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RESEARCH NOTE

REVISED Blood-derived extracellular proteins as potential biomarkers for the diagnosis of early ER+ breast cancer and detection of lymph node involvement [version 2; peer review: 1 approved, 1 not approved]

Previously named: Blood-derived extracellular vesicle proteins as potential biomarkers for the diagnosis of early ER+ breast cancer and detection of lymph node involvement

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Abstract

Extracellular vesicles (EV's) are membrane surrounded structures released by different cell types and are emerging as potential therapeutic and diagnostic targets in cancer. In the present study, plasma samples derived from 7 patients with metastatic and non-metastatic ER+ (estrogen receptor positive) breast cancer (BC) were collected and their respective (EVs) isolated and the protein content analyzed by mass spectrometry and FunRich analysis. Here we report on the presence of two putative plasma EV biomarkers (which were absent in healthy controls samples) that could be used to detect early ER+ breast cancer and for those with lymph node (LN) involvement. However, given the preliminar nature of the work, further investigation in a larger patient cohort is warranted to corroborate these findings. If confirmed, these biomarkers could be incorporated into simple blood test kit for the early detection of those with ER+ breast cancer and lymph node involvement.

Keywords

ER+ breast cancer, extracellular vesicles, plasma, biomarkers, diagnostic, lymph node involvement, metastases

Open Peer Review

Approval Status

	1	2
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(revision)		
10 May 2018		view
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06 Mar 2018	view	view

1. **Shweta Aras** , Temple University, Philadelphia, USA

2. **Matthew J. Shurtleff** , University of California, San Francisco (UCSF), San Francisco, USA

Any reports and responses or comments on the article can be found at the end of the article.

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Author roles: **Tucker R:** Conceptualization, Data Curation, Formal Analysis, Funding Acquisition, Methodology, Supervision, Validation, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing; **Pedro A:** Conceptualization, Data Curation, Formal Analysis, Funding Acquisition, Investigation, Methodology, Validation, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing

Competing interests: No competing interests were disclosed.

Grant information: This work is supported by the Foundation of Science and Technology of Portugal [RECI/BIM-ONC/0201/2012], Lyden lab (Weill Cornell Medical College, USA), Champalimaud Foundation Portugal, and Romã Laboratories Ltd
The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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REVISED Amendments from Version 1

In this new version we removed the interpretation that the biomarkers we found are EV-based as the EV isolation method used is insufficient to purify EVs from other contaminants. Also, it is not intuitive that EVs would be likely to contain a histone (usually confined to the nucleus) or hemoglobin and therefore the biomarkers found are now referred to as “extracellular proteins” and not as “EV-based biomarkers”. We have removed the word “vesicle” from our article title.

See referee reports

Extracellular vesicles (EVs) are membrane surrounded structures released by different cell types that are involved in cellular communication and are emerging as potential therapeutic and diagnostic targets in cancer¹ as in the case of early pancreatic cancer². Structurally, EVs are contained within a phospholipid bilayer, whose composition is very similar to the cell membrane. EVs carry proteins, nucleic acids (DNA, mRNA, and miRNA), and lipids and are classified into three types: exosomes, ectosomes (microvesicles, MVs) and apoptotic bodies.

Tumor-derived EVs are also critical components for preparing the tumor microenvironment because they enable tumor cells to escape from the immunological surveillance³ and help in the setting of a pre-metastatic niche for the engraftment of detached cancer cells⁴. Both exosomes and MVs have been extensively studied and attributed various important physiological roles in cancer^{5,6}. For instance, EVs have been found to play an important role in every phase of cancer development from cancer initiation, invasion and metastasis⁷. For these reasons, EVs are potential therapeutic and diagnostic targets in cancer and EV-derived biomarkers maybe useful for predicting future metastatic development and identify metastasis sites⁸.

ER+ (estrogen receptor positive) breast cancer (BC) represents 60–80% of all BC cases^{9,10}. Here we describe our preliminary findings exploring the role of tumour derived EVs biomarkers that could ultimately be used as part of a test kit for the detection of early ER+ BC and lymph node involvement.

