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KINASE SUPPRESSOR OF RAS 2 IS INVOLVED IN REGULATION OF CELL PROLIFERATION AND IS UP-REGULATED IN HUMAN INVASIVE DUCTAL CARCINOMAS OF BREAST

*M. Jia, S. Souchelnytskyi**

Department of Oncology-Pathology, Karolinska Biomics Center, Z5:01, Karolinska University Hospital, Solna, SE-17176, Karolinska Institutet, Stockholm, Sweden

Aim: **To study the expression of Kinase Suppressor of Ras 2 (KSR2) in human breast tumors and its effect on proliferation of breast epithelial cells. We reported previously that KSR2 was up-regulated in immortalized human breast epithelial cells.** ***Methods:*** **Proteomics technologies, systems biology tool for a KSR2 network analysis, immunoblotting, siRNA technology, overexpression of KSR2, cell proliferation assays and immunohistochemistry of tissue microarray of human breast tumors and normal breast tissue were used.** ***Results:*** **In conditionally immortalized primary epithelial cells KSR2 expression was shown to be up-regulated. The involvement of KSR2 in regulation of cell proliferation was predicted by a KSR2-centered network analysis. We observed that KSR2 down-regulation with specific siRNA inhibited cell proliferation. By immunohistochemistry of tissue microarray it was demonstrated that KSR2 expression was enhanced in human invasive breast carcinomas.** ***Conclusion:*** **Our findings propose KSR2 as a new marker of immortalization, which has an impact on cell proliferation.**

Key Words: **KSR2, proliferation, human breast epithelial cells, breast cancer, invasive ductal carcinoma.**

Acquisition of immortalization by somatic cells is one of the hallmarks of tumorigenesis [1, 2]. Immortalization is a complex process, which involves not only activation of telomerase (TERT), but also changes in a number of signalling mechanisms related to regulation of cell proliferation and apoptosis [3–5]. Among such cell cycle regulators are cyclin-dependent kinase inhibitor $p16^{INK4a}$, p53, MAP kinase and pRb (reviewed in [3–5]). Further studies are expected to unveil the whole complexity of immortalization, and to describe all involved proteins and genes.

Kinase Suppressor of Ras 2 (KSR2) is a scaffold protein involved in activation of MAP kinase pathway [6, 7]. KSR2 was found to mediate A-Raf signalling. However, a screening for KSR2 interacting proteins showed that KSR2 may play a broader role in regulation of cell proliferation, by forming high-order complex(es) including kinases (MEK1, MEK2, Erk2, p38, cdk4, PI3K) and phosphatases (PP2A, PP6), translational, ribosomal, transport and structural proteins [8]. These findings indicated that KSR2 may play a role in tumorigenesis.

A model of immortalization of human breast epithelial cells was recently developed using conditional immortalization of primary cells [9]. Temperature-sensitive construct of SV40 LT in combination with hTERT allowed controlled immortalization of human primary cells [9]. This model of immortalization was found suitable for studies of signalling mechanisms of the transition from immortalization to senescence. Proteome profiling of these cells showed that immortalization-to-senescence transition leads to the changes in expression of a number of proteins [10]. Some of them, like KSR2, could be directly involved in regulation of cell proliferation.

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*Correspondence: Fax: +46 (0) 8–517–71 000
E-mail: serhiy.souchelnytskyi@ki.se

Abbreviations used: hTERT – catalytic subunit of human telomerase; IDC – invasive ductal carcinoma; KSR2 – kinase suppressor of Ras 2; LT – simian virus 40 large-tumor antigen; MAPK – mitogen activated protein kinase.

MATERIAL AND METHODS

Cells. 184A1 and MCF10A cells were obtained from ATCC (Manassas, USA), and were cultured in media recommended by ATCC. Conditionally immortalized human primary epithelial cells were generated using constructs and methods described early by O´Hare *et al.* [9]. Notably, the catalytic subunit of human telomerase (hTERT) and a temperature-sensitive mutant of simian virus 40 large-tumor antigen were used (non-DNA binding non-Bub1 binding thermolabile T antigen; [9]). The luminal 226Lts4 and 226LU19 cells were used. Expression of LT antigen was confirmed by immunoblotting, and hTERT was monitored by TRAP assay (O'Hare and Jat; manuscript).

Proteomics. Proteome profiling of conditionally immortalized cells, two-dimensional gel electrophoresis, gel image analysis and MALDI TOF mass spectrometry for identification of differentially expressed proteins were described by Jia *et al.* [10].

Network analysis of KSR2 for interactors. FunCoup tool was used to identify KSR2 interactors (*http://funcoup.sbc.su.se*). FunCoup operates with databases which have been selected for their thorough analysis and incorporation of experimental data. This ensures that only confirmed by multiple laboratories results are taken into consideration for building a network. Settings for the network analysis were as follows: confidence score 0.10, only direct one-step interactors observed in humans were considered.

Transfections and immunoblotting. Cells were transfected in 6-wells plates by LipofectAMINE 2000 reagent, as recommended by the supplier (Invitrogen, Carlsbad, USA). pcDNA3-KSR2 construct was kindly provided by Dr. Jiahuai Han. siRNA to KSR2 (ID # 41069)

was obtained from Ambion (Austin, USA). Control scrambled siRNA (sc-37007) were obtained from SantaCruz Biotech (Santa Cruz, USA). Media was changed 6 hs after transfection. For immunoblotting, cell lysates were resolved on SDS polyacrylamide gels and transferred onto Hybond P membranes (GE Healthcare, Piscataway, NJ). Membranes were blocked with 5% (v/v) BSA and then incubated with a primary antibody against KSR2 (H00283455-A01; Abnova, Taiwan) as recommended by manufacturer, and followed by an HRP-conjugated secondary antibody (GE Healthcare, Uppsala, Sweden). The proteins were visualized using Western Blotting Luminol Reagents (Santa Cruz Biotechnology Inc.). For transfection with siRNA, cells were seeded in 24-well plates, and transfection procedure was performed the next day, as recommended by the siRNA suppliers. After transfection, cells were cultured in a medium supplemented with 10% FBS before assays.

Cell proliferation assays. Cell proliferation was measured using [^{3}H]thymidine incorporation assay and CellTiter 96® Non-Radioactive Cell Proliferation Assay (MTT assay) (Promega, Promega Biotech AB, Stockholm, Sweden). 184A1 and MCF10A cells were seeded in plates for proliferation assays. Cells were incubated with 0.1 μCi/ml of [^{3}H]thymidine for the last 24 hs of the 72 hs incubation time period. Radioactivity incorporated in DNA was measured as described earlier [11]. MTT assay was performed in parallel with [^{3}H]thymidine-incorporation test, except that no radioactivity was added. Cells were grown for the 72 hours, and MTT assay was performed according to the manufacturer's recommendations. Statistical significance of observed differences was evaluated using Student's t-test.

Immunohistochemistry. AccuMax breast cancer arrays (ISU ABXIS Co., Ltd, Seoul, South Korea) were used. Each array slide contains 45 cases of cancer tissues and 4 non-neoplastic tissues. Arrays were stained with anti-KSR2 antibody (H00283455-A01; Abnova) at a dilution of 1:250. Antigen retrieval was performed using DakoCytomation target retrieval solution high pH (DAKO, Carpinteria, CA, USA). The slides were stained with VECTASTAIN Elite ABC kits (Vector Laboratories Inc., Burlingame, CA, USA) following the manufacturer's instruction, counterstained with hematoxylin and mounted with Fluoromount G (Southern Biotechnology, Birmingham, AL). The stained tissues were photographed using a Leica DFC camera and images were acquired with Leica QWin Standard software (Leica Microsystems Imaging Solutions Ltd, Cambridge, UK). Intensity of staining was evaluated as absent ((-), no staining or weak staining in fewer than 5% of cells) middle/present ((+), staining in > 5% but < 50% of cells), and strong ((++), staining in > 50% of cells). Staining was evaluated in tumor cells and epithelial cells of normal tissues.

