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ORIGINAL ARTICLE

Characterization of kinase suppressor of Ras-1 expression and anticancer drug sensitivity in human cancer cell lines

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Abstract Previous studies have indicated that the ERK1/2 MAP kinase signaling pathway plays an important role not only in cell growth, cell cycle regulation, and differentiation, but also in determining the sensitivity of cells to anticancer agents as well. Furthermore, expression of kinase suppressor of Ras-1 (KSR1), a molecular scaffold that modulates signaling through the ERK1/2 MAP kinase pathway, has been shown to influence the cellular sensitivity to the anticancer agent cisplatin. To further define the role of KSR1 expression on drug sensitivity, the expression of KSR1 was examined in the NCI60 anticancer drug screen, a panel of cancer cell lines representing nine tissue types, established by the Developmental Therapeutics Program (DTP) at the National Cancer Institute (NCI). The expression of thousands of molecular targets has been examined in the NCI60 panel as well as the cellular toxicity for greater than 400,000 compounds. KSR1 expression varied almost 30-fold difference between the highest and lowest expressing cell lines in the NCI60. Using the COMPARE analysis algorithm, KSR1 expression was correlated with sensitivity of the compounds screened by DTP and several novel agents were identified whose sensitivity correlated with KSR1 expression in the NCI60 panel. Cytotoxicity of two agents, cytochalasin H and tunicamycin, identified through the COMPARE analysis of KSR1 expression and drug sensitivity, was also examined in wild type ($KSR^{+/+}$) mouse embryo fibroblasts (MEFs) and MEFs deficient in KSR1 expression ($KSR1^{-/-}$). These studies demonstrated enhanced sensitivity, as well as increased ERK activation, in $KSR^{-/-}$ MEFs following exposure to tunicamycin or cytochalasin H compared to $KSR^{+/+}$ MEFs. Furthermore, restoration of KSR1 expression in $KSR^{-/-}$ MEFs following stable transduction of cells with a KSR1 expression vector, enhanced sensitivity of cells to tunicamycin and cytochalasin H and decreased ERK1/2 activation following exposure to these drugs. In addition, the sensitivity to cytochalasin H and tunicamycin of breast cancer cell lines with low KSR1 expression, (HS578T and MDA-MB-231/ATCC), was increased relative to the sensitivity of breast cancer cells with higher levels of KSR1 (MCF7). These studies indicate that KSR1 may play an important role in the determination of cellular sensitivity to anticancer agents.

Keywords KSR1 · NCI anticancer drug screen · Drug resistance · DNA damage · Signal transduction

Introduction

While chemotherapy plays a critical role in the treatment of cancer, the development of drug resistance ultimately limits the successful treatment of many patients. Previous studies have identified multiple mechanisms associated with the development of resistance to anticancer agents including decreased drug accumulation, decreased drug activation, enhanced cellular repair, changes in target expression, and altered downstream effectors (reviewed in [37]). Alterations in the intracellular signal transduction pathways, including the ERK1/2 mitogen activated protein (MAP) kinase pathway, have also been observed in the development of drug resistance [37]. For example, previous studies have shown that alterations in the ERK1/2 MAP kinase signaling

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cascade including the growth factor receptors, Ras, Raf and ERK1/2, have been found to be associated with the development of resistance to chemotherapy and radiation ([11, 14, 29, 45, 50, 56, 60]).

Previous studies have also demonstrated that KSR1, initially discovered through genetic screens of *Drosophila melanogaster* and *Caenorhabditis elegans*, can function as a scaffold that modulates signaling through the ERK1/2 MAP kinase signaling cascade [25, 51, 54]. KSR1 has been shown to interact with Raf, MEK, and ERK, as well as several other regulatory proteins including BRAP2 [32], C-TAK1 [39], and 14-3-3 [6, 62]. KSR1 has been shown to play a role in cell proliferation [28, 46], differentiation [57–59], oncogenesis [28], adipogenesis [26], and senescence [27]. In addition, down-regulation of KSR1 expression through targeting with antisense oligonucleotides has been shown to inhibit the growth of pancreatic cancer cells [63].

We have previously examined the effects of KSR1 expression on the sensitivity of cells to the DNA cross-linking agent cisplatin [22]. These studies demonstrated that KSR1$^{+/+}$ MEFs were approximately 4-fold more sensitive to cisplatin treatment compared to MEFs lacking KSR1 expression (KSR1$^{-/-}$ MEFs). Furthermore, this increase in cisplatin sensitivity in KSR1$^{+/+}$ MEFs was also associated with increased ERK1/2 activation following cisplatin treatment compared to KSR1$^{-/-}$ MEFs. Both cisplatin-induced activation of ERK1/2 and the increased sensitivity of the cells to cisplatin were abrogated by preincubating MEFs with inhibitors of MEK1/2 kinase. Furthermore, stable transduction of KSR1 in KSR$^{-/-}$ MEFs with a KSR1 expression vector resulted in increased cisplatin-induced ERK1/2 activation and enhanced cisplatin-induced apoptosis. Other studies demonstrated that increased expression of KSR1 in MCF7 human breast cancer cells also resulted in increased cisplatin-induced ERK1/2 activation and apoptosis compared to parental MCF7 cells [22]. Taken together, these studies indicated that KSR1 expression is an important determinant of cellular sensitivity to cisplatin, through modulation of cisplatin-induced ERK1/2 activation.

In order to further explore the possible relationship between KSR1 and drug sensitivity, KSR1 expression was examined in the NCI60 anticancer drug screen maintained by the Developmental Therapeutics Program of the National Cancer Institute. This panel consists of human tumor cell lines isolated from nine tissue types, including breast, ovarian, colon, CNS, renal, lung, skin, leukemia, and prostate. In studies described in this report, the relationship between KSR1 expression and the sensitivity of cells to the various compounds was examined in the NCI60.