Methods

Samples

Plasma samples from 4 control patients (2 adult women and 2 men) which were confirmed as not having any form of BC, ER+ BC metastases, BC1 and BC2 explants EVs, SKBC and parental BC (Lyden lab, WCM, USA). Samples CF37, CF5, CF1, CF25, CF33, CF27 and CF110 were collected at Champalimaud Clinical Centre, Portugal, as part of a study on the role of tumor-derived microvesicles and bone marrow progenitor cells as diagnostic and prognostic biomarkers in advanced BC and inflammatory BC Patients (RECI/BIM-ONC/0201/2012, FCT, Portugal). ER+ BC patient samples were selected based on their stage of disease progression – confirmed by CT-scan and surgery. EVs derived from conditioned media of cells lines SKBr3, MCF7, MDA468, MDA231 and MCF10A were also used in this study (details about these samples can be found in Table 1).

Ethics approval and informed consent

This study was approved by an Ethics Review Board at Champalimaud Foundation, Portugal. All study patients provided their written, informed consent.

EV purification and analysis

EV purification and analysis were performed at the Lyden lab (WCM) accordingly to Andre *et al.* 2016¹¹. Briefly, plasma was pelleted at $500 \times g$ for 10 min, then the supernatant was centrifuged at $20,000 \times g$ for 20 min. Exosomes were then harvested by centrifugation at $100,000 \times g$ for 70 min. The exosome pellet is resuspended in PBS and collected by ultracentrifugation at $100,000 \times g$ for 70 min. The exosome pellet is resuspended in PBS and then stored at -80°C .

Proteomics and proteomic analysis

Proteomic analysis was performed at the Rockefeller University, Proteomics Center as described in Hamidi *et al.*, 2017¹². Proteomic analysis was performed with the help of FunRich Program version 3. Only proteins with Mascot scores of approximately 90 or >90 were considered¹³.

Results and discussion

Clinical data on the EVs isolated from BC patient's plasma samples and cell lines can be found in Table 1. The method used for EV isolation also precipitates lipoproteins and immunocomplexes (IC) which are known possible contaminants¹⁴. However, samples submitted for mass spectrometry analysis showed none of the recognised contaminants of high speed centrifugation. In the two patients with early BC (Table 2a), we detected HCG1745306 isoform CRA-a, a protein from the family of alpha type haemoglobins and for the patient with lymph node involvement, we detected histone H1.2 (Table 2 a–b). HCG1745306 isoform CRA-a was only present in the two patients with early BC with Mascot scores of 3208.8 and 3966.5, respectively and absent in all controls and other patient samples.

Histone H1.2 was also detected in samples from the two patients with bone metastases, a parental primary BC sample and metastatic SKBr-3, MDA468, MDA231 cell lines. However, histone H1.2 was absent from the plasma sample of a patient with multiple metastases, from the non-metastatic MCF7 cell line (a non significant mascot score) and from MCF10A cells EVs (Table 2b). These observation suggests that histone H1.2 might represent a potential marker for LN involvement and metastatic potential. Recent studies suggest histone H1.2 phosphorylation may be useful as a clinical biomarker of breast and other cancers because of its ability to recognize proliferative cell populations. Both MCF7 (expressing an allelic variant A142T) and MDA231, have a greater number of histone H1.2 phosphorylations when compared to MCF10A cell line¹⁵. Curiously, phosphorylation of histone H1.2 at S173 increases during the M phase relative to the S phase, suggesting that this event is cell cycle-dependent and may serve as a marker for proliferation of cancer cells during BC invasion^{16,17}. Also, histone H1.2 is a novel component of the nucleolar organizer regions during mitosis¹⁸ and H1.2 depletion was observed in a human BC cell line caused cell cycle G1-phase arrest¹⁹. Indeed, a higher mitotic index (≥ 7) in primary tumors is significantly associated with LN involvement²⁰ and higher mitotic indices accurately predict axillary LN involvement at operation²¹.

