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14. ABSTRACT

Breast cancer is the most frequently diagnosed cancer and the second leading cause of cancer death among women in the US. Because metastatic disease is the major cause of death, it is crucial to understand the mechanism by which tumor cells metastasize to the distant organs so that we can identify a better therapeutic target. During this funding period, we had a breakthrough finding that the metastasis suppressor gene, NDRG1, can directly interact with the Wnt receptor, LRP6, and block the Wnt signaling. This inhibition leads to down-regulation of the ATF3 gene and thus suppressing metastases. We also found that a combination of NDRG1, PTEN and ATF3 is a good prognostic marker for breast cancer patients. These results suggest that the Wnt and ATF3 pathways are a potential therapeutic target for patients with metastatic disease. We will focus our next year's effort on further clarification of the NDRG1 pathway.

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INTRODUCTION

This is a revised report for our project which was funded by DOD Breast Cancer Research Program. The original report was disapproved because (i) it was not clear whether funds were used for experiments other than breast cancer related research, (ii) some figures are misidentified in the text (iii) key research accomplishment and outcome that have been listed in the previous report do not need to be reported and (iv) reportable outcome need to be appended. Accordingly, we have revised the report and revisions were indicated by underline in the text.

Once breast cancer is diagnosed, the most critical question is whether the disease is localized or has it already metastasized to other organs (1). However, the molecular basis of tumor metastasis is poorly understood as yet. The proposed research in this application aims at elucidating the function of the tumor metastasis suppressor gene, Drg-1/NDRG1, in the hope that we can define a specific target for novel and effective therapies to prevent metastatic disease of breast cancer. We hypothesize that NDRG1 functions as a tumor metastasis suppressor in breast cancer (Task 1). We also hypothesize that loss of tumor suppressor PTEN down-regulates NDRG1 gene which leads to metastases (Task 2). We also plan to assess NDRG1 as a diagnostic or prognostic marker to accurately predict metastatic disease. Our ultimate goal is to develop a novel therapeutic method which mimics the function of the NDRG1 gene. We believe that the knowledge gained from the proposed study will eventually be translated into clinical trials.

BODY

Task 1-a.

To examine the effect of NDRG1 on tumor metastases in nude mouse model by injecting NDRG1 expressing cells orthotopically as well as intravenously. We will also examine the expression of the metalloprotease genes at the tumor site in the animals.

We first constructed MDA-MB231 cell line which expressed the luciferase gene using a lentivirus system. We then introduced NDRG1 expression vector into this cell line using a flag-tagged NDRG1 gene and obtained 3 independent clones. These cell lines were confirmed for NDRG1 expression by Western blot. At this point we injected one clone and the parental cell line into 5 nude mice/group through i.v., and we are currently checking the mice twice a week using the Xenogen bioluminescent instrument. Initially, we had several small technical problems such as weak signal, method of anesthesia and handling the instrument. However, we are now more confident to accomplish this task in a relatively short time. We are also constructing a cell line that over-expresses NDRG1using T47D which is a more metastatic breast cancer cell, in order to test the effect of NDRG1 in animals.

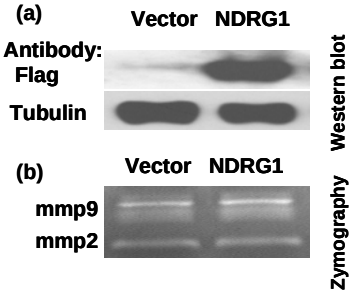


Fig. 1. Expression of NDRG1 does not affect MMP2 or MMP9. (a) NDRG1 was ectopically expressed in MCF7 and cell lysates were prepared. The lysates were then subjected to Western blot (a) and Zymography assays.

We have also constructed MCF7 cell lines that expressed NDRG1. The expression of metalloprotease genes, MMP2 and MMP9 were examined on these clones (Fig. 1). However, our preliminary data indicate that NDRG1 did not activate these metalloproteases. We are currently confirming our results by Zymography.

Task1-b.

To perform metastasis specific microarray analysis using an inducible NDRG1 expression system to understand downstream effectors of Drg-1.

We have established tetracycline-inducible expression of NDRG1 in a tumor cell and performed a microarray analysis using the Affymetrix human gene array. The results of our microarray analyses indicated that the ATF3 gene, a member of ATF/CREB transcription factor family (2, 3), was most significantly suppressed by induction of the NDRG1 gene. To verify the result of the microarray data, the NDRG1 expression plasmid (pcDNA3/NDRG1) or the empty pcDNA3 vector was transiently transfected into the breast cancer (MCF-7 and MDA-435) as well as prostate (PC3 and ALVA) cell lines and the level of ATF3 protein was examined by Western blot (Fig. 2 A). We found that NDRG1 indeed attenuated the ATF3 expression in a dose-dependent manner in all these cell lines, while the empty vector did not have any notable effect. In a complementary approach, we introduced NDRG1

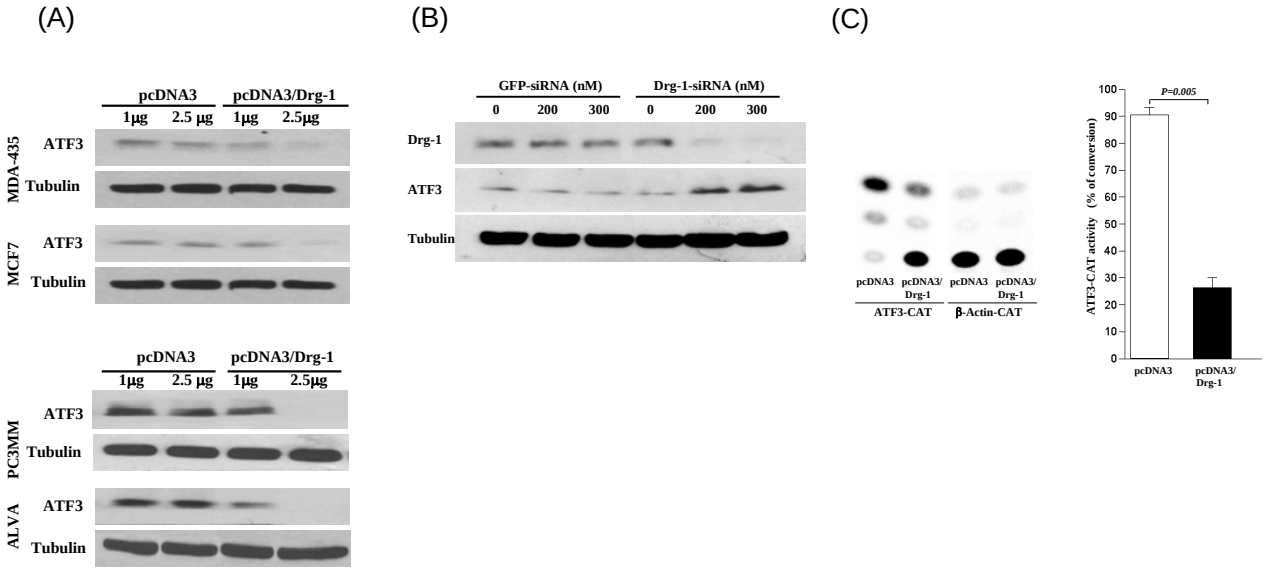


Fig. 2. NDRG1 down-regulates ATF3 expression. (A), Empty vector pcDNA3 or NDRG1 expression vector, pcDNA3/NDRG1, at the indicated amounts, was transfected into the breast cancer cell lines (MDA-435 and MCF7) and prostate cancer cell lines (PC3MM and ALVA). Forty-eight hour post-transfection, cells were lysed and Western blot was performed using antibodies against ATF3 and Tubulin. (B), siRNA for Drg-1 or GFP was synthesized and various amounts of the siRNA, as indicated, were transfected into PC3MM cells. After 72 hours, cells were lysed and the lysates were examined by Western blot with antibodies for NDRG1, ATF3 and Tubulin. (C), A CAT-reporter plasmid (ATF3-CAT) containing the ATF3 promoter region (-1850 to +34) was co-transfected with NDRG1 expression plasmid (pcDNA3/NDRG1) or empty vector (pcDNA3) into the cells. Forty eight hours later, the cells were harvested, lysed and the lysates were then assayed for the CAT activity. Acetylated chloramphenicol was resolved on TLC plate (representative run, left panel) and each spot was quantified (right panel). A reporter plasmid containing the β-actin promoter (βactin-CAT) was used as a control.

siRNA or GFP siRNA in the cancer cells and found that the NDRG1 siRNA specifically abrogated the expression of the NDRG1 gene which led to concomitant up-regulation of the ATF3 expression in these cells (Fig. 2B). These data strongly suggest that NDRG1 plays a crucial role in the in regulation of the ATF*3* gene, and down regulation of Drg-1 in tumor cells results in augmentation of ATF3 expression. To further examine whether down-regulation of ATF3 expression by NDRG1 is mediated at the transcriptional level, tumor cells were co-transfected with NDRG1 expression vector (pcDNA3/NDRG1) or an empty vector (pcDNA3) and ATF3-CAT reporter plasmid, and the CAT reporter assay was performed. We found that the ATF3-CAT reporter activity was significantly attenuated by NDRG1; thereby strongly suggesting that NDRG1 negatively controls the expression of the ATF3 gene at the transcriptional level (Fig. 2C).

To corroborate the above in vitro results, we established prostate tumor cell lines that expressed the ATF3 gene and they were then injected to SCID mice. The growth of primary tumor was measured for a period of 3 weeks and mice were then sacrificed to examine the metastatic lesions in the lungs. We found that growth rate of primary tumor did not change notably between the tumors with and without expressing ATF3. However, the number of metastatic lesions in the lungs were significantly increased in the mice that received tumor cells over-expressing ATF3, suggesting that ATF3 indeed is capable of promoting the tumor metastases.

Table 1. Effect of ATF3 on tumor metastasis

Cell line	ATF3 [a]	Tumor incidence [b]	*In vivo* doubling time	Lung metastasis (mean+/−S.E.) [c]	P value
AT2.1	−	5/5	4.1+/−0.5	5+/−1.9	
Vector only	−	5/5	3.9+/−0.3	5.2+/−3.3	0.96
ATF#9	−	4/4	3.7+/−0.8	2.5+/−0.9	0.51
ATF#4	+	5/5	4.3+/−0.3	39+/−11.3	0.02*
ATF#111	+	5/5	3.7+/−0.2	32+/−9.0	0.02*
ATF#207	+	5/5	2.5+/−0.2	46+/−3.2	<0.001*

a. ATF3 expression was examined by western blot.

b. Number of tumor-bearing SCID mice / no. of tumor-inoculated SCID mice.

c. Number of metastatic lesions on lungs per SCID mouse.

To further elucidate the mechanism of NDRG1 as a metastasis suppressor, we performed the Yeast two hybrid analysis. After screening approximately 1.2 million c-DNA clones of normal human tissues, we obtained four clones that reproducibly interacted with NDRG1 (Fig.3). Among these clones, LRP6, which is known to be a co-receptor of the Wnt protein, showed the strongest interaction with NDRG1, and we decided to proceed with further analyses. To show the interaction of NDRG1 and LRP6 in mammalian cells, we first tagged NDRG1 and LRP6 with Flag and HA, respectively, for the purpose of a co-immunoprecipitation experiment. Flag-Drg1was subcloned into the tetracycline inducible system followed by transfecting and establishing a cell line. The expression plasmid of HA-LRP6 was then transiently transfected into the tet-inducible Flag-NDRG1 cells, followed by induction of NDRG1 by tetracycline. The cells were lysed and proteins were precipitated by using anti-HA antibody. The sample was then subjected to Western blot analysis using anti-Flag antibody. As shown in Fig.3B, the results of our co-immunoprecipitation experiment indicate that Flag-NDRG1 was pulled-down with HA-LRP6, suggesting that these two proteins are indeed interacting in the cell. We also performed a similar co-immunoprecipitation experiment using HA-LRP5 and found that NDRG1

also interacts with LRP5 (data not shown). LRP6 functions as an essential co-receptor together with Frizzled for the Wnt signaling pathway. Apparently, LRP6 constitutes the distal signal-initiating

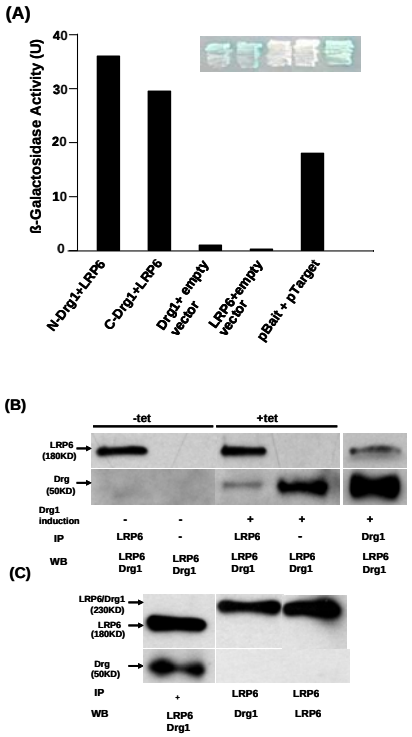


Fig. 3. Drg-1 interacts with LRP6. (A) Using a yeast two-hybrid system, LRP6 was identified as an interactor of Drg-1. The Drg1 was fused with LexA DNA-binding domain at N- (N-Drg1) or C-terminal (C-Drg1), and target LRP6 was fused with B42 activation domain. Appropriate combinations of expression plasmids were transformed in the same yeast strain, Egy48. The resultant transformants were grown and assayed for the β-galactosidase activity (Miller unit) of the reporter LacZ gene. pBait and pTArget are a pair of positive controls. Inset photo shows transformants grown in an agar plate containing X-gal. (B) Co-immunoprecipitation of Drg1 and LRP6 in mammalian cells. For lanes 1-4, PC3mm with the tet-inducible Flag-tagged Drg1 gene was transiently transfected with the expression plasmid of HA-tagged LRP6. Cells were then treated with (Lanes 1 and 2) or without (lanes 3 and 4) teteracycline to induce Drg1. Cells were then lysed and the proteins were precipitated with anti-HA antibody and Protein-G agarose (lanes 1 and 3). The precipitated proteins were detected by Western blot with HA antibody for LRP6 and Flag antibody for Drg-1. Lanes 2 and 4 show control Western blot without immunoprecipitation. For lane 5, PC3mm cells were tranfected with the expression plasmid of HA-LRP6 and immunoprecipitation was done using anti-HA antibody. Western blot was then performed to detect LRP6 and endogenous Drg1 using anti-HA and anti-Drg-1 antibodies, respectively. (C) Co-immunoprecipitation of crosslinked Drg1 and LRP6. PC3mm cells with the tet-inducible Flag-tagged Drg1 gene were tranfected with HA-LRP6 expression plasmid and further incubated for 48 hrs. Cell were then treated with (lanes 2 and 3) or without (lane 1) non-cleavable and membrane permeable cross-linker DSS for 1 hrs. Cell lysates were prepared and subjected to immunoprecipitation using anti-HA antibody followed by Western blot using both anti-Flag (lane 1 and 2) and anti-HA (lane 1 and 3) antibodies. Note that band on lanes 2 and 3 were shifted due to LRP6-Drg1 cross-linked complex.

component. The Wnt gene was originally identified as a developmental gene in Drosophila but it was later found to play a key role in mammalian embryogenesis as well as in tumorigenesis in various types of human cancers. Recently, the Wnt pathway has also been found to play a critical role in epithelial-mesenchymal transition (EMT). The EMT is a typical characteristic change of tumor cells from primary to metastatic cell, which accompany β-catenin re-localization, loss of E-cadherin and ability to invade ECM. In fact, over-expression of the Wnt ligand or the receptor has been shown to promote invasiveness of tumor cell and the following metastases. Therefore, it is conceivable that NDRG1 suppresses the metastatic process by blocking the Wnt signal by binding to LRP6.