RESULTS AND DISCUSSION

Using proteomics approach KSR2 was identified as an up-regulated protein in immortal human primary epithelial cells [10]. Protein spot containing KSR2 was detected only in immortal cells, which indicated up-regulation of at least more than 2 fold (Fig. 1). Conditionally immortalized cells were generated by expression of hTERT and temperature-sensitive mutant (U19tsA58) of simian virus 40 large-tumor (LT) antigen, as described by O´Hare *et al.* [9].

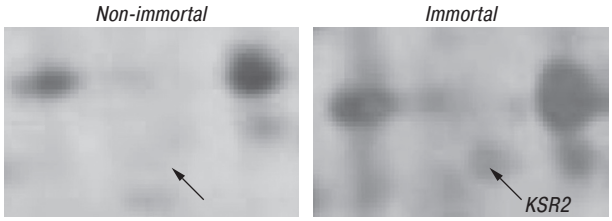


Fig. 1. Images of inserts of 2D gels which show the protein spots of KSR2. Images show the KSR2 spot in 2D gels from cells cultured under permissive (Immortal) and non-permissive (Non-immortal) conditions. Arrows indicate migration position of the protein spot of KSR2.

KSR2 is a scaffold protein in mitogen-activated protein kinase (MAPK) pathways [6–8]. To explore potential impact of KSR2 on cell proliferation and generic MAPK pathways in unbiased way, we used FunCoup tool (*http://funcoup.sbc.su.se*). The generated network (Fig. 2) showed potential involvement of Raf-1, mitogen-activated kinases 1 and 3 (MAPK1 and MAPK3), mitogen-activated kinase kinase 1 (MAP2K1) and MAPK scaffold protein 1 (MAPKSP1) (Fig. 2). These molecules provide further links to regulators of the cell cycle, p53 and hTERT (data not shown). A number of other KSR2 interactors involved in regulation of the cell cycle were reported, although they have to be validated [8]. Thus, analysis of the KSR2-centered network indicated that KSR2 may have an impact on cell proliferation.

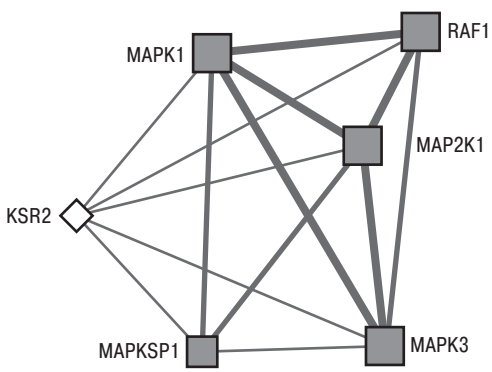


Fig. 2. KSR2 forms a network with components of Raf/MAP kinase signalling. The network of interacting proteins was built with KSR2 as a hub, using FunCoup tool (*http://funcoup.sbc.su.se*).

To manipulate KSR2 level in cells subjected to cell proliferation assays, we enhanced expression of KSR2 by transfecting cells with a specific vector or down-regulated KSR2 with a specific siRNA (Fig. 3). We studied two human breast epithelial cell lines, MCF10A and 184A1, which are both non-tumorigenic and considered to have normal phenotype of human breast epithelial cells. These cells can be cultured *in vitro*, and therefore are immortalized. Both cell lines also express KSR2 (Fig. 3). However, 184A1 cells may undergo immortalization crisis, and have lower proliferation potential, as compared to MCF10A cells [12].

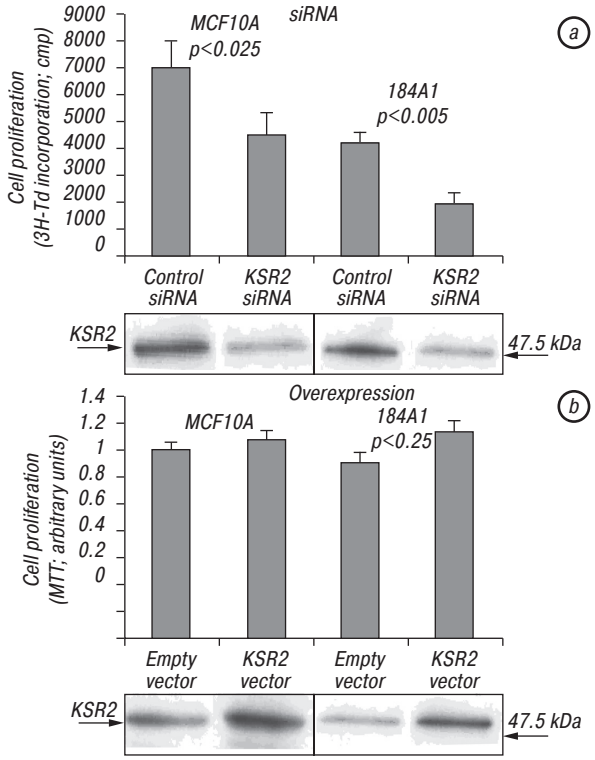
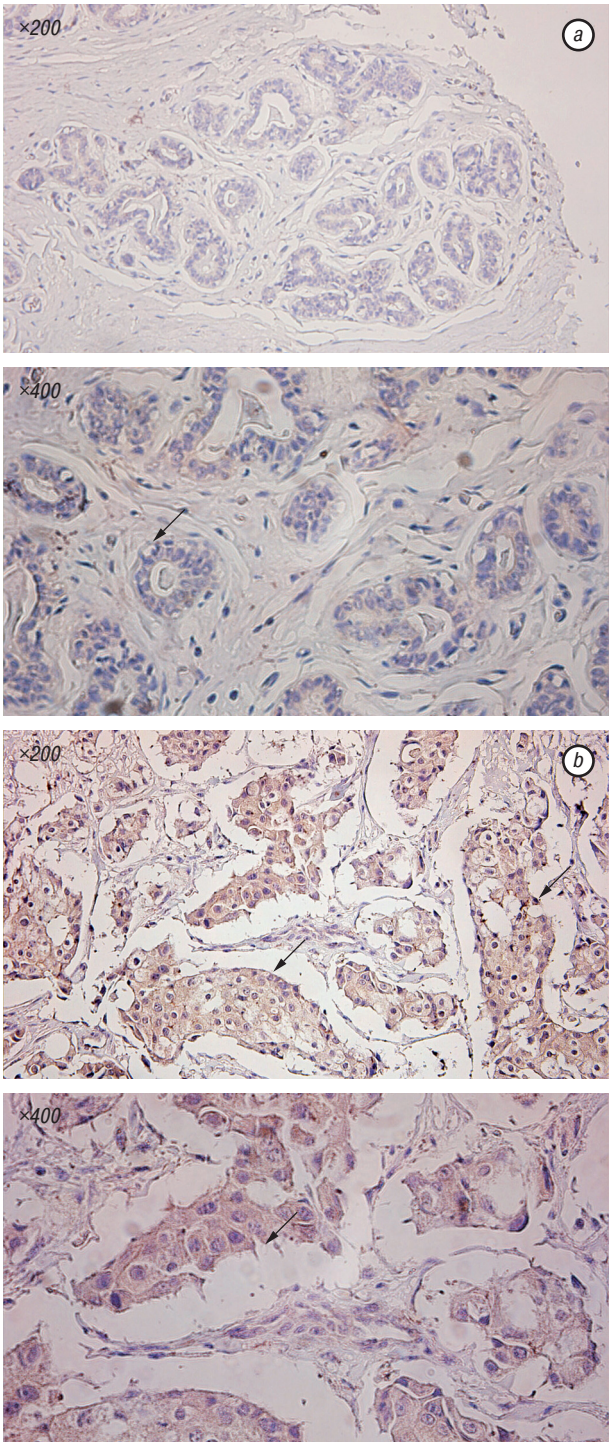


Fig. 3. Down-regulation of KSR2 resulted in inhibition of the cell proliferation. (*a*) 184A1 and MCF10A cells were transfected with siRNA to KSR2, and with control siRNAs, as indicated. Proliferation of cells was monitored by measurement of [3H]thymidine incorporation. (*b*) 184A1 and MCF10A cells were transfected with KSR2 expressing vector, and with control empty pcDNA3 vector, as indicated. Cell proliferation was measured by MTT assay. Expression of KSR2 (*a,b*) was monitored by immunobloting of the whole cell extracts. Migration position of a 47.5 kDa marker is indicated on the side of gel panel. Statistical significance in assays was calculated by Student's t-test.

We observed that down-regulation of KSR2 had a strong inhibitory effect on proliferation of both cell lines (Fig. 3*a*). KSR2 overexpression had rather weak effect on cell proliferation, which was more pronounced for 184A1 cells, but not significant for MCF10A cells (Fig. 3*b*). This could be explained by the presence of endogenous KSR2 in a quantity already sufficient for its normal functions. On the other hand, decrease in KSR2 expression by siRNA, had an inhibitory impact on proliferation of both cell lines, indicating the rate-limiting role of KSR2. Thus, KSR2 is involved in maintaining the cell proliferation rate.

To explore whether KSR2 expression could be altered in breast tumors, we performed immunohistochemistry study of human breast tissue microarray. The microarray consisted of 29 cases of infiltrating ductal carcinomas (IDC), 8 papillary carcinomas, 4 cases of infiltrating lobular carcinomas (ILC) and 4 normal breast tissues. We observed an increase of KSR2 staining in epithelial cells of IDC tumors, as compared with normal breast tissue (Fig. 4*a, b*). We observed KSR2 staining in all 29 cases of IDC, 4 cases of ILC and 8 cases of papillary carcinomas (Fig. 4*c*). All samples of normal breast tissue did not show KSR2 staining. KSR2 staining in tumor samples was detected mainly in cytoplasm of cells. No correlations of KSR2 expression with TNM gradation of tumors were observed.



Samples	Staining		
	(-)	(+)	(++)
Invasive Ductal Carcinoma (29 cases)	0	10	19
Invasive Lobular Carcinoma (4 cases)	0	2	2
Papillary Carcinoma (8 cases)	0	6	2
Normal (4 cases)	4	0	0

Fig. 4. Expression of KSR2 was increased in human infiltrating ductal carcinomas. Representative images of immunohistochemistry stainings for KSR2 are shown: (a) staining of normal human breast tissue; (b) staining of an infiltrating ductal carcinoma case. Magnifications are X200 (top images in panels) and X400 (lower images in panels). (c) Quantification of KSR2 staining in human breast cancer. Evaluation of staining was performed as described in the material and methods section. Numbers indicate numbers of cases with corresponding detection of staining (as (-), (+) and (++)), and the total number of cases is mentioned in the left column.

KSR2 plays an important role in activation of various signalling events, with reported role in signalling by p38 MAPK and MAP2K3 [6–8]. We found that KSR2 is involved in regulation of cell proliferation, and is upregulated in tumor epithelial cells in human invasive ductal, invasive lobular and papillary carcinomas of breast. In conclusion, our findings provide evidence of a potential role of KSR2 in tumorigenesis, indicating that expression of KSR2 may be changed already upon acquisition by cells the ability to non-limited proliferation.

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