The Clinical Implications of RSK1-3 in Human Breast Cancer

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Abstract. *Background/Aim: The ribosomal S6 protein kinase (RSK) family is an important effector of extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) that could influence tumour metastasis by phosphorylating proteins in both the nuclear and cytoplasmic compartments. Aberrant expression of RSK is evident in certain malignancies but the role played by RSK in breast cancer is still not clear. This study aimed to examine the expression of RSK in human breast cancer specimens and its role to breast cancer metastasis. Materials and Methods: The expression of RSK1 to -3 were separately examined in human breast cancer tissues (normal, n=33; cancer, n=112) using quantitative real-time polymerase chain reaction (Q-PCR) and immunohistochemistry. Migration and adhesion of breast cancer cells treated with the RSK inhibitor SL0101 were investigated by electric cell impedance sensing (ECIS). The effect on growth and invasion of RSK1-3 was then investigated using in vitro models. Results: The clinical data and immunohistochemistry revealed that expression of RSK1 and RSK3 were less in tumour tissues than normal. mRNA expression of RSK2 was negatively correlated with grade, TNM staging, and survival rate. SL0101 inhibited adhesion of the MCF-7 and MDA-231 breast cancer cell lines. SL0101 suppressed MDA-231 invasion and the alternate RSK inhibitor BRD7389 inhibited the invasion of MCF-7 and MDA-231 cells. Conclusion: RSK1 and 3 but not RSK2 are down-regulated in breast tumour and are associated with disease progression. RSK may be a key component in the progression and metastasis of breast cancer.*

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Breast cancer is the most common carcinoma in women, with low survival rates in patients due to metastasis. Bone metastasis is the most common complication in breast cancer (1). Understanding the mechanisms of metastatic disease has an important significance in formulating treatment and prevention strategies.

Ribosomal S6 protein kinase (RSK) comprises a family of serine/threonine protein kinases activated downstream of the mitogen-activated protein kinase (MAPK) pathway. There are four main members of the RSK family, named: RSK1, RSK2, RSK3 and RSK4. The RSK family share 75-80% sequence homology and have a high degree of conserved functional motifs. The RSK isoforms are characterized by two distinct and functional kinase domains that are activated by a series of phosphorylation events in a sequential way. The mRNAs coding for RSK1-3 are expressed across all cell line and tissues, but the expression of RSK4 is regulated to a greater extent (2-5).

RSK has multiple functions that are involved in cancer cell proliferation, invasion and migration. Although some RSK isoforms promote cell invasion and motility by altering integrin and transcription activity, others reduce cell motility and invasion through affecting the actin cytoskeleton. The RSK action mechanism is on the basis of different isoforms and cancer types (4). Some chemical inhibitors of RSK have proven effective in blocking invasion and metastasis of several solid tumour types in pre-clinical models (6). RSKs play a key role in tumour metastasis. A previous study showed that RSK2 facilitated the metastasis of head and neck squamous cell carcinoma (HNSCC). Importantly, the effect was only mediated through RSK2, whereas RSK1 did not influence HNSCC metastasis (7). Similarly, RSK1 was

mentioned acting as a negative regulator in non-small cell lung cancer metastasis (8). Recently, some research about RSK in breast appeared (9). For example, depending on protein/RNA interference factor that control the migration of immortalized breast epithelial cells (MCF-10A), RSK1 was alternatively identified to be pro-migratory (10). Another study showed treatment with several RSK inhibitors (including FMK and SL0101) impaired cell line migration in an *in vitro* breast epithelial model, whereas RSK4 expression was not detected in breast epithelial cells (11). However, a report from Thakur and colleagues found that RSK4 inhibited both metastasis and proliferation of MDA-MB-231 breast cancer cells (12), further highlighting the potential effect of other isoforms. Moreover, some studies have shown the effect of RSK inhibitors on tumour metastasis, such as FMK and SL0101 (13-16). These findings demonstrate that RSK may be a potential therapeutic target against tumour progression. In this study, we detected the expression of RSK1-3 in breast cancer tissues and determined the effect of RSK on breast cancer cells using RSK inhibitors.