In order to examine the effect of NDRG1 in a signal pathway, we prepared the lysate of the cells which had the tetracycline-inducible NDRG1 gene after treatment with or without tetracycline. This pair of cell lysate was tested for a screening "activated" signal molecules using 26 different

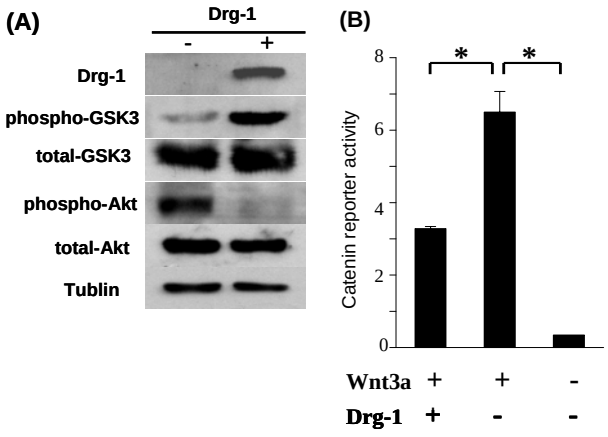


Fig. 4. Drg1 blocks the Wnt signal by augmenting GSK3 Tyr-phosphorylation and inhibition of AKT serine phosphorylation. (A) Cells with the tet-inducible expression plasmid of NDRG1 were treated with or without tetracycline and cell lysates were prepared. The samples were subjected to Western blot analyses using antibodies against phospho-AKT (ser473), total AKT, Drg1, and phospho-GSK3b (Tyr279/216). (B) NDRG1 suppresses β-catenin activity. Cells with the tet-inducible expression plasmid of NDRG1 were transfected with the reporter plasmid containing catenin/TCF binding sites (TOPflash system) and the luciferase gene in the presence or absence of Wnt followed by the treatment with or without tetracycline to induce NDRG1 expression. The cells were then harvested and the luciferase activity of the cell lysates was assayed. Renilla luciferase activity was used as an internal control.

phosphor-specific antibodies (Kinexus) by Western blot analysis. We found that, among these molecules, GSK3β was significantly phosphorylated at Tyr279/216 while Akt was strongly de-phosphorylated at Thr308. To confirm these results, we have performed Western blot analysis using the lysate prepared from cells with or without induction of NDRG1. As shown in Fig. 4A, Akt was significantly de-phosphorylated by over-expression of NDRG1 while total Akt showed no difference. On the other hand, GSK3β was significantly phosphorylated by induction of NDRG1. These results suggest that NDRG1 activates GSK3β but inactivates Akt.

GSK3β is known to be a key signal mediator of the Wnt pathway. The protein makes a complex with APC and this complex stimulates ubiquitination of β-catenin followed by their degradation. When Wnt binds to the receptor, Frizzled and LRP5/6, GSK3β is de-phosphorylated at tyr437 residue and "inactivated". As a consequence, β-catenin accumulates and is transported to the nucleus followed by activation of various "pro-oncogenic" genes. Since our results indicate that NDRG1 binds to LRP6 and also phosphorylates GSK3β at Tyr279/216 which activates this molecule, it is plausible that the interaction of NDRG1 to LRP6 blocks the process of GSK3β de-phosphorylation in the Wnt pathway. It should be also noted that Akt phosphorylates GSK3β at serine residues which results in inactivation of GSK3β. As shown in Fig.4, NDRG1 appears to de-phosphorylate Akt and "inactivate" this protein kinase, suggesting that Drg1 further "activates" GSK3β function by down-modulation of the Akt activity. To test the possibility that NDRG1 indeed blocks the Wnt pathway, we examined the β-catenin/TCF activity using the Topflash reporter plasmid which contains 8 tandem repeat sequences of the TCF binding site upstream of the luciferase reporter gene. The reporter plasmid was transfected to the cells containing tetracycline-inducible NDRG1 plasmid in the presence or absence of Wnt, followed by a treatment of the cells with and without tetracycline. The cell lysates were then assayed for luciferase activity. The results of the experiment showed that the luciferase activity was up-regulated more than 30 times in the presence of Wnt, while the induction of Drg1 significantly suppressed the reporter gene activity (Fig. 4B). These results strongly support our notion that NDRG1 blocks the Wnt pathway by GSK3β activation.

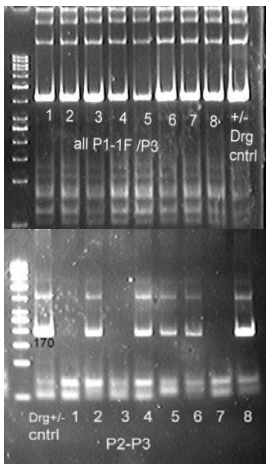
Task 2-a.
To identify the PTEN responsive region on NDRG1 promoter and the factors responsible for the activation

The reporter plasmid of NDRG1 was successfully constructed and we also generated systematic deletion mutants of the promoter region of the NDRG1. The PTEN expression vector is in our hands. We are now conducting a series of experiments to determine the location of PTEN responding region on the promoter of NDRG1 gene. We expect that we will obtain the results of this experiment shortly. Once we identify the region, we will then introduce site-specific mutations to validate the results of deletion analysis.

Task 2-b.
To examine whether the down-regulation of NDRG1 by PTEN indeed leads to metastasis in an animal model

We have not yet pursued this Task at this point extensively. However, we have recently obtained the NDRG1 knockout mouse from Japan and we are establishing a colony of this knockout mouse at SIU (Fig. 5). These mice will be used for this task in the future.

(a)



(b)

	P1/P3	P2/P3
+/+	2kb	170bp
+/-	2kb/400bp	170bp
-/-	400bp	—

(c)

#	Tag		Genotype
1	1179	♂	-/-
2	1180	♂	+/-
3	1181	♂	-/-
4	1182	♂	+/-
5	1183	♂	+/-
6	1184	♀	+/-
7	1185	♀	-/-
8	1186	♀	+/-

Fig. 5. Establishment of an NDRG1 knockout mouse colony. After 3 cycles of backcross breeding, the first generation of homozygous knockout animal was obtained. (a): Results of genotyping by PCR analysis, (b): Expected size of PCR product for homozygous and heterozygous knockout mice, (c): Summary of the first generation of NDRG1 knockout mouse colony.

Task 3-a.

To examine paired samples of primary and lymph node metastases of breast cancer patients for the expression of the NDRG1 gene.

We performed immunohistochemical analyses for NDRG1 and other clinical parameters on both breast and prostate cancers. As shown in Fig. 6, in both breast and prostate cancers, a significant level of differential expression of NDRG1 was observed between the patients with organ-confined disease and those with metastasis to lymph node or bone. In the case of breast cancer, while 89.7% patients were positive for NDRG1 expression out of 29 localized cases, 60.7% were positive for NDRG1 expression among 56 patients with metastases (Fig. 6A). These results strongly suggest the negative involvement of NDRG1 in the process of metastasis in breast cancer. In the case of prostate cancer, while 28 cases (70%) were positive for NDRG1 out of 40 localized prostate cancer cases, only 5 (25%) were positive for NDRG1 expression out of each of the 20 and 19 cases with lymph node and bone metastasis. Thus, the negative correlation of NDRG1 with metastatic spread to lymph node and

(A)

Factor	Total (85)	Drg-1 expression positive	reduced	P value
Age				
< 51	33	25 (75.8%)	8 (24.2%)	
>51	52	35 (67.3%)	17 (32.7%)	0.56
Histological grade				
I/II	30	24(80.0%)	6(20.0%)	
III	55	36(65.5%)	19(34.5%)	0.16
P53				
Wild type	57	40 (70.2%)	17 (29.8%)	
mutant	28	20 (71.4%)	8 (28.6%)	0.99
ER				
Positive	40	27 (67.5%)	13 (32.5%)	
Negative	45	33 (73.3%)	12 (26.7%)	0.73
Tumor status				
$T_{1-2}N_xM_x$	64	46 (71.9%)	18 (28.1%)	
$T_{3-4}N_xM_x$	21	14 (66.7%)	7 (33.3%)	0.86
Metastasis status				
$T_xN_0M_0$	29	26 (89.7%)	3 (10.3%)	
$T_xN_{1-2}M_{0-1}$	56	34 (60.7%)	22 (39.3%)	0.01*

(B)

	All (62)	Drg-1 expression positive (34)	reduced (28)	P value
Age				
(mean +/- S.E. yrs.)	72.0 +/- 1.0	72.6 +/- 1.3	71.2 +/- 1.6	0.9
Gleason grade				
≤ 7	38	26	12	
> 7	24	8	16	0.015*
P53				
Wild type	59	32	27	
Mutant	3	2	1	0.8
Differentiation				
Well	16	14	2	
Moderate	19	14	5	
Poor	27	6	21	<0.001*
Nuclear grade				
I	32	22	10	
II / III	30	12	18	0.044*
Metastasis status				
Organ confined	40	28	12	
Lymph node metastasis	20	5	15	0.003*
Bone metastasis	19	5	14	0.006*

Fig. 6. Association of Drg1 with various clinical parameters. The results of immunohistochemical examination for the expression of NDRG1 in breast (A) and prostate (B) cancer patients were analyzed for the association with various clinical parameters. In each case, chi-squared test was performed to test the significance of association. * indicates statistically significant correlation ($P<0.05$).

bone is highly significant (P= 0.003 and 0.006 respectively), and in fact, is much stronger than the positive correlation with Gleason scores.

Task 3-b.

The relationship between the expression of the NDRG1 gene and recurrence of the disease will be examined retrospectively in patients over a 10 year period

Our Kaplan-Meier analysis on 85 patients of breast cancer for a period of 5 years indicate that patients with NDRG1 positive expression had significantly more favorable prognosis than those with reduced expression of the gene (P=0.002, log rank test, Fig. 7). Thus, the reduced expression of NDRG1 can be a strong predicator of lymph node and bone metastasis and, in turn, of survival. In multivariate Cox regression analysis involving NDRG1 expression status, primary tumor size and metastasis status, NDRG1 emerged as an independent statistically significant prognostic factor (Table 1). The odds ratio for NDRG1 is 2.435 (95%CI 1.030-5.760, P=0.043), implying that the death risk of patients with reduced NDRG1 expression within a specific time was 2.4 times higher than the risk of patients to die within the same time course with NDRG1 positivity.

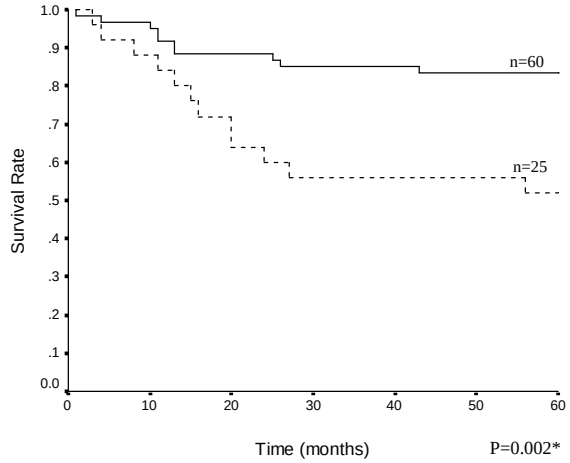


Fig. 7. NDRG1 expression is correlated with survival rate in breast cancer. Disease-free survival rate over a period of 5 years was analyzed in 85 patients in relation to NDRG1 expression. Solid line and dotted line indicate Drg-1 positive patients and patients with reduced expression of NDRG1, respectively. P value was determined by log rank test.

Table 2. Multivariate Cox regression analysis

Variables	reference level	b	SE	Wald's x^2	Hazard ratio	95% CI	P
Drg-1	**positive**	**0.890**	**0.439**	**4.107**	**2.435**	**1.030 – 5.760**	**0.043***
Tumor status	**$T_{1\text{-}2}N_xM_x$**			**2.264**			**0.132**
Metatsases	**$T_xN_0M_0$**	**1.513**	**0.760**	**3.963**	**4.538**	**1.024 – 20.117**	**0.046***

Thus, the reduced expression of NDRG1 can be a strong predicator of lymph node and bone metastasis and, in turn, of survival. Therefore, these data underscores the clinical relevance of this gene in advancement of breast cancer.

Task 3-c.

To evaluate the status of PTEN, and NDRG1 expression and their relation to survival of breast cancer patients

We have so far performed an immunohistochemical analysis on an archive of more than 80 breast and prostate cancer tissue samples for which we have 5-year survival data. The results showed that NDRG1 was expressed strongly in the epithelial cells of normal ducts and glands in breast tissue sections, while the poorly differentiated tumor cells in the same specimen had significantly reduced level of NDRG1 (Fig. 8). We also found that the expression of PTEN followed a pattern similar to that of NDRG1 (Table 3), which strongly supports our working hypothesis. Although the number of processed samples is too small to draw any conclusions, we observed that patients who have positive staining of both Drg-1 and PTEN tend to have better prognosis. We will continue to process more number of samples this year.

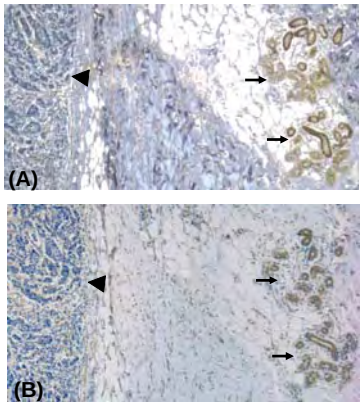


Fig.8. Immunohistochemical analysis of NDRG1 with respect to PTEN and other clinico-pathological parameters in human breast cancer. Immunohistochemistry for Drg-1 and PTEN was performed on paraffin tissue sections. A representative field from a breast cancer specimen immunostained with NDRG1 (A) and PTEN (B) antibodies.

Table 3. Relationship between NDRG1 and PTEN

		Drg-1 expression		P value
Breast Cancer	**All (85)**	**positive (60)**	**reduced (25)**	
PTEN status				
Positive	**62**	**51 (82.3%)**	**11 (17.7%)**	
Negative	**23**	**9 (39.1%)**	**14 (60.9%)**	**<0.001***
Prostate Cancer	**All (81)**	**positive (51)**	**reduced (30)**	
PTEN status				
Positive	**63**	**44 (69.8%)**	**19 (30.16%)**	
Negative	**18**	**7 (38.89%)**	**11 (61.11%)**	**0.03***

KEY RESEARCH ACCOMPLISHMENTS

1. We found that ATF3 is indeed capable of promoting tumor metastasis without affecting primary tumor growth in an animal model. This was statistically verified during this grant cycle.
2. Using the Yeast two-hybrid system, we identified the receptor of Wnt, LRP6, as the direct target of NDRG1. Binding of NDRG1 indeed blocked the signaling of the Wnt pathway.
3. We have examined the expression of NDRG1 in tumor tissues from breast cancer patients and found that the expression of NDRG1 is inversely correlated with 5-year survival of patients and that NDRG1 can be a strong predicator of lymph node and bone metastasis and, in turn, of survival.
4. The expression of PTEN is significantly correlated with the NDRG1 level and their expressions are inversely correlated with patient survival.

REPORTABLE OUTCOMES

Peer reviewed publications
(The following works were directly or partly supported by the current grant)

1. Iiizumi, M., Bandyopadhyay, S. and Watabe, K. (2007) Interaction of DARC and KAI1: a critical step in metastasis suppression. *Cancer Res* 15, 1411-14
2. Wen Liu, Juhi Bagaitkar, Kounosuke Watabe (2007) Roles of AKT signal in breast cancer. Frontier in Bioscience. 12:4011-9.
3. Sonia Mohinta, Hailong Wu, Priyasri Chaurasia, Kounosuke Watabe (2007) Wnt Pathway and Breast Cancer. Frontier in Bioscience. 12:4020-33
4. Megumi Iiizumi, Sonia Mohinta, Sucharita Bandyopadhyay and Kounosuke Watabe. (2007) Tumor - endothelial cell interactions: Therapeutic potential. Microvascular 74:114-20.

Abstract/presentation

1. Milinda Esquivel, Roslyn Alonzo, Elissa Donzis, Kounosuke Watabe, Therese Commes, and Linda A. deGraffenried (2007) The Novel Metastasis Suppressor Drg-1 Reverses the Invasive Potential Conferred by Akt in Breast and Prostate Cancer Cells. American Association for Cancer Research. Los Angeles.
2. Eiji Furuta, Rui Zhan, Sucharita Bandyopadhyay, Shigeru Hirota, Sadahiro Hosobe, Misako Watabe, Sudha K. Pai, Megumi Iiizumi, Sonia Mohinta, Wen Liu, Kounosuke Watabe (2007) Hypoxia up-regulates the fatty acid synthase gene in breast cancer cells American Association for Cancer Research Los Angeles
3. Sonia Mohinta, Megumi Iiizumi, Sucharita Bandyopadhyay, Ying Wang, Rui Zhan, Sudha K. Pai, Misako Watabe, Eiji Furuta, Wen Liu, Shigeru Hirota, Sadahiro Hosobe, Taisei Tsukada, Kunio Miura, Yukio Takano, Ken Saito, Therese Commes, David Piquemal, Tsonwin Hai and Kounosuke Watabe (2007) Drg-1 suppresses tumor metastasis by blocking the Wnt pathway American Association for Cancer Research Los Angeles
4. Megumi Iiizumi, Sucharita Bandyopadhyay, Sudha K. Pai, Misako Watabe, Shigeru Hirota, Sadahiro Hosobe, Taisei Tsukada, Kunio Miura, Ken Saito, Eiji Furuta, Sonia Mohinta, Wen Liu, Rui Zhan, Kounosuke Watabe (2007) Roles of RhoC in tumor progression in prostate cancer American Association for Cancer Research Los Angeles
5. Watabe K (2007) Functions of metastasis suppressor genes, NDRG1 and KAI1. University of Chicago, Medical School Chicago IL.

Employment

1. Dr. Megumi Iiizumi (Postdoc) has been supported by the current grant.
2. Dr. Eiji Furuta (Postdoc) has been partly supported by the current grant.