Materials and methods

Cell lines and reagents

MCF-7, HS578T, and MDA-MB-231/ATCC cells were obtained from Dr. Dominic Scudiero of the Developmental Therapeutics Program at the National Cancer Institute (DTP, NCI). A2780 cells were obtained from Dr. Thomas Hamilton at the Fox Chase Cancer Center. These cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal calf serum and 100 μg/mL gentamicin at 37°C with 5% carbon dioxide. KSR$^{-/-}$ and KSR$^{+/+}$ mouse embryonic fibroblasts (MEFs), as well as KSR1$^{-/-}$ cells infected with retrovirus carrying a bicistronic vector expressing KSR1 and GFP (KSR1/GFP), were obtained from Dr. Robert Lewis at the University of Nebraska Medical Center, which were prepared as described previously [28, 41]. MEFs were maintained in DMEM containing 10% fetal calf serum, 100 μg/mL gentamicin, and 0.1 mM nonessential amino acids. Compounds from the NCI screen were obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, NCI, courtesy of Dr. Robert Schultz. These compounds were dissolved in DMSO and stored at −80°C.

Western blotting

Frozen cell pellets of the NCI60 panel of cell lines were obtained from the NCI and stored at −80°C until analysis. Cells were lysed in EBC buffer (40 mM Tris–HCl, pH 8.0, 120 mM NaCl, and 0.5% NP-40) and protein concentration determined using a protein assay solution (Bio-Rad Laboratories, Hercules, CA, USA). Cytosols containing 25 μg of protein per sample were separated by SDS-polyacrylamide gel electrophoresis on 10% gels, and then transferred to nitrocellulose membranes (Bio-Rad Laboratories). Primary antibodies directed against KSR1 (C-19) and actin (I-19) were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Donkey anti-goat secondary antibodies conjugated to Alexa Fluor 680 or Alexa Fluor 800, were obtained from Molecular Probes (Invitrogen, Carlsbad, CA, USA). Western blots were analyzed using a Li-Cor Odyssey infrared imaging system (Li-Cor Biotechnology, Lincoln, NE, USA). Expression data from the western blot analysis of KSR1 expression in the NCI60 anticancer drug screen were analyzed using the COMPARE algorithm by Dr. Susan Holbeck (DTP, NCI).

For ERK1/2 activation studies, cells were plated on 60 mm tissue culture plates and incubated at 37°C for 24 h. For experiments using the MEK inhibitor U0126 (CALBIOCHEM, San Diego, CA, USA), cells were preincubated with 50 μM U0126 for 2 h prior to treatment and

then treated with the corresponding agents as indicated. Following treatment, cells were collected by scraping with a rubber policeman in lysis buffer containing 50 mM Tris–HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 1 mM DTT, 1% Triton X-100, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 10 mM sodium 2-gycerolphosphate, 0.1 mM PMSF, and 1× protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO, USA). Fifty micrograms of protein were separated by SDS-PAGE using 4–20% acrylamide gels (Invitrogen) and then transferred to nitrocellulose membranes (Bio-Rad). KSR1 expression in MEFs was detected using mouse anti-KSR1 antibody (BD Biosciences Pharmingen, San Jose, CA, USA). Antibodies for pERK1/2 (I-4), ERK2 (C-14-G), Grp78 (H-129), and donkey anti-goat secondary (I-19) were obtained from Santa Cruz Biotechnology Inc., while donkey anti-mouse secondary antibody was obtained from Jackson Laboratories (Bar Harbor, ME, USA). Western blots were visualized on autoradiography film (Molecular Technologies, St Louis, MO, USA) using ECL solution (Pierce Biotechnology, Rockford, IL, USA).

NCI60 assays and COMPARE analysis

The analysis of drug sensitivity using the NCI60 anticancer drug panel using the COMPARE algorithm have been described previously [35, 43, 47]. Briefly, based on the relative growth rate of each cell line in the panel, approximately 5,000–40,000 cells in 100 μL of medium were plated in 96-well microtiter plates and incubated overnight at 37°C in an atmosphere containing 5% carbon dioxide in air. Aliquots of the compound to be tested beginning at a maximum dose of 10^{-4} M and the cells and then serial dilutions of each agent spanning over a 5 log range were added to corresponding wells. The cells were then incubated for an additional 48 h at 37°C and cytotoxicity assessed by using sulforhodamine B assay and an automated microtiter plate reader [35].

The COMPARE algorithm analyzes the correlation between expression of a specific target in each cell line with the growth inhibitory effect of each compound in the database. The correlations are then assessed and ranked by COMPARE using the Pearson correlation coefficient. A positive Pearson correlation coefficient predicts that higher expression of a molecular target (in this case, KSR1) in each cell line is correlated with increased sensitivity to the given agent, while a negative value predicts that higher expression results in increased resistance to the specific agent. These analyses were also used to evaluate the relationship between KSR1 expression and the expression of other molecular targets that have already been examined in the NCI60 panel.

Cytotoxicity assays

Cell viability was determined by 3-(4-5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously [12]. Cells (2,000/well) were seeded in 96-well tissue culture plates and incubated at 37°C overnight. Following exposure to each drug at increasing concentrations for 5 days, 100 μg of MTT in PBS was added to each well and the plates incubated at 37°C for 4 h. Plates were centrifuged at 500×*g* for 5 min and the medium was removed by inverting the plate and shaking twice. DMSO (120 μL) was then added to each well and following incubation at room temperature for 30 min with gentle rocking, the absorbance at 570 nm was determined using an ELx 807 Ultra Microplate Reader utilizing KCjunior software (Bio-Tek Instruments, Winooski, VT, USA). Relative survival represents the results obtained for treated cells divided by that obtained for untreated cells and the data expressed as percent relative survival.

For short-term MTT assays, 3,000 per well were plated and treated as described above. For experiments utilizing U0126, the inhibitor (50 μM) was added to each corresponding well for 2 h prior to treatment with 10 μM cytochalasin H or tunicamycin at 37°C for 30 h, and then analyzed as described above. Relative survival represents the value obtained for cells treated with U0126 relative to control cells.

Statistical analysis

The results for all MTT cytotoxicity assays are expressed as the mean and SEM of three independent experiments. For the 5-day MTT assays, Student's *t* test was conducted using SigmaStat software (Systat, San Jose, CA, USA) to determine the level of significance between average means. *P* values <0.05 were deemed to be statistically significant.

Results

Previous studies from our laboratory had identified a correlation between the expression of KSR1 and the cellular sensitivity of MEFs to cisplatin [22]. These studies also revealed that the increased cisplatin sensitivity in $KSR1^{+/+}$ MEFs compared to $KSR1^{-/-}$ MEFs involved enhanced signaling through the ERK1/2 MAP kinase pathway following exposure of cells to cisplatin. In order to further elucidate the possible role of KSR1 expression on cellular sensitivity to other anticancer agents, the expression of KSR1 was examined in the NCI60 anticancer drug screen panel of cell lines. Using western blot analysis to assess KSR1 expression, the level of KSR1 in each cell line of the NCI60 anticancer drug screen panel was examined. In order to

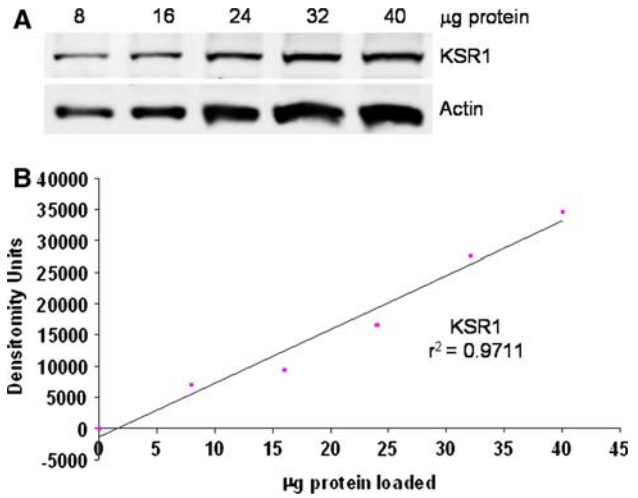

Fig. 1 Standard curve and assay validation. Increasing amounts of protein from A2780 ovarian cancer cells were evaluated by SDS-PAGE and immunoblotted with antibodies for KSR1 and actin (**a**). The immunoblots were evaluated as described in "Materials and methods". The densitometry results are depicted along with the correlation coefficient from the linear regression analaysis (**b**)

determine the linear range for detection of KSR1 expression, increasing amounts of cell lysate from A2780 human ovarian cancer cells were analyzed for KSR1 expression (Fig. 1a). Shown in Fig. 1b are results of KSR1 expression in increasing amounts of lysate from A2780 cells. Linear regression analysis indicated that values obtained were linear across the entire range examined (r^2 of 0.9711).

Using western blot analysis, KSR1 and actin expression was then demonstrated in 58 cell lines of the NCI60 panel (MDA-N and RXF 393 cell lines were not available). As shown in Fig. 2, detectable levels of KSR1 expression were observed in all cell lines examined, with the exception of the HL-60(TB) human promyelocytic leukemia cell line. KSR1 expression was not detected in this cell line even when 100 µg of cell lysate was used in the analysis (data not shown). As described above, an aliquot from A2780 cells lysate was loaded onto each gel as an internal control for blot to blot variations. As shown in Fig. 3, the ratio of KSR1:actin expression for the A2780 sample on each blot was similar in each of the five blots used to examine the NCI60 cell lines. For each blot shown in Fig. 2, the ratio of KSR1:actin expression was determined for each cell line. The results shown in Fig. 4 depict the KSR1:actin ratio for each cell line, relative to the value obtained for A2780 cells obtained on the same blot. The results shown in Fig. 4 demonstrate a wide range (over 30-fold) in KSR1 expression in the NCI60 cell lines.

The COMPARE analysis was first used to examine the possible correlation between KSR1 expression and the expression of the other molecular target data maintained in the DTP database. Shown in Table 1 are the molecular targets whose expression exhibited the highest correlation with the expression of KSR1. For example, expression of

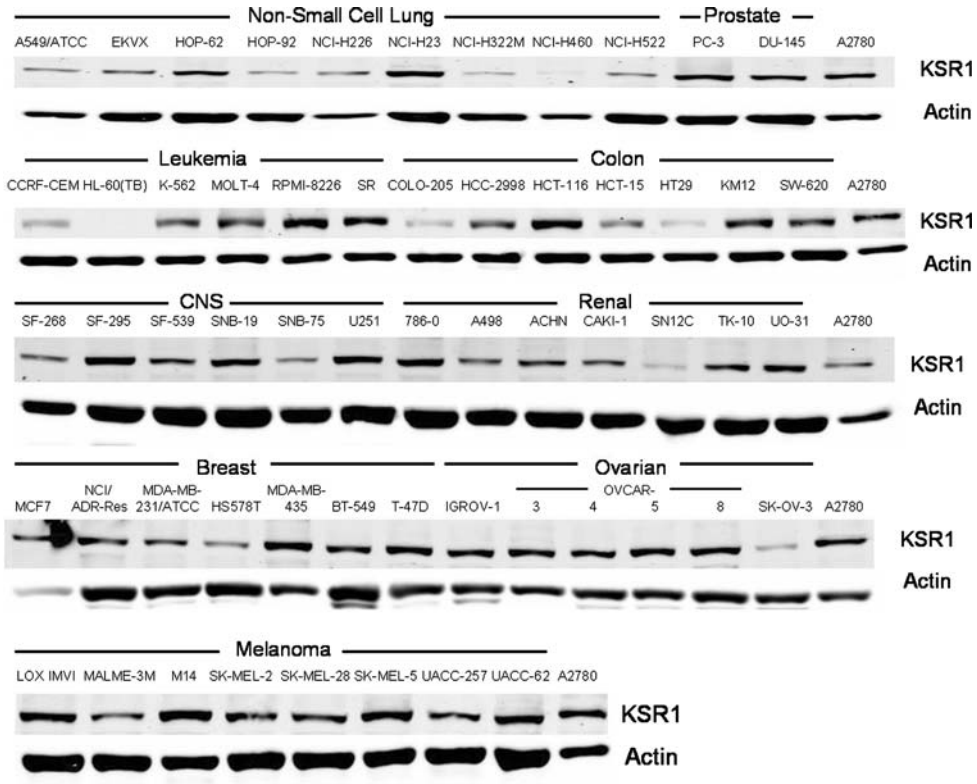

Fig. 2 Expression of KSR1 in the NCI60 anticancer drug panel screen. Samples for all cell lines included in the NCI60 anticancer drug panel screen were evaluated by western blot analysis for KSR1 and actin as described in "Materials and methods". A sample from the ovarian cell line A2780 was run on each western blot as an internal control

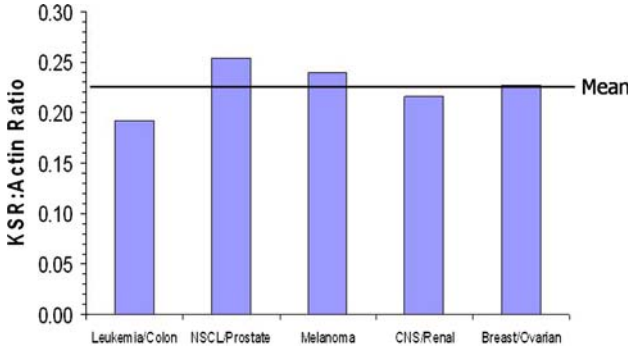

Fig. 3 Variation in KSR1 to actin levels in control samples. KSR1 and actin ratio levels were determined for each control sample from A2780 cells on the five individual blots needed to examine the entire NCI60 panel of cell lines. The mean KSR1:actin ratio for each A2780 sample is presented along with the mean for all five control samples

the reduced folate carrier and the ABCA12 multidrug resistance transporter were found to be positively correlated with KSR1 expression in the NCI60 cell line panel. In addition, the expression of Fgr, a Src-family tyrosine kinase that contributes to integrin-mediated signal transduction, demonstrated a negative correlation with KSR1 expression. There was no significant correlation between the expression of KSR1 and the expression of other members of the ERK1/2 signaling cascade, including Ras, Raf, MEK, and ERK (data not shown).

The correlation between KSR1 expression and drug sensitivity was next assessed using the COMPARE algorithm. Shown in Table 2 are the results of the COMPARE analysis of the databases of cytotoxicity assays completed by the NCI. The database of clinical agents includes compounds currently used clinically, as well as those that have entered any clinical trails. The Open database includes an unrestricted examination of compounds in the database, whereas the biological evaluation committee (BEC) database includes compounds the BEC has selected for more extensive analysis.

Table 1 COMPARE analysis of KSR1 protein expression versus other molecular target data

Molecular target	PCC
Reduced folate carrier	0.51
Fgr	−0.51
ABCA12	0.45

The results shown in Table 2 indicate a correlation between the level of KSR1 expression in the NCI60 panel of cell lines and cytotoxicity to several agents in the NCI database. As expected, some agents exhibit a positive correlation between KSR1 expression and increased sensitivity, while other agents display an inverse correlation between cytotoxicity and KSR1 expression. In order to further evaluate the role of KSR1 expression in determining cytotoxicity, two compounds (tunicamycin and cytochalasin H) identified from the studies presented in Table 1 were obtained and their cytotoxicity was examined in MEFs originating from KSR1-deficient mice ($KSR1^{-/-}$) and wild type ($KSR1^{+/+}$) mice. Shown in Fig. 5a is the western blot of $KSR^{-/-}$ and $KSR^{+/+}$ MEFs, which demonstrates the lack of KSR1 expression in $KSR1^{-/-}$ MEFs. As shown in Fig. 5b and c are the results of cytotoxicity assays in $KSR^{-/-}$ and $KSR^{+/+}$ MEFs of two agents, cytochalasin H and tunicamycin. These results shown in Fig. 5b and c indicate that MEFs deficient in KSR1 expression ($KSR1^{-/-}$) are more sensitive to cytochalasin H (Fig. 5b) and tunicamycin (Fig. 5c) relative to $KSR1^{+/+}$ MEFs.

Since KSR1 has been shown to modulate signaling through the ERK1/2 pathway, we also examined ERK1/2 activation following treatment of $KSR1^{-/-}$ and $KSR1^{+/+}$ MEFs with tunicamycin or cytochalasin H. The time course

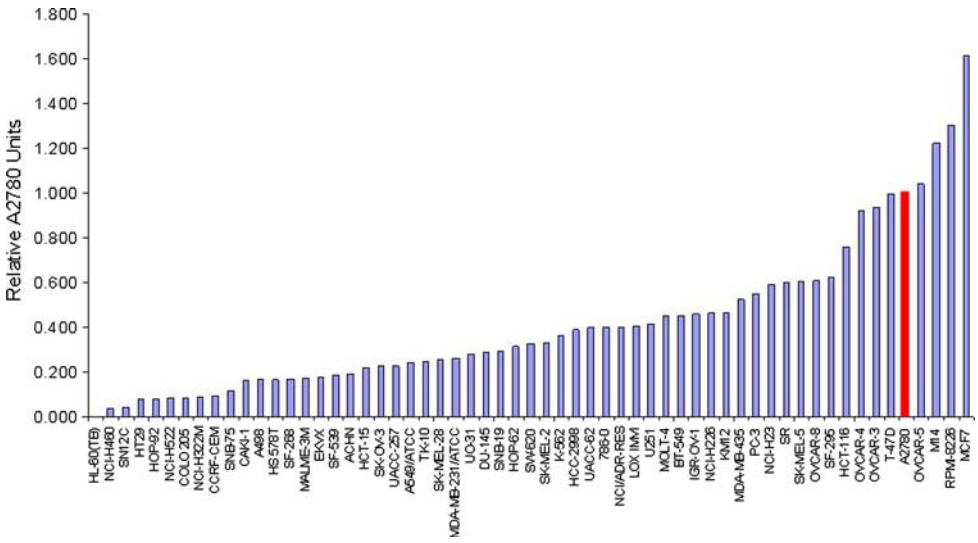

Fig. 4 KSR1 expression in the NCI60 anticancer drug panel screen. Following correction for background density, the ratio of KSR1 to actin band density was determined for each cell line of the NCI60 panel. The results for the KSR1:actin ratio determined for A2780 ovarian cancer cells was set to one for standardization

Table 2 COMPARE analysis of KSR1 protein expression versus drug sensitivity databases

Clinical agents database		Open database		BEC database	
Name	PCC	NSC number	PCC	NSC number	PCC
Tamoxifen	0.411	660647	0.618	677813	0.557
A-TGDR	0.272	700238	0.592	700497	0.503
6-Mercaptopurine	0.266	678007	0.572	628930	0.467
B-TGDR	0.243	108944	0.581	674272	0.455
Phosphotrienin	0.242	683610	0.583	701994	0.442
Actinomycin D	0.232	677813	0.557		
Diglycoaldehyde	0.227	666981	0.585	674674(Pectenotoxin 1)	−0.437
				305222(Cytochalasin H)	−0.382
06-Methylguanine	−0.276	685991	−0.517	177382(Tunicamycin)	−0.377
Bryostatin 1	−0.270	312621(Prieurianin)	−0.480	626369(Illudin S)	−0.393
Batracylin	−0.264	629487	−0.547	682771	−0.350
Mitindomide	−0.241				
L-Asparaginase	−0.230				
Rhizoxin	−0.220				
Mitozolamide (Azolastone)	−0.188				

of ERK1/2 activation in KSR1$^{-/-}$ and KSR1$^{+/+}$ MEFs was examined following exposure to cytochalasin H (Fig. 6a) and tunicamycin (Fig. 6b). Within 2 h following exposure to either agent, there was a marked increase in ERK1/2 activation in KSR1$^{-/-}$ MEFs. Drug-induced activation of ERK1/2 was observed for up to 24 h following exposure of KSR1$^{-/-}$ MEFs to either of these agents. In contrast, there is little if any activation of ERK1/2 following exposure of KSR1$^{+/+}$ MEFs to cytochalasin H (Fig. 6b) or tunicamycin (Fig. 6c). These results suggest that KSR1 expression is associated with inhibition of cytochalasin H- or tunicamycin-induced activation of ERK1/2 and decreased sensitivity to cytochalasin H and tunicamycin.

To further examine the effects of ERK1/2 activation on cytotoxicity of these agents, KSR1$^{-/-}$ MEFs were incubated with cytochalasin H or tunicamycin in the presence or absence of the MEK1/2 inhibitor U0126. As shown in Fig. 6c, pretreatment with U0126 completely abrogated ERK1/2 activation following exposure to cytochalasin H or tunicamycin. In addition, the results shown in Fig. 6d demonstrate that inhibition of ERK1/2 in KSR1$^{-/-}$ MEFs following incubation with U0126 also inhibited cytochalasin H- and tunicamycin-induced cell death.

Previous studies have demonstrated a role for the glucose response protein (Grp78) in the cellular response of melanoma cells to tunicamycin [18]. These studies have shown a relationship between Grp78 expression and ERK1/2 activation in the cellular response to tunicamycin, where pretreatment with U0126 not only blocked ERK1/2 activation, but also Grp78 expression and enhanced tunicamycin-induced apoptosis [18]. To examine the effect of tunicamcyin treatment and ERK1/2 activation on Grp78 expression in KSR1$^{-/-}$ MEFs, cells were exposed to no treatment, tunicamycin (10 μM) or tunicamycin following pretreatment with U0126 (50 μM for 2 h) for 24 h. Data presented in Fig. 6e shows that Grp78 expression increases following treatment with tunicamycin, however, this increase in Grp78 expression was not blocked by pretreatment with U0126. These data, in addition to the data shown in Fig. 6c and d, indicate that in KSR1$^{-/-}$ cells, tunicamycin-induced Grp78 expression is independent of ERK1/2 activation, and acts independently of ERK1/2 in affecting tunicamycin-induced cellular sensitivity.

To confirm that KSR1 expression is associated with increased resistance to cytochalasin H and tunicamycin, KSR$^{-/-}$ MEFs which had been transduced with a bicistronic vector containing full-length KSR1 were assayed for toxicity to both of these agents. Shown in Fig. 7a is the comparison of KSR1 expression in KSR1$^{-/-}$ MEFs and KSR1/GFP transduced MEFs. Cytotoxicity studies indicate that cells expressing KSR1 (KSR1/GFP) were more resistant to treatment with cytochalasin H (Fig. 7b) or tunicamycin (Fig. 7c), relative to KSR1$^{-/-}$ MEFs. These results lend further support to the hypothesis that KSR1 expression is associated with the development of increased resistance to both cytochalasin H and tunicamycin.

We also evaluated the sensitivity of several breast cancer cell lines in the NCI60 panel to cytochalasin H and tunicamycin. As shown in Fig. 8, cytotoxicity assays indicated that breast cancer cells expressing low levels of KSR1 (HS578T and MDA-MB-231/ATCC cells) had increased sensitivity to both cytochalasin H (Fig. 8a) and tunicamycin (Fig. 8b) compared to breast cancer cells expressing higher levels of KSR1 (MCF7 cells). These studies also support

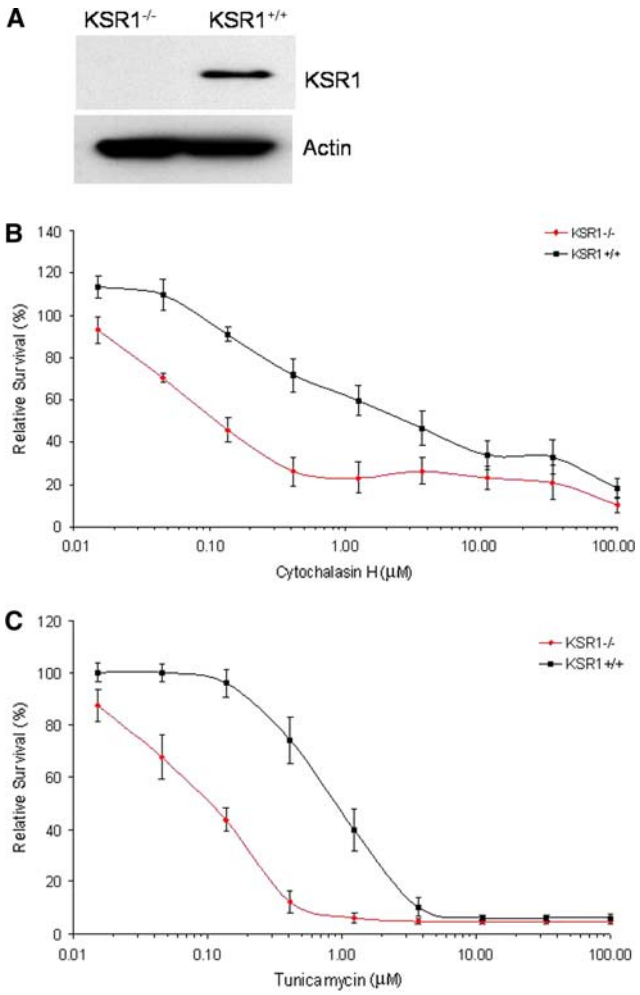

Fig. 5 Sensitivity of KSR1$^{-/-}$ and KSR$^{+/+}$ MEFs to agents identified from COMPARE data. Expression of KSR1 was determined in KSR1$^{-/-}$ and KSR$^{+/+}$ MEFs by western blotting (**a**). The sensitivity of KSR1$^{-/-}$ and KSR$^{+/+}$ MEFs to cytochalasin H (**b**) or tunicamycin (**c**) was determined by MTT assay as described in "Materials and methods". The survival for cells treated with each agent was compared to untreated cells and the data expressed as the relative percent survival relative to untreated cells. The results are expressed as the average and standard error of the mean for three independent experiments

the role of KSR1 expression with the development of resistance to cytochalasin H and tunicamycin.

Discussion

The use of human cell lines for anticancer drug screening began in the 1980s with the assembly by the NCI of a panel of human leukemia cell lines. The panel was subsequently expanded to include cell lines derived from 9 tissue types, and currently continues to be used by the NCI for drug screening [4]. This panel of cell lines has been extensively characterized, including the analysis of approximately 1,500 molecular targets in the NCI60 cell lines, assessed at the level of RNA or protein expression (http://www.dtp.nci.nih.gov/). In addition, the sensitivity of the cell lines in the NCI60 to approximately 400,000 compounds, including a wide range of natural products, as well as approximately 200 clinically useful anticancer agents [49]. COMPARE, a method of pattern recognition analysis, allows delineation of the possible relationship between the expression of molecular targets in the cell lines and their sensitivity to specific agents [43].

Studies on the NCI60 panel have led to the identification of compounds that have subsequently shown to be clinically useful. For example, flavopiridol, an inhibitor of cyclin-dependent kinases, was noted to have activity in vitro in the NCI60 panel and subsequent clinical studies have demonstrated activity of flavopiridol in a number of tumor types [7]. UCN-01 another inhibitor of cyclin-dependent kinases, as well as protein kinase C, was also noted to have activity in the NCI60 panel and subsequent clinical studies have identified clinical activity of this agent in the treatment of lymphomas [48]. In addition, 17-AAG, an agent derived from Geldanamycin, is an inhibitor of Hsp90, a molecular chaperone that interacts with a range of protein kinases including members of the ERK1/2 MAP kinase and phosphotidylinositiol 3-kinase (PI3K) families [34]. Results from cytotoxicity of 17-AAG in the NCI60 panel led to clinical trials with 17-AAG.

In addition, valuable information has been gleaned from the analysis of the myriad of potential molecular targets in the NCI60 cell lines. For example, studies on the expression of the multidrug-resistant transporter MDR-1 in the NCI60 led to the identification of compounds whose cytotoxicities were affected by this transporter [2]. Evaluation of the expression of *ras* genes in the NCI60 panel led to the observation that 17 of 60 cell lines in the panel harbored activating mutations in at least one *ras* allele. Correlation of the pattern of *ras* mutations with the sensitivity databases identified several groups of anticancer agents that displayed enhanced sensitivity in cells harboring *ras* mutation [24]. These studies have demonstrated the utility of the NCI60 panel.

The ERK1/2 MAP kinase pathway has been shown to play a role in cell growth, differentiation, cell cycle progression and apoptosis and several studies have implicated the activation of the ERK1/2 pathway in the development of resistance to anticancer agents. For example, increased expression of the HER-2/neu receptor [1, 13] and EGFR [9, 10] has been associated with increase resistance to some anticancer drugs. Other studies have linked the expression of Ras and Raf with increased expression of the MDR-1 multidrug resistance transporter [5], and increased Raf activity has been associated with resistance to doxorubicin and paclitaxel [33]. These studies suggest a role for the