Materials and Methods

Human breast specimens. Breast tissue samples were collected from patients with breast cancer after surgery and snap-frozen in liquid nitrogen until further use. The samples included breast tumour tissues (n=112) and normal adjucent in paired tissues (n=33) obtained from the same patients. The size of these specimens was verified by a pathologist and the normal tissues were free from tumour deposits. All protocols were approved by the local Ethics Committee. Full details of patient clinical data are shown in Table I.

Table I. *Breast cancer patient clinical data details*.

Clinical data	No. of samples
Tissue sample	
Normal	33
Tumour	112
NPI	
1	58
2	34
3	15
Tumour grade	
1	19
2	36
3	55
TNM staging	
I	62
II	34
III	7
IV	4
Survival status	
1	81
2	6
3	4
4	16

NPI: Nottingham prognostic indicator.

Cell culture. Breast cancer cell lines MCF-7 and MDA-MB-231 were acquired from the European Collection of Animal Cell Cultures (ECACC; Salisbury, UK). These two wild-type cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 with L-Glutamine (Sigma-Aldrich, Poole, Dorset, UK) supplemented with 10% fetal calf serum (FCS; PAA Laboratories, Somerset, UK), penicillin and streptomycin (Sigma-Aldrich), in an incubator at 37.0˚C, 95% humidity and 5% CO_2.

Immunohistochemical staining of RSK1-3 in breast specimens. Frozen sections of breast tumours and normal tissues were sectioned at a thickness of 6 μm using a cryostat [Leica Microsystems (UK) Ltd., Bucks, UK] and placed in phosphate-buffered saline for 5 min. Briefly, immunohistochemistry was performed as described previously (17). Primary antibodies were diluted to 1:100 and secondary antibody to 1:100 in buffer. Diaminobenzidine chromogen (Vector Labs, Peterborough, Cambridgeshire, UK) was then added to the sections, that were incubated in the dark for 5 min. Independent observers carried out counting of microvessels as we reported previously (18).

Real-time reverse transcription quantitative polymerase chain reaction (QPCR). Real-time quantitative PCR was based on the Amplifluor™ technology and primers were designed by Beacon Designer software and consisted of complementary sequence to universal Z probe (Intergen Inc., Oxford, UK). Primer sequences are shown in Table II. Q-PCR was used to quantify the level of mRNA expression of *RSK1, RSK2* and *RSK3* from the cDNA samples of breast tissues. Real-time PCR was carried out using an IcyclerIQ™ (Bio-Rad, Hemel Hempstead, UK) with the following cycling conditions: 94˚C for 12 min, 60 cycles of 94˚C for 15 s, 55˚C for 40 s (data-capture step) and 72˚C for 20 s.

Electric cell-substrate impedance sensing (ECIS) based analyses on cell adhesion and cell migration. An ECIS Ztheta instrument (Applied Biophysics Ltd, Troy, NJ, USA) was used to evaluate cell migration as previously described by Jiang *et al*. (19). Special 96-well W96E1 microarrays were needed for ECIS. DMEM (100 μl) with different concentration of RSK inhibitors (SL0101: 90 nM (R&D Systems, Abingdon, Oxfordshire, UK) and BRD7389: 2 μM (R&D Systems, Abingdon, Oxfordshire, UK)) was placed in a 96-well W96E1 microarrays, then MCF-7and MDA-231 were seeded into the wells of array at approximately 10^5 cells and placed in the ECIS array holder.

In vitro cell-invasion assay. Transwell inserts with an 8 μm pore size were coated with 50 μg Matrigel/100μl (BD Matrigel™ Basement Membrane Matrix (BD Biosciences, Oxford, UK)) and air-dried. Following rehydration and preparation of medium with different concentration of inhibitors, 30,000 cells/200 μl per well were added to the top wells with 0.5% fetal calf serum and 1 ml with 10% fetal calf serum medium was placed in the bottom wells. After 48 h, the cells that migrated through the matrix and pores were fixed with 4% formalin, stained in crystal violet counted.