CONCLUSIONS

During this 3rd year funding period, our major effort was focused on Task 1b, Task3b and Task 3c. We also obtained promising preliminary results in other tasks. Our finding in Task 1b indicates that NDRG1 directly interacts with the Wnt receptor, LRP6, and this interaction blocks the Wnt signaling. We believe this is a break-through discovery for understanding the suppressor function of NDRG1. Therefore, we plan to further investigate the role of NDRG1 in the Wnt pathway. We also found that ATF3 is the target of NDRG1 and that ATF3 indeed promotes metastases in an animal model, suggesting that ATF3 and NDRG1 serve as prognostic markers and therapeutic targets for metastatic disease. Because ATF3 is a transcription factor, further down-stream target is of paramount interest. We are currently trying to screen potential targets by promoter scanning. We have also shown that PTEN positively regulates the expression of NDRG1 and a combination of these two markers serves as a useful predictor of breast cancer patients. We have requested a non-cost extension of this grant, and we will focus our next year's effort on further clarification of the NDRG1 pathway.

So what?
Metastatic disease remains the primary cause of death for breast cancer patients. Therefore, it is crucial to identify specific target molecules for better treatment of the patients. Our finding suggests that NDRG1 suppresses tumor metastases by blocking the Wnt pathway followed by inhibiting the function of ATF3. Our results also indicate that PTEN up-regulates the expression of NDRG1. Therefore, a combination of PTEN, NDRG1 and ATF3 can be used for diagnostic/prognostic markers as well as for therapeutic targets. The role of NDRG1 in Wnt signaling is also interesting and our results suggest that this signal pathway is crucial to understand the NDRG1 function. Further understanding of the mechanism of NDRG1 function and its relationship to Wnt signal may reveal more rationale targets for the treatment of metastatic disease.

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Justification of the expenditure

The fund of this grant has been used only for the breast cancer related research as originally described in the proposal. We have included some data using prostate cancer cells because the direct comparison of the data from both breast and prostate cancer often presents interesting similarity and/or contrast. Particularly, the similarity between these two organ systems provides additional evidence to validate

our data of breast cancer. However, these prostate cells and reagents were existing materials in our lab or purchased by using different grant funds that were received for our prostate cancer projects, and they are “spilled over” materials.

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Tumor–endothelial cell interactions: Therapeutic potential

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Abstract

Metastasis is the primary cause of death in cancer patients. However, the molecular mechanism of the metastatic process is poorly understood because it involves multiple steps with a high degree of complexity. A critical step for successful establishment of secondary colonization is the hematogenous dissemination of malignant cells. During this process, the attachment of cancer cells to the endothelial cells on microvasculature is considered to be an essential step and many adhesion molecules as well as chemokines have been found to be involved in this process. This interaction of cancer–endothelial cell is considered not only to determine the physical site of metastasis, but also to provide the necessary anchorage to facilitate tumor cell extravasation. However, recent evidence indicates that this interaction also serves as a host defense mechanism and hinders the process of metastasis. The tumor metastases suppressor gene, KAI1, has been known to block metastatic process without affecting the primary tumor growth, and this protein has been found to be able to bind to the chemokine receptor, Duffy antigen receptor for chemokines (DARC), which is expressed on endothelial cells. Importantly, this interaction markedly induces senescence of tumor cells. This novel finding is not only significant in the context of molecular dissection of metastatic process but also in the therapeutic implication to develop drugs inhibiting metastasis.
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Keywords: Endothelial cell; Metastasis suppressor; KAI1; Senescence; DARC

Contents

Metastatic disease is the major cause of cancer death

After decades of extensive effort and investment in cancer research, we are finally observing a declining trend in cancer death, which is mainly attributed to early detection and preventive measures for various types of cancer.

However, cancer still accounts for the second leading cause of death, and more than half a million people succumb to the disease every year in the USA alone (American Cancer Society, 2006). At the time of diagnosis of cancer, the most critical question for the treatment is whether the tumor is localized or has it already metastasized. Unfortunately, there are no effective

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treatment options available for patients with metastastic cancer, and more than 90% of patients ultimately die due to sequelae of metastatic disease. Although the clinical importance of tumor metastasis is well recognized, advances in understanding the molecular mechanism involved in metastasis formation have lagged behind other developments in the field of cancer research. This is attributed to the fact that metastasis involves multiple intricate steps with a high degree of complexity.

The first step in metastasis is the detachment of tumor cell from primary tumor mass. A major factor that enables tumor cells to become motile is decreased adhesiveness which is mediated by hydrolyses originating from the central necrotic area of large tumors (Sylven, 1973). In addition, certain cellular factors such as the autocrine motility factor (AMF) (Stracke et al., 1987) and hepatocyte growth factor (HGF) (Michalopoulos, 1990) are also found to be active players in this process. The tumor cell detachment from the primary tumor mass is followed by intravasation during which neoplastic cells must traverse barriers of collagen and elastic structures in the interstitial tissues and basement membrane, a hallmark of invasion front in all forms of cancer (Beitz and Calabresi, 1993). Enzymes capable of degrading the extracellular matrix are important in this process. A positive association with tumor aggressiveness has been reported for various classes of degradative enzymes including serine-, thiol-proteinases (Reich et al., 1988; Recklies et al., 1982; Sloane and Honn, 1984), heparanases and metalloproteinases such as matrix metallopeptidase 2 (MMP2) and matrix metallopeptidase 9 (MMP9) (Wang et al., 1980). Protease secretion by invading cells is often coupled to changes in cell shape and locomotion (Kalebic et al., 1983). The regulation of proteolysis can take place at many levels because proteinase inhibitors can be produced by the host and by the tumor cells themselves (Murphy et al., 1981). Such proteinase inhibitor proteins including tissue inhibitors of metalloproteinases (TIMP) and plasminogen activator inhibitors (PAIs) are considered to act as metastasis suppressor proteins although there are some controversies about the role of PAIs in metastases. Once a neoplastic cell has invaded the host circulatory system, it must survive in this hostile environment that includes mechanical damage, lack of growth factor from the original environment and the host immune system (Nicolson and Poste, 1983). Tumor cells that have survived and reached the endothelium of a distant organ extravasate using the same hydrolytic enzymes used for the initial step of invasion and form a secondary metastatic tumor. Therefore, tumor cells must undergo complex steps that involve many factors for successful metastasis.

Metastatic tumor cell interacts with the endothelium in vascular system

When the primary tumor grows more than 1 mm^3 in size, it can no longer obtain oxygen and nutrients by diffusion and hence initiates the process of angiogenesis. Therefore, many types of cancers secrete angiogenic factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) and stimulate the vascular formation around the tumor (Folkman, 1990; Ferrara, 2002). The tumor vasculature appears to have distinct features compared to the normal vessels in its structure and cellular components. The majority of tumor vessels have abnormal branching pattern with thin and leaky walls (McDonald and Baluk, 2002; Maniotis et al., 1999; Folberg et al., 2000; Hashizume et al., 2000). However, a functional vessel-network is essential not only for providing nutrients for tumor growth but also for metastatic spread of tumor cells to distant organs (Yano et al., 2003). In general, cancer cells acquire their invasive property at a relatively early stage, during which many tumor cells are constantly shed into the circulatory system in patients (Hashizume et al., 2000; Daldrup et al., 1998; Ellis and Fidler, 1996). However, considering the number of tumor cells in the circulation, an incidence of successful metastasis to distant organs is a very rare event. This is mostly due to the body's immune system including cytotoxic T-lymphocyte (CTL) and innate immune cells that effectively eliminate the circulating tumor cells. Cancer cells, on the other hand, utilize a number of strategies to evade the immune surveillance, such as down-regulation of major histocompatibility complex (MHC) and tumor-specific antigens and formation of tumor-cell–platelet aggregates (Sadelain et al., 2003; Nieswandt et al., 1999).

The tumor cells that survive the harsh environment and finally reach the distant organs still need to extravasate to establish secondary colonization. How the tumor cells initiate this process is a critical question and it is yet to be clarified. However, two possible mechanisms have been proposed. One is tumor cell arrest at the capillaries (Chambers et al., 2002). Tumor cells often aggregate with platelets, and due to the size of their mass they are physically "trapped" in the capillaries of the distant organs. In fact, using fluorescence-tagged tumor cell and a video-capturing image technique, Weiss et al. found that many tumor cells injected into mice intraperitoneally were indeed "arrested" in capillaries (Weiss et al., 1992). These "arrested" tumor cells either remain dormant at the site or start growing and eventually extravasate by rupturing the blood vessel or by inducing the secretion of proteolytic enzymes. However, the majority of cancer cells that are "arrested" in capillaries appear to die due to deformation and surface-membrane rupture (Weiss et al., 1992). Therefore, this process may not be an efficient strategy for tumor cells to establish metastatic colonization at a distant site.

The other proposed mechanism of extravasation of tumor cells mimics the infiltration of leukocytes to the inflammatory site, and this process requires the adhesive property of tumor cells to bind to the endothelial cells of blood vessels. When leukocytes are attracted to the site of inflammation via chemokine gradient, their extravasation occurs in four steps (Dunon et al., 1996; Goodman et al., 2003). The first step is "rolling and adhesion" involving selectins which is a reversible process. Leukocytes adhere to the vessel wall via selectin molecules and "roll" along the endothelium. The second step is "tight binding" of leukocytes to the endothelium through other adhesion molecules on the endothelial cells such as integrins, intercellular adhesion molecule 1 (CD54), human rhinovirus receptor (ICAM1) and platelet/endothelial cell adhesion

molecule (CD31 antigen) (PECAM1). The third step is "diapedesis" and the leukocytes start extravasating or crossing the endothelial wall. This step also involves adhesion molecules such as leukocyte functional antigen-1 (LFA-1) and macrophage receptor 1 (MAC-1) and this interaction enables the leukocytes to migrate through the endothelial cell junction or squeeze their cell bodies through narrow pores (Engelhardt and Wolburg, 2004). The final step is the migration of leukocytes to the inflammatory site by various types of chemokines including interleukin 8 (IL8). Because many cancer cells express similar adhesion molecules that are also expressed on the migrating leukocytes, it is thought that cancer cells use a similar strategy for adhesion to the endothelial cells during metastasis. Initial studies to demonstrate the adhesive property of cancer cell to endothelial cells were done in tissue culture systems which were essentially static systems devoid of shear forces associated with physiological blood flow. In this *in vitro* system the cancer cells were added to monolayers of endothelial cells and the number of adhered cells was quantified in the presence and absence of various factors. It was found that there was a marked increase in adhesion of cancer cells to endothelial cells in the presence of cytokines such as interleukin-1 beta (IL-1β) (Okada et al., 1994), interleukin 1 (IL-1) (Lafrenie et al., 1994) and tumor necrosis factor alpha (TNF-α) (Okada et al., 1994; Sheski et al., 1999) that are known to induce the expression of adhesion molecules. Hence, these data serve as important evidence that cancer cells indeed bind to the endothelial cells. This interaction of cancer–endothelial cells is mediated by various adhesion molecules expressed on both cancer and endothelial cells. E-selectin which is expressed on endothelial cells binds to its ligand sialyl Lewis-X (SLE) or A-antigen expressed on cancer cells of colon and renal cell carcinoma (Lafrenie et al., 1994; Steinbach et al., 1996; Tozawa et al., 1995; Ye et al., 1995). Among the immunoglobulin superfamily, vascular cell adhesion molecule 1 (VCAM-1) expressed on endothelial cells was also found to bind to α4 integrins expressed on renal cell carcinoma (Lafrenie et al., 1994; Steinbach et al., 1996; Taichman et al., 1991; Tomita et al., 1995) and α4β1 expressed on melanoma (Garofalo et al., 1995) and sarcoma cells (Paavonen et al., 1994). The integrin adhesion molecules like α6 on endothelial cell mediate the adhesion of α6β1 on highly metastatic B16/129 melanoma cells to the luminal surface (Ruiz et al., 1993). In another similar instance, αvβ3 on endothelial cells have also been shown to bind to small cell carcinoma cells (Sheski et al., 1999). A more recent evidence shows that the expression of αvβ3 integrin (CD51/CD61) on human melanoma cells is required for adhesion of cancer cells with human Thy-1 cell surface antigen (THY1) expressed on the activated endothelium. Thus various molecules expressed on endothelial cells ranging from selectins, integrins and immunoglobulins superfamily are necessary for adhesion to the cancer cells for their emigration and subsequent formation of metastatic tumors. To mimic the cancer–endothelial cell interaction under a physiological condition of blood flow which generates shear force, the static system was modified by introducing fluid flow which can simulate the physical properties of microvascular blood flow. By using this system, E-selectin-dependent 'rolling' has been observed in colon, ovarian, breast and melanoma cancer cells under dynamic flow condition (Brenner et al., 1995; Giavazzi et al., 1993; Tozeren et al., 1995). To further validate cancer–endothelial cell interaction in living organisms, Al-Mehdi et al. recently used the intravital videomicroscopy technique, generally used to observe adhesion of circulating intravascular cells, and indeed detected metastatic tumor cells attached to the endothelium of pre-capillary arterioles and capillaries in intact mouse lungs (Al-Mehdi et al., 2000). They injected Green Fluorescent Protein (GFP)-tagged metastatic cells into a mouse and traced the behavior of these tumor cells and found that the majority of the cells were attached to the endothelium of pre-capillary arterioles although rolling-like movement was not observed during that time period. Therefore, they concluded that tumor cells were not "arrested" at the capillaries, rather they preferentially attached to the endothelium before proceeding towards extravasation. They also found that tumor cells attached to the endothelium proliferate before extravasation. Therefore, this "attachment" of tumor cells to the endothelium appears to be an advantage for the tumor cells because it not only determines the physical site of metastasis, but also provides the necessary anchorage that prevents anoikis and facilitates cell proliferation.

Tumor metastases suppressor, KAI1, interacts with DARC on endothelial cells

The attachment of tumor cells to the endothelium appears to provide a platform for tumor cells to extravasate and proliferate for successful metastases. However, recent evidence indicates that this is not always the case and that the interaction of tumor–endothelial cells is rather a host defense mechanism to block metastasis process when tumor cells possess the metastases suppressor protein, KAI1, on the cell surface (Bandyopadhyay et al., 2006). The KAI1 gene was originally isolated as a prostate-specific tumor metastasis suppressor gene, using the microcell-mediated chromosome transfer method (Dong et al., 1995). The KAI1 gene is identical to CD82 which is located in the p11.2 region of human chromosome 11 (Dong et al., 1997). The protein has four hydrophobic and presumably transmembrane domains, two extracellular domains and three short intracellular domains (Engel and Tedder, 1994). When the KAI1 gene is transferred into a highly metastatic cell, metastatic ability of KAI1-expressing cell is suppressed in mice, whereas their primary tumor growth is not affected (Dong et al., 1995). Consistent with the *in vitro* and *in vivo* results, the immunohistochemical analysis of various types of human cancers revealed that KAI1 expression is significantly reduced in advanced and metastatic tumors (Rinker-Schaeffer et al., 2006; Furuta et al., 2006).

To elucidate the molecular mechanisms of metastases suppression by the KAI1 gene, Bandyopadhyay et al. recently utilized the yeast two hybrid system to screen interacting proteins of KAI1 and found that the KAI1 protein is capable of binding to the promiscuous chemokine (C-X-C motif) (CXC) receptor, DARC, which is highly expressed on the surface of endothelial cells of lymphoid and blood vessels, particularly in

small veins and venules (Bandyopadhyay et al., 2006). Immunohistochemical analyses indeed indicate that the pattern of expression of DARC and KAI1 is opposite in endothelium and cancer cells. KAI1 is highly expressed in normal epithelial cells but undetectable in endothelial cells, while DARC is strongly expressed in the endothelium and not in epithelial cells (Bandyopadhyay et al., 2006). The results of a series of experiments including *in vitro* binding assay and co-immunoprecipitation as well as antibody inhibition assay demonstrated that KAI1 indeed binds to DARC on endothelial cells (Bandyopadhyay et al., 2006). Therefore, it is reasoned that KAI1 on cancer cells interacts with DARC only when they intravaste into the blood vessel. To test this hypothesis, tumor cells ectopically expressing the KAI1 gene were injected into wild type or DARC knockout mice subcutaneously or intravenously, and the incidence of lung metastases was examined (Bandyopadhyay et al., 2006). The result of this *in vivo* experiment was striking. The number of pulmonary metastases was significantly increased with the KAI1+ cells that were injected into the DARC knockout mice compared to the wild type animals. These results strongly support the notion that the metastasis suppressor function of KAI1 is dependent upon the interaction of KAI1 and DARC on endothelial cells. Their results also suggest that the inefficiency of metastasis through the "capillary arrest" is partly due to the trapping of tumor cells by endothelium via KAI1–DARC interaction.

