

Decreased expression of the Augmenter of Liver Regeneration results in increased apoptosis and oxidative damage in human-derived glioma cells

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The mammalian growth factor *erv1*-like (*GFER*) gene encodes a sulfhydryl oxidase enzyme, named Augmenter of Liver Regeneration (ALR). Recently it has been demonstrated that ALR supports cell proliferation acting as an anti-apoptotic factor. This effect is determined by ALR ability to support the anti-apoptotic gene expression and to preserve cellular normoxic conditions. We recently demonstrated that the addition of recombinant ALR (rALR) in the culture medium of H₂O₂-treated neuroblastoma cells reduces the lethal effects induced by the hydrogen peroxide. Similar data have been reported in the regenerating liver tissue from partially hepatectomized rats treated with rALR. The purpose of the present study was to evaluate the effect of the *GFER* inhibition, via the degradation of the complementary mRNA by the specific siRNA, on the behaviour of the apoptosis (apoptotic gene and caspase expression and apoptotic cell number) and of the oxidative stress-induced parameters (reactive oxygen species (ROS), clusterin expression and mitochondrial integrity) in T98G glioma cells. The results revealed a reduction of (i) ALR, (ii) clusterin and (iii) bcl-2 and an increase of (iv) caspase-9, activated caspase-3, ROS, apoptotic cell number and mitochondrial degeneration. These data confirm the anti-apoptotic role of ALR and its anti-oxidative properties, and shed some light on the molecular pathways through which ALR modulates its biological effects.

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Gliomas represent the most frequently diagnosed tumours of the central nervous system. With a highly invasive phenotype, gliomas diffusely infiltrate into various regions of the brain,^{1,2} rendering their surgical resection impossible; therefore, the prognosis for patients with gliomas is poor even in response to multidisciplinary treatment.³

Augmenter of Liver Regeneration (ALR), a sulfhydryl oxidase enzyme⁴ encoded by the growth factor *erv1*-like (*GFER*) gene, is present in all mammalian cells^{5–7} and, compared with tissues from healthy subjects, is significantly overexpressed in tissues of patients affected by cell-proliferating diseases, such as hepatocellular carcinoma (HCC), chronic hepatitis (CH), cholangiocellular carcinoma⁸ or in regenerating liver after partial hepatectomy in rats.^{9,10} We recently demonstrated that ALR protects human-derived neuroblastoma cells from H₂O₂-induced apoptosis, reducing mitochondrial swelling, cell death and apoptotic cell number, inhibiting cytochrome-c mitochondrial release and upregulating the bcl-2/bax gene-transcription protein ratio. Similar data

are reported in the literature in different experimental models, *in vivo* and *in vitro*.^{11–15}

Various reports reveal that a defective or inefficient apoptosis is an acquired hallmark of cancer cells,¹⁶ which renders any pharmacological treatment almost ineffective.¹⁷ Apoptosis is characterized by two different molecular pathways: (i) the extrinsic pathway, which is triggered by the presence of transmembrane death receptors (Fas, TNF receptor and TRAIL receptors) and their respective ligands on cellular membranes and (ii) the intrinsic pathway, known as mitochondrial apoptosis, which requires the disruption of the mitochondrial outer membrane and leads to the apoptotic caspase cascade.¹⁸ Mitochondrial apoptosis is regulated by the anti-apoptotic bcl-2 family of proteins, which prevents mitochondrial swelling.

In the present study, in human-derived glioma cells, we investigated the effect of *GFER* silencing, by specific siRNA, on apoptotic gene expression, caspase-9 and activated caspase-3 expression, apoptotic cell number, reactive oxygen

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Abbreviations: ALR, Augmenter of Liver Regeneration; rALR, recombinant ALR; *GFER*, growth factor *erv1*-like; PBS, phosphate buffer saline; FBS, foetal bovine serum; ROS, reactive oxygen species; siRNA, small interfering RNA; TNFR, tumour necrosis-factor receptor; TRAIL, tumour necrosis factor-related apoptosis inducing ligand receptors; HSS, hepatic stimulatory substance; HCC, hepatocellular carcinoma; CH, chronic hepatitis; 7-AAD, 7-aminoactinomycin D; EDTA, ethylene diamine tetra acetate; SDS, sodium dodecyl sulphate; IGEPAL, octylphenoxypolyethoxyethanol; IMS, intermembrane space; OXPHOS, oxidative phosphorylation; mtTFA, mitochondrial transcription factor A; PH, partial hepatectomy; PFA, paraformaldehyde; BSA, bovine serum albumin; HRP, horseradish peroxidase; DCFH-DA, 2,7-dichlorofluorescein diacetate; LPO, lipid peroxidation; Mia40, mitochondrial intermembrane space import and assembly protein 40

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species (ROS) levels, clusterin expression and mitochondrial morphology.

Results

H₂O₂-induced oxidative stress. Figure 1 reports the behaviour of H₂O₂-induced ROS in glioma cells maintained in cell culture and the effect of the increasing doses of recombinant ALR (rALR) to the culture medium. We have previously and broadly described the effects of ALR both on apoptosis and on the endocellular redox state altered by H₂O₂ addition in cell culture of neuroblastoma cells.¹² In our present experimental conditions, a five- to six-fold increase, statistically significant ($P=3.7 \times 10^{-5}$), of ROS levels was registered in H₂O₂-treated cells compared with H₂O₂-untreated cells. The presence of increasing doses of rALR in the culture medium counteracted the effect induced by H₂O₂.

Effect of GFER silencing

ALR expression. Figure 2 reports ALR expression in siRNA/ALR-treated cells and control cells evaluated both using western blot analysis (Figure 2a) and confocal microscopy (Figure 2b). The western blot analysis revealed the presence of two forms of the protein, one of 21 and one of 23 kDa,¹⁰ markedly reduced in siRNA/ALR-treated cells compared with control cells (Figure 2a, upper-right). The normalization of ALR expression, with respect to β -actin level, revealed a statistically significant ($P=6.7 \times 10^{-5}$) reduction of ALR in siRNA/ALR-treated cells compared with control cells. The confocal microscopy immunodetection revealed a considerable presence of ALR in control cells, which resulted significantly reduced in siRNA/ALR-treated cells (Figure 2b). For each determination, the nuclear identification, evidenced by TO-PRO-3 (Invitrogen srl, Milan, Italy), the specific ALR immunodetection, revealed by Alexa Fluor 488 (Invitrogen srl), and the 'merge' are reported.

Clusterin expression. Figure 3 reports the clusterin expression in siRNA/ALR-treated and control glioma cells evaluated using western blot analysis (Figure 3a) and confocal microscopy (Figure 3b). A marked reduction of clusterin in siRNA/ALR-treated cells, compared with

control cells, was evidenced (Figure 3a, upper-right). The normalization of clusterin expression, compared with β -actin level, revealed a statistically significant ($P=5.4 \times 10^{-3}$) reduction of the protein. Clusterin immunodetection using confocal microscopy revealed a considerable amount of this factor in the cytosol of control glioma cells, which dramatically reduced in siRNA/ALR-treated cells (Figure 3b). Nuclear identification, evidenced by TO-PRO-3, and the specific clusterin immunodetection, evidenced by Alexa Fluor 488, are reported.

ROS levels. Figure 4 reports the level of ROS determined by the GFER siRNA treatment and the protective effect of rALR when it was introduced into the culture medium. A statistically significant ($P=1.1 \times 10^{-4}$) increase of >4-fold of the ROS was present in siRNA/ALR-treated cells compared with control cells. The presence of increasing doses of rALR (1–100 ng/ml) in the culture medium counteracted the siRNA/ALR-induced oxidation in a dose-dependent way.

Apoptotic gene expression. Figure 5 reports the effect of GFER siRNA treatment on the expression of apoptotic genes, *bcl-2* and *bax* (Figure 5a), *caspase-9* (Figure 5b) and activated *caspase-3* (Figure 5c) in siRNA/ALR-treated and control glioma cells. The western blot analysis of *bcl-2* and *bax* (Figure 5a) showed a significant reduction of *bcl-2* expression in siRNA/ALR-treated cells, compared with control cells, without a notable variation of *bax* between the two experimental conditions. The densitometric analysis of the *bcl-2* signals, normalized to β -actin expression, demonstrated that the *bcl-2* expression was significantly reduced in siRNA/ALR-treated cells ($P=4.1 \times 10^{-5}$). Figure 5b reports *caspase-9* expression revealing its increase in siRNA/ALR-treated cells, compared with control cells. The densitometric analysis, normalized to β -actin, revealed this variation statistically significant ($P=1.4 \times 10^{-3}$). Figure 5c reports the expression of activated *caspase-3*. An increase was detected in siRNA/ALR-treated cells, compared with control cells, and the densitometric analysis of the protein-related signals, normalized with that of the β -actin, revealed the increase statistically significant ($P=1.3 \times 10^{-5}$).

Cytofluorimetric analysis. Figure 6 reports the cytofluorimetric analysis of siRNA/ALR-treated and control glioma cells. In the upper part of the figure (Figure 6a), the biparametric histograms LOG FL1 versus LOG FL4 are reported, distinguishing four cell populations: the viable cells (low FITC and low 7-AAD), the secondary necrotic cells (high FITC and high 7-AAD), the early apoptotic cells (high FITC and low 7-AAD) and a possible fourth cell population corresponding to the damaged viable cells (low FITC and high 7-AAD). An increase in the percentage of early apoptotic cells was observed in the culture of siRNA/ALR-treated glioma cells and the comparison of the data from three different experiments (Figure 6b) revealed a statistically different ($P=1.9 \times 10^{-8}$) percentage of Annexin V⁺ cells in the culture of siRNA/ALR-treated cells (40.72 ± 3.5) compared with the percentage of Annexin V⁺ cells

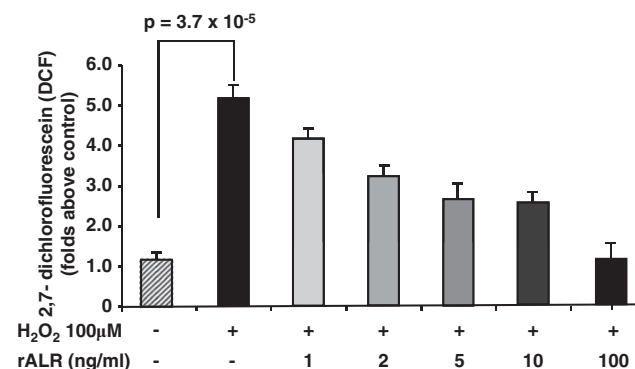


Figure 1 Effect of rALR treatment on H₂O₂-induced ROS levels on glioma cells. Human T98G glioma cells, treated with H₂O₂, were incubated with different concentrations of rALR (1–100 ng/ml). The means \pm S.D. of six triplicate independent experiments are shown

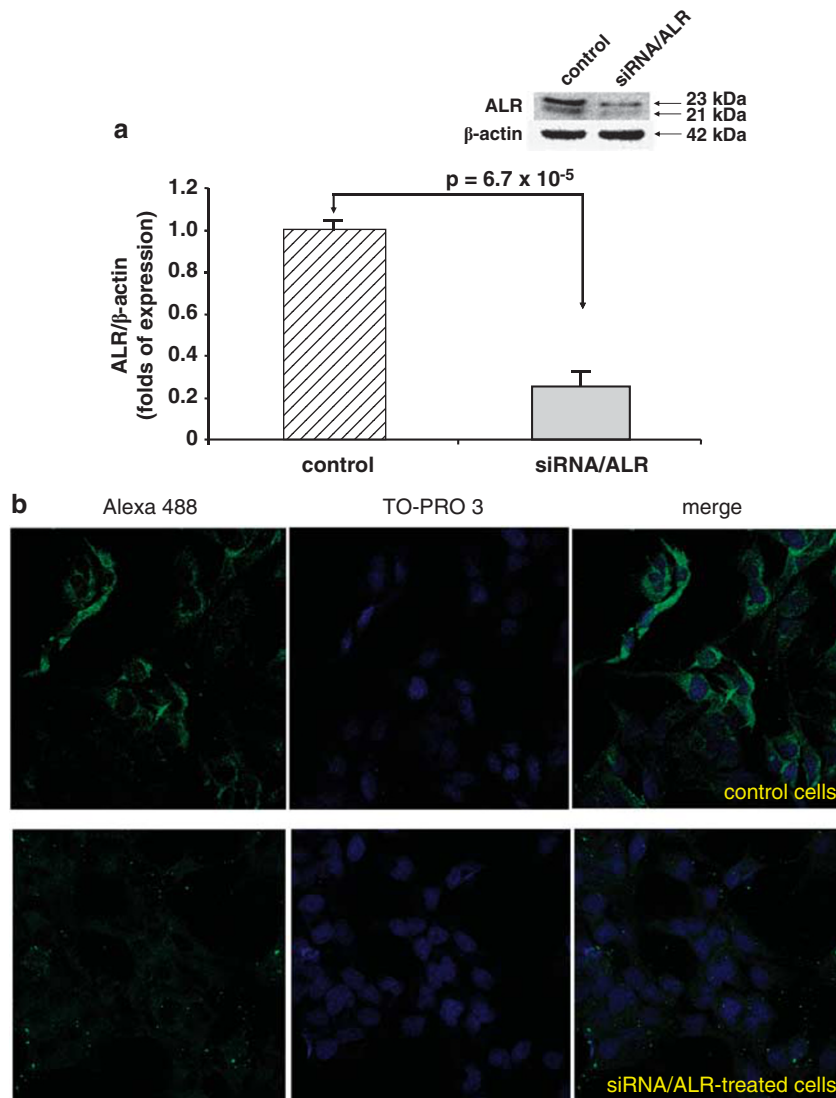


Figure 2 Effect of siRNA/ALR treatment on ALR expression. ALR expression in siRNA/ALR-treated and control human T98G glioma cells, determined using western blot analysis (a) and confocal microscopy (b, green colour), are reported. The data shown are means \pm S.D. from five independent experiments

detected in the culture of control cells (5.63 ± 1.55). No necrotic or damaged cells were detected in any evaluation.

Electron microscopy (EM) analysis. Figures 7 and 8 report the analysis, performed via EM, of control glioma (Figure 7) and siRNA/ALR-treated cells (Figure 8). The control cells appear elongated with peripheral cytoplasmic expansion and the nuclei contain finely aggregated chromatin (down-left inset) and voluminous nucleoli (arrows). The cytoplasm contains numerous mitochondria of normal appearance (up-right inset). Instead, the siRNA/ALR-treated cells present a more abundant cytoplasm with degenerated organelles. The nuclei are extremely irregular and the mitochondria appear swollen or fragmented (down-right inset). The cells present electron-dense granules resembling lysosome structures (up-left inset). Vacuolar dilatations of the endoplasmic reticulum are present (arrows).

Discussion

More than 10 years ago, our group identified a new protein, the ALR,^{5,19} initially known as HSS,²⁰ which increases hepatocyte proliferation when administered to liver cells already primed to proliferate;²¹ the exact role of ALR in the cellular metabolism is still not well defined. Only recently, some of the metabolic functions of ALR have been revealed by *in vivo* and *in vitro*^{10–15} experiments. In a recent study, in partially hepatectomized rats,¹⁰ we demonstrated a rapid increase of ALR in the regenerating liver in the first 6–12h after the surgery, which favours the anti-apoptotic gene expression, a molecular event, at this time of the regenerative process, crucial for the sustention of liver-mass recovery after the surgery.^{9,22} In the same study, we identified two ALR protein isoforms, with molecular weights of 21 and 23kDa, respectively.¹⁰ the 21-kDa form significantly increased after partial hepatectomy (PH), supporting the notion that this could

