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Coordinate expression of the PI3-kinase downstream effectors serum and glucocorticoid-induced kinase (SGK-1) and Akt-1 in human breast cancer

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Abstract

The phosphatidylinositol 3-kinase (PI3-K) signalling pathway has been implicated in breast cancer development and resistance to therapy. Akt-1 and serum and glucocorticoid-induced kinase-1 (SGK-1) are homologous kinases which are important downstream effectors of PI3-K signalling. We sought to determine the individual expression patterns of these two kinases in order to better understand their respective roles in PI3-K signalling in breast cancer. To this end, we examined the expression of both p-Akt-1 and SGK-1 in 40 breast cancers. p-Akt-1 expression was seen in 58% of tumour samples, while SGK-1 overexpression was detected in 48%. Interestingly, a highly significant association was found between the expression of p-Akt-1 and SGK-1 ($P = 0.002$), suggesting complementary physiological functions in PI3-K signalling. This finding is consistent with recent genetic data from *Caenorhabditis elegans* suggesting that both SGK-1 and Akt-1 are required for signalling downstream of insulin receptor activation.

Keywords: Akt-1; SGK-1; PI3-kinase; Breast cancer; Insulin signalling; Immunohistochemistry

1. Introduction

Activation of the PI3-kinase (PI3-K) pathway is a common event in breast cancer [1]. By far the most studied downstream effector of this pathway is Akt-1, also known as protein kinase B (PKB). Akt-1 plays an important role in regulating mammary epithelial cell survival [2]. Endogenous Akt-1 activity in breast cancer cell lines promotes cancer cell survival and chemotherapy resistance [3]. Activation of Akt-1 in breast cancer also predicts a worse outcome among endocrine-treated patients [4]. Like Akt-1, serum and glucocorticoid-inducible kinase-1 (SGK-1) is also a downstream effector of the PI3-K pathway and can contribute to survival signalling in mammary epithelial cells [5,6]. In contrast to Akt-1, SGK-1 appears to be degraded very rapidly and steady-state levels are not readily detectable in mammary epithelial cell lines by Western blot analysis unless a proteasome inhibitor is added to the culture media [7]. Therefore, total SGK-1 protein homeostasis is tightly regulated and when the protein is expressed, SGK-1 is constitutively phosphorylated via a PI3-K dependent mechanism [6].

Although the expression of these related kinases has been studied extensively in cell lines, there have been surprisingly few reports examining either SGK-1 or

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Akt-1 protein expression in human breast cancers. In this study, we examined 40 human breast cancer samples for both p-Akt-1 and SGK-1 expression using immunohistochemistry to evaluate their overall frequency of overexpression and subcellular localisation. We hypothesised that SGK-1 overexpression may substitute for p-Akt-1 expression in breast cancers due to their common downstream substrates (*e.g.*, FKHRL-1 [8] and GSK3 [9]). Surprisingly, we instead discovered a highly significant positive association between SGK-1 and p-Akt-1 expression, suggesting that Akt-1 and SGK-1 may have complementary functions in executing downstream PI3-K signalling.

2. Materials and methods

2.1. Patients and tumour specimens

A total of 40 independent breast cancer samples from 34 patients treated at the University of Chicago from 1996 to 1999 were included in this retrospective study. Six of the tumour samples represented asynchronous metastatic tumours from the same patients whose prior tumours were available for examination. All patient identifiers were removed from the samples and the University of Chicago's Institutional Review Board reviewed the experimental protocol.

2.2. Immunohistochemical analysis

Immunohistochemical staining was performed on 4 μm sections obtained from formalin-fixed, paraffin-embedded tissue blocks. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide. The sections were subjected to antigen retrieval by boiling in 0.01 mol/l citrate buffer (Dako, pH 6.0) for 20 min in a pressure cooker. Primary antibody recognising p-Akt-1 (rabbit polyclonal anti-pSer473 Akt from Biosource International, Inc.) was used at a 1:80 dilution. The anti-SGK-1 antibody (IgG rabbit polyclonal antibody against the C-terminus of SGK-1 from Stressgen) was used at a 1:300 dilution. Diluted primary antibody was applied to the slides for 1 h at room temperature in a humidified chamber and washed thoroughly with 1X Tris buffered saline (TBS). Slides were then incubated with the pre-diluted secondary anti-rabbit antibody provided with the biotin-free horseradish peroxidase EnVision plus detection system (DAKO, Carpinteria, CA). Antigen–antibody binding was detected by diaminobenzidine substrate chromogen system. The slides were briefly immersed in hematoxylin for counterstaining and evaluated under light microscopy. Negative controls using rabbit IgG followed by secondary antibody were performed for each experiment.