The physiological relevance of the KAI1–DARC interaction is of paramount interest. Crucial clue to answer this question came from the recent study of T-cell activation. When T and B cells are activated, KAI1 (also called CD82) is significantly up-regulated while it is undetectable at resting stage. Interestingly, co-engagement of KAI1/CD82 and T-cell receptor (TCR) by anti-KAI1 mAb and anti-CD3 mAb, respectively, was able to activate T cells *in vitro*. When KAI1/CD82 is bound to the antibody, KAI1 appears to transmit an intracellular signal to phosphorylate tyrosine followed by rapid increase in intracellular Ca2+ level and interleukin-2 (IL-2) production (Lebel-Binay et al., 1995). Importantly, this activation triggered changes in cell morphology and inhibition of cell proliferation (Lagaudriere-Gesbert et al., 1998). To test whether the activated KAI1 signal can also block the cell proliferation of epithelial tumor cells, the effect of the same antibody on the KAI1+ tumor cells was examined (Bandyopadhyay et al., 2006). The results of this experiment revealed that the addition of anti-KAI1 antibody to the culture of KAI1+ cells significantly suppressed the growth of the tumor cells, and the same effect was observed when KAI1+ cells were co-cultured with endothelial cells. These results strongly suggest that the activation of KAI1 via antibody or DARC triggers a growth inhibitory signal of tumor cells. The next question is as to what signals and genes are responsible for the growth inhibition. To explore this question further, GFP-labeled tumor cells with or without expressing KAI1 were co-cultured with human endothelial cells and the degree of apoptosis and senescence was examined. The results clearly indicated that the growth inhibitory effect by the KAI–DARC interaction was not due to apoptosis but senescence and that the induction of senescence was associated with the up-regulation of senescence-controlling genes, T-box 2 (TBX2) and p21 genes (Bandyopadhyay et al., 2006). Therefore, these *in vitro* and *in vivo* results are consistent with the notion that KAI1 suppresses metastases by interacting with DARC on endothelial cells after intravasation followed by induction of cell senescence and that this senescence is triggered by activation of TBX2 and p21 (Fig. 1). The tumor cells at the senescent stage are expected to be cleared swiftly by immune cells in the blood vessels as Xue et al. recently reported that senescent tumor cells trigger innate immune responses which target these tumor cells and that this mechanism was dependent on transient expression of the p53 gene in tumor cells (Xue et al., 2007). In this context, it is noteworthy that the expression of

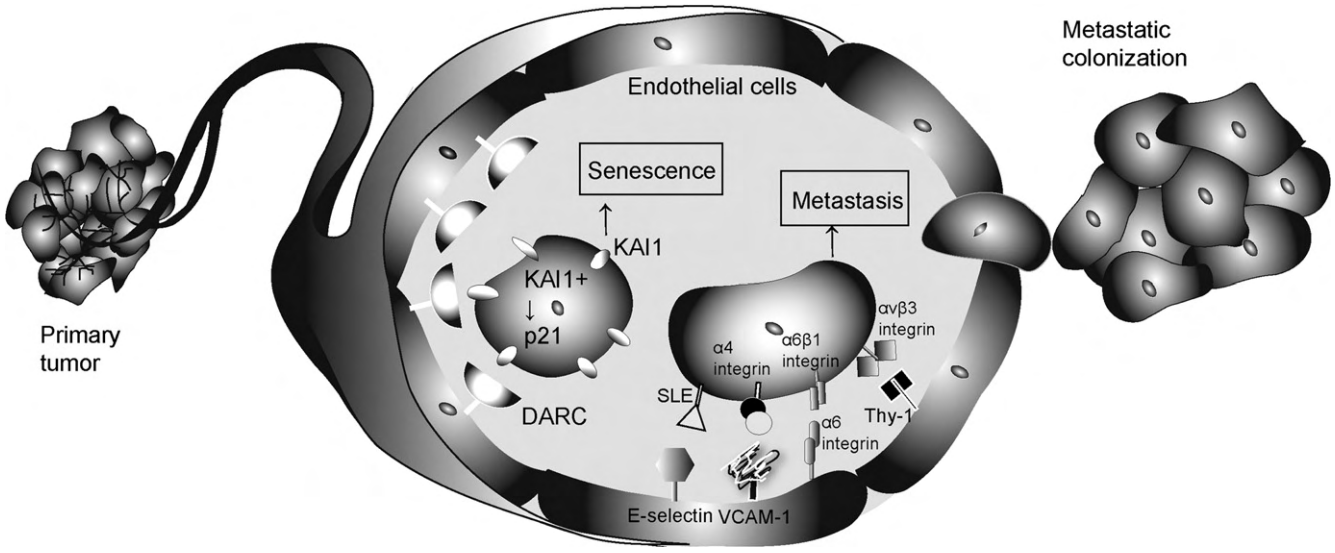

Fig. 1. Interaction of tumor–endothelial cells. Tumor cells in blood vessels attach to endothelial cells through various adhesion proteins. The KAI1 protein on the tumor cell also interacts with DARC on the endothelial cells which induces tumor cell senescence, while a tumor cell without KAI1 successfully extravasates and colonizes in a distant organ.

KAI1 is partly controlled by p53 as reported previously (Mashimo et al., 1998). These results clearly illustrate the importance of interplay between p53, KAI1 and DARC for blocking tumor metastases in the circulatory system.

Signaling of KAI1–DARC interaction

KAI1 is a member of the transmembrane 4 superfamily which is known to form a large complex with multiple proteins which is referred to as tetraspanin web (Rubinstein et al., 1996). KAI1 was indeed found to be associated with many different proteins. Lee et al. recently reported that KAI1 interacts with a tetraspanin, Kitenin, whose over-expression promoted increased tumorigenicity and metastasis *in vivo* (Lee et al., 2004). Kitenin is considered to block the suppressor function of KAI1 which renders the tumor cell more invasive, although the exact molecular mechanism of the Kitenin function is yet to be elucidated. KAI1 was also found to be associated with other tetraspanins, CD9 and CD63 whose functions are not well understood, although these genes are correlated with poor prognosis and increased metastasis (Higashiyama et al., 1995). In addition, Odintsove et al. also reported that KAI1 is able to bind to the EGF receptor and block the EGF-induced cell migration, although the physiological relevance of this observation still needs to be investigated *in vivo* (Odintsova et al., 2000). Like other tetraspanin proteins, KAI1 is also associated with various integrins including $\alpha3\beta1$, $\alpha4\beta1$, $\alpha5\beta1$ and $\alpha6\beta1$ integrins (Iiizumi et al., 2007). It should be noted that interaction of KAI1 and $\alpha3\beta1$ was found to block the fibronectin/$\alpha3\beta1$-induced cell invasion through inhibition of the cytoskeletal system (Zhang et al., 2001). Therefore, KAI1 appears to form a multi-protein complex. However, how this complex affects the interaction of KAI1–DARC and transmits the senescence signal remains to be elucidated. Zhang et al. previously found that KAI1 is also associated with a key signal molecule, protein kinase C (PKC), which controls cell cycle, migration and invasion. It appears that this interaction facilitates recruitment of PKC into proximity with specific integrins such as $\alpha3\beta1$ and $\alpha6\beta1$ that are known to be strongly associated with KAI1. Therefore, it is plausible that, when KAI1 is activated by DARC on endothelial cells, the KAI1–integrin–PKC complex transduces a signal to induce cell senescence via activation of p21 and TBX2 genes. In this context, it should be noted that PKC is capable of directly controlling p21 expression and activity which is the hallmark of KAI-induced senescence (Deeds et al., 2003). More recently, Sridhar et al. reported that KAI1 is able to suppress integrin-dependent activation of the receptor kinase c-Met *in vitro* (Sridhar and Miranti, 2006). The ligand of c-Met is HGF which is capable of both stimulating and arresting the cell cycle and these effects in either case are mediated through the regulation of p21. Whether this pathway is involved in the KAI/DARC-induced senescence is an interesting question and needs to be clarified.

Therapeutic implication of KAI1–DARC interaction

The ability of DARC to trigger the KAI1 signal which leads to tumor cell senescence suggests an attractive possibility to develop anti-tumor strategy. DARC is known to be a receptor of Plasmodium vivax, a malaria parasite, and more than 70% of West African descendants lack DARC expression and hence are resistant to the infection (Sanger et al., 1955; Tournamille et al., 1995). Epidemiological data indicate that these same populations have significantly higher incidence of various types of cancers as well as higher rate of metastatic disease in comparison to the white population (Luo et al., 2000), suggesting that DARC plays a role in tumor progression. In fact, Shen et al. recently reported that growth of prostate tumor was significantly faster in DARC-deficient mice than in wild type animals (Shen et al., 2006). Wang et al. also found that ectopic expression of DARC in breast cancer cells significantly suppressed the incidence of spontaneous pulmonary metastases in mice (Wang et al., 2006). These observations strongly support the notion that DARC functions as a suppressor for both tumor growth and metastases, and therefore a molecule mimicking the function of DARC may serve as an effective anti-cancer as well as anti-metastatic drug. One approach toward this end would be to define the structure of the interaction domains of KAI1 and DARC, which may provide crucial information to design a small DARC-like peptide capable of engaging in the activation of the KAI1 signal. Another approach is to use chemical libraries to screen compounds that are able to activate the KAI1 signal using senescence markers for the KAI1 activation. These approaches are expected to be effective therapeutic strategy not only for the metastatic disease but also for primary tumors as long as KAI1 is expressed in tumor cells. However, in most patients with metastatic disease, the KAI1 gene is down-regulated although the gene is still intact (Dong et al., 1995). One possible explanation for the down-regulation is the mutations of p53 in these tumor cells. In fact, we have shown that the activation of p53 by etoposide indeed restored the expression of KAI1 *in vitro* (Mashimo et al., 2000), which suggests that it is possible to restore the expression of KAI1 in patients. Therefore, the strategy of restoration of KAI1 expression followed by activation of the KAI1 signal holds a great promise for the treatment of patients even without KAI1 expression. It is expected that further understanding of the nature of KAI1–DARC interaction and its signaling pathway should provide more information regarding other specific targets and strategic options to develop effective anti-metastatic drugs.

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Interaction of Duffy Antigen Receptor for Chemokines and KAI1: A Critical Step in Metastasis Suppression

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Abstract

Tumor metastases suppressor protein KAI1/CD82 is capable of blocking the tumor metastases without affecting the primary tumor formation, and its expression is significantly down-regulated in many types of human cancers. However, the exact molecular mechanism of the suppressor function of KAI1 remains elusive. Evidence from our laboratory supports a model in which tumor cells dislodge from the primary tumor and intravasate into the blood or lymphatic vessels followed by attachment to the endothelial cell surface whereby KAI1 interacts with the Duffy antigen receptor for chemokines (DARC) protein. This interaction transmits a senescent signal to cancer cells expressing KAI1, whereas cells that lost KAI1 expression can proliferate, potentially giving rise to metastases. Our model of the mechanism of action of KAI1 shows that metastasis suppressor activity can be dependent on interaction with host tissue and explains how KAI1 suppresses metastasis without affecting primary tumor formation. Taken together, *in vitro* and *in vivo* studies identify the KAI1-DARC interaction as a potential target for cancer therapy. [Cancer Res 2007;67(4):1411–4]

KAI1 Blocks Metastases without Affecting Primary Tumor Formation

When cancer is diagnosed, the most critical question is whether the disease is localized or has it already disseminated to other parts of the body. Unfortunately, the majority of patients already have a clinically undetectable metastatic disease at the time of a visit to the clinic, and >90% of cancer patients ultimately succumb to sequelae of metastatic disease. Following primary tumor formation, a population of tumor cells can acquire molecular and cellular changes, which enable cancer to spread to distant sites. These include invasive phenotype that results in the loss of cell-cell adhesion and cell-extracellular matrix adhesion followed by proteolytic degradation of the matrix. Additional changes are needed in order for cells to intravasate into neighboring blood and lymphatic vessels and disseminate through the circulation. Those cells that survive in the circulation are arrested at distant organ sites, extravasate, and lodge at the secondary sites, where the cells must also proliferate and colonize for successful metastasis. The molecular mechanism(s) regulating acquisition of metastatic ability remains poorly understood despite the urgent need for development of novel treatment options for patients with metastatic disease. The discovery of a series of metastasis suppressor genes in the past decade has shed new light on many crucial aspects of this intricate biological process. The metastases suppressor genes and their encoded proteins, by definition, suppress the process of metastasis without affecting tumorigenesis. To date, more than a dozen of these genes have been identified and include *nm23, KAI1, Kiss1, BRMS1, MKK4, RhoGDI2, RKIP, Drg-1, CRSP3, SSeCKs, TXNIP/VDUP-1, Claudin-4*, and *RRM1* (1).

The *KAI1* gene was originally isolated as a prostate-specific tumor metastasis suppressor gene using the microcell-mediated chromosome transfer method followed by Alu-PCR. It is located in the p11.2 region of human chromosome 11 (2, 3). When the *KAI1* gene was transferred into highly metastatic Dunning rat prostatic cancer cells, KAI1-expressing cancer cells were suppressed in their metastatic ability in mice, whereas their primary tumor growth was not affected (2). The DNA sequencing analysis of the *KAI1* gene revealed that it is identical to *CD82*, a surface glycoprotein of leukocytes (3). The protein has four hydrophobic and presumably transmembrane domains, two extracellular domains, and three short intracellular domains and belongs to the family of tetraspanins. Immunohistochemical analysis of human prostate tumor samples revealed that the KAI1 expression was down-regulated in >70% of the primary tumors (4). Similar results were also observed in other types of tumors, including lung, breast, pancreatic, colon, bladder, ovarian, hepatocellular carcinoma, and melanoma (1, 5). *KAI1* gene expression is correlated with poor survival in patients with these types of cancers. Therefore, the KAI1 is a bona fide metastasis suppressor protein in multiple cancer types. This raises the intriguing question of how the *KAI1* gene suppresses the metastasis process.

Duffy Antigen Receptor for Chemokines on Endothelial Cell Plays a Key Role in the Suppressor Function of KAI1

To understand the mechanism of KAI1 in metastasis suppression, a yeast two-hybrid system was used to systemically screen interacting proteins of KAI1 and found that KAI1 was physically associated with the Duffy antigen receptor for chemokines (DARC; ref. 6). DARC is a promiscuous CXC chemokine receptor that is strongly expressed on the endothelial cells of lymphatic and blood vessels as well as on RBCs. Immunohistochemical analyses confirmed that DARC is highly expressed in the endothelium, particularly in the small veins and venules as well as lymphatic vessels in both normal and tumor tissue of the prostate and breast, whereas its expression is undetectable in the epithelial cells or stroma. On the other hand, KAI1 is highly expressed in the normal epithelial cells in these organs, and the expression is significantly reduced in carcinoma. Based on this spatial localization, we hypothesized that KAI1 on epithelial cells interacts with the DARC when cancer cells expressing KAI1 intravasate and encounter the endothelial lining of small blood vessels. A series of experiments

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done to test this putative interaction using human endothelial cells found that tumor cells expressing KAI1 indeed bound to endothelial cells and this binding was blocked by KAI1 antibody. These results prompted the question "what is the physiologic outcome of the interaction between KAI1 expression tumor cells and DARC on endothelial cells?" Results from recent studies on T-cell activation provided clues crucial to answering this question. KAI1/CD82 is barely detectable on resting peripheral T and B lymphocytes, whereas its expression is highly up-regulated on activation of these cells. This up-regulation is associated with morphologic changes and expression of activation markers, such as CD82 and MHC II antigens. Lebel-Binay et al. described that the coengagement of KAI1/CD82 and T-cell receptor by anti-CD82 monoclonal antibody (mAb) and anti-CD3 mAb, respectively, was able to activate T cells *in vitro*. Specifically, when T cells are stimulated *in vitro* by anti-KAI1/CD82 mAb, KAI1/CD82 seems to transmit a signal that results in tyrosine phosphorylation, a rapid increase in intracellular Ca^{2+} level, and interleukin-2 production (7). Interestingly, this activation was associated with a change in cellular morphology and inhibition of cell proliferation (8). Therefore, we hypothesized that engagement of KAI1/CD82 on cancer cells may also activate a similar signal pathway, which results in growth arrest of tumor cells. Consistent with this hypothesis, the addition of anti-CD82 antibody in the culture of KAI^+ cells resulted in significant growth suppression of tumor cells, which was also observed when the tumor cell was cocultured with human endothelial cells. Therefore, our data strongly suggest that growth suppression is determined by dynamic and reciprocal interaction of KAI1 on cancer cells and DARC on endothelial cells in the vasculature (Fig. 1).

To further corroborate these findings, melanoma cells with or without expression of KAI1 were transplanted into DARC knockout as well as wild-type mice and resultant overt lung metastases were quantitated. Primary tumors developed in all mice without significant changes of growth rate regardless of the KAI1 level in the tumor cells and DARC status of the mice. However, the KAI1-positive cell lines developed significant number of pulmonary metastases in the DARC knockout mice, whereas metastasis was almost completely abrogated when the same cell lines were injected into the heterozygote and wild-type littermates. Thus, in the absence of DARC, tumor cells expressing high level of KAI1 formed metastases, supporting our model that the metastasis suppressor function of KAI1 is dependent on the interaction of KAI1 and DARC on endothelial cells. The biochemical nature of this growth suppression through the KAI1-DARC interaction is of significant interest. To explore this, green fluorescent protein–labeled tumor cells were cocultured with endothelial cells and found that KAI1-positive cells became senescent without a sign of apoptosis. Further, senescence was associated with down-regulation of

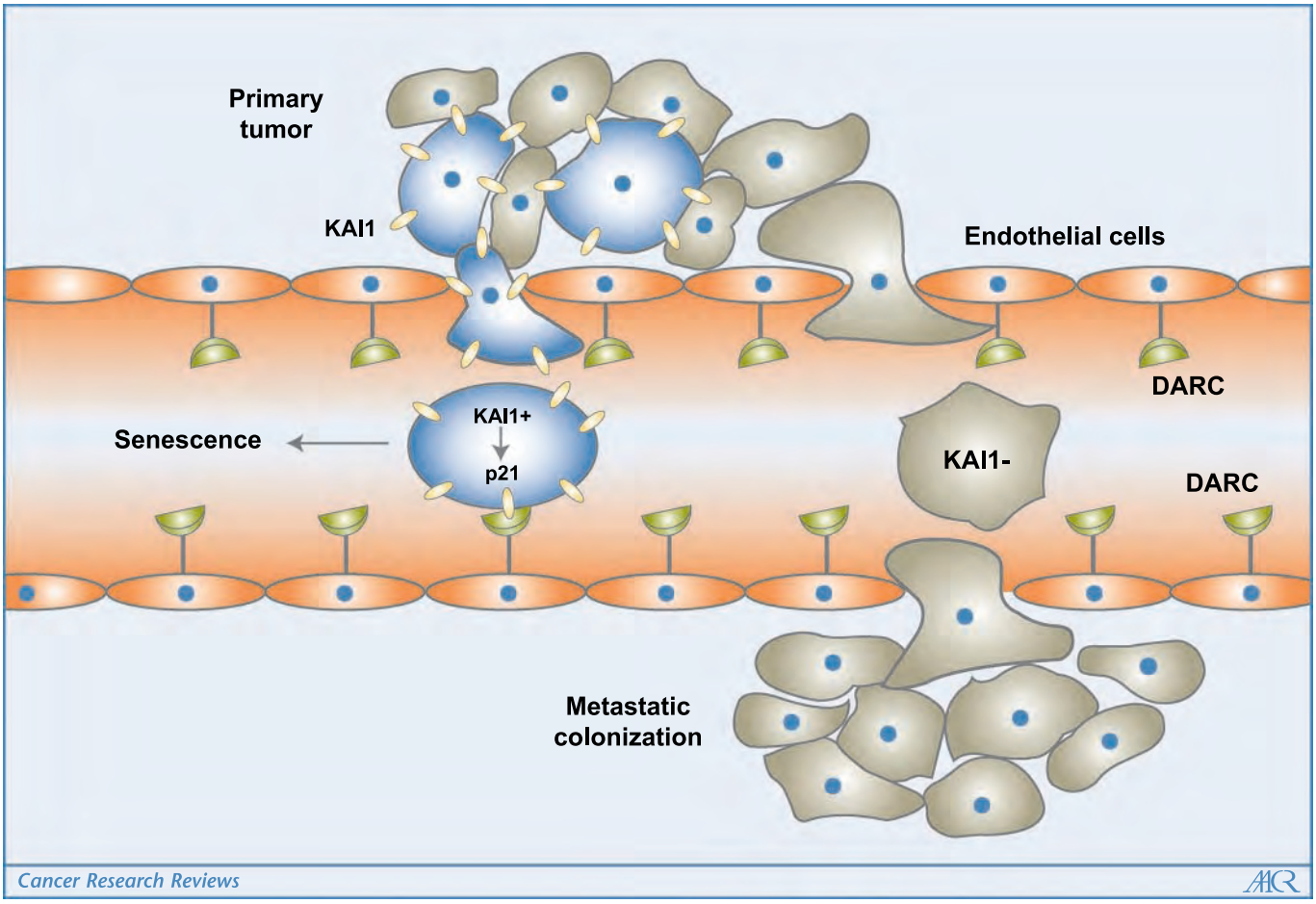

Figure 1. KAI1-DARC interaction blocks tumor metastasis.

TBX2 and up-regulation of the cyclin-dependent kinase inhibitor p21. These studies suggest that growth suppression induced by the KAI1-DARC interaction is due to the activation of p21 followed by cellular senescence.

DARC Signal for Cancer Therapy?

Our model of the mechanism of action of KAI1 explains how KAI1 suppresses metastasis without affecting primary tumor formation. However, it has also provoked many critical questions about (*a*) whether KAI1 function requires other "cofactors," (*b*) what cellular signal is induced by DARC and KAI1 interaction, and (*c*) whether KAI1-DARC signal be targeted for cancer therapy.

KAI1 has been reported to be associated with many different membrane proteins, including integrins, Kitenin, epidermal growth factor receptor (EGFR), CD63, CD9, EW12, and c-Met (9–14). KAI1 belongs to the transmembrane 4 superfamily, which is known to form a multiprotein complex referred to as tetraspanin web (9) that also interacts with integrins. KAI1 was indeed found to be associated with various integrins, including $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, and $\alpha_6\beta_1$, and the complex of integrin $\alpha_3\beta_1$ and KAI1 was reported to suppress fibronectin/$\alpha_3\beta_1$-induced cell invasion through inhibition of the cytoskeletal system (10). KAI1 was also found to be associated with other tetraspanins, including CD9 and CD63 (11). The functions of these molecules are not well understood; however, the loss of expression of CD9 and CD63 correlates with poor prognosis and increased metastasis (12). Therefore, these two tetraspanins may also play an important role in the KAI1 function. More recently, Lee et al. (13) found that KAI1 is associated with another tetraspanin molecule, Kitenin, whose overexpression promoted increased tumorigenicity and metastasis *in vivo*. The exact molecular function of Kitenin is yet to be understood; however, it was proposed that Kitenin decreases the metastasis suppressor functions of KAI1 and/or cytoplasmic signaling pathway that shifts the invasive/anti-invasive balance toward invasion. In addition to integrins and tetraspanins, Odintsova et al. (14) recently found that KAI1 physically associates with the EGFR and rapidly desensitizes the EGF-induced signal, which could lead to suppression of cell migration, although it is as yet unclear whether this mechanism indeed accounts for the metastasis suppression *in vivo*. Nevertheless, KAI1 seems to be able to interact with various proteins on the membrane. It is yet to be determined whether all these components indeed form a "multiprotein complex" and whether they are all necessary or sufficient for the metastasis suppressor function of KAI1 because these proteins associated with KAI1 have been identified in different systems.

DARC-KAI1 interaction seems to transduce cytoplasmic signal to nucleus to modulate TBX2 and p21 expression and induce senescence. The signal transduction mechanism involved in this senescence pathway is a crucial question. Thus far, however, little information is available about the signals related to the KAI1 function. Zhang et al. (10) recently reported that protein kinase C (PKC) is associated with various tetraspanins, including KAI1, and that these tetraspanins act as linker molecules to recruit PKC into proximity with specific integrins. It was also shown that only those integrins ($\alpha_3\beta_1$ and $\alpha_6\beta_1$) that strongly associated with tetraspanins, such as KAI1, were in association with PKC. Therefore, KAI1 may act as a modulator of the PKC signal, which plays a crucial role in cell cycle progression, migration, and invasiveness as well as in cell cycle arrest (15, 16). Interestingly, PKC was found to be able to directly modulate p21 expression and activity (15), which is the hallmark of KAI1/DARC-induced senescence. More recently, KAI1 has been shown to suppress integrin-dependent activation of the receptor kinase c-Met (17). The ligand of c-Met is hepatocyte growth factor, which is capable of both stimulating and arresting cell cycle, and these effects in either case are mediated through the up-regulation or down-regulation of p21. Therefore, PKC and c-Met pathway may play key roles in the KAI1/DARC-induced signaling, although this possibility needs to be tested directly.

DARC is the receptor of the malaria parasite *Plasmodium vivax*. Approximately 70% of West African descendants have lack of expression of DARC on erythrocytes, thereby resistant to malaria infection. Interestingly, the same population showed significantly higher incidence of both prostate and breast cancer as well as higher rate of metastatic disease than white (18). DARC also serves as promiscuous receptor for both C-C and CXC chemokines, which is believed to function as "decoy" of excess chemokines. Therefore, DARC is proposed to play a role as antimetastatic molecule by clearing angiogenic CXC chemokines. In fact, Shen et al. (19) recently have shown that growth of prostate tumor was significantly augmented in DARC-deficient mice compared with the wild-type. More recently, it was also reported that overexpression of DARC in breast cancer cells significantly suppressed the spontaneous pulmonary metastasis (20). Therefore, DARC may function as a metastases suppressor by two different mechanisms (i.e., by inducing KAI1 signal in tumor cells and by sequestering angiogenic factors). This raises an attractive possibility of developing antimetastatic drugs that mimic the action of DARC. Further understanding of the biochemistry of the interaction of KAI1-DARC and their cofactors as well as the downstream signal should lead to effective therapeutic strategy against metastatic cancer.

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Wnt pathway and breast cancer

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1. ABSTRACT

Breast cancer is one of the most debilitating human carcinomas with second highest mortality rate after lung cancer in women. Recent advancement in genetic and biochemical analyses has deciphered the molecular pathways involved in breast cancer development. Wnt signal has long been established to play a critical role in normal development as well as in tumorigenesis. In this review, we summarize the role of Wnt signal in the development of mammary carcinoma, the molecular mechanism via which Wnt signal exerts its malignant potential and various nodal points in the Wnt cascade that can be targeted for drug development and cancer treatment.

2. INTRODUCTION

The combination of Drosophila segment polarity gene *Wingless* (1) and mouse proto-oncogene *Int-1* (2, 3) led to the development of the term 'Wnt'. At present 19 Wnt genes are identified and these proteins constitute the key factors in the regulation of signal transduction in the embryonic development of the metazoan (4, 5). The origin of the pathway can be traced back to show that it is evolutionarily conserved from primitive diploblast hydra (6) to higher order mammals and even in plants (7, 8). Over the past 20 years, numerous data revealing the importance of the Wnt pathway has been generated not only in the context of development but also in cancer pathogenesis and therefore redefining cancer as a result of dysregulation of

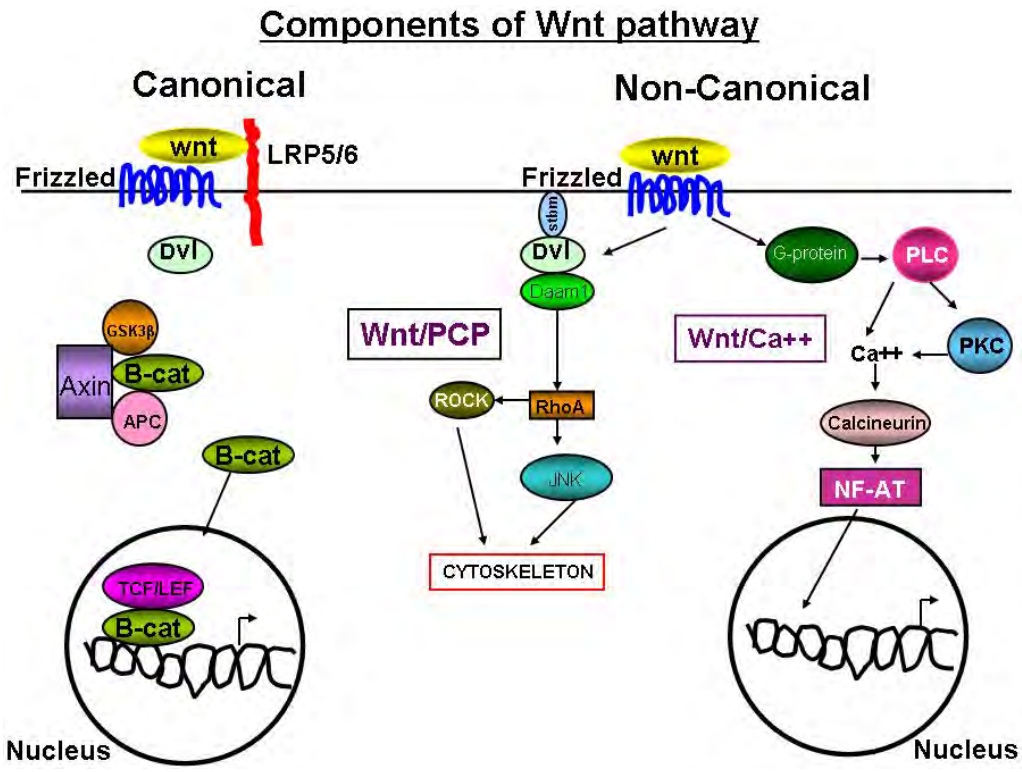

Figure 1. Components of Wnt pathway.

developmental processes. There has been an explosion of essential information regarding the Wnt pathway in recent years which on one hand increases the complexity of the Wnt pathway to an unfathomable extent and, at the same time, leads to the emergence of potential players in the development of human disease. Recently, a genome-wide RNAi (interference RNA) screening of the Drosophila cells led to the identification of 238 new regulators of the Wnt pathway. Fifty percent of them had human orthologs and out of which 18% were associated with human disease, thus adding a higher degree to the already existing intricacy of the Wnt pathway (9).

The Wnt pathway has been implicated in the specification of cell and tissue polarity, mitogenic stimulation and differentiation and also adult tissue homeostasis (10-12). Wnt proteins are palmitoylated on conserved cysteines which are essential for signal transduction (13). The Wnt proteins are also glycosylated on conserved N-linked glycosylation sites (14). A number of degenerative human diseases arise due to the dysregulation of the Wnt pathway. For example, a mutation of LRP5 causes increased bone density (15, 16), vascular defects in the eye called OPPG (osteoperosis-pseudogliioma syndrome) (17) and FEVR (familial exudative vitreoretinopathy) (18). A mutation in Axin2 leads to the development of tooth defects (19), predisposition to colon (19, 20) and liver cancer (21). Wnt signaling cascade is also an essential regulator of stem cell proliferation and self-renewal, which is supported by the fact that Wnt3a protein, *in vitro* promotes the self-renewal of hematopoietic stem cells (13). With the increasing amount of information obtained in the last few decades, it is clear that aberrant Wnt signal plays a central role not only in various human degenerative diseases but also in tumorigenesis and tumor progression. It is well established that the Wnt signaling is emerging out to be a major pathway in its contribution to the development of human cancer. In this review, we will focus on how aberrant activation of the Wnt cascade affects human breast cancer.

3. TYPES OF WNT PATHWAY

The Wnt pathway primarily consists of canonical and non-canonical pathway (see Figure 1).

3.1. Canonical pathway

The canonical pathway involves beta-catenin as the key component which is conserved from plants to higher animals and asserts its actions by transcriptional activation of target genes in the nucleus. Once Wnt is secreted, it binds to various factors like SFRP (secreted frizzled-related sequence protein) and WIF (Wnt inhibitory factor), however genetic and biochemical evidence show Frizzled (Fz) to be the primary receptors of Wnt proteins. Frizzled are seven-transmembrane receptors with CRD (cysteine rich domain) at the N-terminal for Wnt to directly bind to it (22-24). In canonical Wnt pathway, there is another single-pass transmembrane receptor called LRP6/5 which forms a trimeric complex to transduce the signal (25, 26). Frizzled is required for multiple Wnt pathways (27, 28), but LRP6/LRP5 on the other hand is specifically

required for the Wnt/β-catenin mediated pathway (29, 30). The binding of Wnt to Frizzled configures its heptahelical structure to bind and hyperphosphorylate Dishevelled (Dsh in Drosophila and Dvl in vertebrates) (31) that transduces the signal. Dvl binds to Axin at the C-terminus via its DIX (Dishevelled homologous) domain and N-terminus via its PDZ (acronym from 3 proteins: Post synaptic density protein [PSD95], Drosophila disc large tumor suppressor [DlgA], and Zo-1 protein) domain along with GSK3beta to form a ternary complex which enables Dvl to recruit FRAT1 in the absence of Wnt signal. In the presence of Wnt this complex is disrupted and signal cannot be transduced (32). The cytosolic domain of LRP6 which contains PPP(S)P motif reiterated 5 times, can also activate the Wnt pathway by phosphorylation even at a single PPP(S)P motif (33). It has been shown that LRP6/5 binds to Axin, a scaffolding protein, and localizes it to the plasma membrane followed by its degradation and thus leading to the dispersal of the beta-catenin destruction complex (33, 34). LRP6 is phosphorylated by both GSK3beta and CKI (casein kinase I) (35) as well as by CK1 gamma (36). However, the importance of the intracellular domain of LRP6 has also been shown to constitutively activate the Wnt pathway via Wnt3a-induced LEF1 irrespective of its membrane localization (37). Recent studies report that Wnt-3a triggers the internalization of LRP6 by its interaction with caveolin which facilitates the recruitment of Axin to its phosphorylated PPP(S)P domain, leading to the accumulation of beta-catenin in the cytosol (38). In the cytoplasm, Axin forms a multi-protein complex with APC (adenomatous polyposis coli) (39-41), GSK3beta and CKI (42-45). This complex facilitates beta-catenin phosphorylation by CKI and GSK3beta which enables an F-box protein in the E3 ubiquitin ligase complex, containing beta-transducing repeats, to bind and mark beta-catenin for proteasomal degradation (46-49). GSK3beta is a widely expressed Ser/Thr protein kinase which phosphorylates a variety of substrates at both primed and unprimed sites. Results of an yeast two hybrid analysis showed that GSK3beta interacts with LRP6 at the C-terminal to phosphorylate at the PPP(S)P motif to attenuate GSK3beta activity (50). In response to Wnt signal, titration of Axin from the APC-Axin-GSK3beta complex results in the disruption of the complex as it binds to the phosphorylated PPPSP motif of LRP6 and thus causing beta-catenin to accumulate in the cell cytoplasm.Finally, beta-catenin is translocated to the nucleus and binds to TCF/LEF (51, 52). The TCF family (TCF-1, LEF1, TCF-3,4) contains high mobility group box (HMG) which is responsible for binding to the target DNA (53). The beta-catenin-TCF complex is converted from a transcriptional repressor to transcriptional activator by displacing Groucho and its recruitment of HDAC (histone deacetylase) (54). The displacement of Groucho leads to the recruitment of histone acetylase CBP/p300 (cyclic AMP response element binding protein) which acts as a co-activator (55, 56). Two other protein components, BCL9 and Pygo, are also shown to potentiate the transcriptional activation of beta-catenin-TCF complex (57). The beta-catenin-TCF complex also interacts with various proteins like ICAT (58, 59) which leads to an inhibition of beta-catenin and TCF interaction and also dissociates LEF (lymphocyte enhancer factor) and CBP/p300 from the activating complex (59, 60). Therefore, a controlled mechanism exists inside the nucleus for the tight regulation of Wnt target gene expression.

3.2. Non-canonical pathway

The non-canonical Wnt pathway can be further divided into two categories: Wnt/PCP (Planar Cell Polarity) and Wnt/Ca++ pathways (61). Both pathways utilize Wnt, Frizzled and Dvl proteins as ligands and receptors, but without the involvement of beta-catenin. In Wnt/PCP pathway, Frizzled activates JNK through Strabismus (Stbm), Dvl, Daam1 and GTPase RhoA and Rho-associated Kinases (62) which modulates the cytoskeletal organization. On the other hand, the Wnt/Ca++ pathway works through the release of calcium via phospholipase C (PLC) and protein kinase C (PKC). The elevated level of cytosolic calcium activates calcineurin phosphatase which in turn dephosphorylates NF-AT and leads to its accumulation in the cytoplasm followed by its translocation to the nucleus to activate the target genes. Given the non-canonical pathway does not require the participation of either LRP6 or beta-catenin, in this review we will focus on the canonical Wnt pathway in relation to breast cancer progression.

4. ROLE OF WNT IN MAMMARY GLAND DEVELOPMENT

Several lines of recent evidence show that the Wnt pathway is critical in the development of normal mammary gland. The most prominent example is the role of Wnt-4 in the lobular development. When mammary epithelial buds from Wnt-4KO (knockout) mice were implanted in the post-natal mammary fat pad, devoid of endogenous epithelium of wild-type mice, it showed a significant reduction of lobular branching (63). Supporting this finding, the overexpression of Wnt-4 in virgin mice induced a pregnancy-like growth pattern in reconstituted mammary gland (64). In addition, Wnt signaling is also required for the development of the bud stage. In this stage, the invagination of epithelial layer takes place to give rise to a bud like structure which serves as the foundation for the mammary gland. Apart from Wnt-4, Wnt10b (previously known as Wnt-12) is also required for mammary bud development as shown by the whole mount *in situ* hybridization (65). The most convincing evidence of beta-catenin involvement in development of mammary gland came from the fact that mammary development is defective in mice with disrupted LEF1 (66). LEF1 is a transcription factor of the TCF family that associates with beta-catenin to stimulate the expression of Wnt target genes. In this context, the secretion of PTHrP by mammary epithelium is essential for the induction of LEF1 expression (67). The growth of the mammary gland requires epithelial-mesenchymal interactions that is critical for its development (68). The mesenchyme surrounding the mammary bud is required for the mammary epithelial cell fate and is mediated by the paracrine signaling of the PTHrP secreted proteins and PTHR1 receptor. Therefore, PTHrP also facilitates the induction of LEF1 for the downstream activation of Wnt signaling mediated by beta-catenin. Hence, these lines of evidence clearly indicate a

Table 1. Dysregulation of beta-catenin in clinical samples

IHC staining of Wnt component	% of cases	Type of cancer	Reference
Reduced membrane beta-catenin Reduced cytoplasmic beta-catenin. Nuclear staining beta-catenin	36 72	Breast Carcinoma Phyllodes tumor	73 74 75
Reduced immunostaining Wnt-1	95	Invasive Breast carcinoma	76
Reduced immunostaining Wnt inhibitory factor (WIF)	60	Breast carcinoma	77, 78
Reduced immmunostaining adenomatous polyposis coli (APC)	35	Primary breast cancer	74

Table 2. Animal models of Wnt pathway

Wnt Protein	Mouse model	Mammary phenotype	Status in breast cancer	Reference
Wnt1	MMTV-Wnt1	Adenocarcinoma Breast cancer	Upregulated in grade I tumor Anti-estrogen resistant ER-positive	76, 78, 82, 83 84
Wnt10b	MMTV-Wnt10b	Adenocarcinoma	_	85
Axin	MMTV-axin	Lack of alveoli	Mutation	86, 87
Beta-catenin	MMTV-ΔN89/Δ90 beta-catenin	Precocious alveolar development Adenocarcinoma	Upregulated in cytoplasm of ductal & lobular carcinoma Poor prognosis	69, 75, 88, 89
T-cell factor (TCF)	Tcf1 knockout	Adenocanthomas	_	90, 91
Lymphocyte enhancer factor (LEF)	Lef1 knockout	Arrest of mammary development at E13	_	66
Glycogen synthase kinase3-beta (GSK3-beta)	MMTV-LTR-KImGSK3beta (Kinase inactive)	Mammary tumor with upregulation of beta-catenin and Cyclin-D	Dominant negative	92
Casein kinase2-alpha (CK2-alpha)	MMTV-CK2-alpha	Adenocarcinoma	Overexpression	93, 94

direct involvement of the Wnt signaling pathway in the complex process of normal mammary gland development.

5. ROLE OF WNT PATHWAY IN MAMMARY GLAND CARCINOGENESIS

5.1. Clinical significance of the Wnt pathway in breast cancer

In contrast to the normal breast tissue, there was a remarkable difference in staining pattern of beta-catenin in malignant tissue (69, 70). Alteration in the level of beta-catenin in various stages of breast cancer tissue shows a clear dominance of dysregulation of Wnt pathway. The localization of beta-catenin in the cytoplasm or nucleus is another important criterion to determine the aberrant activation of the Wnt pathway. In addition, immunohistochemical studies have given an apparently contrasting prognostic value of phospho-beta-catenin based on its subcellular location (71). The cytoplasmic localization is associated with prolonged disease free survival whereas nuclear localization has an aggressive and reduced disease-free survival. Thus a more complicated role is played by the stoichiometry, modification and spatial regulation of beta-catenin (see Table 1).

Other than beta-catenin, human breast cancer in relation to canonical Wnt pathway shows deregulation in several stages. Recent study has shown a redundant expression of Wnt ligands for example, Wnt3a, Wnt10b, Wnt6 etc in breast cancer cell lines (72). Furthermore, immunohistochemical studies on primary breast cancer tissues have shown an elevated expression of Cyclin D1 and c-Myc both of which are direct transcriptional targets of canonical Wnt pathway. Immunostaining of Wnt-inhibitory proteins (WIF) can be a reliable parameter to assess the cancer phenotype and is exhibited by reduced immunostaining patterns. The abnormality of APC in the dysregulation of the Wnt pathway is primarily manifested by the truncation mutation though a reduced immunostaining has also been observed in breast cancer specimens. Thus the Wnt signal in the context of mammary gland has dual functions: on one hand, it is essential for the normal development of mammary gland and on the other hand, aberrant Wnt signal in mammary gland is manifested in the form of cancer. Hence, an optimal activation of the Wnt signal with tight control mechanism is necessary for bypassing tumorigenesis and malignant breast carcinoma.

5.2. Animal model of breast cancer for Wnt pathway

Wnt signal orchestrates a complex network of developmental programs but its aberrant signaling has been observed in many human cancers. Most of the common human cancers caused by Wnt signaling relates to the mutation of its various components of canonical Wnt pathway for example, beta-catenin, APC and Axin. However, evidence of mutation in Wnt pathway responsible for breast cancer is unexpectedly lacking.

A number of transgenic and knockout animal modelshave been employed to study the intricate role of Wnt pathway in tumorigenesis. Table 2 shows the mouse models with various Wnt component manipulations that lead to the mammary development defects and tumorigenesis. The first transgenic mouse was constructed with Wnt-1 having an MMTV (mouse mammary tumor virus) mediated insertional mutagenesis. This results in the transcriptional activation of Wnt-1 gene that leads to the development of lobuloalveolar hyperplasia and eventually cancer (2). Furthermore, epigenetic changes or mutation at any nodal point of the Wnt pathway also leads to the development of cancer (79-81). It is well established that dysregulation in components of the Wnt pathway are responsible for tumorigenesis, and among them, beta-catenin/TCF component has been widely used in transgenic

mouse model to elucidate and characterize canonical Wnt pathway. Other models with Axin mutation showed defective alveoli formation. Similar dysregulation of mammary development occurred in the LEF-1 knockout mice. A dominant-negative form of GSK3beta also led to the formation of breast tumor with the upregulation of beta-catenin and its downstream target Cyclin-D.

To gather further insights into the role of Wnt pathway in breast carcinoma it is essential to make animal models to delineate the precise role of each of the components which is responsible for the cause and insidiousness of the disease.

5.3. How dysregulation of Wnt pathway leads to tumorigenesis and malignant progression?

The downstream effector of the Wnt signaling, beta-catenin, is the primary component for the Wnt-mediated mammary oncogenesis. Beta-catenin is responsible for the upregulation of cell cycle regulatory molecules such as c-Myc and Cyclin D1 (88, 89). Cyclin D1 is frequently overexpressed in breast cancer and plays a major role in the mammary cell proliferation (95). Cyclin D1 is a target not only of the Wnt signaling pathway but also of other mitogenic signaling pathways (95). It was previously shown that TGF alpha and Wnt cooperatively induce mammary tumorigenesis (96) mediated by the direct interaction between beta-catenin and EGFR/erbb2 heterodimers (97). Recent evidence has shown that the dysregulation of Wnt signaling is coupled with other signaling pathways which lead to breast cancer. It has been found that increased expression of Wnt-1 in HMEC (human mammary epithelial cells) leads to activation of the Notch signaling pathway mediated by DNA damage response. The notch signaling is upregulated by the aberrant expression of notch ligands, Dll1, Dll3, Dll4, that lead to the tumorigenic transformation of the HMEC cells (98). A clear evidence of the crosstalk between erbB and Wnt signaling was shown when Wnt overexpression in HC11 mammary epithelial cells or treatment with conditioned medium from cells expressing Wnt-1 or Wnt-5a increased the expression of Cyclin D1 via the induction of EGFR (99). Cyclin D1 is required for the mitogenic signaling via EGFR in mammary tumor cells (100). The expression of Cyclin D1 was repressed when EGFR kinase activity was inhibited suggesting that Wnt-1 and Wnt-5A activated the MAPK signaling pathway by EGFR and induced mammary tumorigenesis.

Beta-catenin degradation is dependent on GSK3beta activity that is regulated by AKT protein kinase as well as Wnt ligands. Thus, a crosstalk between PI3K pathway and Wnt pathway is relevant for breast cancer progression. It has also been shown that beta-catenin is induced by the PI3K/AKT pathway in the presence of growth factors like insulin, IGFI, FGFI (101, 102). Thus, the degree of complexity of the Wnt pathway increases manifold with the involvement of other pathways and opens up new avenues to develop preventive measures to cure cancer.

A number of recent studies indicate that the Wnt pathway is also responsible for EMT (epithelial mesenchymal transition). EMT is required for gastrulation, neural crest formation, organ morphogenesis and wound healing. It is found that the EMT is also a key regulator in the process of acquisition of invasive property of cancer cells via which it traverses the ECM (extracellular matrix) during dissemination leading to metastasis. During EMT, the epithelial cells are transformed to the mesenchymal, fibroblast-like property characterized by the loss of cell adhesion and increased cell motility. It has been well documented that beta-catenin which interacts with adherens junction molecule, E-cadherin, is responsible for maintenance of tight cell-cell interaction. Due to aberrant Wnt signaling, the titration of membrane associated beta-catenin is reduced, resulting in the increased cytosolic beta-catenin which subsequently enters the nucleus to upregulate the Wnt target genes. Results of a recent immunocytochemical analysis of breast carcinoma specimens showed that there was a significant reduction of E-cadherin with concomitant increase in the expression of Snail and Slug, both of which are zinc-finger transcription factors that bind to the E-boxes in the E-cadherin promoter to repress its expression (103). This was also accompanied by the aberrant expression of MMP9 (matrix metalloprotease) that is responsible for the degradation of basement membrane of ECM (104). In another study, elevated expression of frpHE (human stromal protein of the secreted frizzled gene family) mRNA has also been observed in the stroma of *in situ* and infiltrating breast carcinoma (105).

Among other components, Axin acts as a negative regulator of the Wnt cascade. LOH (loss of heterozygosity) at a region of human chromosome 17q23-q24, where the Axin gene is located, has been observed in breast as well as other forms of cancer (106). On the contrary, Axin2 homologue has been shown to play a positive role in breast cancer by stabilizing the transcription factor Snail-1, a key regulator of EMT, in a Wnt-Axin2-GSK3beta cascade (107). Thus, it is clearly evident that dysregulation of multiple nodal points in the Wnt cascade leads to tumorigenesis and pathogenesis of cancer.

5.4. Wnt pathway, stem cells and breast cancer

Stem cells are defined as those cells which are endowed with the property of self renewal that are able to generate daughter cells, capable of giving rise to a repertoire of cells found in mature tissue. There are two different types of stem cells: a) those responsible for tissue renewal and b) those requiring appropriate stimulus for repairing of damaged tissue. The stem cells usually have a slow cycling with innumerable replicative potential and thus are favorable for the development of cancer. Several lines of evidence that show the existence of stem cell niche in mammary gland (108-112) and thus it is postulated that there is a direct relationship between mammary stem cells and breast cancer. A recent concept of cancer stem cell is redefining the origin and development of cancer. Cancer stem cell is defined as a cell within a tumor with the ability to self-renew and to cause the heterogeneous lineages of cancer. Cancer stem cells can only be defined experimentally by their ability to recapitulate the generation of a continuously growing tumor. Cancer stem

cell can also be called as "tumor initiating cell'' and ''tumorigenic cell.''

Wnt has been a subject of great attention in recent years for its multifunctional property in cell fate decision during development as well as in self renewal of cells. It has long been established that Wnt signaling plays an important role in the process of self renewal in hematopoietic stem cells (HSC) where elevation of beta-catenin takes place with the activation of TCF/LEF-1 promoter activity (113). Studies with purified Wnt-3a proteins have shown to induce self renewal properties in HSC (13) and TCF-4 which has also been shown to be essential for the maintenance of crypt stem cells in the small intestine (114). It has been recently shown that a single mammary cancer stem cell which has the power of self-renewal and multipotency, is capable of developing a complete mammary gland in premalignant tissue in MMTV-Wnt-1 mice (115). Other studies have also shown a direct role of Wnt signaling in self renewal in epidermal (116) and gut cells (117). Hence, these recent advancements in the development of cancer stem cell theory is interesting as numerous approaches can be developed to target specific cancer stem cells to curtail the development of cancer.

6. TARGETING WNT PATHWAY FOR POTENTIAL THERAPY

It is conceivable that targeting various components of the Wnt pathway, which is dysregulated in the process of cancer progression, would provide a rationale for pharmacological intervention. In the past few decades, a number of attempts have been made to curb aberrant Wnt signaling that is responsible for a wide spectrum of human cancers. The Wnt pathway has been targeted at various levels: a) the extracellular Wnt ligands b) intracellular protein level of various Wnt components, c) aberrant expression of the critical mediator, beta-catenin level and d) downstream targets of the Wnt pathway. There are also various natural inhibitors of Wnt signaling pathway and a rational approach to potentially downregulate the activated Wnt pathway. In this section, we look into the various approaches key regulators of Wnt signal for cancer therapy in general.

6.1. Beta-catenin

A multiple number of cancers arise due to beta-catenin abnormality and thus it is one of the most promising targets of the Wnt pathway. A number of strategies including RNAi, antisense and protein knock-down have been developed. Antisense approach has been used in colon cancer which resulted in the reduction of beta-catenin both at the mRNA as well as protein level that subsequently affects its downstream targets TCF and Cyclin D1 by reducing their expression (118, 119). Similar results were obtained by RNAi method not only in colon cancer (118, 120) but also in esophageal cancer (121), leukemia and lymphoma cell lines (122). NSAIDS (non-steroidal anti-inflammatory drugs) like celecoxib approved by FDA (food and drug administration) as well as EMEA (European Medicines Agency) (123, 124) are effective in decreasing the nuclear levels of beta-catenin and subsequently reduce the formation of multiple polyps in FAP patients (125). In a cell-based small molecule screening process hexachlorophene has been found to degrade beta-catenin expression by a Siah-1 (126) mediated pathway in colon cancer cells (127).

