Silencing of Selected Glutamate Receptor Subunits Modulates Cancer Growth

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Abstract. *Background: Emerging evidence supports a role for glutamate in the biology of cancer. We studied the impact of glutamate receptor subunit silencing on cancer phenotype. Materials and Methods: Different fragments of the coding region for ionotropic glutamate receptor AMPA 4 (GLUR4), ionotropic glutamate receptor N-methyl D-aspartate 1 (NR1), ionotropic glutamate receptor kainate 5 (KA2) and ionotropic glutamate receptor N-methyl D-aspartate 2D (NR2D) were stably transfected into human TE671, RPMI8226 and A549 cell lines. Resulting changes in cell proliferation, migration and mRNA expression of genes that determine cancer phenotype were assayed. Results: Decreased expression of GLUR4 markedly increased cancer cell proliferation, whereas decreased expression of NR1 markedly reduced the propensity of cancer cells to proliferate. Knockdown of KA2 and NR2D did not influence cancer phenotype. Gene silencing of GLUR4 modulated the mRNA expression of various genes in these cancer cell lines, as determined with the Human Cancer PathwayFinder™ PCR Array. Knockdown of GLUR4 influenced the expression and function of genes involved in invasion and metastasis, tumour suppressor genes, oncogenes and adhesion genes. Conclusion: The findings suggest that glutamate receptor subunits on cancer cells are linked to biochemical pathways that regulate malignant phenotype.*

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Glutamate (Glu) is a major neurotransmitter in the central nervous system and is also involved in regulation of tumor growth (1-4). It has been reported that glutamate receptor (GluR) antagonists limit tumor growth (2, 5, 6) and that blocking the expression of selected GluR subunits inhibits proliferation of cancer cells *in vitro* (7, 8).

GluRs consist of two main groups: ionotropic receptors (iGluRs) and metabotropic receptors (mGluRs). The iGluRs form ion channels, the mGluRs belong to the superfamily of G-protein-coupled receptors (9, 10). The iGluRs form ion channels that may also possess metabotropic properties (11-14). The iGluRs are divided into three groups based on structural similarities and are named according to the type of synthetic agonist that activates them. N-Methyl-D-aspartate (NMDA) tetrameric receptors consist of two obligatory NR1 subunits and two of four types of regulatory subunits, NR2A, B, C and D. The NR1 subunit is necessary for calcium conductivity of the channel, while the NR2 and NR3 subunits determine electrophysiological and pharmacological properties of the receptor (9, 15-19). α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptors (AMPARs) are homo- or hetero-tetramers composed of subunits GLUR1-GLUR4 (20, 21). AMPARs gate Na^+/Ca^{2+} in response to ligand binding. The GluR2 subunit determines Ca^{2+} permeability of AMPARs (9, 11). 2-Carboxy-3-carboxymethyl-4-isopropenylpyrrolidine receptors (kainate, KARs) are formed as tetrameric assemblies of GluR5-7 and KA1/2 subunits. KA1 and KA2 subunits do not form homomeric functional receptors. Similar to GluR2, GluR5 and GluR6 undergo glutamine/arginine editing (22).

Experimental evidence supports the role for Glu and its receptors in peripheral tissues (23, 24) and in cancer (25-28). It has been demonstrated that GluR subunits are expressed in a variety of cancer types, *e.g.* gliomas and other brain tumors,

squamous cell carcinoma, colon cancer and osteosarcoma (1, 7, 26, 30-38).

Cancer cells display various phenotypic alterations, for example sustaining proliferative signaling, evading growth suppressors, activating invasion and metastasis and enabling replicative immortality (39). Previously we systematically studied expression of various GluR subunits in human cancer cell lines and in human brain tumors and reported variable expression of all studied subunits (36, 40). Functional studies show that inhibition of NMDA receptor activity leads to negative regulation of cell viability and proliferation of various cancer cell lines (3). It has been hypothesized that silencing of glutamate receptor subunit expression in cancer cells might influence carcinogenesis.

Here we explored the functional role of selected GluR subunits in human cancer cells by silencing their expression. The results suggest that certain GluR subunits are involved in controlling biochemical pathways that regulate tumor survival, proliferation and propensity to metastasize.

Materials and Methods

Cell culture. Human rhabdomyosarcoma/medulloblastoma (TE671) and human multiple myeloma cells (RPMI8226) were obtained from the European Collection of Cell Cultures (Center for Applied Microbiology and Research, Salisbury, UK). Human Caucasian lung carcinoma cell line (A549) was obtained from the Institute of Immunology and Experimental Therapy Polish Academy of Sciences, Wrocław, Poland. The cell lines TE671 and A549 were grown in monolayer culture and the RPMI8226 cells in suspension culture. TE671 and A549 cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) (PAA Laboratories, Pasching, Austria). Iscove's medium (Biochrom AG, Berlin, Germany) was used for culture of the RPMI8226 cell line. All media were supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin (all from PAA Laboratories). The cultures were kept at 37˚C in a humidified atmosphere of 95% air and 5% CO_2.

shRNA-retroviral vectors and transduction. For the transduction of DNA-sequences encoding shRNA molecules, the self inactivating retroviral Moloney murine leukemia virus backbone pRVH-1-puro, described previously, was used (41). This vector contains a puromycin resistance gene driven by a CMV immediate early promoter and a H1 polymerase III promoter for the expression of shRNA molecules in reverse orientation. The following *GRIK2* (NCBI Gen Bank accession number NM_002088) and *GRIN2D* (NCBI Gen Bank accession number NM_000836) target sequences were identified using an algorithm provided by Ambion Inc. (www.ambion.com) and synthesized by Eurofins MWG Biotech, Ebersberg, Germany. After annealing, the upper and bottom strands were ligated into the *BglII*/*SalI*-restrictions sites of pRVH-puro. The sequences were as follows: *GRIK2* shRNA (372-392 bp) 5'-AAGGAGATCCCCCACATCAAG-3'; *GRIN2D* shRNA (1184-1204 bp) 5'-AACATCACGTGGGATAACCGG-3'. As control, we included a hairpin against luciferase, according to Elbashir *et al*. (42). Retroviral particles were generated as described previously by Soneoka *et al*. (43). Briefly, 293T cells were cotransfected with an expression construct for gag-pol (pHIT60), the MoMuLV-based retroviral vectors and the vesicular stomatitis virus G-protein (pMD.G2). Viral supernatants were harvested 48 and 72 h after transfection. Target (10^5) cells were plated in 30 mm dishes the day before transduction, then transduced with retroviral supernatants and selected with puromycin (0.5 μg/ml; Sigma-Aldrich, Taufkirchen, Germany).