Table 1. Clinical data for different patient samples and cell lines.

Sample ID	Menopausal status	ER/PR/Her2 status (%)	Metastases pattern	Sample type
CF5	pre	100/95/-	LN+	Plasma
CF37	pos	100/-/-	LN-	Plasma
CF110	pos	100/100/-	Locally advanced	Plasma
CF1	pre	100/100/-	LN, liver	Plasma
CF25	pos	75/25/-	LN, liver, cartilage, skin	Plasma
CF33	pos	100/?/-	LN, liver, bone, skin, lung, brain	Plasma
CF27	pos	100/1/-	LN, lung, bone	Plasma
SKBC	?	?	Multiple metastasis	Plasma
BC1	?	ER+	Bone	Bone metastasis explant conditioned media
BC2	?	ER+	Bone	Bone metastasis explant conditioned media
Parental breast cancer	?	?	Primary tumor	Primary breast cancer conditioned media
SKBr3 (metastatic in mice) ²²	? (43y)	HER2+	Metastasis	Pleural effusion (ATCC) Conditioned media from cell line culture
MDA468 (metastatic in mice) ²³	? (51y)	TN (triple-negative)	Metastasis	Pleural effusion (ATCC) Conditioned media from cell line culture
MDA231 (highly metastatic in mice)	? (51y)	TN	Metastasis	Pleural effusion (ATCC) Conditioned media from cell line culture
MCF7 (poorly metastatic in mice)	pos	ER+	Metastasis	Pleural effusion (ATCC) Conditioned media from cell line culture
MCF10A	pre	Benign -fibrocystic disease	-----	Mammary gland; breast (ATCC) Conditioned media from cell line culture

Table 2. a–b, Plasma EV-derived candidate biomarkers for early ER+ breast cancer and LN involvement. Also, represented the Mascot scores for each protein in each sample.**a**

	Female1	Female2	Male1	Male2	CF37 (LN-)	CF5 (LN+)	CF110	CF1	CF25	CF27	CF33
G3V1N2 (HCG1745306, isoform CRA_a) Early breast cancer	0	0	0	0	3208.75461	3966.542	0	0	0	0	0
P16403 (Histone H1.2) Early breast cancer, LN involvement	0	0	0	0	0	325.1718	0	0	0	0	0

b

	SKBC	BC1	BC2	Parental BC	SKBr3	MDA468	MDA231	MCF7	MCF10A
P16403 Histone H1.2	0	638.4	117.38	102.9	154.45	427.1	90.35	NS	0

Our study does suffer from a number of recognised limitations. Firstly, the isolation method used is insufficient to purify EVs from other contaminants. Indeed, the EV literature is quite confusing due, in part, to the over-interpretation of observations from samples prepared using inadequate isolation methods. Secondly, it is not intuitive that EVs would be likely to contain a histone (normally confined to the nucleus) or haemoglobin, therefore our current data does not support the idea that the biomarkers we found are EV-associated and may just represent blood-derived extracellular proteins. Although we only have data for a small number of patients, a strength of our study is that samples were drawn from those with confirmed non-metastatic and metastatic disease at different sites and so are likely to be representative patients.

Nonetheless, our initial observations suggest the possibility that HCG1745306 isoform CRA-a could be used as a potential biomarker for the detection of early ER+ BC. Moreover, histone H1.2 might represent a marker for LN involvement. Further work in a larger cohort of patients clearly needed to refute or confirm these initial findings.

Dataset 1. The mass spectrometry analysis results from all patient samples

<http://dx.doi.org/10.5256/f1000research.14129.d196597>

Conclusion

Our early results suggest that blood- derived extracellular biomarkers could represent a means of identifying both patients with early ER+ BC and those in which the disease has spread to the sentinel nodes. Confirmation of these findings in a larger patient cohort might lead to the development of an antibody-antigen based test kit which could be used to provide an instant diagnosis of BC for patients visiting their primary care physician with a suspicious lump, thereby avoid the need for mammography and invasive biopsy.