The beta-catenin–TCF complex in the nucleus is responsible for the modulation of Wnt target genes. Hence, targeting this complex would be the most appropriate approach to develop cancer therapeutics. Several studies show the presence of a constant level of beta-catenin-TCF complex in human cancer. Rational design combined with high-throughput screening can lead to the development of drugs which can disrupt the beta-catenin-TCF complex (128). The elucidation of the crystal structure of beta-catenin-TCF has probed into the molecular mechanism by which it interacts to form a stable transcription factor complex (129-131). Thus drug development utilizing the disruption of beta-catenin-TCF complex holds great promise. There has been a great deal of speculation regarding the use of NSAIDS to treat cancer as it inevitably causes serious side-effects including alimentary canal and kidney damage. Therefore, there is a considerable amount of skepticism regarding the use of NSAIDS. However, new generation NSAIDS like NO-releasing aspirin (NO-ASA) has been shown to arrest growth in colon cancer cells by inhibiting beta-catenin-TCF interaction (135,136), with a thousand-fold more efficacy than traditional aspirin administration (137-139). However, it should be noted that beta-catenin also interacts with overlapping domains with E-cadherin (132) and APC (133) and these interactions are negative contributors to the Wnt signaling. Therefore, it is a real challenge to develop small molecules which can effectively and selectively disrupt beta-catenin-TCF complex without affecting its interaction with E-cadherin or APC.

There are three natural compounds PKF115-584, PKF-222-815 and CPG049090, which are obtained from high-throughput screening (HTP) of natural compounds, which has shown to inhibit Wnt signaling in colon cancer cells (134) and also in Xenopus embryo (51). It is well established that recruitment of various co-activators are necessary for efficient activation of beta-catenin-TCF target Wnt genes. Thus if co-activators like CBP (CREB-binding proteins) and BCL9 (B-cell lymphoma)/pygopus are effectively inhibited to interact with beta-catenin-TCF complex, it would lead to the downregulation of Wnt pathway. Indeed a small molecule inhibitor, ICG-001, which selectively binds to the CBP resulted in the titration of CBP from beta-catenin-TCF complex followed by reduction of Wnt signaling effectively in colon cancer cell. Furthermore, the inhibition of Wnt signaling was accompanied by the reduced expression of anti-apoptotic gene, survivin (135).

In an adenoviral based approach, the FADD (Fas-associated via death domain) gene under the tight control of promoters containing TCF-responsive elements was introduced in colon cancer cells which effectively killed the cells, substantiating the validity of the approach.

Other viral based approaches include the generation of oncolytic viruses. The tumor cells that exhibit higher beta-catenin-TCF activity have augmented therapeutic effect of the viruses by replication in the target tumor (136). This was validated by engineering replicating viruses which express the viral E1B and E2 genes from promoters containing TCF-response elements. This was highly effective in colon cancer cells but showed a 50-100 fold decrease in lung cancer cells and normal fibroblast lacking an active beta-catenin-TCF signaling (136).

6.2. Extracellular components of Wnt pathway

6.2.1. sFRP

The sFRP (secreted Frizzled–related proteins) comprises a family of five glycoproteins that binds to the Frizzled receptor and antagonizes the Wnt signal. Reduction in sFRP's has led to the development of various types of cancer including breast and it has been shown that restoration of sFRP inhibited the growth and promoted apoptosis (76, 78, 137-143). Hence, sFRPs are potential targets to curb adverse effects of Wnt pathway by inducing apoptosis and restricting cell growth.

6.2.2. Wnt

Various methods have been employed to knock down Wnt-1 expression by antisense RNA (144). Apart from that, a monoclonal antibody (145, 146) which can neutralize the effect of Wnt-1 has also been developed. This antibody proved to be effective in a number of human cancers like breast and non-small cell lung cancer by inducing apoptosis accompanied by reduction in tumor growth in animal models (146). Similar results were observed when Wnt-2 monoclonal antibody was used to treat melanoma (147) and non-small cell lung carcinoma (148) that effectively induced apoptosis resulting in the inhibition of malignant progression.

6.3.3. Dkk

Dkk is another antagonist of Wnt pathway which prevents binding of Wnt to LRP5/6 (30) and thus has a considerable potential to serve as a therapeutic target. Dkk-1 is the most important member of the Dkk family of proteins that includes Dkk2, Dkk3, and Dkk4 (149, 150). It has been shown that expression of exogenous Dkk3 leads to cell growth inhibition in non-small cell lung carcinoma (151) as well as reduced invasion and motility in osteosarcoma cells (152).

Therefore, multiple strategies are applied to treat aberrant Wnt signaling and subsequently curb specific human cancer. However, most of the drugs are still at an infant stage. Due to the highly complex nature of Wnt signal, it is imperative to develop drugs with high specificity and efficacy.

7. CONCLUSION AND FUTURE DIRECTION

The complexity of the Wnt pathway is increasing with the identification of more key regulators and its cross-talk with other major pathways. In the context of breast cancer, evidence regarding the mutational status of various components of the Wnt pathway is sparse. Yet, dysregulation of Wnt pathway is emerging as a major cause in the development of breast cancer. The crucial questions which need to be addressed include what factors mediate the hyperactivation of Wnt pathway and what mediators stabilize beta-catenin, thereby activating the downstream effectors and Wnt target genes as well. More fundamental questions need to be elucidated, as how the negative and positive regulators co-ordinate and integrate in the cellular milieu to constitutively activate the Wnt pathway. Identification of the crucial nodal points which are responsible for the etiology of breast cancer can provide therapeutic targets to develop drugs with high efficacy opening new avenues in the treatment of breast cancer.

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Roles of AKT signal in breast cancer

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1. ABSTRACT

The PI3K/Akt pathway plays a central role in a variety of cellular processes including cell growth, proliferation, motility and survival in both normal and tumor cells. The PI3K/Akt pathway is also instrumental in epithelial mesenchymal transitions and in angiogenesis during tumorigenesis. Many of the transforming events in breast cancer are a result of enhanced signaling of the PI3K/Akt pathway. Akt therefore is considered to be a rational target for cancer therapies and inhibitors of the PI3K/Akt pathway have been identified. In this review, we discuss the recent information about the functional roles of PI3K/Akt pathway in tumorigenesis and progression of breast cancer.

2. INTRODUCTION

Breast cancer is one of the most common malignancies in the United States (1). The development of breast cancer from *in situ* malignancy to metastasis requires changes in signaling pathways, which increase cell motility, enhance tumor cell survival and increase the ability to undergo epithelial-mesenchymal transition. Akt/protein kinase B (PKB) is a serine/threonine kinase and was first identified by Tsichlis *et al.* as a pro-survival protein in a PI3K-dependent manner (2). Mammals have 3 isoforms of Akt (Akt1, 2, 3), encoded by 3 different genes. In both normal and cancer cells, various stimuli activate PI3K, a lipid kinase that catalyzes the synthesis of the membrane phospholipid PI(3,4,5)P3 from PI(3,4)P2, which

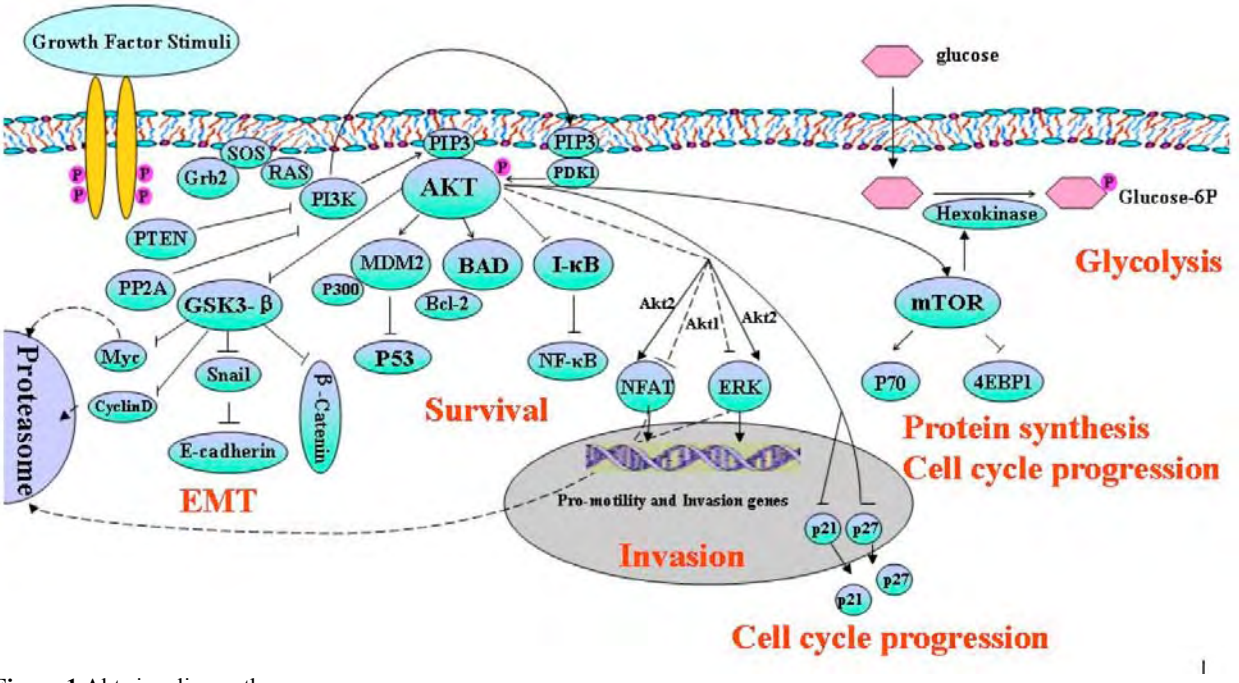

Figure 1 Akt signaling pathway.

can effectively recruit Akt to the plasma membrane by direct interaction with the Akt pleckstrin homology domain (3). Subsequently, Akt is phosphorylated at Thr308 by PDK1 and at the Ser473 via autophosphorylation or other kinases to reach maximal activation (4). Once phosphorylated and activated, Akt is transferred to several subcellular locations where it can phosphorylate its targets in various pathways. Akt plays a critical role in cell survival by interacting negatively with apoptosis promoting proteins like BAD, MDM2, NF-kappaB, Caspase-9 and Forkhead, and with proteins involved in cell proliferation (p21, p27), cell growth (mTOR), cell motility and invasion (GSK-3beta). These proteins also play critical roles in tumorigenesis and metastasis.

Akt has been previously implicated in various types of tumors including prostate, ovarian, breast and thyroid cancer (4, 5, 6). Remarkable increase in the Akt kinase activity has been found in approximately 30% to 40% of breast cancer specimens (7, 8). The clinical importance of Akt is further substantiated by the observations that: (i) constitutive activation of Akt and other components in the Akt pathway are seen both in *in situ* breast carcinoma and invasive breast cancer; (ii) the activation of Akt plays an important role in conferring resistance to anti-estrogens such as tamoxifen, a gold standard therapeutic drug for hormone receptor-positive breast cancer; and (iii) Akt is involved in a broads pectrum of chemoresistance as well as radioresistance of breast cancers. Therefore, Akt is considered to be a novel target for therapies for breast cancer. In this review, we will focus on the roles of Akt in the progression of breast cancer and on the prognostic and therapeutic utility of this protein.

3. FUNCTION OF AKT SIGNAL

3.1. Akt acts as a cell survival factor through various signal pathways

Enzymes that modulate the Akt signaling, such as MDM2, alpha6beta4 integrin, the tumor suppressors PTEN and PHLPP are frequently mutated in human tumors, resulting in up-regulation of the Akt activity and increased growth and survival of tumor cells (9-12). Upon stimulation by growth factors, activated Akt detaches from the inner surface of the plasma membrane and re-localizes to the nucleus, suggesting that targets of Akt are also located in the nucleus (Figure 1). Akt indeed has been shown to influence the cellular localization and functions of cell cycle inhibitors p21 and p27 (13, 17), which induce cell growth arrest by inhibiting the functions of cyclin-dependent kinases in the nucleus. Akt can phosphorylate p21 on a consensus threonine residue Thr145 in the nuclear localization signal, leading to the cytoplasmic localization of p21 (13). By similar strategy, Akt can also phosphorylate p27 at Thr157 and transport the protein to cytoplasm, thus enhancing cell proliferation (17). It has also been found that PKCalpha activity, which can inhibit cyclin D1 activity in the early stage of colon cancer, is dependent on PI3K/Akt pathway (15). These results suggest a possibility of cross-talk between the Akt and other pathways in regulating the cell cycle program. As a survival factor, Akt is an important determinant of anti-apoptosis in tumorigenesis of various cancers by either directly or indirectly disrupting apoptosis pathways, which are induced by Bad or p53, or by rescuing survival factors like NF-kappaB (14, 16, 17).

3.2. Akt functions in cell motility and EMT

Growth and survival are not the only phenotypes that prevail in various carcinomas; cell motility and invasion through basement membrane are also important

Table 1. Significance of Akt pathway in breast tumorigenesis and progression

	Primary tumor [7,8,44,46,82,83,84]	Overall survival [50,66,85]	Relapse [50,66]
pAKT	33%-40%	P=0.0406	35%
PTEN abnormality	26%-48%	35% P<0.05	90%
PI3K abnormality	35%	P<0.052	
p-mTOR	32%-44.9%	P<0.01	43.5%
ErbB2 over-expression	30%	P<0.05	50.9%
ER positive	60%	P<0.05	31.7%

phenotypes that are ultimately responsible for the progression of primary tumors into metastases (18). Numerous studies have provided overwhelming evidence that efficient signaling through the PI3K/Akt axis promotes cell motility and invasion, although the three Akt isoforms (Akt1, 2, 3) have specific and non-redundant roles in regulating invasion. Mercurio *et al.* previously reported that the alpha6beta4 integrin, a tumor-associated antigen, promoted breast and colon cancer cell migration and invasion by activating PI3K (19). Other groups have also reported that ectopic expression of Akt increased cell migration and invasion in certain cells through secretion of matrix metalloproteases or activation of various small GTPases, like Rac. This leads to degradation of the extracellular matrix or remodeling of the actin cytoskeleton followed by the enhancement of cell motility (19, 20). Expression of Akt can also promote epithelial-mesenchymal transition (EMT), a process closely associated with tumor progression to invasive and metastatic carcinoma (21). One of the possible mechanisms by which Akt mediates the EMT program is through crosstalk with the Wnt pathway. Zhou *et al.* found that Akt phosphorylated GSK-3beta, a negative regulator of beta-catenin/TCF transcription factor, at Ser9 to inactivate this protein. GSK-3beta has been known to activate a transcriptional factor, Snail, which can down-regulate E-cadherin and thus regulate epithelial-to-mesenchymal transition (EMT) (22). In human cancer, dominant transcriptional repression is largely responsible for the loss of E-cadherin expression, which is required for maintaining the properties of epithelial cells and for the interaction with neighboring epithelial cells (23). Thus, Akt-induced EMT and invasion have emerged as a critical process during cancer progression and metastasis.

3.3. Differential roles of Akt isoforms

Humans have three Akt isoforms, Akt1 (PKB-alpha), Akt2 (PKB-beta), and Akt3 (PKB-gamma), which are encoded by distinct genes localized on different chromosomes. Although they share similar structures with approximately 80% amino acid identity, their expression patterns and biological activities differ significantly (24 - 26). Akt1 is significantly expressed in various tissues and was initially cloned as a homolog to the viral Akt8 oncogene (10, 27). The Akt1-knockout mouse is viable but small in body size (28). In normal physiology, Akt1 is known to be involved in placental development and maintenance. On the other hand, Akt2 is preferentially expressed in insulin-responsive tissues, involved in glucose metabolism, adipogenesis and B-cell function (29). Akt2-knockout mouse exhibits defects mainly in glucose homeostasis and develops type II diabetes (30). The expression of Akt3 gene is limited to the brain, heart, and kidneys (22, 31) and plays a role in postnatal development of the brain (30).

