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14. ABSTRACT

Tumor metastasis is an extremely inefficient process and only a fraction of cells in the primary tumor can successfully establish metastatic colonization. These cells by definition have a stem-like ability, but they also need to have an ability of metastasizing to other organs. Therefore, in addition to a tumor stem cell, an existence of a metastatic stem cell is predicted. Despite the critical importance of the concept, this idea has not been rigorously tested due to a lack of an appropriate experimental system. We propose to take an innovative approach to challenge this question by isolating stem cell population from a unique set of breast tumor cell lines and by examining their metastatic behavior in an animal model. The overall objective of our project is to identify metastatic stem cells of breast cancer and define basic characteristics of these cells. To test our hypothesis, we will (i) isolate stem-cell population from non-metastatic and metastatic cells of a pair of syngenic breast tumor cell lines, and test their metastatic ability in an animal model, and (ii) examine their gene expression profiles by microarray analysis and verify the results in tumor stem cells of human breast cancer specimens.

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None provided.

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INTRODUCTION

More than 90% of deaths caused by breast cancer is attributed to metastatic disease (1). However, the exact molecular mechanism of tumor metastasis is still poorly understood. It has been well recognized that only a fraction of cells in the primary tumor eventually metastasizes to the distant organs; however, the origin and nature of these cells are still unclear (2). The purpose of this project is to test our novel hypothesis that metastatic cells are originated from a distinct tumor cell population which has both stem-like properties and an invasive ability. The overall objective of our project is to identify metastatic stem cells of breast cancer and define basic characteristics of these cells. To test our hypothesis, we will (i) isolate stem-cell population from non-metastatic and metastatic cells of a pair of syngenic breast tumor cell lines, and test their metastatic ability in an animal model, and (ii) examine their gene expression profiles by microarray analysis and verify the results in tumor stem cell of human breast cancer specimens.

The grant was funded for one year period, and the project is ongoing as proposed. However, we had an initial delay of setting up the account and hiring personnel, and therefore, the project is behind schedule. Accordingly, we have requested no-cost extension to DOD in July 2010, although the approval of request is still pending. Therefore, for the purpose of this progress report, we assume that our request is approved.

BODY

Task 1. To isolate stem-cell population of non-metastatic and metastatic cells from a pair of syngenic breast tumor cell lines and test their metastatic ability in an animal model.

(a) "Label" MDA-MB231 and BoM cells with the luciferase gene either wild type or mutant.
We have generated lentivirus containing the luciferase gene and the virus was infected to MDA-MB231, MB231BoM and MB231BrM. The latter two cell lines were originally isolated by Dr. Massagu's group from metastasized tumors in bones and brain after injecting MDA-MB-231 into animals (3). They are highly metastatic to bones and brain, respectively, when they are transplanted into immunodeficient mice. After infection of the luciferase lentivirus at m.o.i of 5, the efficiency of infection and labeling was examined using the Xenogen bioluminometer. We found that nearly 100% cells were labeled with the luciferase gene, and these cell were used for the following experiments.

(b) Isolate tumor stem cells (CD24-, CD44+ and ESA+) from both cell lines.
Flow cytometric analysis of these cell lines (MDA-MB231, MB231BoM and MB231BrM) using previously identified surface markers for cancer stem-like cells (CD24, CD44 and ESA) (4) indicate that these cell lines contain a minor population (3-8%) of CD24-/CD44+/ESA+ cells (Fig. 1). We then isolated cancer stem-like cell from these cell lines, using respective antibodies by the magnetic sorting system (MACS).

(c) Test their metastatic ability in DOD-SCID mice.
To test the tumor initiating abilities of the isolated tumor stem-like cells, we injected these cells into nude mice at low doses, and the results of our limiting dilution analysis indicate that the tumor stem-like cells from each line showed significantly stronger ability of tumorigenesis than the corresponding non-stem cell populations and unsorted populations (Table 1). When they were intracardially injected into nude mice, we found that they were highly metastatic to bone and brain.

We consider that all aims of Task 1 were successfully accomplished.