Transfection and establishment of stable transfectants. For silencing of the *GRIA4* and *GRIN1* target genes, we used the p*Silencer*™ puro expressions vector system (Ambio Inc., Austin, TX, USA) according to the manufacturer's instructions. The shRNA sequences were designed to target human *GRIA4* (NCBI Gen Bank accession number NM_001077243) and *GRIN1* (NCBI Gen Bank accession number NM_007327) using the shRNA target finder and design algorithm for p*Silencer*™ plasmid. The sequences were as follows: *GRIN1* shRNA (625-645 bp) 5'-AACGACCACTTCACTCCCACC-3'; *GRIA4* shRNA (1989-2009 bp) 5'-AAGCCCTTCATGAGTTTGGGC-3'. For the negative control, p*Silencer*™ 3.1-H1 puro negative control plasmid (named scramble) was used. This shRNA sequence does not match any known human, mouse or rat genes. The oligonucleotides were ligated in the p*Silencer*™ puro plasmid and sequenced. The expression vectors were transfected into cancer cells using FuGENE 6 transfection reagent (Roche, Mannheim, Germany). Transfectants were selected by exposure to puromycin (0.5 μg/ml) for 14 days.

Semiquantitative reverse transcription polymerase chain reaction (RT-PCR). The cDNA (1-2 μl) was amplified by endpoint polymerase chain reaction for *GluR1, GluR2, GluR3*, synapse-associated protein 97 (*SAP97*), glutamate receptor binding protein (*GRIP*), AMPA receptor binding protein (*ABP*) and protein interacting with protein kinase C 1 (*PICK1*). In brief, cDNA was subjected to PCR in a 25 μl reaction mixture, containing 5.0 μl 5X PCR buffer with 1.5 mM $MgCl_2$, 3 mM dNTPs, 0.4 μM of forward and reverse primers and 0.5 U Go Taq® DNA Polymerase (all materials obtained from Promega Mannheim, Germany). The cDNA was amplified in 30-33 cycles, consisting of denaturing over 30 s at 94˚C, annealing over 60 s at 60˚C, and primer extension over 60 s at 72˚C. Amplified cDNA was subjected to polyacrylamide gel electrophoresis and subsequent silver staining. As a positive control, commercially available RNA obtained from human brain (HB) (Ambion, Austin, TX, USA) was used. The housekeeping gene human ribosomal protein L41 (*hRPL41*) was co-amplified in multiplex PCR as an internal control.

Real time PCR. The cell pellets were snap-frozen and stored at –80˚C until analysis. Total RNA was extracted by selective binding properties of a silica based membrane using the Qiagen RNeasy mini kit (QIAGEN, Hilden, Germany) including DNaseI treatment. The concentration of RNA was determined spectrophotometrically, and the integrity of all samples was confirmed by electrophoresis in ethidium bromide-stained 1.0% agarose gels. RNA (1 μg) was reverse-transcribed with Moloney murine leukemia virus reverse transcriptase, RNasin and dNTPs (all Promega, Mannheim, Germany) in 35 μl of reaction mixture. The resulting cDNA was used for PCR with a primer pair for human β-actin (ACTB) (forward primer: ATCATGAAGTGTGACGTGGAC, reverse primer AACCGACTG CTGTCACCTTCA) to confirm the outcome of the cDNA synthesis. Real-time PCR was performed in order to obtain a quantitative

relationship between PCR product obtained from the genes of interest and from the housekeeping gene (*hRPL41*). PCR primer sequences were retrieved from the online Primer Bank database (http://pga.mgh.harvard.edu/primerbank/index.html). Quantitative RT-PCR assays were carried out by using 7300 Real-Time PCR System (Applied Biosystems, Darmstadt, Germany) with SYBR green fluorescence. The amplification of PCR products was performed in 20 μl of reaction mixture containing 1 μl of cDNA, 0.5 U Platinum® *Taq* DNA Polymerase (Invitrogen, Karlsruhe, Germany), 1 × PCR Buffer with SYBR green, 2 mM $MgCl_2$ (Invitrogen), dNTPs each 62.5 μM (Promega), 1 × ROX (Invitrogen), 0.5 μM of forward and reverse primers and 1 μl of cDNA. The rhodamine derivative ROX is the passive reference label in this reaction. PCR was carried out beginning with 3 min at 95˚C followed by a denaturation step at 95˚C for 10 s, an annealing step at 60˚C for 15 s, and an extension step at 72˚C for 30 s for 40 cycles. The identity of the PCR products were assayed with a dissociation curve analysis (95˚C for 15 s and from 60˚C to 99˚C at each temperature for 15 s) and by PAGE-electrophoresis with silver staining. For the quantification of each PCR product, the threshold cycle (ct), which determines the cycle when sample fluorescence exceeds a chosen threshold above background fluorescence, was used. Δct value was calculated from the difference of the ct for the gene of interest from the ct of the housekeeping gene.

Preparation of protein extracts. The cultured cells were harvested, washed with cold PBS and lysed in modified RIPA buffer, 1% IGEPAL® R CA-630, 1.0% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4, 20 mM NaF, 0.5 mM DTT, 1 mM PMSF and protease inhibitor cocktail (Roche, Mannheim, Germany) in PBS, pH 7.4. After 30 min incubation period, cell lysates were centrifuged at 3,000 × g for 10 min. Total protein content was measured in the supernatant fractions by MicroBC Assay Protein Quantification Kit (Interchim, Montluçon Cedex, France).

Western blotting. Western blotting analysis was used to detect shRNA-mediated inhibition of target genes. Equal amounts of cellular proteins (40 μg) were solubilized in 3 × Laemmli sample buffer (30% glycerol, 3% SDS, 0.19 M Tris-HCl, pH 6.8, 3% β-mercaptoethanol, 0.015% Bromphenol blue) and boiled for 5 min at 95˚C. Protein samples were electrophoretically separated on a 7.5% SDS-polyacrylamide gel under reducing conditions and transferred by electroblotting onto a PVDF membrane (Roth, Karlsruhe, Germany). Membranes were blocked for 1 h at room temperature with 1 × Animal-free Blocking (Vector Laboratories Inco., Burlingame, CA, USA) and then incubated with primary antibody in blocking solution overnight at 4˚C. The conditions of the primary antibody were the following: GRIN2D (1:200; Santa Cruz Biotechnologies Inc., Heidelberg, Germany), GRIK2 (1:250; Millipore, Schwalbach, Germany), GRIA34 (1:300; Santa Cruz, Biotechnologies Inc., Heidelberg, Germany), GRIN1 (1:300; Santa Cruz, Biotechnologies Inc., Heidelberg, Germany) in blocking buffer. Horseradish-conjugated immunoglobulins were used as secondary antibody (1:3000; Sigma-Aldrich, Steinheim, Germany) in blocking buffer, and proteins were detected using ECL system and autoradiography film (Hyperfilm ECL; GE Healthcare, Munich, Germany).

