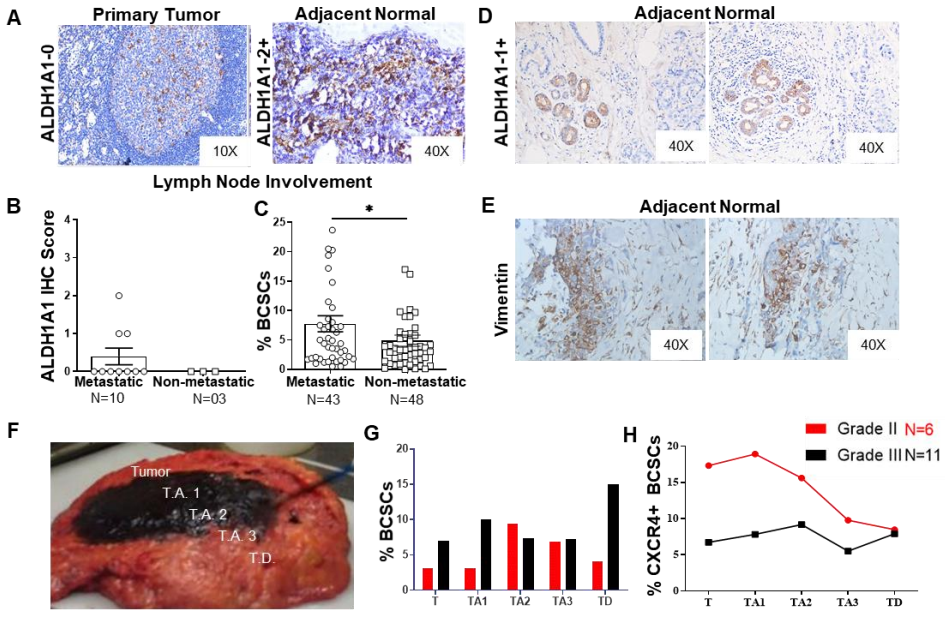
Comment 8: Does the (new) Title really reflect what's reported in the paper?

Response 8: Title has been changed. Now it reads as, "**Evaluation of breast cancer stem cells in human primary breast carcinoma and their role in aggressive behavior of the disease"**

Comment 9: Figure 2F, why do the authors switch to histograms instead of the cytoplots in Figure 1?

Response 9: Figure 2F represented the histograms of CXCR4 expressing BCSCs at different tissue interval. Histogram graph was chosen as we wanted to determine the MFI Median Fluorescent Intensity of CXCR4 expression on BCSCs and it is a univariate analysis graph, whereas, dot plot is a bivariate graph based on gating BCSCs on CD44 and CD24 parameters.

Figure 2F has been removed in updated figure.



Comment 9: Table 3 could be made Supplemental. If I understand correctly, these are the

same data that are used in Figure 3? Same applies to Table 4, doesn't that show pretty much the same as the plots in Figure 4?

Response 9: Suggested changes has been made. Table 3 and table 4 has been added to supplementary data file. Please refer to Appendix 3: Supplementary Table 4A-B, Table 5

Comment 10: Figure 3B and E, how are these samples and genes clustered? Figure 3C and F, how are these genes ranked (why not by expression in stroma), and why is the heat map only two colors?

Response 9: Few changes in Fiues have been made**.** Earlier figure 3B and 3E makes figure 3B now. Similarly, earlier figure 3C and 3F are part of figure 3A.

Figure 3A heatmap represents the average gene expression profile of Cancer cells and stromal cells in Hi-BCSCs and Lo-BCSCs tumors.

The clustergram in Figure 3B is generated by RT² Profiler PCR Array analysis platform. The clustergram is based on non-supervised hierarchical clustering of the entire dataset to display a heat map.

4th Editorial decision
07-Sep-2021

Ref.: Ms. No. JCTRes-D-21-00020R3
Evaluation of breast cancer stem cells in human primary breast carcinoma and their role in aggressive behavior of the disease
Journal of Clinical and Translational Research

Dear authors,

I am pleased to inform you that your manuscript has been accepted for publication in the Journal of Clinical and Translational Research.

You will receive the proofs of your article shortly, which we kindly ask you to thoroughly review for any errors.

Thank you for submitting your work to JCTR.

Kindest regards,

Michal Heger
Editor-in-Chief
Journal of Clinical and Translational Research

Comments from the editors and reviewers: