Indian Journal of Experimental Biology
Vol. 60, June 2022, pp. 386-396

Mild overexpression of a novel transcription factor Grainy head like-2 (Grhl2) elicits hybrid phenotype in human astrocytoma cells

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Received 23 September 2020; *revised 16 February 2022*

The Grainy head like-2 (Grhl2) transcription factor plays a major role in embryonic and cancer development. The role of Grhl2 has been intensively studied in various cancers but not for brain cancer. Hence, in this study, we provide a preliminary understanding on the role of Grhl2 that regulate the transition of astrocytoma cells. The human A172 astrocytoma cell line, a mesenchymal cell characterized by mild overexpression of Grhl2 transcription factor, was used in this study. At first, the Grhl2 stably overexpressing A172 clones into three types i.e., Grhl2+ (mild), Grhl2++ (moderate) and Grhl2+++ (high) based on mRNA and protein expression levels of Grhl2 were characterized. Phenotypic characteristics of vector and Grhl2+ cells were observed using phase contrast microscopy. Quantitative PCR (qPCR), Western blot and immunofluorescence were used to detect the level of mesenchymal markers (N-cadherin/vimentin) and also epithelial markers (E-cadherin/ β-catenin) in vector and Grhl2+ cells. The migration and invasion characteristics of vector and Grhl2+ cells were determined by scratch assay and Boyden chamber assay. Further, the Grhl2+ cells were characterized to determine the effect of temozolomide chemotherapy drug which were widely used in treating brain cancer. As expected, in phase contrast image, we observed the mesenchymal characteristic of A172 cells becomes hybrid phenotype i.e., mixture of mesenchymal (spindle-like fibroblast morphology) and epithelial (cobblestone like appearance) cells upon Grhl2 mild expression (Grhl2+) when compared to vector cells. Further, we found that there was a significant upregulation of E-cadherin at both mRNA and protein levels in Grhl2+ cells when compared to vector cells. There was a significant upregulation of β-catenin, N-cadherin and vimentin at mRNA levels, but there was no significant upregulation at the protein levels in Grhl2+ cells compared to the vector cells. The migration and invasion were diminished in Grhl2+ cells when compared to the vector control cells. We observed that the Grhl2+ were sensitive to the temozolomide compared to the vector cells. This infers that the Grhl2+ cells were unable to attain complete transition of mesenchymal to epithelial state, and hence we categorized the Grhl2+ cells as hybrid phenotype. The results provide a better understanding of the largely unknown function of Grhl2 in human astrocytoma cells as tumor progression or suppression.

Keywords: Boyden chamber assay, Brain **c**ancer, Cell migration, Chemotherapy, Embryonic development, Epithelial markers, Invasion, Mesenchymal markers, Scratch assay, Temozolomide, Tumor progression/suppression

Transcription factors have a domain that interacts with various promotor regions or RNA polymerase II and consequently regulates the amount of messenger RNA (mRNA) produced by the gene[1]. There are various transcription factors like TWIST, Slug, Snail and ZEB1 which were mainly involved in the embryonic development and various cancers[2-5]. These transcription factors also play a major role in brain cancer pathogenesis[6-9].

Emerging evidence enlightened that Grainy head like 2 (Grhl2), a novel transcription factor, play an important role in embryonic development viz., neural tube closure, wound healing and maintenance of epidermal integrity[10-12] and in various cancers, such as breast, colon, oral, ovarian, pancreatic, prostate cancers as indicated in Table 1[13-25]. The Grhl2 transcription factor is one of the 12 genes localized to an amplified region of chromosome 8q22 associated with poor prognosis and chemo-resistance in breast cancer[26]. A study comprising of 351 breast tumor expression datasets found elevated expression of several genes localized to 8q22, including Grhl2, specifically in high-grade tumors[27]. Several reports indicate the role of Grhl2 in cancer development and progression independent of 8q22 amplification[28].

The Grhl2 also plays a unique role in the control of cellular proliferation and differentiation through transcriptional regulation of its target genes, e.g.,

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Table 1 — Functions of Grainy head like 2 [Grhl2] transcription factor in various cancers

Cancer	Function of Grhl2
Breast cancer	Oncogene and Tumor suppressor[13]
Breast cancer	Tumor suppressor[14]
Colorectal cancer	Oncogene[15,16]
Gastric cancer	Tumor suppressor[17]
Human oral squamous cell carcinoma	Oncogene[18]
Oral cancer	Oncogene[19]
Ovarian cancer	Oncogene[20]
Ovarian cancer	Oncogene and Tumor suppressor[21]
Pancreatic cancer	Oncogene and Tumor suppressor[22]
Pancreatic cancer	Oncogene[23]
Prostate cancer	Oncogene and Tumor suppressor[24]
Sarcoma	Tumor Suppressor[25]

hTERT[18] and p63[29] and epidermal differentiation complex (EDC) genes[30]. The gain of Grhl2 has also been associated with oral squamous cell carcinoma[18], hepatocellular carcinoma[31], and murine follicular thyroid carcinoma[32] whereas, in silencing of this factor induced apoptosis through death receptor FAS ligand expression in fibrosarcoma (HT1080) cell line[33]. The Grhl2 has been shown to induce both epithelial to mesenchymal transition (EMT) and mesenchymal to epithelial transition (MET) in various cancers[13-25].