There are a number of recent reports that demonstrate physiologically distinct functions of Akt1 and Akt2 in regulating cell migration and EMT (31-33). The expression of Akt2 increases invasion of cancer cells *in vitro*. In contrast, Akt1 has been reported to potently block the *in vitro* migration and invasion of three distinct breast cancer cell lines (31). Two possible mechanisms have been suggested for this phenomenon. First, over-expressed Akt1 can block ERK activity and cell migration and also inhibit EMT (34). Secondly, Akt1 can stabilize MDM2 followed by ubiquitination and degradation of NFAT (35). This action, therefore, inhibits the transcription of genes involved in migration and invasion such as the pro-motility factor autotoxin/ENPP2 (35, 36). On the other hand, Akt2 is the predominant isoform that is amplified in breast and ovarian tumors (37). Mutations that activate Akt2 have also been detected in colon cancer (38). Irie *et al.* reported that down-regulation of Akt2 reverted all aspects of IGF-IR-induced phenotypic changes in 3-D acinar structures (31). They also found that specific down-regulation of Akt1 induced a dramatic phenotype resembling EMT and enhanced growth factor-stimulated migration. However, Maroulakou *et al.* recently reported that Akt1 ablation in their transgenic mouse model significantly inhibited the development of mammary adenocarcinomas, while Akt2 ablation accelerates the tumorigenesis (39). These observations apparently contradict with previous results of *in vitro* studies and suggest that the function of Akt is cell context dependent and the expression of Akt is controlled by autocrine and paracrine stimuli that may be missing in the *in vitro* systems. Therefore, all Akt isoforms do possess the ability to transform various cell types in an isoform-specific manner, but the exact roles of each Akt isoform in tumorigenesis need to be further clarified.

4. ROLE OF AKT SIGNAL IN TUMORIGENESIS AND TUMOR PROGRESSION OF BREAST CANCER

4.1. Akt signal is upregulated in human breast cancer

The regulation of cell proliferation and cell survival in breast cancer is a complex interplay between steroid hormones, growth factors, and their receptors including IGF (insulin-like growth factor) receptors and members of EGF receptor family (40). In breast cancer, the PI3K/Akt pathway can be activated by membrane receptors such as the ErbB family of growth factor receptors and the estrogen receptor (ER) (41). Over-expression of ErbB2 also known as HER-2/neu has been found in approximately 30% of human breast cancers (Table 1) (7). Recently, Tokunaga *et al.* examined 252 human primary breast

carcinoma specimens from 138 patients and found that 84 cases (33.3%) were positive for pAkt expression and that pAkt was significantly associated with ErbB2 over-expression (P<0.0001) (7). Similarly, Bacus *et al.* also reported a significant correlation between ErbB2 and Akt2 expression in breast cancer specimens (42). Previously, the amplification and/or over-expression of Akt2, but not Akt1, were considered to play an important role in human breast malignancy. However, Cheng *et al.* later reported that the elevated Akt1 kinase was an essential requirement for the oncogenic activity of Akt in prostate, breast and ovarian carcinomas (43). Stal *et al.* also discovered the correlation between ErbB2 and Akt expression only for tumors that co-expressed Akt1 and Akt2 (44). In addition, they found that the increased expression of Akt2 occurred frequently in ER-negative tumors. In contrast, Akt1 was expressed with similar frequency in both ER-positive tumors and in ER-negative tumors. The other isoform, Akt 3, was found to be associated with ER-negative breast cancer in Nakatani's study (9). Therefore, different Akt isoforms may function differentially in breast malignancy in conjunction with the ER status of the patients. It has been known for a long time that the aberrant activation of Akt in breast carcinoma was associated with the status of ER (45), PR (progesterone receptor) and ErbB2 over-expression (46). In past years, many studies have been focused on the regulation of ER-alpha transcriptional activity by PI3K/Akt signaling, but much less is known about the regulation of ER-beta. Recently Duong *et al.* used a tissue microarray for twenty-nine infiltrating breast carcinoma to examine the relationship between the expression of the activated ER-beta and Akt (45). They found that there was a positive correlation between phosphorylated Akt and ER-beta protein levels in a clinical setting, in which the largest population consisted of high expression of ER-beta and pAkt. This is the first report showing that Akt regulates several components of ER-beta-mediated transcription. This sheds light on a significant determinant of ER-beta in breast malignancy.

Another major mechanism of Akt activation is through the loss of function in the tumor suppressor gene, PTEN (47). Several studies have demonstrated that PTEN abnormalities such as reduced PTEN expression, or PTEN mutation, e.g. PIK3CA (mutation of the catalytic subunit-alpha of PI3K), in breast cancer are associated with stage, grade, lymph node metastases, and steroid receptor status (46). Recently, Shoman *et al.* examined the prognostic significance of reduced PTEN expression in ER-alpha-positive breast cancer patients and the potential association with the resistance to tamoxifen (48). They found that ER-alpha-positive breast tumors with reduced PTEN expression had significantly shorter relapse-free survival, which suggests a poor response to tamoxifen therapy. Based on their discovery, it was proposed that reduced PTEN expression could result in increased activity of PI3K/Akt-mediated anti-apoptotic pathway, which interfered with cellular actions of tamoxifen and resulted in tumor recurrence.

Among the downstream targets of Akt, p21 is a critical modulator of cell cycle and cell survival. Hung *et al.* examined 130 breast cancer specimens for the correlation among the expression status of the key biological markers in the ErbB2-Akt-p21 pathway (49). They concluded that both cytoplasmic localization of p21 and over-expression of phospho-p21 were associated with high expression of ErbB2 and phosphorylation of Akt, which correlated with worse overall survival. Another key target of Akt, mTOR which is an Akt-activated serine-threonine kinase and a target of rapamycin, is involved in a variety of functions including transcriptional and translational control such as phosphorylation of p70S6K (40S ribosomal protein S6 kinase) and 4EBP1 (eukaryotic initiation factor 4E-binding protein-1). Zhou *et al.* reported that expression of mTOR, phosphorylated Akt, and 4E-BP1 increased progressively as proliferation and invasion increased in breast cancer (50). Therefore, Akt activation, regardless of the ER status, appears to be a common event in human breast cancer.

4.2. Roles of Akt signaling in breast tumorigenesis and metastasis

High levels of phosphorylated Akt, mTOR, and 4EBP1 have been found in IDH (intraductal hyperplasia) and DCIS (ductal carcinoma *in situ*) (50). Clinical-pathological data also demonstrate that over-expression of ErbB2 leads to increased p-mTOR and p-4EBP1 levels in cultured breast cancer cells (50). Therefore, the Akt/mTOR/4EBP1 pathway plays a crucial role during the development and progression of breast malignancy. In addition, phosphorylation of Akt, mTOR and 4EBP1 increase progressively in breast cancer in association with the over-expression of ErbB2, both of which give rise to poor disease-free survival (50). This observation indicates the utility of these pathway components in predicting the prognosis of patients with breast cancer, especially for those treated with mTOR inhibitors.

In light of homeostasis of breast epithelial cells in controlling proliferation and apoptosis, Akt appears to function as a survival factor during breast tumorigenesis. Activation of the PI3K/Akt pathway causes phosphorylation of Bad, leading to modulation of cellular apoptosis (51, 52). Cannings *et al.* recently examined the expression of Bad, pBad (ser112), Bcl-2, Bcl-xl and Bax on 402 ER positive breast cancers (16). They found that activation of the PI3K/Akt pathway by either heregulin or estrogen had no effect on the expression of Bad, Bcl-2, Bax or Bcl-xl; however, heregulin increased pBad expression. In addition, the active signaling of ErbB2 in the precursor lesions of breast cancer induced the expression of FAS (Fatty acid synthase) which plays a synergistic pro-tumorigenic role in the early phases of breast cancer (53). Therefore, the nutritional insensitivity of FAS in transformed cells may be driven by a constitutive activation of PI3K/Akt signaling pathways in response to oncogenic changes such as over-expression and/or activation of growth factor receptors.

The PI3K/Akt pathway also plays a pivotal role in increasing invasion and migration and in promoting the EMT program of breast tumor cells. Several reports demonstrated the enhancement of nuclear Akt activity in

breast cancer cells at the leading edge of invasive fronts (14, 54, 55). It has been reported that transcriptional activity of NFAT (Nuclear factor of activated T cell) is elevated in highly invasive breast cancer cell lines and that blocking of NFAT activity resulted in the decreased invasion of tumor cells *in vitro* (30). Activated Akt1 significantly suppressed the activity of NFAT, whereas Akt1 knockdown induced by siRNA increased this activity. In contrast, Akt2 positively regulated NFAT and facilitated invasion. Irie *et al.* also reported that suppression of Akt1 expression increased the migration and EMT of MCF-10A cells by suppressing EGF-stimulated migration and ERK activity (31).

Abundant evidence indicates that Akt mediates resistance to endocrine therapy in breast cancer, and this effect is related to the ability of PI3K/Akt pathway to regulate both ER-alpha and ER-beta activity (45, 56, 57). Although it is known that constitutively activated mutant Akt decreases ER-alpha protein expression, Akt can also upregulate the ER-alpha activity in the absence of estrogen and render human breast tumor cells resistant to tamoxifen-induced apoptosis (58). Given the clinical observations of deregulated PTEN/Akt and reduced ER or PR expression in human breast cancer, the PTEN/Akt pathway appears to be utilized by breast cancer cells to acquire a growth advantage and perhaps develop hormone independence. Recently, Duong *et al.* also found that Akt can regulate the expression of ER-beta in breast cancer, which suggests that Akt can function on both subunits of ER (45). These discoveries highlight the importance of the Akt pathway in the process of tumorigenesis and suggest that appropriate inhibition of any of the critical factors in the Akt pathway may be a rational therapeutic approach.

5. THERAPEUTIC IMPLICATION OF AKT PATHWAY FOR CANCER

Predictive markers that inform treatment choice are of critical importance in the validation and application of novel therapeutics. It is increasingly recognized that tumor profiling with multiple markers may aid patient stratification. Until now, endocrine therapy for breast cancer is still the most effective systemic treatment for patients with hormone receptor positive breast cancer. Major clinical trials have shown that the ER status is the strongest and the most reliable predictor of the response to endocrine therapy (59). ER activity is often potentiated to a higher level with the combination of EGF, IGF-1 and E2 through the downstream kinase pathways such as ERK/MAPK and PI3K/Akt (56, 57). Recent studies suggest that Akt activity increases as breast cancer malignancy intensifies, resulting in a poor prognosis (7, 50). In addition, the activation of Akt has been reported to be associated with resistance to anti-estrogens such as tamoxifen, a gold standard endocrine therapy for hormone receptor-positive breast cancer (58, 60). Among pre-menopausal patients treated with tamoxifen, those with activated Akt were more prone to relapse with distant metastasis (44). On the other hand, among post-menopausal patients, those negative for Akt showed significant benefit from tamoxifen (61). Therefore, tumor resistance associated with endocrine therapy, acquired either *de novo* or during the treatment, has been found to be related to activated Akt.

A common mechanism of resistance to chemotherapy is a profound resistance to apoptosis. Therefore, the fundamental question for an anti-tumor drug is whether malignant cells will effectively lose their resistance to apoptosis when Akt is inhibited. The importance of this effect has been demonstrated by an experiment in which apoptotic resistance was restored when performing treatment with LY294002, the inhibitors of PI3K/Akt pathway (57). On the other hand, when cells are transfected with a constitutively active form of Akt, such drugs lose their effect. Therefore, the inhibition of the Akt signaling pathway may improve the efficiency of endocrine therapy for metastatic breast cancer. Several currently ongoing or scheduled phase II/III clinical trials of endocrine therapy for locally advanced or metastatic breast cancer are listed below (62).

5.1. Akt inhibitors

Akt can be activated by a variety of factors, thus designing drugs specific to Akt protein kinase may sound theoretically appropriate but there are many practical problems. Since Akt protein shares homology with other members of the kinase family with respect to its ATP binding domain, targeting Akt in a manner to specifically reduce cell survival with minimal toxicity remains a challenge. Most kinase inhibitors bind to the ATP binding site in the protein molecule, thereby inhibiting their activation. Staurosporine, tri-substituted imidazoles and pyrroles have been previously reported to be competitive and irreversible inhibitors of Akt (63, 64). Also, Triciribine/AP-2 was a non-specific isoform inhibitor of Akt which has not been fully developed due to high hepatotoxicity and hyperglycemia. Another Akt inhibitor is Perifosine which is now in Phase II clinical trials. Perifosine is believed to block Akt activity by hindering localization of Akt to the membrane and inducing dephosphorylation (65).

5.2. PI3K inhibitors

Two compounds, Wortmannin and LY294002, have been used as inhibitors of PI3K in recent studies (36, 66). Wortmannin is a fungal metabolite and a potent inhibitor of PI3K. It binds irreversibly to the p110 catalytic subunit at nanomolar concentrations and reduces Akt cellular activity by 50% (66). However, the major disadvantage of Wortmannin is its lack of stability in an aqueous environment. Currently, water-soluble Wortmannin derivatives are being developed to circumvent this issue (66). LY294002 is a flavanoid derivative and a reversible, competitive inhibitor for the ATP binding site of PI3K (66). It has both anti-proliferative and pro-apoptotic activity. Both Wortmannin and LY294002 act in concert with other cytotoxic drugs, radiation or antibodies, enhancing their therapeutic efficacy (36). Transient expression of a constitutively active Akt in non-small cell lung cancer cells with low Akt activity conferred these cells with resistance to chemotherapy- or radiotherapy-induced apoptosis. Moreover, treatment of the cells with LY294002

sensitized the cells to chemotherapeutic agents or radiotherapy.

5.3. Rapamycin analogs

Akt signaling in the tumor vascular stroma is sensitive to rapamycin, suggesting that rapamycin blocks tumor growth through multiple mechanisms. Firstly, VEGF-A-induced acute permeability is dependent on Akt signal and downstream activation of endothelial eNOS (67-69). Therefore, sustained endothelial Akt activation causes increased size of tumor blood vessels. Rapamycin can inhibit endothelial Akt signaling, Akt1-dependent vascular changes, tumor growth and tumor vascular permeability (70). Secondly, the mTOR kinase can regulate the expression of nutrient transporters in response to nutrient availability (71). Withdrawal of growth factors also results in diminished uptake of glucose and other nutrients essential for cell growth including amino acids and low-density lipoprotein-cholesterol, but these effects are attenuated by a constitutively active Akt (72). The maintenance of nutrient uptake by Akt can be eliminated in the presence of rapamycin, suggesting that mTOR is necessary for this effect. Finally, rapamycin markedly diminishes the ability of constitutively active Akt to suppress apoptosis (73). The mechanism of this effect remains to be established; however, Harada *et al.* reported that one possible pathway is through the phosphorylation of Bad by p70S6K, an mTOR downstream target (52).

5.4. Trastuzumab and other EGFR inhibitors

The monoclonal antibody trastuzumab, also known as Herceptin (Genentech Inc, South San Francisco, CA) inhibits the activity of ErbB2 by binding to the extracellular portion of the receptor and inducing its degradation. Trastuzumab suppresses the PI3K/Akt pathway, resulting in TRAIL-induced apoptosis in a cell-specific manner (74). Trastuzumab appears to be beneficial for the treatment of metastatic disease which is resistant to chemotherapy (75). It has also been shown to enhance the tumoricidal effects of other chemotherapeutic agents, in part by enhancing their apoptotic effects (76). A number of agents targeting the EGF receptor are also either in phase III trials (Erlotinib), or are clinically available (Cetuximab and Gefitinib). Gefitinib has been shown to inhibit Akt in a breast cancer cell line, which can be recovered by a constitutive expression of the active form of PI3K.

6. CONCLUSIONS AND FUTURE DIRECTIONS

Dysregulation of the PI3K pathway through PTEN abnormalities occurs in up to 50% of breast cancer cases. When abnormalities of other upstream and downstream factors are included, the majority of breast tumors have activation of the PI3K/Akt pathway, making this pathway an attractive target for pharmacologic interventions. However, there are still many issues that remain to be elucidated. Firstly, although the correlation between Akt and those generally identified prognostic-markers for breast cancer like ER status have been extensively examined, there are still debates on the correlation loop among ErbB2, Akt and ER. Several reports on breast cancer indicate a significant positive correlation between active Akt and over-expression of growth factor receptors, especially ErbB2, whereas little correlation was found between ER and Akt activation. Although over-expression of ErbB2 stimulates ER expression by activating Akt, it has also been reported that there is no significant correlation between ER status and phosphorylated Akt or ErbB2 over-expression (77-79). Therefore, more clinical examination is needed to clarify this intriguing loop among ErbB2, Akt and ER status. Further, there are different perspectives on how to effectively use phosphorylated Akt as a "diagnosis marker" for predicting the development of breast cancer, the survival rate and the curative effect of endocrine therapy. It has recently been recognized that the phosphorylated Akt did not correlate with the overall survival in breast cancer patients, although the high pAkt level did correlate with poor prognosis for post-operative endocrine therapy patients (78, 80). These results indicate that pAkt may be better used as a diagnosis marker for metastatic breast cancer rather than for overall breast cancer survival. Last, although the frame of the versatile function of the PI3K/Akt pathway has been well established, further details of the underlying mechanisms are still unclear. For example, more explicit explanation is needed for the various and non-overlapping functions of Akt isoforms in breast cancer. In addition, the mechanism for Akt nuclear import remains uncertain as neither Akt nor its chaperones contain recognizable nuclear localization signals. Recent intriguing discovery is that mTOR can function as an upstream activator as well as a downstream target of Akt, which makes the anti-tumor drug of mTOR inhibitors like RAD001 more effective by repressing the Akt pathway (81). Further understanding of the PI3K/Akt pathway and elucidating the precise role of each component involved in this pathway should aid in the development of more efficient and specific therapeutic strategies for breast cancer patients.

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