Data availability

Dataset 1: The mass spectrometry analysis results from all patient samples [10.5256/f1000research.14129.d196597](https://doi.org/10.5256/f1000research.14129.d196597)²⁴

Competing interests

No competing interests were disclosed.

Grant information

This work is supported by the Foundation of Science and Technology of Portugal [RECI/BIM-ONC/0201/2012], Lyden lab (Weill Cornell Medical College, USA), Champalimaud Foundation Portugal, and Romã Laboratories Ltd.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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[Data Source](#)

Open Peer Review

Current Peer Review Status:

Version 2

Reviewer Report 01 May 2018

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Matthew J. Shurtleff

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The authors have not made a true attempt to satisfy my reservations. They have only parroted my reservations in their response and discussion, and removed the word "vesicle" from their title. They have not attempted to revise the content of their article at all. Indeed, the first two words of the text remain "extracellular vesicles". I do not consider this work satisfactory for indexing.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Version 1

Reviewer Report 16 April 2018

<https://doi.org/10.5256/f1000research.15368.r32581>

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Matthew J. Shurtleff

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Tucker and Pedro present results from proteomics studies of material precipitated from the plasma of breast cancer patients and healthy controls, and from the media of explant cultures and

cell lines. They observe CRA-a (HBA2, alpha hemoglobin) as being present in precipitated material from the plasma of patients with early breast cancer and Histone H1.2 as being present in samples with lymph node involvement (plasma), and the media of explant cultures of bone metastases and metastatic cell lines.

While the results are clearly presented, the interpretation that these are EV-based biomarkers is not supported. The EV isolation method used is insufficient to purify EVs from other contaminants. Furthermore, it is not intuitive that EVs would be likely to contain a histone (normally confined to the nucleus) or hemoglobin. The authors' discussion of this major concern is insufficient ("However, samples submitted for mass spectrometry analysis showed none of the recognised contaminants and it was therefore concluded that the main EV type present in these samples is the MV.").

The EV literature is quite confusing due, in part, to the over-interpretation of observations from samples prepared using inadequate isolation methods. Therefore, in its current version, I do not recommend indexing of this report. Ideally, the authors should repeat the work with approaches that better enrich for EVs over non-EV protein contaminants (e.g. density-gradient based ultracentrifugation or immuno-affinity purification) and protease protection assays in the presence/absence of detergent should be used to evaluate if the proteins are indeed associated with EVs and if they are on the surface or interior of vesicles. If further experiments are not possible, I recommend revising the paper to remove the unsupported conclusion that the proteins identified are EV-associated. (For example the title could be: Blood-derived extracellular proteins as potential biomarkers for the diagnosis of early ER+ breast cancer and detection of lymph node involvement).

For reference, I direct the authors to an updated publication prepared by members of the EV community on the minimal information for studies of EVs in the *Journal of Extracellular Vesicles*¹.

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Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

No

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Not applicable

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

No

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 26 March 2018

<https://doi.org/10.5256/f1000research.15368.r32046>

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Shweta Aras

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In this research note by Tucker et al., authors have identified 2 novel biomarkers potentially involved in early breast cancer development as well as lymph node metastasis using extracellular vesicles from patient samples along with adequate controls of metastatic and non-metastatic cell lines. Several reports in the literature have already suggested the importance of EVs in cancer initiation and progression in various types of cancers. Although the novelty of this publication is limited, identification of 2 new molecules differentially expressed in primary versus metastatic breast cancer patients opens up the possibility of them being used in a simple prognostic blood test for detecting early BC development as opposed to mammography and other invasive techniques. A probable limitation of the study would be lack of identification of molecular mechanisms through which this upregulation of CRA-a and histone H1.2 phosphorylation plays a role in metastasis, specifically in which step of metastatic cascade e.g. intravasation, EMT, extravasation etc? But overall, the authors have done a great job and the article is a good addition to the field.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

I cannot comment. A qualified statistician is required.

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

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