Epithelial-mesenchymal plasticity (EMP) has recently emerged in the field of cancer biology which encompassing bidirectional transitions among epithelial (E), mesenchymal (M), and one or more hybrid E/M phenotypes[34]. The Grhl2 denoted as phenotypic stability factors (PSF) which stabilize the hybrid E/M phenotype during the partial EMT[35]. This phenotypic heterogeneity (EM phenotype) was also associated with EMT and leads to cancer progression[36]. However, Grhl2 transcription factor is essential for the protection of epithelial phenotype, that activates by upregulating epithelial marker E-cadherin (CDH1) and microRNA-200 family members, that can suppress EMT associated transcription factor ZEB1, and force MET[14,28,37]. However, the overexpression of Grhl2 or E-cadherin may not always be enough to make complete MET[21,25,38]. These observations strengthen the feature that cells may navigate through various paths in the multi-dimensional landscape of EMP to undergo EMT or MET in a context-dependent manner. The alteration of Grhl2-associated RAD21 enrichment in epithelial genes is crucial to redefine the transition of cellular states along the EMT spectrum[39].

Although Grhl2 expression pattern was demonstrated by Riethdorf *et al.*[40] in normal and various malignant brain tumor (astrocytoma, oligodendroglioma, ependymoma and medullo blastoma) cells of non-epithelial origin and its Grhl2 expression was higher i.e., 93% percentage in astrocytoma compared to other malignant brain tumors, there is no study to our knowledge available elucidating the role of Grhl2 in brain cancer until now. Hence, the present study, we focused on brain astrocytoma based on the Grhl2 high expression than the other brain tumors.

Materials and Methods

Cell culture

Human A172 astrocytoma (ATCC, CRL-1620) cell lines were cultured as suggested by ATCC. Briefly, A172 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA, 11965-092) supplemented with 10% (v/v) heat inactivated fetal bovine serum (HI-FBS) (Gibco, USA, 10082147) and 1% penicillin-streptomycin-amphotericin B (Gibco, 15240062) and cultured in a humidified atmosphere containing 5% CO_2 at 37°C[41].

Plasmid

Full-length human Grhl2 cDNA clone was purchased (Open Biosystems, MMM1013-9201448), linked in frame with an HA-tag sequence at the 5' end, and sub-cloned into pcDNA 3.1 plasmid (Invitrogen, V79020). The pcDNA tagged with vector and Grhl2 were resolved in 1% agarose gel electrophoresis to validate the construct[42] (Suppl. Fig. S1. *All supplementary data are available only online along with the respective paper at NOPR repository at http://nopr.res.in*).

Stable overexpression cell line generation

Stable overexpression clones were generated by transfection of A172 cells with Grhl2 or empty vectors using Lipofectamine 3000 reagent (Invitrogen, L3000008), and supplementing the culture media with 1 mg/mL of G418 [gentamicin disulfate salt] (Sigma, A1720). After three weeks of selection, individual clones were isolated using cloning cylinders (Corning, CLS31666). Single cell-derived clones were subsequently expanded in selection media so as to involve in experiments. The maintenance culture medium was supplemented with 1 mg/mL of G418. At least 6 individual clones were characterized for all stable cell lines[42].

Cell morphology

Confluent vector control and Grhl2+ cells were trypsinized, counted and plated in 6-well culture plate

with complete medium at a density 1×10^4 cells/mL for 24 h. Cell morphology was accessed using phase contrast microscope (Leica DMIL LED) at 40X magnification. Three independent experiments were carried out to confirm cell morphological changes[2].

Quantitative real time polymerase chain reaction (RT-PCR)

Total ribonucleic acid (RNA) was extracted from human A172 vector control and Grhl2 over-expressed cells using TRIzol reagent (Sigma, T9424). The RNA was further purified by treating with deoxyribo nuclease (DNase) from TURBO DNase kit (Invitrogen, AM1907). To measure the gene expression, equal amounts of total RNA were converted to first-strand cDNA using SuperScript III First-Strand Synthesis System (Invitrogen, 11752250). The resulting cDNAs were used for real-time polymerase chain reaction (PCR) using TB Green Premix Ex Taq II mix (Takara, RR820A) in triplicates, which were performed using QuantStudio5 Real-time PCR System (Applied Biosystems) with the pre-set PCR program. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was used as an internal control. The sequences of primers used for real-time PCR experiments are shown as supplementary information (Suppl. Table S1.). PCR data analysis was performed using the log delta CT method normalized with GAPDH as an endogenous control gene. Fold change was calculated from gene expression for Grhl2+ cells when compared to vector control cells normalized to GAPDH as an endogenous control gene. The mean and standard deviation of fold change in expression of each gene was calculated[43].

Western blot analysis

Human A172 vector control and Grhl2+ cells were harvested and lysed proteins were extracted using nuclear protein buffer as described by Andrews & Faller[44]. The extracted nuclear protein was quantified by Lowry's method[45]. Proteins (20 µg) were subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membrane (GE Healthcare, 10600023)[46]. The membrane was blocked with either 5% BSA / nonfat dry milk powder in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 h at 25°C, and washed with PBS (X3) for 5 min followed by incubation with respective primary antibodies (Table S2) for 16 h at 4°C. Blots were washed with TBST for 30 min (X6) and incubate with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies for 2 h at 25°C. Blots were washed with TBST for 30 min (X6) and protein antibody complexes were visualized by enhanced chemi-luminescence (ECL) reagent (solution A & B, 1:1 ratio) (GE healthcare, RPN3245) in Fluorchem M instrument (Protein Simple) at 1 min exposure. The relative band intensity was analyzed using ImageJ software (Version 1.6.0_20) (NIH, Bethesda, MD)[47] and the data were shown as fold change of target protein / β-actin protein expression. Further, for stripping, the blots were incubated for 30 min at 25°C in stripping buffer (25 mM glycine, 1% SDS, pH 2.0) and blots re-probed with primary antibody appropriately. Blots were then incubated for 1 h at 25°C with HRP-conjugated secondary antibody and re-imaged.

Immunofluorescence analysis

Human A172 vector control and Grhl2+ cells (1×10^4 cells) were seeded in 8-well culture chamber (BD Falcon, 354108). After 24 h of incubation, cells were washed with ice-cold PBS (X3) and fixed with 4% paraformaldehyde for 15 min. Cells were then washed with PBS (X3) for 5 min and permeabilized with 0.25% Triton X-100 for 5 min at 25°C. The non-specific binding sites were blocked with blocking reagent (10% BSA and 5% horse serum in PBS) for 90 min at 25°C and then washed with PBS (X6) for 30 min, and incubated in respective primary antibodies (Table S2) for 16 h at 4°C. Slides were incubated in Alexa Fluor conjugated secondary antibodies for 2 h at 25°C. These cells were then washed with PBS (X6) for 30 min and a drop of gold anti-fade DAPI (4′,6-diamidino-2-phenylindole) reagent (Invitrogen, 879916) was added in each section of air dried slides. The cover slips were placed on the slides and air bubbles were removed gently. The target proteins in the cells were visualized under a fluorescence microscope (Leica DMIL LED) at 40X magnification, with excitation 340 nm/ emission 425 nm for blue filter and excitation 450 nm/ emission 530 nm for green filter. The corrected total cell fluorescence (CTCF) was calculated using Image J analysis (NIH, USA)[2].

Migration and invasion assays

Human A172 vector control and Grhl2+ cells were seeded in 6-well culture plate (Corning, 3506) at a density of 1×10^5 cells per well. A scratch was made with 200 µL sterile tips at 24 h later in the central area of the confluent culture, and cells were incubated for additional 72 h in fresh media after removing the

detached cells by careful washing. Cultures were observed at regular intervals and pictures of the wound area were taken using microscope[2].

Boyden chamber assays for quantitation of cell migration and invasion were performed using 96-well BME Cell Invasion Assay System (Cultrex, 3455-096-K) according to the manufacturer's instructions. Briefly, human A172 vector control and Grhl2+ cells 5×10^4 cells were seeded in triplicate in the top chamber on an 8 micron polyethylene terephthalate (PET) membrane, either uncoated for measuring migration, or pre-coated with basement membrane extract for measuring invasion. The plates were incubated for 48 h, further plates were washed with cell dissociation solution and the number of cells in the lower chamber was quantified by adding Calcein-AM solution, incubated additionally for 1 h, followed by measuring fluorescence at 485 nm excitation and 520 nm emissions to obtain relative fluorescence units (RFU) using TECAN instrument (Infinite 200 PRO) and the percentages of the cell migration and invasion was calculated and charting them against a standard curve prepared from known number of cells[2].

Cytotoxicity assay

Human A172 vector control and Grhl2+ cells (1×10^4) were seeded in the 96-well plate (Tarsons, 941196) for 12 h and then chemotherapy drug; temozolomide [TMZ] (GLIOZ-20-5519, Dr. Reddy's Laboratory) purchased from Christian Medical College Hospital Pharmacy, Vellore was treated with various concentrations ranging from 0 to 50 µM and incubated for 48 h in a CO_2 incubator. After incubation, the media and removed media and added MTT solution (SRL, 33611) (5 mg/mL in PBS) was added in each well and incubated for 4 h at 37°C. The MTT solution was removed and purple formazan crystals were dissolved with DMSO to each well and kept for 10 min at 25°C. The absorbance was measured at 570 nm[48] using Thermo MULTISKAN GO instrument. The optical density (OD) value was used to calculate the percentage of cell viability using the following formula.

$$\text{Percentage of cell viability} = \frac{\text{OD value of TMZ treated sample}}{\text{OD value of untreated sample}} \times 100$$

Statistical analysis

The statistical analysis was carried out in SPSS software (Version 16.0), Graph Pad Prism (Version 5.0) and Instat (Version 3.0). Data were represented as mean ± standard deviation. One way ANOVA using Dunnett's post test and student's "t" test were used to calculate the statistical significance between the two groups of data. A value of P <0.05 was considered statistically significant.

Results

Mild overexpression of Grhl2 in A172 cells elicits hybrid phenotype

To determine the importance of Grhl2-mediated cellular transformation, the oncogenic property was characterized with different levels of Grhl2 by stably expressing A172 cell line. We categorized the Grhl2 stably expressing A172 clones (at 11th passage) into three types i.e., Grhl2+ (mild), Grhl2++ (moderate) and Grhl2+++ (high) based on mRNA and protein expression levels of Grhl2. The schematic representation of different clones was shown in Fig. 1A. The different A172 clones elicit different level of Grhl2 mRNA and protein expression which was shown by quantitative PCR (Fig. 1B) and by Western blot (Fig. 1C) and immunofluorescence (Fig. 1D) respectively when compared to vector control A172 cells. Our results showed that, there was a significant upregulation in mRNA and protein level of Grhl2 overexpression of Grhl2+ (mild), Grhl2++ (moderate) and Grhl2+++ (high) in A172 stable clones when compared to A172 vector clones.

We hypothesized that Grhl2+ mild overexpression may lead to hybrid cellular transformation and might play a significant role in tumor suppression. Our results showed that Grhl2+ cells have changed the morphology of A172 vector cells from mesenchymal (Fig. 1E) to hybrid phenotype i.e., a mixture of both mesenchymal cells (spindle-like appearance leads to induction of cell motility) and epithelial cells (cobblestone-like appearance leads to gain of cell-cell adhesion) (Fig. 1F). Similarly, Grhl2++ and Grhl2+++ moderate and high expression also changed the morphology of A172 cells from mesenchymal to epithelial cells (cobblestone-like appearance) (Fig. S2).

Emergence of epithelial and decline of mesenchymal markers in mild overexpression of Grhl2

The emergence and decline of mesenchymal and epithelial markers (mRNA levels by qPCR and protein levels by western blot/immunofluorescence, respectively) were investigated in Grhl2+ A172 cells. Based on the results, significant upregulation of epithelial marker; E-cadherin at both mRNA (14 fold increase) and protein (1.2 fold increase) levels in Grhl2+ cells when compared to vector control cells

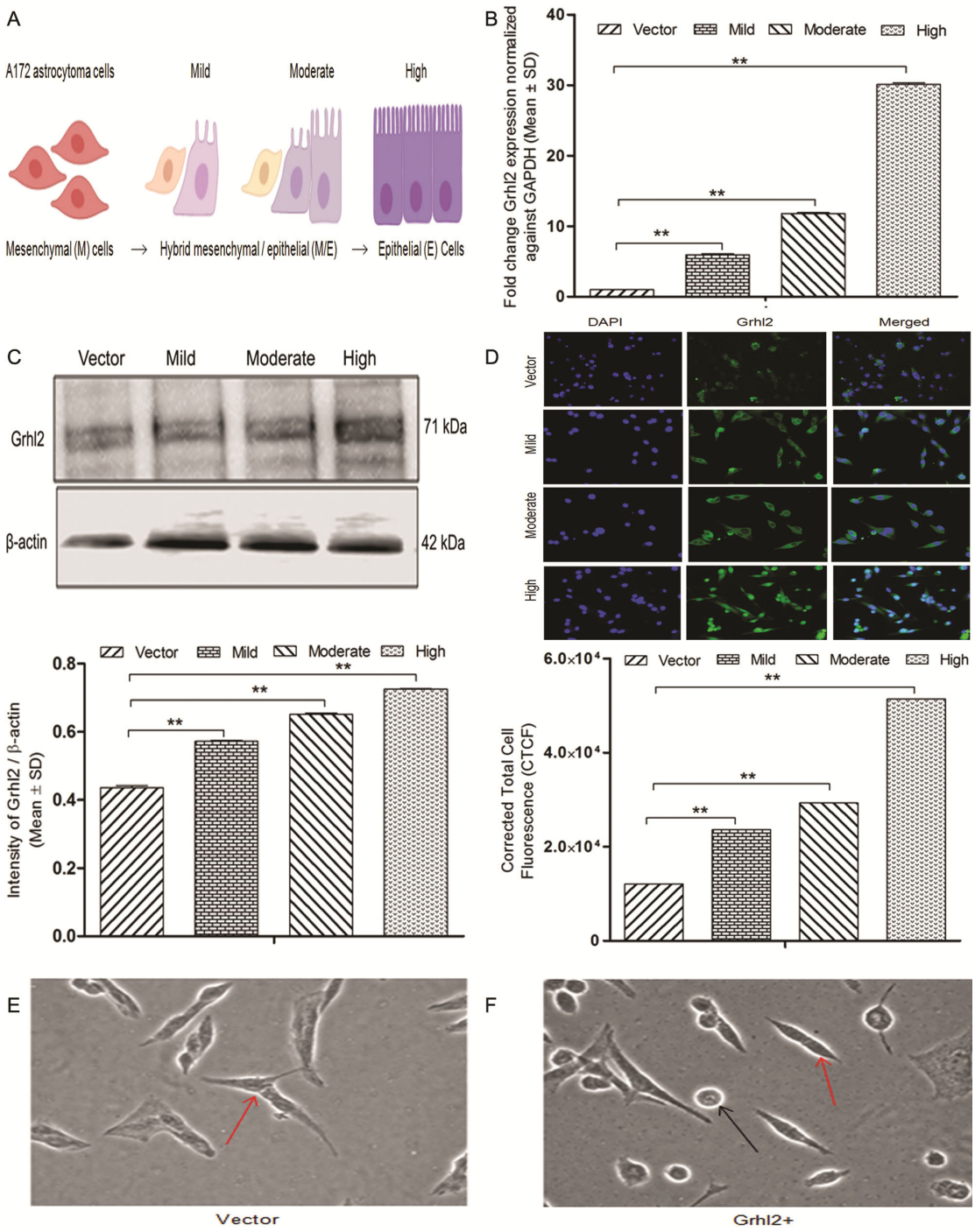

Fig. 1 — Different levels of Grhl2 in A172 astrocytoma cells and their phenotypic feature in mild Grhl2 over expressed cells. (A) Schematic representation of different clones showing mild, moderate and high expression of Grhl2 in A172 cell lines. Illustration created using Biorender.com; (B) qPCR fold change mRNA expression of Grhl2 normalized to GAPDH in Grhl2 transfected A172 cell lines (mild, moderate and high). The data was expressed in mean±SD of three independent experiments (**P <0.01); (C) Representative immunoblot of differential protein expression of Grhl2 in A172 cell line transfected with vector and Grhl2 (mild, moderate and high) and their band intensities were normalized to that of β-actin using Image J software (**P <0.01); (D) Protein expression of Grhl2 transfected (mild, moderate & high) A172 cells by immunofluorescence assay and their corrected total cell fluorescence intensity was calculated using ImageJ analysis software and significant values were calculated by one-way ANOVA using Dunnett's post test compared with vector cells (**P <0.01); (E) Vector cells showing rod shaped mesenchymal cells (red arrow); and (F) Grhl2+ cells showing both rod shape mesenchymal cells and cobble stone shape epithelial cells (black arrow) under phase contrast microscopy [Objective 40X].

were shown. Even though there was a significant upregulation of β-catenin mRNA (2.7 fold increase) were noted, but the protein level was not significantly upregulated in Grhl2+ cells when compared to vector control cells.

Further, there was significant upregulation of mesenchymal markers; N-cadherin (4.1 fold increase) and vimentin (4.6 fold increase) at mRNA level, but not at protein level in Grhl2+ cells when compared to vector control cells (Fig. 2 A-C).

Diminish migration and invasion in Mild overexpression of Grhl2 in A172 cells

Notably, while Grhl2+ cells did not undergo obvious morphological changes and noted as heterogeneity or hybrid phenotype with increase in E-cadherin (epithelial markers) a profile which leads to epithelial state. In this context, it is interest to determine the migration and invasion effect in the hybrid phenotype of A172 cells. The MET is associated with a decreased rate of directional