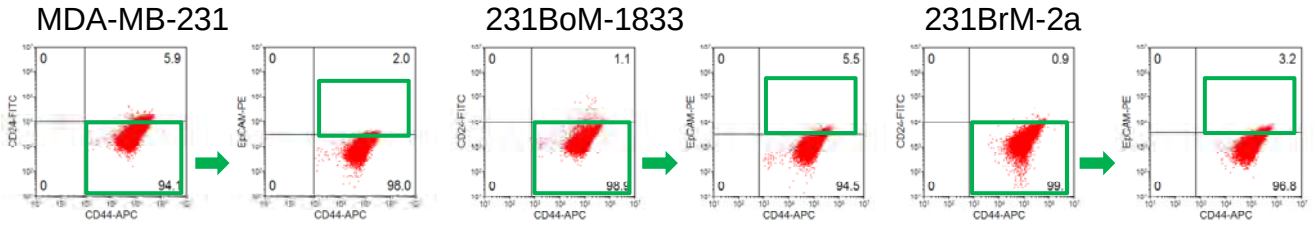


Fig. 1. MB231, MB231BoM and MB231BrM cells were analyzed by FACS for the expression of CD24, CD44 and ESA markers.

Table I. tumor initiation ability of cancer stem-like cells

Strain	Population	Number of tumors/number of injections Cells per injection 10,000	1,000	100	10	Tumor-initiating cell frequency (95% cenfidence interval)	
MDA-MB-231	Unsorted	2/4	1/4	0/2		1/10,720	(1/3,203-1/35,879)
	Stem cells	6/6	5/6	2/6		1/448	(1/183-1/1,097)
	Non-stem cells	1/2	0/2	0/2		1/16,705	(1/2,356-1/118,284)
231BoM-1833	Unsorted	6/6	6/7	3/5	0/3	1/334	(1/140-1/844)
	Stem cells	5/5	11/11	9/11	0/5	1/66	(1/33-1/133)
	Non-stem cells		2/4	0/4	0/4	1/1,671	(1/419-1/6,668)
231BrM-2a	Unsorted						
	Stem cells		4/4	5/6	2/6	1/45	(1/19-1/110)
	Non-stem cells		5/6	1/6	0/6	1/569	(1/236-1/1374)

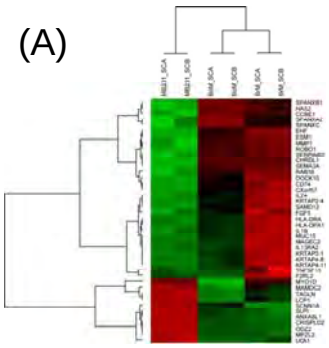
Task 2. To examine their gene expression profiles by microarray analysis and verify the results in tumor stem cell of human breast cancer specimens.

(a) Analyze expression profile of non-metastatic and metastatic stem cells by Affymetrix microarray.

We then performed global expression profile analysis for these stem-like cells using the Affymetrix expression array. Figure 1A shows a heat-map of genes whose expressions were significantly altered by more than 10 times among tumor stem cells from the three cell lines (Total 42 genes). There are 7 genes that are highly over-expressed in both the metastatic tumor stem cells, while 6 genes were found to be significantly down-regulated in both of these cells (Fig. 2A).

To further narrow down the list of genes by considering the clinical significance, we examined the relationship between the expression of these genes and overall- and metastasis-free survival of breast cancer patients using the existing GEO data base (Fig. 2B). Among up-regulated genes of our array analysis, five genes (MMP1, SERPINB2, SPANXB1, HAS2 and ESM1) were all correlated with overall- and metastasis-free survival in at least one cohort data in GEO. On the other hand, the down-regulation of four genes (ODZ2, CRISPLD2 and MAMDC2) was significantly correlated with overall- and metastasis-free survival. We also confirmed the results by qRT-PCR for some of the genes (Fig. 2C). These results suggest that these genes play critical roles in metastatic tumor stem cells in breast cancer progression.

(A)



(B)

Gene	FC in BoM SC	FC in BrM SC	Overall survival P-value	Prognosis	Cohorts	MET-free survival P-value	Prognosis	Cohorts
MMP1	47.46	103.90	0.0034	Poor	5 / 5	0.0004	Poor	3 / 5
SERPINB2	33.11	58.15	0.0253	Poor	1 / 5	0.0485	Poor	1 / 5
EHF	23.31	69.62	ns	None	0 / 5	ns	None	0 / 5
CHRDL1	15.31	17.46	0.0005	Good	1 / 5	0.0027	Good	1 / 5
SPANXB1	18.46	10.37	0.0080	Poor	2 / 5	0.0022	Poor	2 / 5
HAS2	17.77	12.01	0.0124	Poor	2 / 5	0.0166	Poor	1 / 5
CCBE1	16.96	10.33	ns	None	0 / 1	ns	None	0 / 1
ANXA8L1	-11.42	-16.94	ns	None	0 / 5	ns	None	0 / 5
CRISPLD2	-14.90	-17.84	0.0074	Good	1 / 5	0.0293	Good	1 / 5
UCA1	-19.57	-14.81	ns	None	0 / 1	ns	None	0 / 1
ODZ2	-21.35	-26.39	0.0009	Good	2 / 2	0.0024	Good	1 / 2
LCP1	-49.54	-14.16	0.0267	Poor	1 / 5	0.0486	Good	1 / 5
MPZL2	-38.25	-50.60	0.0109	Poor	1 / 4	0.0429	Poor	1 / 4