Statistical analysis. Statistical analysis was performed using the Minitab statistical software package (version 14, MINITAB, Coventry, West Midlands, UK). Data with a non-parametric

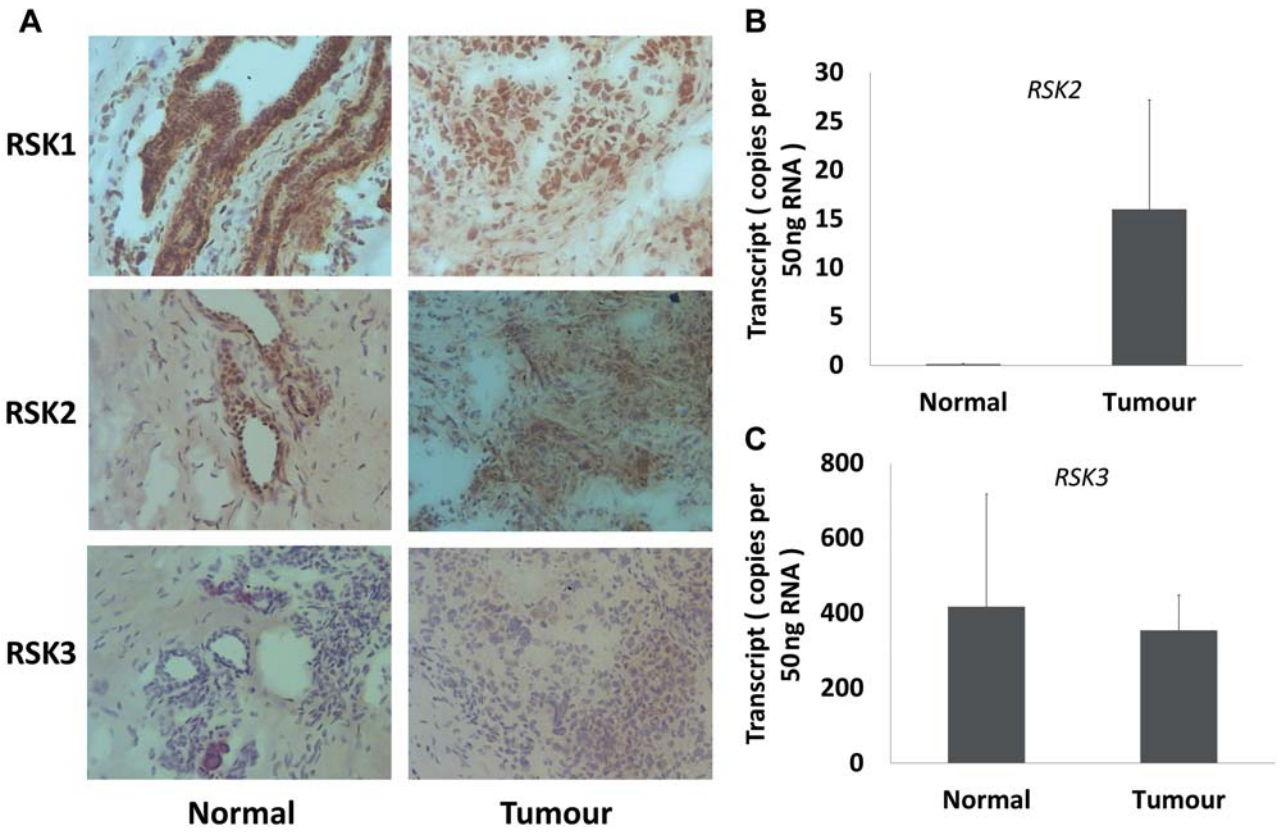

Figure 1. *Expression of RSK1-3 in breast cancer tissues. A: Immunohistochemical staining of RSK1-3 in normal breast tissue (left panel) and tumour tissue (right panel). B: RSK-2 transcripts level was increased in human breast cancer. C: The expression of RSK-3 is higher in normal adjacent tissues.*

Table II. *Primer sequences used in study.*

Gene	Sense primers (5'-3')	Antisense primers (5'-3')
RSK1	GTGGGAAGGTTTACCCTCAGT	ACTGAACCTGACCGTACAGATCATAGCCTTGTCTTTT
RSK2	CGGAAAATGGTCTTCTCAT	ACTGAACCTGACCGTACATAATGATGCAAGACAGTTCC
RSK3	GTGGGCTCCTACTCAGTGT	ACTGAACCTGACCGTACAGGGGTCTCTCTTGCTCTTAC
GAPDH	CTGAGTACGTCGTGGAGTC	ACTGAACCTGACCGTACAGAGATGATGACCCTTTTG

distribution were assessed with the Mann–Whitney test, whilst a two sample *t*-test was used for normally distributed data. Differences were considered to be statistically significant at $p<0.05$.