The immunoreactivity of tumour cells was evaluated by a breast pathologist (S.S.) by analysing both the staining intensity and percentage of immunoreactive tumour cells. Positive staining was defined as any cytoplasmic and/or nuclear staining in more than or equal to 10% of tumour cells. Stromal cells exhibited no significant reactivity with either the anti-p-Akt-1 or anti-SGK-1 antibodies.

2.3. Indirect immunofluorescence microscopy

SK-BR-3 breast cancer cells (3×10^6) were seeded onto glass coverslips. The next day, adherent cells were washed twice in 1X PBS and fixed for 30 min in freshly prepared 4% paraformaldehyde and 5 mM Hepes in Hank's balanced salt solution (HBSS; Life Technologies). Cells were then blocked and permeabilised simultaneously for 30 min at room temperature using a 1% FCS/0.01% saponin/HBSS solution. Cells were then incubated in a humidity chamber for 1 h at 37 °C with anti-pSer473-Akt-1 (1:100), anti-SGK-1 (1:100) antibody, or rabbit IgG (negative control) diluted in blocking/permeabilisation buffer. Following incubation, cells were washed 3 times for 2 min each in blocking/permeabilisation buffer and then incubated in a humidity chamber in the dark for 1 h at 37 °C with the secondary anti-rabbit antibody (1:1500) conjugated to a red spectrum Alexa Fluor 568 (Molecular Probes). Cells were washed 3 times for 2 min using the blocking/permeabilisation buffer, mounted onto slides using Gel Mount (Biomeda), and examined at 400× with a Zeiss Axiovert 200 inverted fluorescence microscope using a Texas Red (533–588 nm) filter; images were captured using an Orca ER camera and analysed using the Openlab® program.

2.4. Statistical analysis

Fisher's exact test was used to examine a possible association between SGK-1 and p-Akt-1 expression, as well as to evaluate any association between SGK-1 or p-Akt-1 and either oestrogen receptor (ER) or tumour grade.

3. Results

Patient ($n = 34$) and tumour ($n = 40$) characteristics are listed in Table 1. Overall, 19 of 40 tumour biopsies (48%) demonstrated increased SGK-1 expression in $\geqslant 10\%$ of tumour cells compared to the negligible staining in stromal cells (Fig. 1A). The remaining 21 samples were negative for SGK-1 immunoreactivity. Out of these 40 samples, 23 (58%) expressed p-Akt-1 (Fig. 1B) and the remaining 17 were negative. Stromal staining was uniformly negative in all samples examined. SGK-1 expression was observed to be exclusively cytoplasmic

Table 1
Patient and tumour characteristics

Patient sample[a]	Age	Tumour type	Grade	SGK-1	p-Akt-1	ER
1A	60	IDC	2	+	+	POS
1B	60	IDC	2	+	+	NA
2A	54	IDC	2	+	+	POS
2B	54	IDC	2	+	+	NA
3	56	IDC	2	+	+	POS
4B	40	IDC	3	+	+	NA
5	52	IDC	3	+	+	POS
6	43	IDC	3	+	+	POS
7	38	IDC	2	+	+	POS
8	77	IDC	3	+	+	POS
9A	59	IDC	3	+	+	POS
10	33	IDC	2	+	+	NEG
11	42	IDC	3	+	+	NEG
12	59	IDC	2	+	+	POS
13	41	IDC	2	+	+	POS
14	34	ILC	2	+	+	NEG
15	57	IDC	2	0	0	NEG
16	49	IDC	2	0	0	POS
17	56	IDC	3	0	0	NEG
18	37	IDC	3	0	0	POS
19	36	IDC	3	0	0	NEG
20	52	IDC	3	0	0	POS
21	56	IDC	2	0	0	POS
22	53	IDC	2	0	0	POS
23	53	IDC	3	0	0	POS
24	55	IDC	3	0	0	POS
25	43	IDC	2	0	0	POS
26A	44	IDC	3	0	0	NEG
26B	44	IDC	3	0	0	NA
9B	59	IDC	3	0	0	NA
4A	40	IDC	3	0	+	NEG
27	34	IDC	2	+	0	POS
28	38	IDC	3	+	0	NEG
29	45	IDC	3	0	+	NEG
30	51	IDC	2	0	+	NEG
31	64	IDC	2	0	+	NEG
32	40	IDC	3	0	+	NEG
33	33	IDC	2	+	0	POS
34A	41	IDC	2	0	+	POS
34B	41	IDC	2	0	+	NA