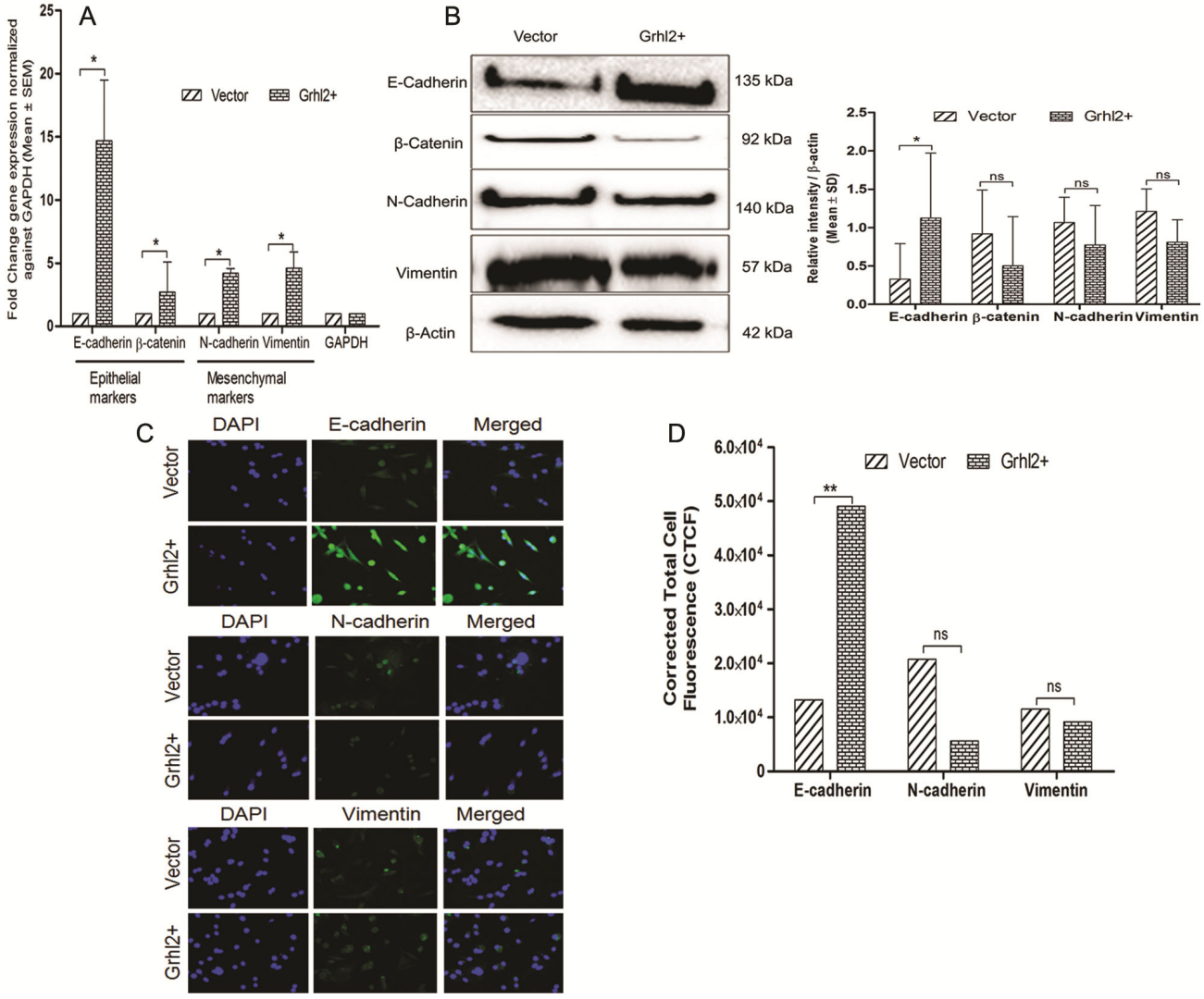

Fig. 2 — mRNA and protein expression of epithelial and mesenchymal markers in Grhl2 mild over-expressed A172 cells. (A) qPCR fold change mRNA expression of epithelial and mesenchymal markers normalized to GAPDH in Grhl2 transfected A172 cells. The data was expressed in mean±SEM of three independent experiments (*P <0.05); (B) Representative Immunoblot of protein expression of epithelial and mesenchymal markers in A172 cell line transfected with vector and Grhl2+ and their band intensities were normalized to that of β-actin using Image J software. The error bar indicates the mean+SD from three independent experiments and significant value were calculated by Student't' test (*P <0.05, ns non-significant); (C) Protein expressions of epithelial and mesenchymal markers for vector and Grhl2+ cells by immunofluorescence assay; and (D) and their corrected total cell fluorescence image intensity were quantified using Image J software and significant values were calculated by one-way ANOVA using Dunnett's post test compared with vector cells (**P <0.01, ns: non-significant) [Objective 40X].

migration in scratch assays. Consistent with this, A172 vector control cells filled up the scratch within 72 h, whereas Grhl2+ cells unable to complete scratch at 72 h (Fig. 3A). A rigorous test was further carried out to determine the potential of migration and invasion in the Boyden chamber assay. We noticed dramatic increase in migration (50.36%) and invasion (24.28%) potential in A172 vector control cells whereas there was significant decrease in migration (14.15%) and invasion (13.6%) in Grhl2+ A172 cells (Fig. 3B). Taken together, these data indicate that Grhl2+ in a mesenchymal cell was capable to diminish the migration and invasion.