Cell viability assay. The transfected and selected cell populations were plated on 96-well microplates (Greiner bio-one, Frickenhausen, Germany) at a density of 2×10^4 cells per well in culture medium for 72 h. Cell viability was determined by addition of the yellow tetrazolium salt 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, Steinheim, Germany), which is metabolized by viable cells to purple formazan crystals for 3 h. Formazan crystals were solubilized with 10% SDS, 0.6% acetic acid in DMSO and the product was spectrophotometrically quantified by measuring absorbance at 570 nm by using Infinite® 200 reader (Tecan, Crailsheim, Germany).

Cell proliferation assay. Quantification of cell proliferation was based on the measurement of BrdU incorporation during DNA synthesis. Cultured cells were incubated with BrdU-labeling solution (Cell Proliferation ELISA, BrdU; Roche) for 2 h. Afterwards the cells were fixed with FixDenat solution and incubated with anti-BrdU-HRP-Fab-fragments to bind the BrdU in the newly synthesized cellular DNA. The immune complexes were detected by using 3,3',5,5'-tetramethylbenzidine (TMB) substrate and quantified by measuring the absorbance at 370 nm (Infinite® 200 reader; Tecan.).

Cytotoxicity assay. Detection and quantification of cytotoxicity in the cell population after transfection and selection were performed with Cytotoxicity Detection Kit^{Plus} (Roche) based on measurement of lactate dehydrogenase (LDH) activity through a coupled enzymatic reaction, whereby yellow tetrazolium salt is reduced to red formazan salt. The LDH reaction mixture was directly added to the cultured cells on a 96-well microplate. The reaction was stopped with stop solution after incubation at room temperature for 20 min. The amount of formazan salt is directly related to LDH activity in the supernatant. Absorption of reaction product was measured spectrophotometrically at 490 nm (Infinite® 200 reader; Tecan).

Transwell assay for determination of cellular migration. We evaluated the effect of *GRIA4* shRNA on invasiveness properties of TE671 cells using transwell migration assays. These were performed in a 24-well Transwell chamber system (Corning, Chorges, France) fitted with a polycarbonate membrane with 12 μm pore size. Cells for migration assay were trypsinized and resuspended in FCS-free DMEM. For each well, 5×10^5 cells were plated in the upper chamber. The lower wells were treated with 200 μg per well Matrigel™ (BD Bioscience, San Diego, USA) in DMEM with 10% FCS as chemoattractant. After 48 h incubation, cells that did not migrate through the pores of the membrane were mechanically removed from the upper surface of the membrane; the migrated cells on the lower surface of membrane were stained with MTT for 1 h, photographed and then lysed with 10% SDS and 0.6% acetic acid in DMSO. For spectrophotometric detection a wave length of 570 nm was used. Data were normalized to the measured value of shRNA control.

Wound healing assay. Cell migration ability was monitored using a wound healing assay. Stably transfected cells of parental line TE671 were plated on 6-well plates and grown to 90% confluence in native culture medium. The layer of cells was mechanically injured by scoring a line with a sterile pipette tip. Loose cells were removed by washing the culture well with native medium. The cells were cultured for a further 48 h. At 0 h, 24 h and 48 h photographic images were captured (Axiovert, Zeiss) under ×40 magnification.

PCR array. The Human Cancer PathwayFinder™ RT^{2}Profiler PCR Array analyzes the expression of 84 genes representative of six biological pathways involved in transformation and tumorigenesis. The

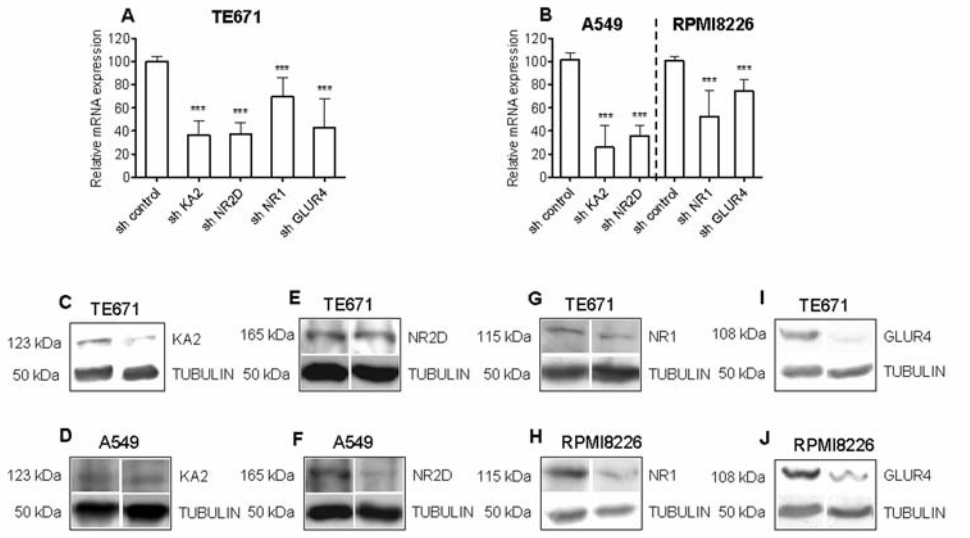

Figure 1. *Expression of receptor subunits KA2, NR2D, NR1 and GLUR4 in TE671, A549 and RPMI8226 cell populations after stable knockdown of KA2, NR2D, NR1 or GLUR4. Quantitative real-time PCR analysis of mRNA expression for KA2, NR2D, NR1 and GLUR4 in TE671 cells (A) and (B) in A549 or RPMI8226 cells. The relative mRNA levels in the shRNA control populations were set as 100%. Results represent the mean±SD. KA2 expression at the protein level was assayed using Western blot in KA2 shRNA transduced TE671 cells (C) and A549 cells (D). Protein level of NR2D in stable NR2D knockdown of TE671 cells (E) and A549 cells (F). Detection of NR1 protein in NR1 stable knockdown TE671 populations (G) and RPMI8226 cells (H). Western blot analysis of protein expression for GLUR4 in TE671 (I) and RPMI8226 cells (J) with stable knockdown of GLUR4 and (J). As loading control, detection of tubulin was used in all samples. Data of real-time PCR represent the mean mRNA expression±SD from three independent experiments, normalized to the shRNA control. Images of Western blot show one representative experiment.*

qRT-PCR arrays were performed with Human Cancer PathwayFinder™ RT2Profiler PCR Array using the qRT-PCR Master Mix (Promega) on a 7300 Real-Time PCR System (Applied Biosystems, Darmstadt, Germany). Five housekeeping controls and PCR controls were included in every array. Expression of analyzed genes was normalized with respect to each housekeeping gene using the ΔΔct method for comparing relative expression (up-regulation >2.0 and down-regulation <0.5). The PCR array results were verified using quantitative real-time PCR with synthesized specific primer for several regulated genes (Primer Bank database, http:// pga.mgh.harvard.edu/primerbank/ index.html) and housekeeping gene hRPL13a.