(C)

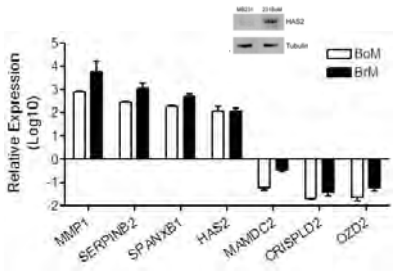


Fig.2. HAS2 gene is upregulated in stem cells from metastatic breast cancer cells
(A) Expression array analysis of breast cancer stem cells. Cancer stem cells from MDA-MB-231, 231BoM1833 and 231BrM-2a were prepared, and their RNAs were subjected to Affymetrix expression array analysis. A heatmap was generated for the genes that were up- or down-regulated in each cell line by comparing tumor stem cell. The cut off threshold is 10 times and only those genes significantly changed ($P<0.05$) were chosen. (B) Survival analysis of breast cancer patients. Genes that were up- or down-regulated more than 10 times in tumor stem cells from both 231BoM-1833 and 231BrM-2a compared to that of MDA-MB-231 were chosen. Overall or metastasis free survival of breast cancer patients who have altered expression of these genes were examined using existing GEO database. A number of cohort data which showed significant difference were recorded. (C) The six genes were chosen and their expressions were examined by qRT-PCR in tumor stem cells from the three cell lines. Fold-changes were indicated which are compared to the gene expression of cancer stem cells from MDA-MB-231. (Inset) Western blotting of HAS2 protein is shown.

(b) Establish "metastatic signature" of stem cell by data analysis.

On going.

(c) Isolate tumor stem cells from both primary and lymphnode metastatic lesions and examine the expression of "metastatic signature" genes in these cells.

On going

Therefore, we consider that the subaim (a) of Task 2 was successfully accomplished.

KEY RESEARCH ACCOMPLISHMENTS

1. We have successfully isolated tumor stem-like cell population from highly metastatic breast cancer cell lines.

2. We have performed global gene expression analysis for the stem-like cells and found that the expressions of a total of 42 genes were significantly altered in these stem-like cells.

3. The results of patient survival analysis for these genes using GEO data base indicated that MMP1, SERPINB2, SPANXB1, HAS2 and ESM1 are significantly correlated with overall- and metastasis-free survival. Therefore, we will focus on these genes for further analysis.

REPORTABLE OUTCOMES

Peer reviewed publications
At this point, we do not have a published manuscript; however, the following manuscript is in preparation and we are expecting to submit it by the end of this year.

Hiroshi Okuda, Aya Kobayashi, Xia Bo, Misako Watabe, Sudha K Pai, Shigeru Hirota, Fei Xing, Wen Liu, Puspa R Pandey, Kounosuke Watabe. Roles of HAS2 in cancer stem-like cells: Hyarulonic aicd promotes interaction of cancer stem-like cells with tumor associated macrophage and endothelial cells in bone.

Employment
Postdoctral fellow: Dr. Hiroshi Okuda

CONCLUSIONS

We were able to isolate highly metastatic tumor stem-like cell population from metastatic breast cancer cell lines. Basedon on the results of Affymtrix gene expression analysis and patient cohort analysis, we identified that 5 genes, MMP1, SERPINB2, SPANXB1, HAS2 and ESM1 are highly expressed in metastatic stem cells. We are currently focusing our effort to understand how the HAS2 gene promotes metastatic ability of tumor stem cells. We are also trying to identify metastatic signature of tumor stem cells based on our results for potential clinical application.

SO WHAT?

Our results clearly indicate that metastatic tumor stem-like cells exist and they have distinct gene expression profiles. The most important implication of our finding is that these genes may serve as potential therapeutic target to treat/prevent metastatic disease. Considering that tumor stem cells are generally drug resistant and they may contribute to recurrent disease, it is of significant interest to elucidate the role of these genes in the self-renewal ability of stem-like cells. We hope to clarify this key question by focusing on HAS2 gene.

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