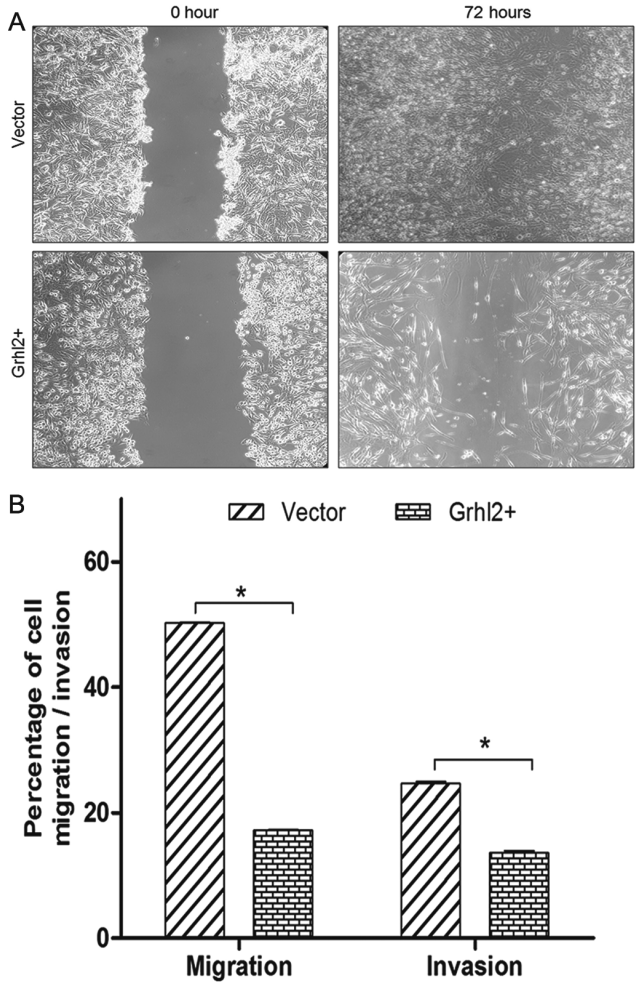

Fig. 3 — Migrations and invasion in Grhl2 mild over-expressed A172 cells. (A) Photomicrograph illustration of vector and Grhl2+ cells using scratch assay experiment [Objective 10X]; and (B) Bar represents the cell migration and invasion analysis of vector and Grhl2+ cells using Boyden chamber assay. [The data are expressed in mean±SD of three independent experiments (*P <0.05)]

Grhl2+ in A172 cells elicits chemosensitive to temozolomide drug

Finally, we determined whether Grhl2+ cells have an effect in chemotherapy; we treated the Grhl2+ A172 cells with temozolomide chemotherapy drug, which were routinely used to treat the astrocytoma patients. Our result shows, Grhl2+ cells are highly sensitive to temozolomide in a dose dependent manner and were statistically significant from low to high dose compared to vector control cells (Fig. 4). This suggests that Grhl2+ cells may have a good prognostic effect in brain astrocytoma cells.

Discussion

Grainy head like 2 (Grhl2) transcription factor was described as a dual role[13] and double edge sword[49] characteristics in many cancers especially in breast cancer there are contradictory reports which were arising in Grhl2 overexpression and knock-down studies[13,50-52]. Eventhough there are controversial reports, several studies exploit that the level of Grhl2 play a major role to define the fate of cellular function i.e., to switch towards either good or poor prognosis in cancer[15,26,31]. Yang *et al*.[53] demonstrated that the identification of new biomarker, Grhl2 together with other six genes CDH2, FN1, CITED2, MKI67 *vs*. CTNNB1 and CTNNA3 play a prognostic role in breast cancer and also they reported new prognosis marker Grhl2 collaborated with the five mesenchymal markers as a poor prognosis gene-set and while two epithelial markers perform a good prognosis gene-set. Menke *et al*.[54] shown that Grhl2 is important for non-neural tissue/fore-brain development. However, there

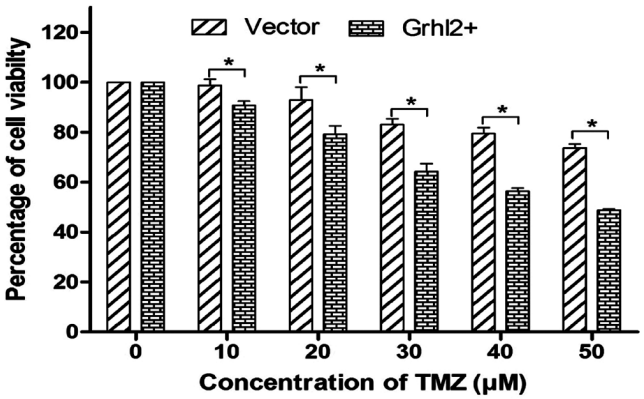

Fig. 4 — Effect of temozolomide drug in Grhl2 mild overexpressed A172 cells. Bar represents the percentage of cell viability of the temozolomide drug in Grhl2+ cells, relative to vector cells. [The error bar indicates the mean+SD from three independent experiments and significant value were calculated by Student's 't' test (*P <0.05)]

were no functional studies to understand the role of Grhl2 in brain cancer[51]. The Grhl2 level in various cancer cells is the decision maker and elucidates the phenotypic characteristic as heterogeneity i.e., the hybrid phenotype of epithelial and mesenchymal (EM) cells.

Grhl2 has different expression level and characteristic phenotypic heterogeneity in brain astrocytoma. Using clones of different levels of Grhl2 expression in human A172 astrocytoma cell line, we classified as Grhl2+ (mild), Grhl2++ (moderate) and Grhl2+++ (high) cells, and made an attempt to provide a preliminary report with mild Grhl2 (a novel transcription factor) overexpression and elucidate the cellular fate of A172 astrocytoma cells. We took advantage of using pcDNA stable clones which overexpress the target genes and able to achieve the stable expression clones with different levels of Grhl2. Our preliminary study is to explore on Grhl2+ in A172 astrocytoma cells. Characteristics of A172 cells are monomorphic mesenchymal fibroblast-like cells in nature[55]. As expected, we found there was a mixture of EM cells in mild overexpression of Grhl2 in A172 astrocytoma cells.