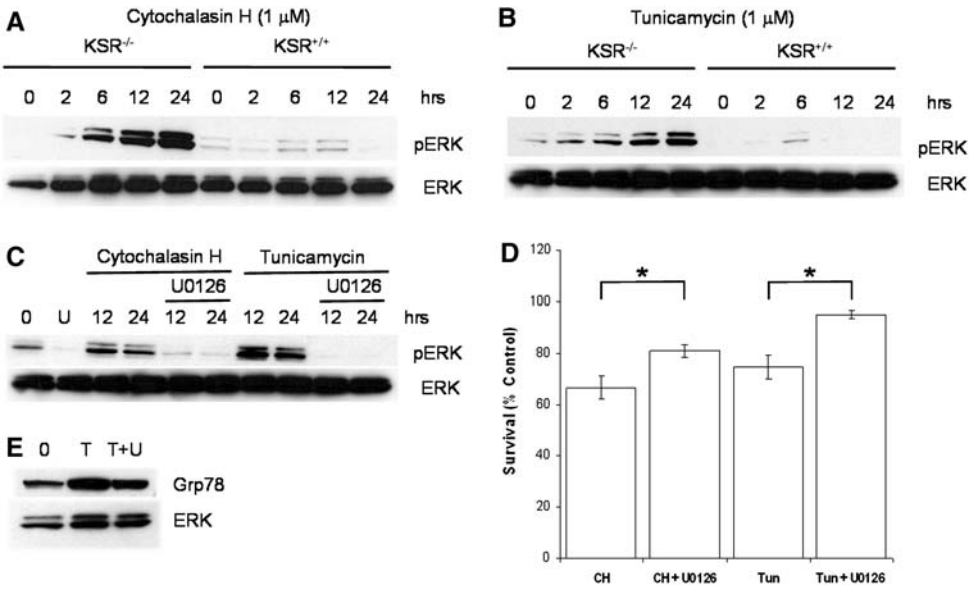

Fig. 6 Effect of cytochalasin H and tunicamycin treatment on ERK1/2 activation in MEFs. To evaluate the effect of cytochalasin H and tunicamycin on ERK1/2 activity, KSR1$^{-/-}$ and KSR$^{+/+}$ MEFs were treated with 1 μM of cytochalasin H (**a**) or tunicamycin (**b**) for the times indicated. The level of phosphorylated p44/p42 ERK1/2 and total ERK was determined as described in "Materials and methods". In order to determine the role of ERK1/2 activation following treatment with cytochalasin H or tunicamycin, KSR1$^{-/-}$ and KSR$^{+/+}$ MEFs were pretreated with U0126 for 2 h prior to treatment and then treated for the times indicated with 1 μM cytochalasin H or tunicamycin. ERK1/2 activation was examined by western blotting (**c**). For determination of the role ERK1/2 activation plays on cell survival following treatment with cytochalasin H or tunicamycin, MEFs (3000/well) plated on 96-well plates were pretreated with our without U0126 for 2 h followed by treatment with 10 μM cytochalasin H (CH) or tunicamycin (T) for 30 h. Cell survival was determined by MTT assay, with the data expressed as percent survival relative to control cells treated with U0126 only or DMSO only as described in "Materials and methods" (DMSO or U0126) (**d**). Data represent the mean average and standard error of the mean for three independent experiments. *Asterisk* indicates $P < 0.05$. To investigate the effect of tunicamycin treatment on Grp78 expression, cells were either exposed to 10 μM tunicamycin (T) for 24 h or cells pre-exposed to U0126 for 2 h followed by treatment with tunicamycin (T+U), compared to untreated control cells. ERK2 was used as a loading control (**e**)

ERK1/2 MAP kinase pathway in the development of resistance to a wide range of anticancer agents.

KSR1 has been shown to function as a scaffold in the ERK1/2 MAP kinase pathway [36] and numerous reports have indicated a identified that at low levels of KSR1 expression, there is a correlation between intracellular KSR1 levels and the cellular response to signal transduction. However at high levels of KSR1 expression, there is a dampening of the activation of the ERK1/2 signal transduction pathway and cell proliferation in response to EGF and PDGF [28]. Other studies have demonstrated that KSR1 expression influences Ras-induced oncogenesis, indicating that KSR1 could also play a role in facilitating the development of cancer [28, 31]. Studies in A431 epidermoid cancer cells found that treatment of cells with phosphorothioate antisense oligonucleotides targeting KSR1 reduced the rate of growth in vitro and in vivo [63]. Similar data were obtained in PANC-01 cells, which express a mutant Ras oncogene [63]. Taken together, these studies suggest that KSR1 expression may be an important therapeutic target for cancer therapy.

Previous reports have demonstrated that the ERK1/2 MAP kinase pathway plays a role in the cellular response to the anticancer agent cisplatin [22, 44, 56]. Using KSR1$^{-/-}$ and KSR1$^{+/+}$ MEFs, our laboratory has previously demonstrated that KSR1 expression was associated with an increase in cisplatin-induced ERK1/2 activation, and that cisplatin-induced ERK1/2 activation was associated with cisplatin-induced apoptosis [23].

In this report, KSR1 expression was examined in the NCI60 anticancer drug panel screen and the results indicate a wide range of KSR1 expression in the cell lines in the NCI60 panel. The only cell line the NCI60 panel with undetectable expression of KSR1 was the HL-60(TB) promyelocytic leukemia cells. It is of interest that previous studies have shown that KSR1 expression is inducible in HL-60 cells following induction of monocytic differentiation following exposure to 1,25-dihydroxyvitamin D3 [58]. In the NCI60 panel, there was no discernable pattern of expression among cell lines of the individual tumor types, and a wide range of expression values was noted among cell lines within specific tissue types.

The COMPARE algorithm identified several compounds for which a correlation between KSR1 expression and cytotoxicity was identified. In order to further clarify the relationship between KSR1 expression and sensitivity to

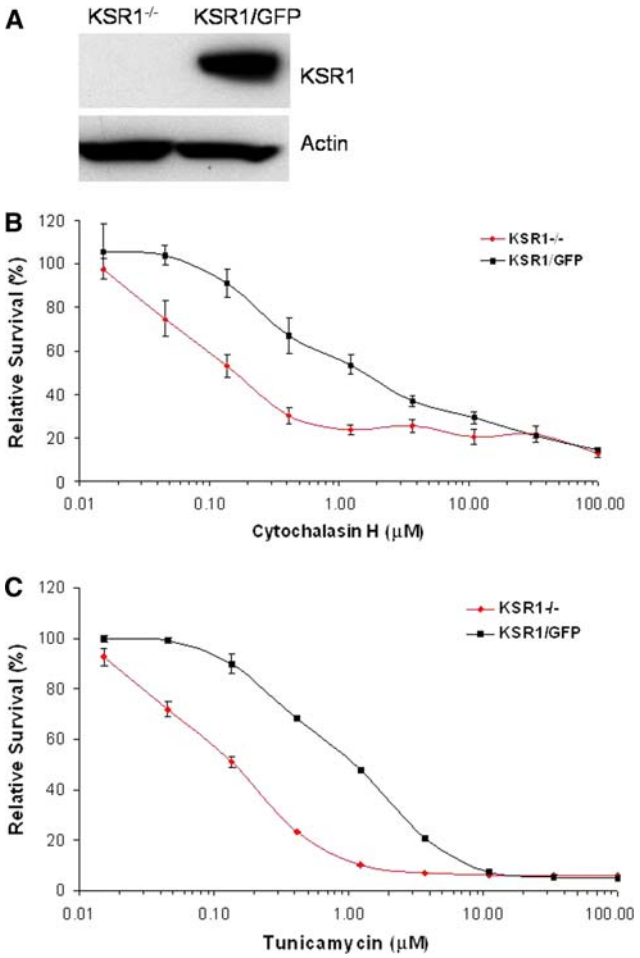

Fig. 7 Effect of KSR1 expression in KSR1$^{-/-}$ MEFs on sensitivity to cytochalasin H and tunicamycin. KSR1 and actin expression was determined by western analysis for KSR1 $^{-/-}$ MEFs and KSR1$^{-/-}$ MEFs stably transduced with an expression vector containing KSR1 (KSR1/GFP) as described in "Materials and methods" (**a**). The sensitivity of KSR1 $^{-/-}$ and KSR1/GFP MEFs to cytochalasin H (**b**) and tunicamycin (**c**) was determined by MTT assay following exposure to each compound for 5 days. The data represent the mean and standard error of the mean for three independent experiments

anticancer agents, two compounds identified by analysis of the NCI60 panel were further studied in KSR1$^{-/-}$ and KSR1$^{+/+}$ MEFs. These studies demonstrated that the cytotoxicity of both cytochalasin H and tunicamycin was greater in cells lacking KSR1 (KSR1$^{-/-}$ MEFs) compared to KSR1$^{+/+}$ MEFs. These studies were consistent with studies performed in the NCI60 panel.