Results

The expression of RSKs in breast cancer. The expression of *RSK1, RSK2* and *RSK3* was examined in breast cancer tissues and adjacent normal tissues using real-time PCR. Analysing our results according to clinical data, we found that expression of *RSK1* and *RSK3* was lower level in tumours than in normal tissues, but that was opposite for *RSK2*. Immunochemical staining of RSKs was examined in human breast tissue sections. RSK1 and RSK3 were expressed at a higher level in breast tumour tissues (Figure 1) than in the normal breast specimens, and this was consistent with the *RSK1* and *RSK3* RNA transcript expression. However, increased levels of RSK2 were found at a higher level in cancer than in normal tissues, that was also consistent with the *RSK2* RNA transcript expression. These results together suggest that RSK1 and RSK3 were expressed at a lower level in breast tumour, while RSK2 was highly expressed.

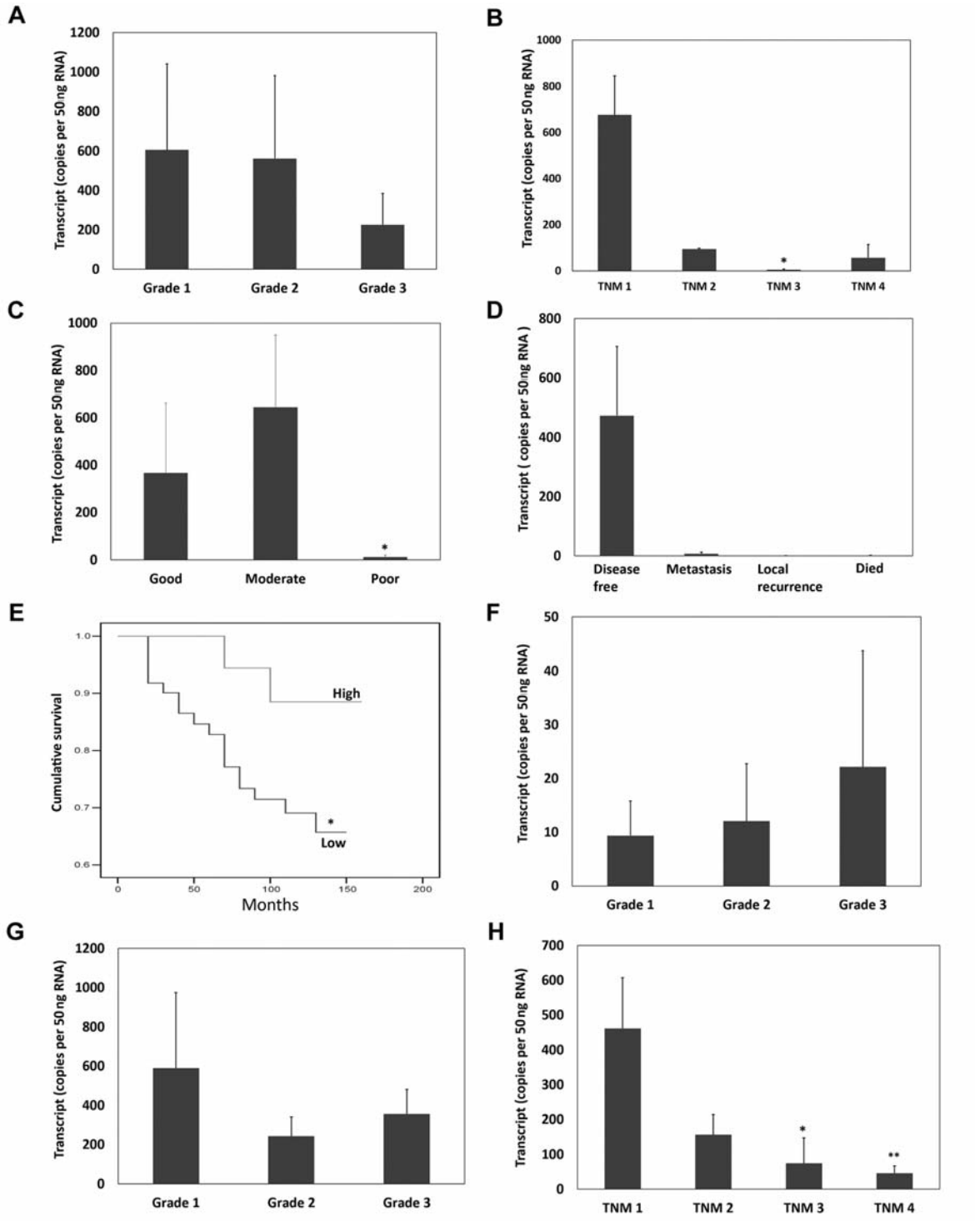

Figure 2. *Correlation of RSK1-3 expression with Nottingham Prognostic Index (NPI), grade, TNM staging and prognosis. Top panel (RSK-1) A: Levels of expression of RSK-1 in breast tumour tissues in connection with grade and B with TNM status (*p<0.05 vs. TNM1 tumour). C: The predicted prognosis was dependent on the NPI value of patient and that good, moderate and poor prognosis had NPI value either <3.4, 3.4–5.4 or >5.4. (*p=0.046 vs. moderate prognostic group). D: Levels of RSK-1 transcript and clinical outcomes. RSK-1 expression progressively decreased in the patients with local recurrence who died of breast cancer, compared to disease-free patients. E: Higher level of RSK-1 expression in primary breast tumours had a positive correlation with longer disease free survival. (*p<0.05; 0.6 represent low level of RSK-1, 1.00 represent high level of RSK-1). Middle panel (RSK-2) F: RSK-2 expression level in breast cancer tissues was highest in grade 3. Bottom panel (RSK-3) G: Levels of expression of RSK-3 in breast tumour tissues in connection with grade status and with TNM status (*p<0.05, vs. ** p<0.01 TNM1 tumour).*