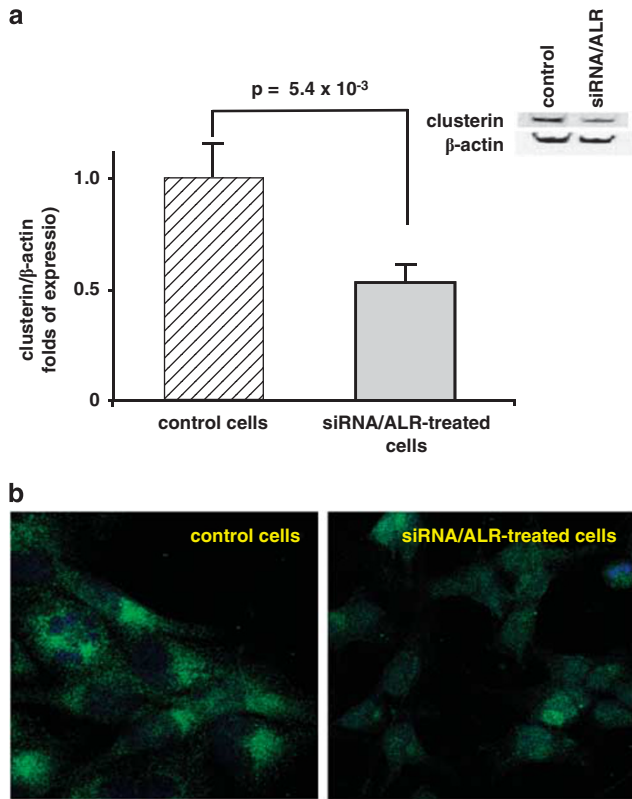


Figure 3 Effect of siRNA/ALR treatment on clusterin expression. Clusterin expression in siRNA/ALR-treated and control human T98G glioma cells, determined using western blot analysis (a, upper right) and confocal microscopy (b, green colour), are reported. The data shown are means \pm S.D. from three independent experiments

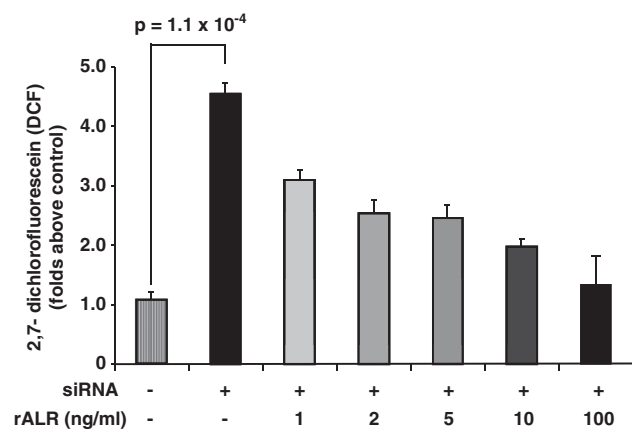


Figure 4 Effect of siRNA/ALR treatment on ROS levels expressed by glioma cells. siRNA/ALR-treated cells were incubated with different concentrations of rALR (1–100 ng/ml), subsequently loaded with DCF-DA and fluorescence intensity measured as described in Materials and Methods. Means \pm S.D. of six triplicate independent experiments are shown

be the ALR protein form that actively participates in the regenerative process after PH, whereas the 23-kDa form remains unchanged. This latter molecular form of ALR has been predominantly recognized in the mitochondrial IMS²³ where it is involved (i) through its FAD molecule^{24,25} in the