Statistical analysis. Experiments were repeated three times and data are expressed as the mean±SD. Data were analyzed by means of one-way ANOVA with Dunnett's multiple comparison.

Results

Procedure of experimental analysis. In previous studies we demonstrated that NMDA, kainate and AMPA receptor subunits are differentially expressed in various cancer cell lines and pediatric brain tumors. Expression of *KA2, NR2D* and *GLUR4* was higher in the high-grade tumors compared to human brain (HB), whereas the GLUR subunit *NR1* mRNA was detected in few tumors. The rhabdomyosarcoma/ medulloblastoma cell line TE671 expressed mRNA for *KA2*, *NR2D, NR1* and *GLUR4* at a high level compared to human brain control. Therefore, this cancer cell line was considered ideal for attempting to silence subunit expression with shRNA. In addition, we used the lung cancer cell line A549, which showed high mRNA levels for *KA2* and *NR2D* and the multiple myeloma cell line RPMI8226 which expresses NR1 and GLUR4 (36, 40). A key question was whether protein expression of *KA2, NR2D, NR1* and *GluR4* subunits would influence malignant phenotype of cancer cell lines *in vitro*.

Transfer of shRNA to cancer cells inhibits expression of mRNA and protein. Levels of mRNA for *KA2, NR2D, NR1* and *GLUR4* were measured using quantitative RT-PCR, as shown in Figure 1A and B. In TE671 cells (Figure 1A) all four shRNAs exhibited a significant silencing effect ($p<0.05$) and knocked down 63.9% to 30.5% of mRNA expression in comparison with shRNA control. The *KA2* and *NR2D* shRNAs led to stronger gene silencing than the shRNAs of the other two receptor subtypes. The knockdown of *KA2, NR2D, NR1* and *GLUR4* showed similar results in A549 and RPMI8226 cells (Figure 1B). The same shRNA sequences led to different degrees of silencing in two different cancer cell lines (for example sh *GLUR4* in TE671 42.9%±25.1 and in RPMI8226 74.6%±10.1). The silencing effect of receptor subunit expression at the protein level was confirmed with Western blot. As loading control, tubulin protein level was used. The immunoblot experiments yielded results similar to the quantitative RT-PCR. The amounts of KA2 (Figure 1 C-

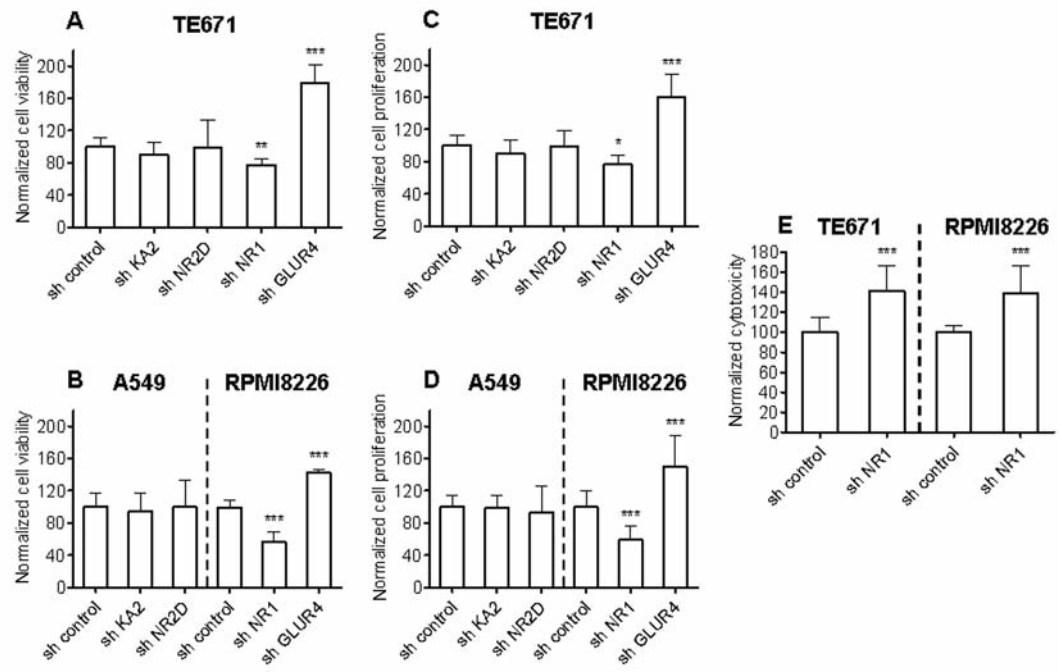

Figure 2. *Determination of phenotype from stable knockdown populations. Normalized cell viability after stable knockdown of NR1, KA2, NR2D or GLUR4 in TE671 (A), A549 and RPMI8226 cells (B). BrdU incorporation was used to analyze cell proliferation following gene silencing in TE671 (C), A549 and RPMI8226 (D) cells. Data represent the mean of cell viability or proliferation±SD from three independent experiments, normalized to the shRNA control. LDH release from cells with stable knockdown of the NR1 subunit was increased (E). Values represent means±SD normalized to the shRNA control from three independent experiments.*

D), NR2D (Figure 1 E-F), NR1 (Figure 1 G-H) and GLUR4 (Figure 1 I-J) proteins were lower in all cancer cell lines compared to shRNA control, however at different degrees. For example, GLUR4 protein was very weakly expressed compared to shRNA control in shRNA transfected TE671 cells, but in transfected RPMI8226 cells more GLUR4 protein was expressed compared to TE671 (Figure 1I-J). The comparison of sh *GLUR4* and shRNA control in RPMI8226 showed silencing of protein expression as well. Collectively, the results indicate that the shRNAs for *KA2, NR2D, NR1* and *GLUR4* were capable of down-regulating mRNA transcripts (*KA2, NR2D, NR1* and *GLUR4*) and the respective proteins.