[a] All female; A, original tumour and B, asynchronous tumour; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; Grade, modified Bloom–Richardson grade; ER, oestrogen receptor; "+", $\geqslant$10% positively staining tumour cells; "0", <10% positively staining tumour cells; NA, not available.

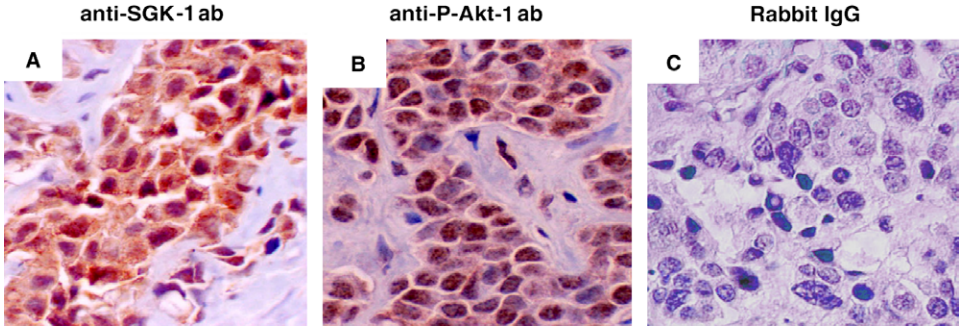

Fig. 1. A. Invasive ductal breast carcinoma demonstrates a predominantly punctate cytoplasmic SGK-1 expression. Stromal immunoreactivity is negligible. B. Anti-p-Akt-1 antibody exhibits predominantly nuclear p-Akt-1 staining in the same tumour. C. Rabbit IgG (negative control) demonstrates no background staining.

in 14 of the 19 positive tumours, while the remaining five SGK-1 positive tumours had mixed nuclear and cytoplasmic staining. In contrast, most p-Akt-1-positive samples exhibited primarily nuclear immunoreactivity (16 of 23); the remaining seven samples showed either cytoplasmic or nuclear/cytoplasmic staining. A negative control with rabbit IgG instead of primary antibody showed no reactivity (Fig. 1C).

Out of the 40 samples, 14 tumours were negative for expression of both SGK-1 and p-Akt-1, while 16 samples co-expressed both SGK-1 and p-Akt-1, suggesting a statistically significant positive association between expression of both proteins ($P = 0.002$, Fisher's exact test) (Table 2). The association was also evident when the same analysis was performed excluding the asynchronous tumours from the six patients who had biopsy available from both primary and metastatic tumours ($P = 0.007$).

We also tested for an association between SGK-1 or p-Akt-1 expression and ER status. ER status was available in all 34 patients' tumour samples. A total of 21 tumours were positive for ER and the remaining 13 were negative. Of the 21 ER-positive tumours, 12 expressed SGK-1 and 11 expressed p-Akt-1. SGK-1 was negative in 11 of the 13 ER-negative tumours and p-Akt-1 was negative in 5 of 13 ER-negative tumours. Thus, neither SGK-1 ($P = 0.09$) nor p-Akt-1 ($P = 0.7$) expression showed a significant correlation with ER status by Fisher's exact test (Table 3).