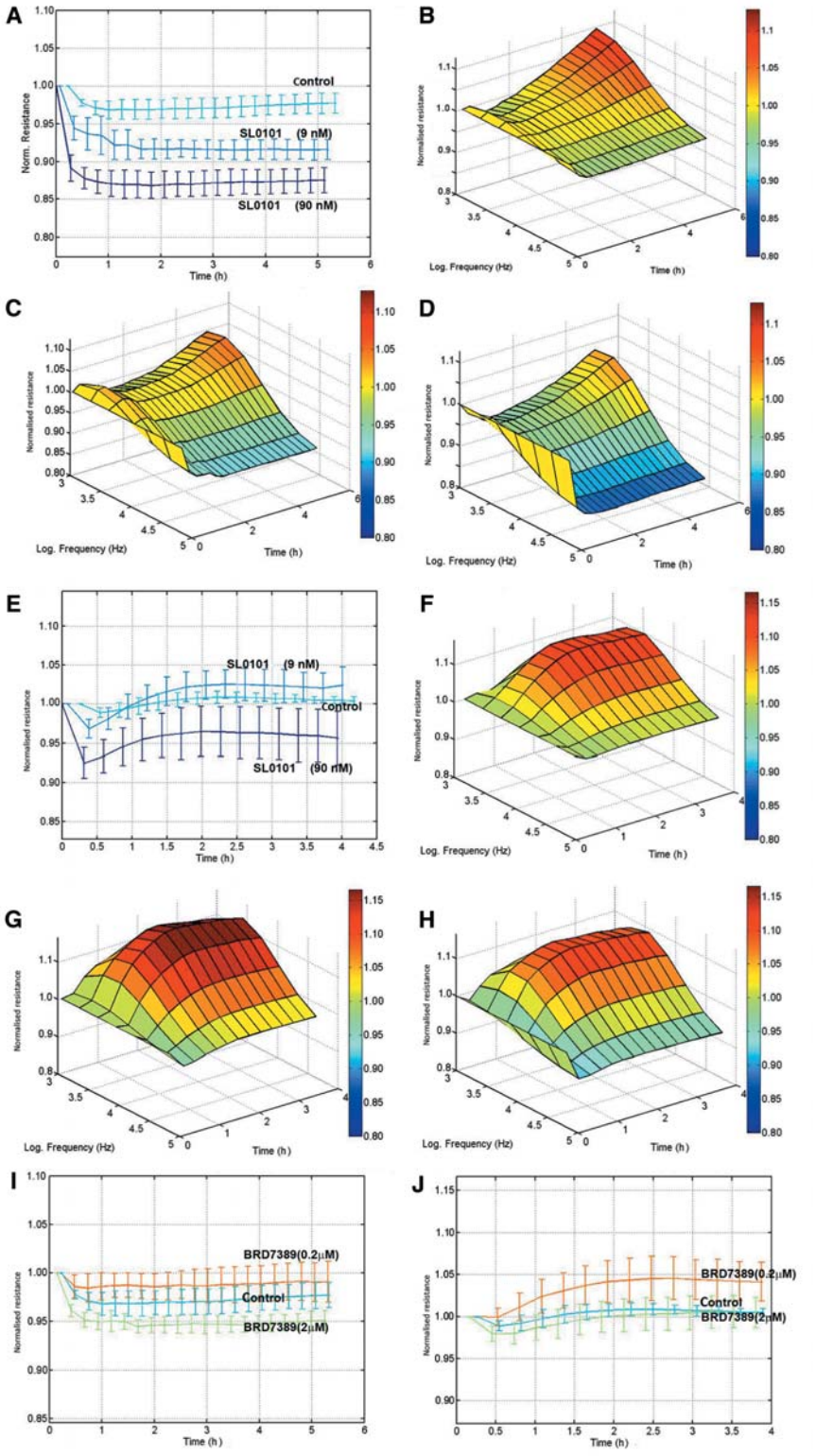

Figure 3. *Effects of RSK inhibitors on the adhesion of breast cancer cells. Top panel: The response of MCF-7 to SL0101-1. A: Traces of MCF-7 cell's response to SL0101-1 at different concentration. B: 3D image of MCF-7 (control); C and D: Response of MCF-7 to SL0101-1 at 9 nM and 90 nM, respectively. Middle panel: The response of MDA MB-231 to SL0101-1. E: Traces of MDA MB-231 cell's response to SL0101-1 at different concentration. F: 3D image of MDA MB-231 (control); G and H: Response of MCF-7 to SL0101-1 at 9 nM and 90 nM, respectively. Bottom panel: The response of MCF-7 (I) and MDA MB-231 (J) to BRD7389 at the concentrations indicated.*

Correlation of RSK expression with Nottingham Prognostic Index (NPI), tumour grade, TNM staging and prognosis. To evaluate the relation of RSK1 expression with disease progression, we analyzed the level of *RSK1* transcript in connection with NPI and TNM staging of breast tumours. We found RSK1 expression decreased from grade 1 to grade 3 tumours, while tumours did not significantly differ in expression (grade 1 *versus* 2, p=0.94; grade 1 *versus* 3, p=0.43; Figure 2A). *RSK1* expression level was decreased in advanced breast cancer, particularly in TNM3. However, a significant difference was only seen in TNM3 tumours when compared to TNM1 tumours (p=0.0047) (Figure 2B).

Based on the NPI values, patients were divided into three groups, those with a good, those with moderate and those with poor prognosis. The lowest level of *RSK1* transcript was found in those with a poor prognostic index compared to those with good and moderate prognosis (p=0.046) (Figure 2C). Regarding the clinical outcomes at the final follow-up, patients were divided into those remaining disease-free, with metastasis, with local recurrence, and those who died of breast tumour after a median of 120 months follow-up. Decreased *RSK1* transcript level was found in patients who developed tumour metastasis and local recurrence, and who died of breast cancer, compared to patients who remained disease-free, but did not reach a statistically significant difference (Figure 2D) (p=0.069, p=0.065 and p=0.065, respectively).

The Kaplan–Meier survival method was used to analyze the disease-free survival status of patients with breast cancer. It demonstrated that patients with tumours with high *RSK1* expression had a longer overall survival compared to those with low *RSK1* expression and this reached statistical significance (p=0.0172) (Figure 2E).