Gene silencing alters viability and cell proliferation. We examined the effect of *KA2, NR2D, NR1* and *GLUR4* knockdown on viability and proliferation of different cancer cell lines. As shown in Figure 2A–D, knockdown of *KA2* and *NR2D* does not change the cell phenotype. Cell viability and proliferation were similar to that of the shRNA control cells in TE671 and RPMI8226 cells. This implies that gene silencing of *KA2* and *NR2D* did not alter phenotype of TE671 and A549 cells. Interestingly, both cancer cell lines demonstrated reduced cell viability in comparison to shRNA control after shNR1 transfection (Figure 2A for TE671, and Figure 2B for RPMI8226). In TE671 cells, viability was 77.63%±7.4 and in RPMI8226 cells 76.7%±11.7 compared to the shRNA control cells. To confirm that silencing of *NR1* indeed reduced cell viability, we analyzed cell proliferation by studying BrdU incorporation into cellular DNA. In both tumour cell lines, cell proliferation was significantly reduced (Figure 2C for TE671 and Figure 2D for RPMI8226). The results indicate that shRNA-mediated specific knockdown of *NR1* inhibited cancer cell growth. We hypothesized that perhaps cytotoxic effects are the reason for the reduced cell viabiltity and proliferation after stable knockdown of *NR1*. To validate the findings, we examined the amount of LDH release (Figure 2E) in both cell lines. LDH release was significantly increased in cell populations transfected with shNR1 compared to shRNA control in TE671 and RPMI8226 cells. Stable knockdown of *NR1* caused an increase of LDH in the supernatant, either due to cell death or plasma membrane damage. We examined the effect of *GLUR4* knockdown on TE671 and RPMI8226 cell viability and proliferation. Stable knockdown of *GLUR4* resulted in a significant increase of cell viability. For instance, the sh *GLUR4* population in both cell lines exhibited remarkably increased viability compared to the shRNA control population (178.5%±22.5 in TE671 and 141.8%±5.0 in RPMI8226). Cell

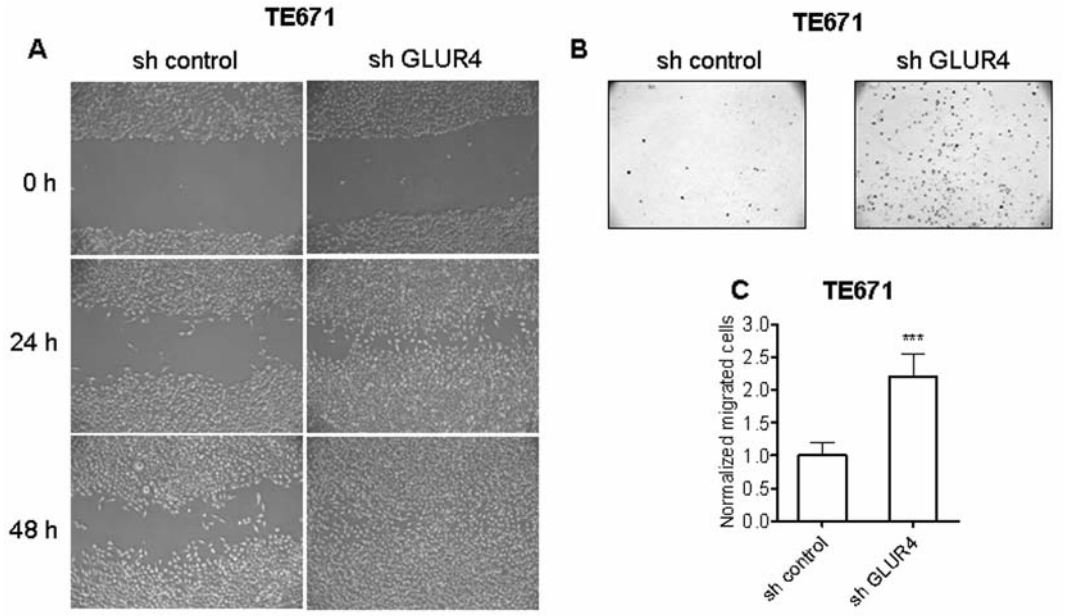

Figure 3. *Effects of knockdown of GLUR4 on migration in TE671 cells. A: Images represent wound-healing assay from two different transfected cell populations (shRNA control and sh GLUR4) of TE671 cells at distinct time points (0, 24, 48 hours). B: Dark stained (dead) and living cells are on the lower surface of the Transwell membrane. After a 48 h migration period, cell viability was assessed with the MTT assay. C: Bars show normalized mean values of migrated cells following a 48 h incubation period. Data represent the means±SD from three independent experiments, normalized to the shRNA control.*

proliferation was also significantly higher in the population sh *GLUR4* compared to the shRNA control. Taken together, these results indicate that silencing of *GLUR4* increases the viability and proliferation of two different cancer cell lines.

Silencing of GLUR4 stimulated migration of TE671 cells. Cancer cell migration is directly related to metastatic potential. We showed that stable knockdown of *GLUR4* promoted cell viability and proliferation *in vitro*. To study whether *GLUR4* silencing would influence cell mobility, we assayed cell migration in the wound healing assay and the transwell culture system. Wound-healing 'scratch' assay, performed under standard cell culture conditions, demonstrated lower migration ability of TE671 cells transfected with shRNA control than those transfected with sh *GLUR4* (Figure 3A) 24 h and 48 h after scratching. The wound closure was nearly complete in the sh *GLUR4* cell population at 48 h. In the transwell culture assay, significantly more cells transfected with sh *GLUR4* were present in the lower compartment compared to shRNA control cells. As shown in Figure 3C, the normalized number of stably transfected cells that passed through the membrane in sh control (1.00±0.19) was remarkably lower than that in the sh *GLUR4* cells (2.19±0.35). The results show that functional knockdown of *GLUR4* increases migratory potential of tumor cells (Figure 3B).

In summary, we demonstrated that plasmid-based RNA interference has the potential to inhibit expression of KA2, NR2D, NR1 and GLUR4 target molecules. A reduced level of GLUR4 in *GLUR4* silenced TE671 and RPMI8226 cells led to a strong increase of viability, proliferation and propensity to migrate. These results provide strong evidence that the knockdown of *GLUR4* supports growth and invasion of TE671 cancer cells. Therefore, we analyzed the effect of *GLUR4* silencing on mRNA expression of other AMPAR subunits (*GLUR1, GLUR2* and *GLUR3*) and AMPA anchor proteins (*SAP97, GRIP, ABP* and *PICK1*) using quantitative real-time PCR. To gain first insight into molecular mechanisms mediating phenotypic changes following stable knockdown of *GLUR4*, we also evaluated its effect on mRNA expression of 84 genes known to modulate viability, proliferation and metastasis in human cancer.