Similarly, correlation between individual tumour grade and SGK-1 or p-Akt-1 expression was analysed. Out of 34 tumour samples, 18 were modified Bloom–Richardson grade II and 16 were grade III. Of the 16 positive SGK-1 cases, 10 were grade II and 6 were grade III tumours. Out of 19 p-Akt-1 positive tumours, 11 were grade II and the remaining 8 were grade III tumours. Therefore, no significant correlation was found between the grade and either SGK-1 or p-Akt-1 expression (Table 4).

Localisation of SGK-1 and p-Akt-1 revealed predominantly cytoplasmic and punctate staining for SGK-1 and a mostly nuclear pattern for p-Akt-1 (Fig. 1A and B). To determine whether this discordant pattern of SGK-1 and p-Akt-1 localisation was present in a breast cancer cell line, we examined the localisation of both kinases in the Her2/neu overexpressing SK-BR-3 breast cancer cell line. As seen in Fig. 2 (top panel), SGK-1 expression in SK-BR-3 cells was cytoplasmic and punctate, while p-Akt-1 expression was both cytoplasmic and nuclear (Fig. 2, middle panel). Rabbit IgG (negative control) showed minimal immunofluorescence (Fig. 2, bottom panel). Overall, these results suggest that SGK-1 expression is predominantly cytoplasmic, while p-Akt-1 staining is both nuclear and cytoplasmic.

Table 2
Significant association of SGK-1 and p-Akt-1 expression in breast cancer samples[a]

SGK-1 expression	p-Akt-1 expression		Total
	Negative	Positive	
Negative	14	7	21
Positive	3	16	19
Total	17	23	40

[a] Fisher's exact test $P = 0.002$.

Table 3
Lack of association between SGK-1 or p-Akt-1 and oestrogen receptor (ER) expression[a]

	ER expression		Total
	Negative	Positive	
SGK-1 expression			
Negative	9	9	18
Positive	4	12	16
Total	13	21	34
p-Akt-1 expression			
Negative	5	10	15
Positive	8	11	19
Total	13	21	34

[a] Fisher's exact test: SGK-1 association $P = 0.120$; p-Akt-1 association $P = 1.000$.

Table 4
Lack of association between SGK-1 or p-Akt-1 expression and tumour grade[a]

	Tumour grade		Total
	Grade II	Grade III	
SGK-1 expression			
Negative	8	10	18
Positive	10	6	16
Total	18	16	34
p-Akt-1 expression			
Negative	7	8	15
Positive	11	8	19
Total	18	16	34

[a] Fisher's exact test: $P = 0.327$ for SGK-1 and 0.730 for p-Akt-1.

4. Discussion

A balance of cell survival and programmed cell death plays an important role in mammary development as well as in the initiation and progression of breast cancer. The PI3-K/Akt-1 pathway is activated frequently in human cancer, and has been implicated in both cell survival and proliferation [10]. Akt-1 is a downstream target of epidermal growth factor and insulin family receptor signalling, two types of receptor-stimulated pathways that are commonly activated in breast cancer.