Further, we found that there was an increase in mRNA of β-catenin (epithelial marker) and N-cadherin and vimentin (mesenchymal markers) in Grhl2+ A172 cells and which may be not sufficient to synthesize the protein for translation process and hence it was unable to detect the significant increase of these proteins. When compared to mRNA level the significant increase in protein expression is important for the functional activity[56,57]. Due to this phenomenon there was a hindrance in complete loss of mesenchymal proteins of N-Cadherin and Vimentin in Grhl2+ A172 cells and the phenotype of the cells tend to be hybrid phenotypic characteristics. Even though there was an incomplete transition in Grhl2+ A172 cells, there was diminish in the cell migration and invasion and were consistent from the other cancer studies where Grhl2 expression prevents cell migration and invasion[20,51].

We further explore the Grhl2+ A172 cells behavior to chemo-drugs which were widely used for chemotherapy to astrocytoma patients and intriguingly we have found that the Grhl2+ cells were sensitive to TMZ drug which may be a good prognosis for human astrocytoma for the patients with mild Grhl2 expression. Further study is needed, to evaluate the efficacy of Grhl2+ expression in different astrocytoma cell lines by treating with different chemotherapeutic drugs as well as radiation treatment. If the level of Grhl2 reaches above the mild level in astrocytoma A172 cells, the cells might be either sensitive or resistant and this needs a further study. Because the high level of Grhl2 expression in breast cancer cell lines were more resistant to doxorubicin, daunorubicin, cisplatin and paclitaxel which were widely used to treat the breast cancer patients[26]. In breast cancer patients if the Grhl2 expression is high, the doxorubicin, daunorubicin, cisplatin and paclitaxel are not worth to treat with these drugs and hence alternative drug is needed to focus to treat those patients.

The enrichment of E-cadherin in Grhl2+ cells may lead to a good prognostic feature in brain astrocytoma as we noted in the cytotoxic studies. The moderate or high Grhl2 overexpression in A172 cells may further enrich or decline the E-cadherin level. If the E-cadherin continues to enrich in either moderate or high Grhl2 overexpression A172 cells which may persist to be a good prognosis. If the E-cadherin reverts to decline in either moderate or high Grhl2 overexpression A172 cells which may persist to be a poor prognosis. Further study is needed to understand the prognostic characteristic in the context of different levels of Grhl2. Our results when compared with previous studies more evidently demonstrate that the level of Grhl2 plays an important role in maintaining the cellular homeostasis (Fig. 5).

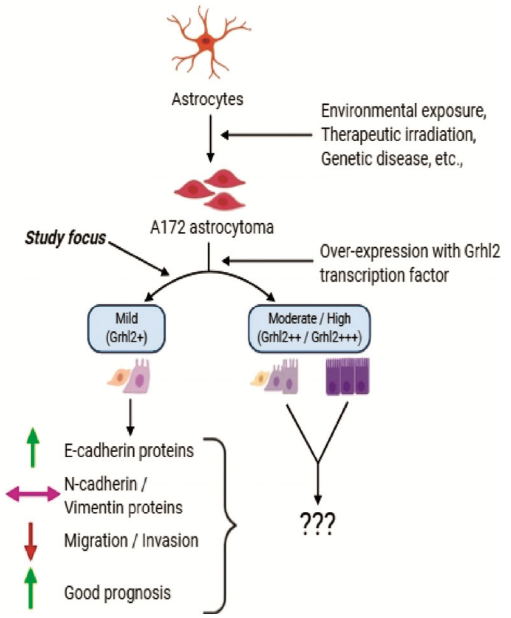

Fig. 5 — Graphical illustration of Grhl2 overexpression in A172 cells. [Model illustrates significant increase in epithelial markers and no significant decrease in mesenchymal markers at protein levels. The Grhl2+ cells diminish migration, invasion and also indicates good prognosis against temozolomide chemotherapeutic drug]

Conclusion

To our knowledge this is the first study to exploit the role of Grhl2 in brain cancer. In conclusion, Grhl2+ A172 cells partially abolishes their cancer cell properties and up-regulates epithelial marker (E-cadherin protein) and no change in mesenchymal markers (N-cadherin and vimentin proteins) with a hybrid phenotype. Hybrid Grhl2+ cells diminish migration / invasion when compared to vector cells and elicits good prognosis against the temozolomide chemotherapeutic drug. Our findings form the basis for understand the role of a novel transcription factor in brain cancer.

Acknowledgement

The authors express their gratitude to the Department of Science and Technology (DST), Science and Engineering Research Board (SERB), Government of India, New Delhi for extending support under National Post Doctoral Fellowship (NPDF) scheme to carry out the research work (Ref. No. PDF/2017/001086 dated 06.10.2017). The authors thank Ms. Betcy Evangeline Pamela and Ms. T. Subashini for their critical input for the manuscript and Dr. RV Shaji, Professor in Hematology, CMCH for providing the gel documentation instrument and Wellcome Biochemistry Laboratory for providing the fluorescence plate reader instrument.

Ethical approval

This study was approved by the Institutional Review Board (IRB), Christian Medical College, Vellore (Ref. No. IRB – 10911 (other) dated: 25.10.2017).

Conflicts of interest

Authors declare no competing interests.

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