We also analyzed the expression levels of *RSK2* and *RSK3* transcript in connection with the grade of breast cancer. *RSK2* expression had a tendency to increase from grade 1 to grade 3 (p=0.83 and p=0.85, respectively) (Figure 2F). However, the highest level of *RSK3* transcript was found in grade 1 compared to grade 2 and 3 tumours, but not with statistical significance (Figure 2G). Furthermore, we analyzed the relationship between *RSK3* and TNM status. Figure 2H clearly shows the expression of RSK3 decreasing stepwise from TNM1 to TNM4 stage of breast tumour and a significant difference was seen in TNM3 and TNM4 stage when compared to TNM1 stage.

ECIS-based cell adhesion assay. We employed ECIS in tracking cell migration and adhesion. The adherence of MCF-7 treated with RSK2 inhibitor SL0101 decreased compared to the control group (no treatment) (Figure 3A). In 3D imaging, it was confirmed (Figure 3B and D). A similar effect was also found in MDA-MB-231 cells in the comparison between the cells treated with RSK2 inhibitor and control group (no treatment), but only when treated with reaction concentration (Figure 3).

Another inhibitor BRD 7389 was used to treat MCF-7 and MDA-MB-231. It showed that the adhesion of breast cancer cells was promoted when treated with low concentration of BRD 7389 (Figure 3I, J).

RSK inhibitors suppress cell invasion in vitro. In searching for the effect of RSK1-3 on the invasion of breast cancer cells, we screened a panel of small-molecule inhibitors to treat the cells. They included RSK2 inhibitor SL0101 and RSK1-3 inhibitor BRD7389. SL0101 only inhibited the invasion of MDA-MB-231, not MCF-7 cells, as shown in Figure 4A and B. BRD7389 suppressed both MCF-7 and MDA-MB-231 cell invasion at concentrations of 0.8 μM and more (Figure 4C and D).

Discussion

In the current study, we demonstrated through q-PCR and immunohistochemical analysis that expression of RSK1 and RSK3 is low in breast cancer compared to normal tissues, but that of RSK2 is not. The expression of RSK was found to be correlated with TNM staging, tumour stage and prognosis of breast cancer.

The link between the level of RSK expression and the clinical outcome is an important observation. An inverse correlation was found between the level of the RSK1 and RSK3 transcripts and tumour grade, TNM and staging. Patients with a low level of RSK1 expression had a poor prognosis and shorter disease-free and overall survival. However, *RSK2* appears to play the opposite role in breast tumours. Based on the clinical data, RSK may act as a potential prognostic indicator in patients with breast cancer. Furthermore, *RSK1* and *RSK3* may act as important protective factors in patients with breast cancer.

RSK2 has been reported as being overexpressed in breast cancer, while RSK3 has been suggested as a tumour suppressor in ovarian cancer (20-21). One study showed that the RSK inhibitor Ac-SL0101 inhibited the growth of the MCF-7 human breast cancer cell line, but did not affect the proliferation of MCF-10A cells, a normal human breast cell line (22). Smith *et al*. also reported that SL0101 inhibited the proliferation of a human breast cancer cell line MCF-7 through blocking the G_1 phase of the cell cycle, but similarly did not alter the proliferation of a normal human breast cell line MCF-10A (6). Recently, Serra *et al*. showed that RSK3/4 mediated resistance to phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) inhibitors in breast tumour cells both *in vivo* and *in vitro* (23). In our study, we detected the effect of RSK on adhesion and invasion in breast cancer cells through RSK