Cytochalasins are fungal proteins that function by binding to actin, thereby blocking a number of cellular events involving the formation and elongation of actin filaments [64]. Several cytochalasins have been noted to possess antitumor activity [20]. Cytochalasin H, a metabolite found in the fungus *Phomopsis paspali* [61], has been previously shown to affect platelet aggregation dynamics [40]. As discussed above, studies in this report demonstrated that MEFs lacking KSR1 expression (KSR1$^{-/-}$) were more sensitive to cytochalasin H treatment relative to KSR$^{+/+}$ MEF and that MEFs expressing KSR1 exhibited reduced ERK1/2 activation following treatment with cytochalasin H. These observations strongly support the role of KSR1 expression in the modulation of cytochalasin H-induced ERK1/2 activation and sensitivity to cytochalasin H.

Tunicamycin is an antibiotic that blocks glycosylation of newly synthesized proteins and previous studies have indicated that treatment of cells with tunicamycin results in the induction endoplasmic reticulum (ER) stress [53]. Other studies have demonstrated that the induction of ER stress by tunicamycin leads to the activation of the ERK1/2 MAP kinase pathway [15, 17, 42] and that ER stress potentiates the ERK1/2 activation induced by hydrogen peroxide [17]. Further studies have shown that inhibition of ERK1/2 activation enhanced tunicamycin-induced cell death in MCF7 cells [15]. In this report, treatment of MEFs with tunicamycin also results in ERK1/2 activation and loss of KSR1 expression was associated with enhanced ERK1/2 activation. Furthermore, inhibition of ERK1/2 was associated with increased survival in KSR1$^{-/-}$ MEFs following exposure to tunicamycin. Taken together, these results in MCF7 cells and in MEFs indicate that the effect of tunicamycin exposure on ERK1/2 signaling is cell type specific. In MEFs, KSR1 expression was associated with decreased activation of ERK1/2 following exposure to cytochalasin H and tunicamycin as well as increased resistance to both agents.

To further define the role of KSR1 expression in determining the sensitivity of cells to cytochalasin H and tunicamycin, cytotoxicity assays were performed in several breast cancer cell lines from the NCI60 panel. The results of these studies indicated breast cancer cells expressing a higher level KSR1 were more resistant to the cytotoxic effects of both cytochalasin H and tunicamycin. These results support the role of KSR1 as a determinant of the sensitivity of cells to both of these agents.

The COMPARE algorithm was also utilized to examine the correlation of KSR1 expression with other molecular targets in the NCI60 panel. These studies identified a correlation between the expression of KSR1 and two drug transport proteins, the reduced folate carrier and ABCA12. The reduced folate carrier is a ubiquitously expressed protein, involved in the transport of reduced folates and antifolates into the cell. Alterations in the activity of this carrier have been shown to be involved in the development of resistance to antifolate analogues, including methotrexate. Previous studies using the NCI60 anticancer drug screen did identify a correlation between the expression of the reduced folate carrier and cellular sensitivity to methotrexate [38].

ABCA12 is a member of the ATP-binding cassette (ABC) family of transporters which are involved in the

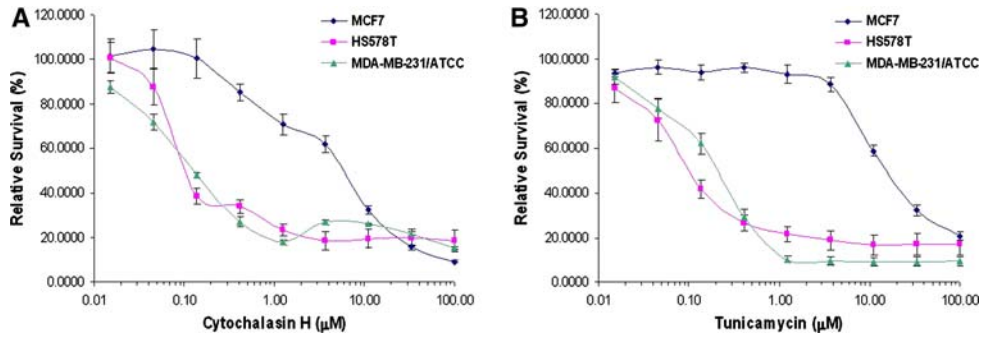

Fig. 8 Sensitivity of select breast cancer cell lines from the NCI60 to cytochalasin H and tunicamycin. To examine the role of KSR1 expression on sensitivity of cell lines in one specific tissue type, cell lines were selected from the breast cancer cell line subset of the NCI60, based on KSR1 expression. MDA-MB-231/ATCC, HS578T, and MCF7 cells were plated on 96-well plates and treated with either cytochalasin H (**a**) or tunicamycin (**b**) for 5 days and cell survival determined by MTT assay. Data are expressed as the mean average and SEM for three independent experiments

transport of a myriad of substrates into the cell [19]. Mutations in the ABCA12 gene are associated with the development of harlequin and lamellar ichthyosis, a dermatological condition involving dry, scaly skin [21, 30]. Previous studies on the expression of ABC transporters in the NCI60 identified an inverse correlation between the expression of a specific ABC transporter (MDR-1) and sensitivity of cells to anticancer agents known to be transported by ABC transporters [52].

A correlation between KSR1 expression and expression of Fgr was also noted in the NCI60 panel. Fgr, a member of the src family of tyrosine kinases, has been shown to be involved in integrin-mediated signaling [3] and recent studies have implicated Fgr signaling in the migration of cell to sites of inflammation [55]. While some studies suggest that the effects of cell migration may, at least in part, involve signaling through PI3K [8], ERK1/2 signaling has also been shown to influence cell migration in various cell types [16]. Thus, it is possible that ERK1/2 signaling may also contribute to Fgr-mediated cell migration. These studies suggest a possible role for KSR1 as a biomarker for prognosis in cancer.

In summary, the results described in this study indicate that KSR1 expression varies considerably in human cancer cell lines and that KSR1 expression is correlated with sensitivity to several agents, including cytochalasin H and tunicamycin. These studies suggest that KSR1 expression may be an important determinant of prognosis following treatment of patients with cancer. Additional studies are needed to determine the precise mechanism by which KSR1 modulates the sensitivity of cells to cytochalasin H and tunicamycin and other anticancer agents.

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