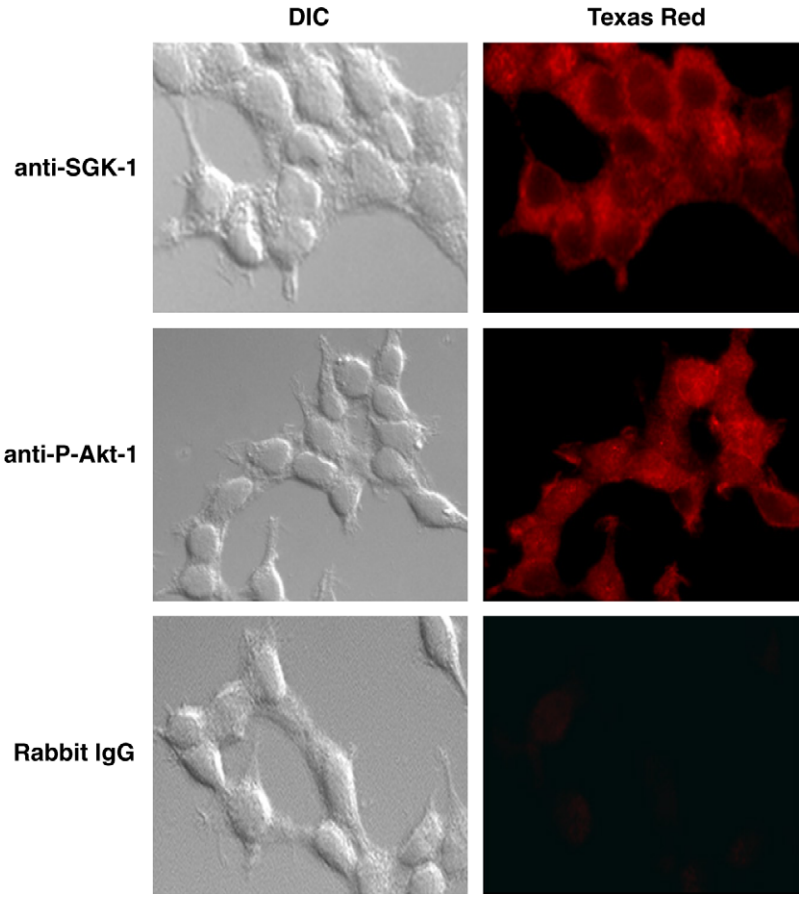

Fig. 2. Indirect immunofluorescence of SGK-1 (top panel) in SK-BR-3 cells reveals a predominantly cytoplasmic localisation. P-Akt-1 localisation (middle panel) is both nuclear and cytoplasmic. Normal rabbit serum exhibits minimal fluorescence (bottom panel).

In addition, Akt-1 activity is often deregulated in a variety of human tumours because of frequent inactivation of the *PTEN* tumour suppressor gene, which negatively regulates PI3-K [11,12]. Similar to Akt-1, SGK-1 also appears to play a role in cell survival downstream of PI3-K signalling and shares at least two important substrates with Akt-1: FKHRL-1 and glycogen synthase kinase-3 (GSK-3) [8,9]. Reflecting its importance, the PI3K-SGK-1/Akt-1 pathway appears to be highly conserved across species, as evidenced by a recent report demonstrating that SGK-1 and Akt-1 are both required for insulin signalling in *Caenorhabditis elegans* [13]. In mammary epithelial cells, SGK-1 expression is associated with resistance to apoptosis following both growth factor withdrawal [5] and chemotherapy [14].

Despite the genetic evidence that SGK-1 and Akt-1 both play central roles in the PI3-K pathway [13], co-expression of SGK-1 and Akt-1 has not been previously examined in primary human cancer samples. While this paper was in preparation, SGK-1 was shown to be over-expressed in 29 of 38 breast cancer samples in the TARP-2 tissue microarray obtained from the United States National Cancer Institute [15]. Here, we find approximately 48% of tumour samples overexpress SGK-1; there also a statistically significant association between SGK-1 and p-Akt-1 co-expression in human breast cancers. This positive association suggests that these two proteins have complementary, rather than redundant, functions. Interestingly, SGK-1 and p-Akt-1 do not appear to co-localise within individual tumour cells; SGK-1 expression is primarily cytoplasmic, while p-Akt-1 expression is predominantly nuclear. Immunofluorescent localisation of endogenous SGK-1 and p-Akt-1 using the same antibodies also suggests distinct localisation patterns in the SK-BR-3 and MDA-MB-231 (data not shown) breast cancer cell lines. Taken together, these data suggest that while SGK-1 and Akt-1 may play complementary roles in PI3-K signalling, they do not necessarily target their substrates to the same location. One possibility, for example, is that Akt-1 primarily phosphorylates FKHRL-1 in the nucleus, while SGK-1 interacts with this substrate in the cytoplasm, preventing re-entry into the nucleus through a complementary phosphorylation event [8].

In summary, we have shown for the first time that SGK-1 and activated p-Akt-1 are co-expressed in approximately 40% of human breast cancers. The coordinate expression of SGK-1 and p-Akt-1 suggests that

these two kinases may play complementary roles downstream of PI3-K signalling. Consequently, attempts to target the PI3-K pathway most effectively may need to block both SGK-1 and Akt-1 activity.

Conflict of interest statement

The authors of this paper have no financial or personal relationships that could bias this work.

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