Modulation of expression of mRNA for AMPAR subunits and AMPA-binding proteins in TE671 and RPMI8226 cells. The expression of mRNA for AMPAR subunits (*GLUR1, GLUR2, GLUR3*) and AMPA binding proteins (*SAP97, GRIP, ABP, PICK1*) was investigated in the cell population stably transfected with sh *GLUR4*. As indicated in Figure 4A, in RPMI8226 cells with knockdown of *GLUR4* expression of *GLUR3* and *ABP* mRNA increased compared to shRNA control. As positive control, RNA of brain from different genders was used. We used quantitative real-time PCR to confirm these results in TE671 cells. The mRNA expression of *GLUR3* (3.0±0.3) (Figure 4B) and ABP (4.3±1.8) (Figure 4C), were increased compared to shRNA control. The

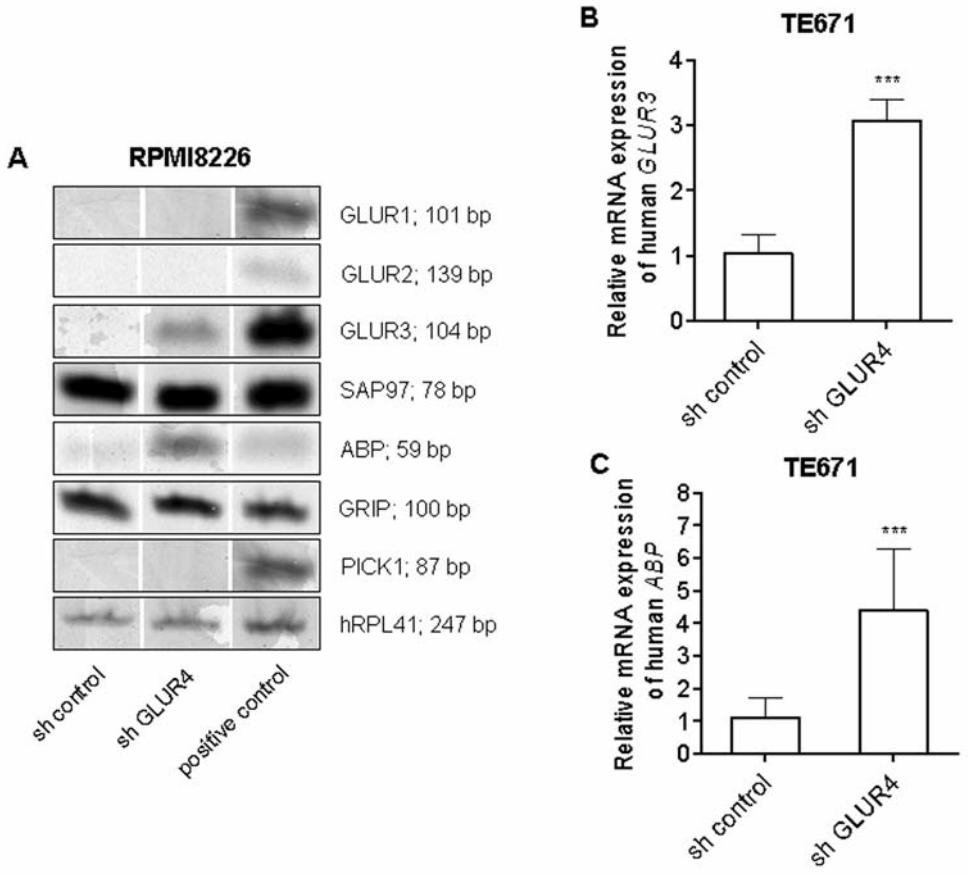

Figure 4. *Gene silencing of AMPA receptor subunit GLUR4 leads to increased gene expression of other AMPA receptor subunits in stably transfected TE671 and RPMI8226 cells. A: Analysis of gene expression of all AMPA receptor subunits and their AMPA-binding proteins after knockdown of GLUR4 in RPMI8226 cells. Polyacrylamide gel electrophoresis with silver staining illustrating representative results of RT-PCR analysis. The housekeeping gene hRPL41 was coamplified as an internal control. Images show one representative experiment. Significant up-regulation of GLUR3 (B) and its AMPA receptor binding protein ABP (C) after knockdown of GLUR4, as detected in TE671 cells by means of quantitative real-time PCR. The relative levels of mRNA in the shRNA control population were set as 100%. Results represent the means±SD from three independent experiments.*

expression of *GLUR1* and *GLUR2* and the expression of AMPA-binding proteins *SAP97, GRIP* and *PICK1* were unaffected by down-regulation of *GLUR4*. The same results were obtained using two different methods, semiquantitative PCR as endpoint PCR, and quantitative real-time PCR. This indicates that silencing of *GLUR4* influences composition of AMPAR complexes and the expression of other functionally related genes and proteins.

Differential gene expression after stable knockdown of GLUR4 in TE671 and RPMI8226 cells. We evaluated the effect of *GLUR4* gene and protein knockdown on expression of selected genes involved in cell cycle control and DNA damage repair, apoptosis and cell senescence, signal transduction molecules and transcription factors, adhesion, angiogenesis, invasion and metastasis. Using the Human Cancer PathwayFinder™ PCR array we analyzed the expression of 84 genes from the knockdown cell population and compared it to the expression in shRNA control cell populations comparing the expression of genes of interest to five different housekeeping genes. We, then, identified genes whose expression was altered after silencing of *GLUR4* and confirmed findings using quantitative real-time PCR. We did not detect changes in the expression levels of fundamental cell cycle regulating genes such as *p53* (NM_000546), cyclin E1 (*CCNE1*) (NM_001238) and cyclin-dependent kinase 2 (*CDK2*) (NM_001798). However, in the stably transfected cell population sh *GLUR4*, we detected a dramatic increase of synuclein gamma (*SNCG*) (NM_003087) (Figure 5A) and matrix metallopeptidase 2 (*MMP2*) (NM_004530) (Figure 5C) expression compared to shRNA control in both cell lines. We also found that nuclear factor of kappa light polypeptide gene enhancer in B-cells (*NFκB*) (NM_003998) was slightly down-regulated. Metastasis associated 1 family, member 2 (*MTA2*) (NM_004739), *SNCG*, integrin beta 3 (*ITGB3*) (NM_000212) and myelocytomatosis viral oncogene homolog

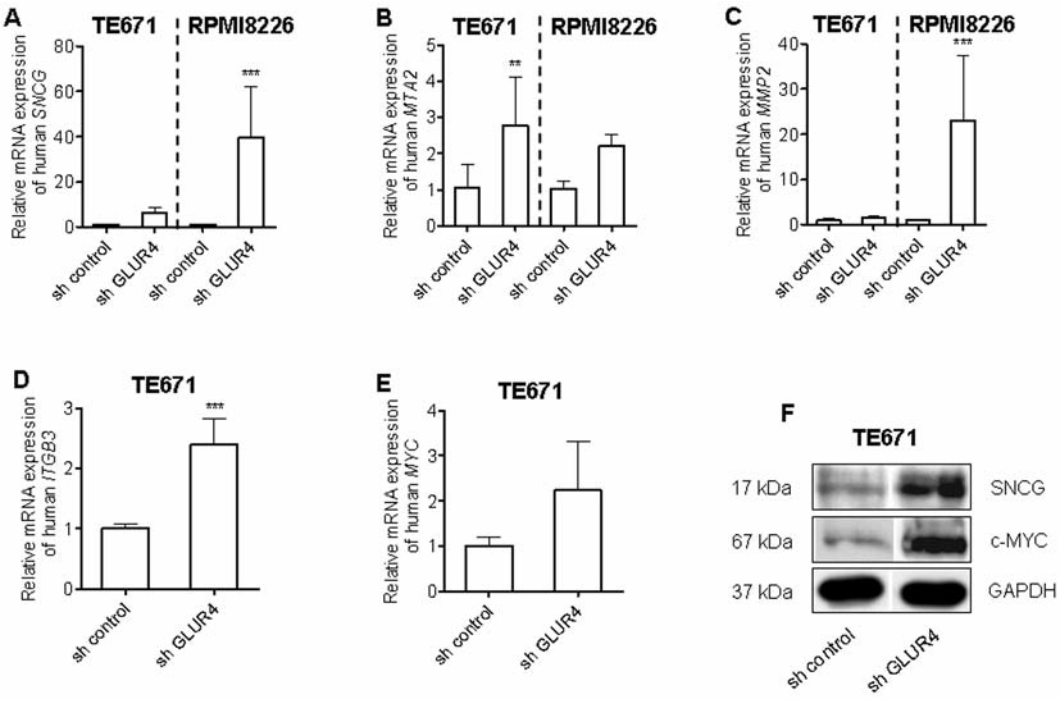

Figure 5. *Quantification of gene expression detected by PCR Array (Human Cancer PathwayFinder™) after knockdown of GLUR4 in human cell lines TE671 and RPMI8226 reveals significant increases for SCNG (A), MTA2 (B), MMP2 (C), ITGB3 (D) and MYC (E) expression. The relative levels of mRNA in the shRNA control group were set as 100%. Results represent the means±SD from three independent experiments. D: Stable knockdown of GLUR4 increased the protein level of SNCG and c-MYC. The housekeeping protein GAPDH was used as internal control. Blots show one of three representative experiments in TE671 cells.*

(*MYC*) (NM_002467) were up-regulated in TE671 cells after knockdown of *GLUR4* (Table I; up-regulation >2.0 and down-regulation <0.5 of relative gene expression). The strong up-regulation of SNCG protein expression on silencing of *GRIA4* was confirmed by Western blot analysis in TE671 cells (Figure 5F). Changes in protein expression in cells stably transfected with shRNA for *GLUR4* in comparison with the shRNA control were observed in both studied cell lines (TE671 and RPMI8226). Interestingly, we observed that the protein level of c-MYC was strongly increased in cells with knockdown of *GLUR4* compared to the shRNA control. No differences were found in the expression of the housekeeping protein GAPDH. We have demonstrated that in TE671 and in RPMI8226 cells with stable knockdown of *GLUR4* the mRNA expression patterns are changed: there is up-regulation of signal transduction molecules (*SNCG* and *MYC*), genes involved in invasion and metastasis (*MMP2* and *MTA2*) and genes that encode adhesion proteins (*ITGB3*).

Discussion

Here we show that knockdown of selected GLUR subunits modulates cancer cell proliferation and invasive behaviour. Reduced expression of GLUR4 markedly increased propensity of cancer cells to proliferate. In contrast, cell viability and proliferation were lower in the cell populations with knockdown of *NR1* in both cell lines. Knockdown of *KA2* and *NR2D* did not influence cancer phenotype in the two studied cancer cell lines, TE671 and RPMI8226. Furthermore, gene silencing of *GLUR4* modulated the mRNA expression of various genes in these cancer cell lines, as determined with the Human Cancer PathwayFinder™ PCR Array. The expression of *GLUR1* and *GLUR2* and the expression of AMPA-binding proteins, *SAP97, GRIP1* and *PICK1* were unaffected by down-regulation of *GLUR4*, whereas expression of *GLUR3* and *ABP* was increased. This indicates that silencing of *GLUR4* influences composition of AMPAR complexes and the expression of other functionally related genes and proteins.

Previous work by our group revealed that glutamate receptor subunits are differentially expressed in a variety of human cancer cell lines and human tumors (36, 40). For the majority of tumours, expression levels of *NR2B, GLUR4, GLUR6* and *KA2* were found to be lower compared to human brain tissue but in some tumours, some subunits were expressed at levels close to or higher than those in human brain tissue. This applies to TE671 and RMPI8226 cells for *GLUR4* and for *KA2*. NMDAR subunits were uniformly

Table I. *mRNA expression of various genes after knockdown of GLUR4 in TE671 and RPMI8226 cells performed with quantitative real-time PCR. The relative levels of mRNA in the sh control population were set as 100%. Results are presented as the mean±SEM from three independent experiments.*

TE 671	sh control	sh GLUR4
SNCG	1,01 (0,08)	6,38 (0,82)***
MTA2	1,06 (0,24)	2,76 (0,50)*
ITGB3	1,00 (0,02)	2,40 (0,15)**
MYC	1,01 (0,06)	2,23 (0,40)
NFKB	1,00 (0,02)	0,76 (0,13)
RPMI 8226	**sh control**	**sh GLUR4**
SNCG	1,05 (0,29)	56,30 (18,39)***
MMP2	1,03 (0,20)	23,12 (5,28)***
ITGB3	1,00 (0,01)	4,53 (1,06)
MTA2	1,02 (0,19)	2,20 (0,12)**
NFKB	1,02 (0,17)	1,90 (0,21)*

expressed at levels much lower than those in human brain. Confocal imaging revealed that selected GLUR subunit proteins were expressed in tumour cells. By means of patch clamp analysis, we were able to show that TE671 cells depolarized in response to glutamate agonists application and that this effect was reversed by GluR antagonists, suggesting that functional Glu gated ion channels are formed by the expressed receptor subunits in tumour cells (36).

It has previously been shown that GluR antagonists (1, 2, 6), and silencing of selected receptor subunits (7, 8) inhibit proliferation of cancer cells. Overexpression of GLUR2 lead to inhibition of cell proliferation and induction of apoptosis in a glioma cell line (45) and knockdown of *GLUR1* reduced proliferation and increased apoptosis in two glioma cell lines (7). In selected gastric cancer cell lines, transfection with *KA2* plasmid influenced cell migration (46). Our findings provide further support for the notion that depending on the type of cancer, GLUR subunits modulate phenotype and malignant behaviour.