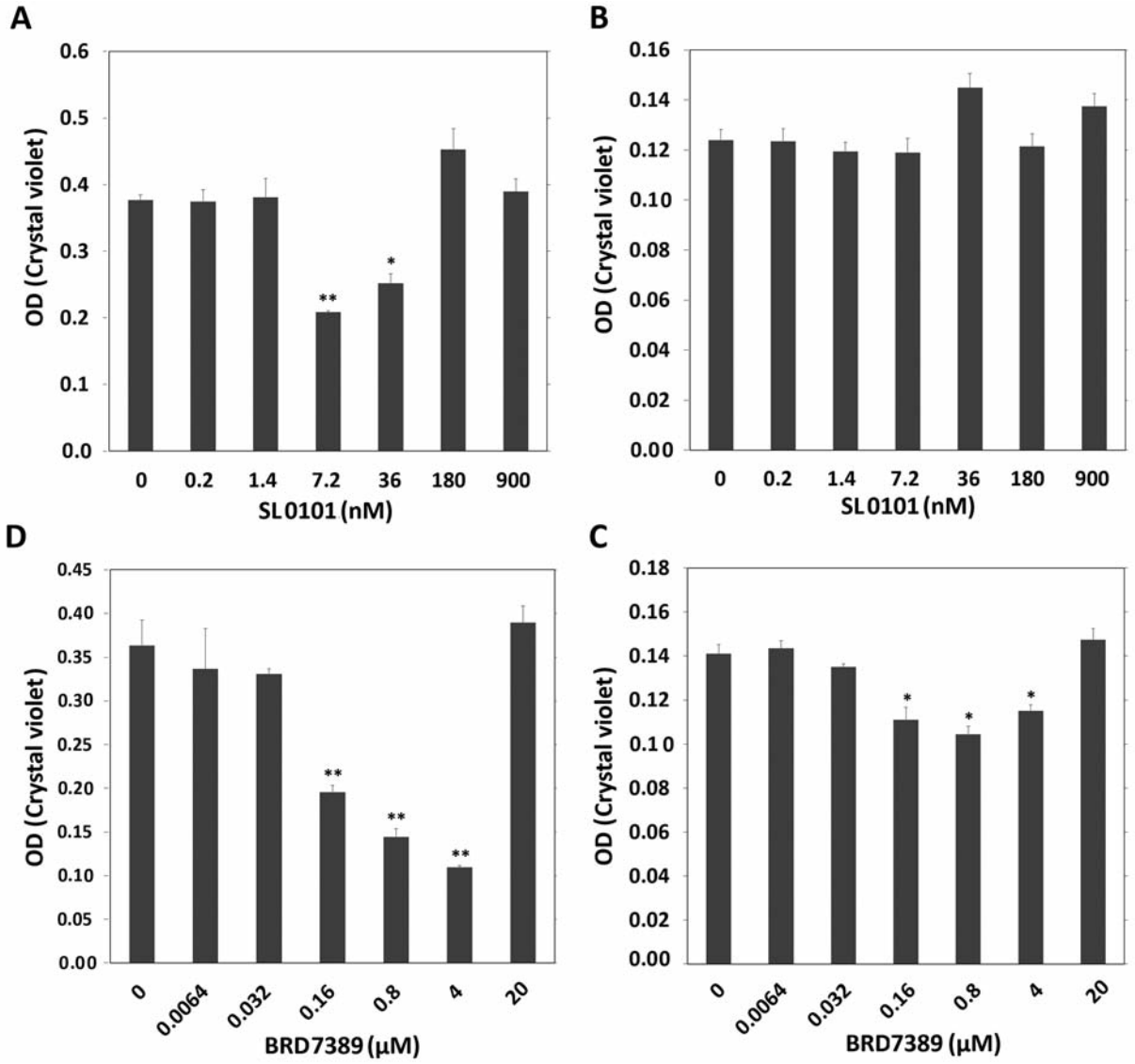

Figure 4. *The effect of RSK1-3 on invasion of breast cancer cells in vitro treated with inhibitor. A: SL0101 inhibited the invasion of MDA-231 but not MCF-7 (B) C: BRD 7389 decreased the invasion of both MDA-231 (C) and MCF-7(D) (*p<0.05, **p<0.01 vs. control without inhibitor).*

inhibitors We found that the adhesion and invasion of MCF-7 and MDA-231 decreased when treated with SL-0101 and BRD7389 at high concentrations, however, a low concentration did not have any influence. Interestingly, the *in vitro* results are consistent with clinical outcome of human breast cancer.

In conclusion, we characterized a pattern of low expression of *RSK1* and *RSK3* in breast cancer that was correlated with metastasis of breast cancer. The results of treatment with RSK inhibitors lead us to predict that RSK1 and RSK3 can inhibit tumour metastasis by reducing growth and invasion. RSK provides a new research direction for the diagnosis and treatment of relevant diseases.

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