To gain insight into potential molecular pathways influenced by GLUR4 gene and protein knockdown we analyzed the expression of selected genes known to influence cancer phenotype using the Human Cancer PathwayFinder™ PCR array. In the stably transfected cell population sh *GLUR4* of RPMI8226 cells, we detected dramatic increase of *SNCG* and *MMP2* gene expression. In this cell line *NFKB* was down-regulated. A similar decrease of *NFKB* gene expression was found in the sh *GLUR4* population of TE671. *MTA2, SNCG, ITGB3* and *Myc* were up-regulated in sh *GLUR4* population of both cell lines after knockdown of *GLUR4*. *MTA2* encodes metastasis-associated protein 2 which has been identified as a component of NuRD, a nucleosome remodeling deacetylase complex identified in the nucleus of human cells. It is strongly expressed in many tissues and represents one member of a small gene family that encodes proteins involved in transcriptional regulation of chromatin remodeling (47, 48). MTA2 includes two DNA-binding domains, a dimerization domain, and a domain commonly found in proteins that methylate DNA. One target protein for this gene product is p53. Deacetylation of p53 is correlated with a loss of growth inhibition in transformed cells supporting a connection between MTA2 and metastasis. In general, MTA family members form independent nucleosome remodeling and deacetylation complexes and repress the transcription of different genes by recruiting histone deacetylases onto their target genes. Overexpression of MAT2 has been associated with hepatocellular carcinoma size and differentiation (49). The ITGB3 protein product is the integrin beta chain beta 3 (50). Integrins are integral cell-surface proteins composed of an alpha and a beta chain. A given chain may combine with multiple partners resulting in different integrins. Integrin beta 3 is found along with the alpha IIb chain in platelets. Integrins are known to participate in cell adhesion, as well as cell surface-mediated signaling. The impact of its up-regulation on tumour phenotype is unclear.

SNCG (synuclein gamma) encodes a member of the synuclein family of proteins, believed to be involved in the pathogenesis of neurodegenerative diseases. Mutations in this gene have also been associated with breast tumor development (33). Liu *et al*. (51) reported that levels of SNCG predict poor clinical outcome in colon cancer with normal levels of carcinoembryonic antigen. Their results suggest that SNCG is a new independent predictor for poor prognosis in patients with colon adenocarcinoma, including those with normal CEA levels. Hu *et al*. (52) reported that SNCG, combined with clinicopathologic features, may be used as an accurate predictor of liver metastasis in colorectal cancer. SNCG is a novel biomarker for aggressive disease and chemoresistance in uterine papillary serous carcinoma and merits further investigation both as a prognostic tool and as a therapeutic target (53). Hibi *et al*. (54) reported that SNCG is closely involved in perineural invasion and distant metastasis in mouse models and is a novel prognostic factor in pancreatic cancer. This is in agreement with our observations that increased expression of SNCG correlated with increased motility of TE671 and RPMI8226 cells. In transfection experiments with ovarian and breast cancer cell lines, overexpression of SNCG enhanced cell motility (55) and resistance to the chemotherapeutic drugs paclitaxel and vinblastine (38, 56). Overexpression of SNCG leads to constitutive activation of mitogen-activated protein kinase 3 (ERK1/2) and down-regulation of Jun N-terminal Kinase 1 (JNK1) (56, 57). Surguchov *et al*. (58) reported that SNCG localizes to spindle poles and associates with centrosome proteins.

These findings underline the role of SNCG in promoting tumor progression, cell proliferation, metastasis, invasion and resistance to chemotherapeutic drugs.

MMP2, a member of the matrix metalloproteinase (MMP) family is involved in the breakdown of the extracellular matrix in normal physiological processes, such as embryonic development, reproduction, and tissue remodeling, as well as in disease processes, such as arthritis and metastasis (59). Most MMPs are secreted as inactive proproteins which are activated when cleaved by extracellular proteinases. MMP2 is an enzyme which degrades type IV collagen, the major structural component of basement membranes. An increase in the expression of MMP2 is expected to increase the metastatic potential of tumour cells. Deb *et al*. (60) demonstrated that β amyloid peptide stimulates expression of MMP2 and MMP9 in astrocytes. In transfection studies, overexpression of SNCG led to up-regulation of MMP2 (61). The functionally active form of MMP2 colocalizes with integrin αvβ3 on the cell surface in melanoma tumours *in vivo* and in endothelial cells of human glioma-associated vasculature (62, 63).

The *MYC* oncogene encodes a transcription factor, c-MYC, that links altered cellular metabolism to tumourigenesis. It has been shown that MYC is a direct activator of RNA polymerase I and III, through its potential to make it a unique transcription factor that is able to regulate all three RNA polymerases (64). Thus it possible that MYC could influence cell growth, cell cycle progression and genome instability as a mechanism for cancer initiation. Wolfer *et al*. (65) demonstrated that c-MYC oncoprotein coordinately regulates the expression of different 'poor-outcome' cancer signatures. Functional inactivation of MYC in human breast cancer cells inhibits metastasis *in vivo* and invasive behavior *in vitro*.

The observed changes in expression of M*MP2, SNCG, MTA2, ITGB3* and *MYC* can explain increased proliferation and migration potential of TE671 and RPMI8226 cells following silencing of GLUR4. We show that stable knockdown of *GLUR4* significantly increased cell viability and proliferation in both cell lines. Furthemore, we observed that knockdown of *GLUR4* in TE671 cells increases cell mobility. These phenotypic traits are possibly the results of the observed changes in mRNA expression patterns after silencing of *GLUR4*.

The study of Ishiuchi *et al*. (1) showed that Ca^{2+}-permeable AMPARs are crucial for agonist-induced proliferation of glioma cells. Apart from classical ionotropic properties, evidence suggests that AMPARs can act through coupling with G-proteins (14) and activation of protein tyrosin kinase Lyn (66). It is plausible that GluRs may activate intracellular signaling pathways (67), which may affect cell growth, survival and proliferation of cancer cells. Causative for the aberrant cellular function of glutamate receptors are changes in genomic sequences, change in the level of expression and aberrant posttranscriptional processing. It has been reported that methylation of gene promoters of *NR2B* in gastric (32) and *NR2A* in colorectal carcinoma cells (68) influenced cancer cell growth, and, as demonstrated by knockout experiments, diminished expression of NR2A and GluR1 subunits and inhibited proliferation of cancer cells (7, 8). In contrast, the presence of rearranged or mutated forms of GLUR subunits may activate cancer cell growth.

There is substantial experimental evidence to suggest that Glu regulates tumour growth. GLUR expression may be particularly important for tumour growth in organs with high concentrations of Glu, and might also influence the propensity of such tumours to set metastases in Glu-rich organs, such as the liver and the brain. In this study, we provide further support for the notion that some GLUR subunits on cancer cells determine their phenotype and propensity to metastasize. Our studies also provide evidence that altered expression of GluR subunits on cancer cells can markedly change expression of genes that regulate DNA repair, angiogenesis, cell proliferation and metastatic potential. How GluR subunits on cancer cells are coupled to intracellular biochemical pathways that determine cancer phenotype, needs to be explored in future studies.

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