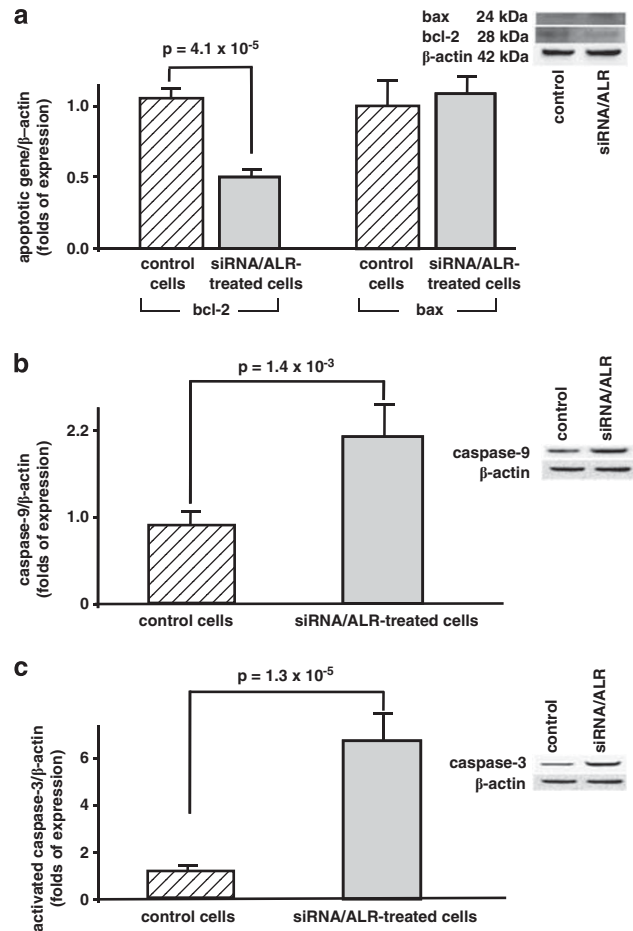


Figure 5 Effect of siRNA/ALR treatment on apoptotic gene expression. bcl-2 and bax (a), caspase-9 (b) and activated caspase-3 (c) expression in siRNA/ALR-treated and control human T98G glioma cells, determined using western blot analysis, are reported. The data shown are means \pm S.D. from five independent experiments

electron pathway, with cytochrome-*c*²⁵ and the Mia40²⁶ as physiological partners and (ii) in the maturation of cytosolic Fe/S proteins.²⁷ Moreover, for the first time *in vivo*, we demonstrated the anti-oxidative capacity of ALR, a biological effect achieved by the induction of clusterin, a secreted chaperone capable of maintaining the physiological cellular redox state.²⁸

In our experiments, as other investigators,^{4,10,12,14,15,29} we used a small 15-kDa recombinant form of ALR that, mainly reported as an extracellular cytokine,^{12,26} represents the smallest yet functional protein capable of participating in the intracellular redox-dependent signalling pathways.¹² Indeed, the small 15-kDa ALR form, similarly to other ALR homologous proteins identified in all organisms from viruses to mammals,⁶ maintains a well conserved amino-acid domain, CxxC, at the C-terminus part of the molecule, responsible for its sulfhydryl oxidase enzymatic activity.^{4,24–27} All these proteins have different molecular weight and cytoplasmic localization, but probably all support similar biological functions as has been reported for some.^{4,23–27} It is noteworthy to underscore the fact that the most important signal structures for the interactions and subcellular distribution of ALR are

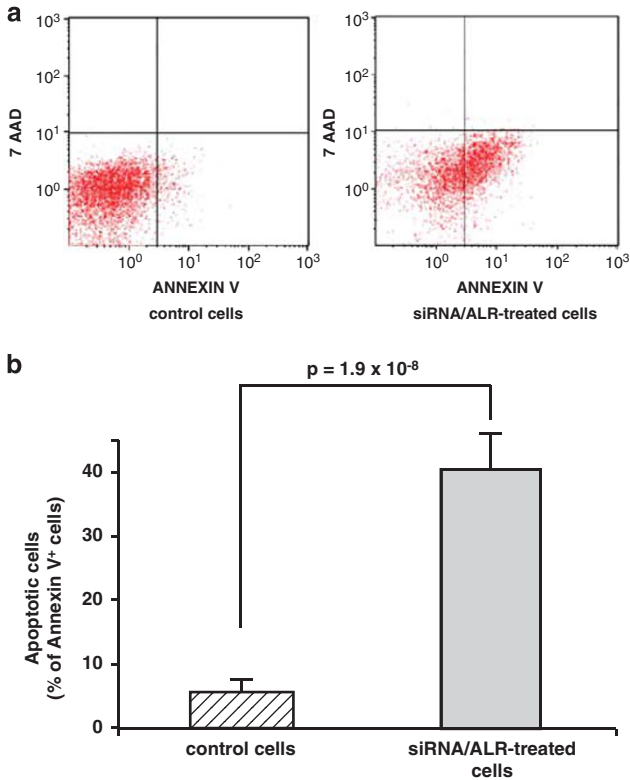


Figure 6 Effect of siRNA/ALR treatment on cytofluorimetric analysis of apoptotic glioma cells. In the upper part of the figure (a), the biparametric histogram of LOG FL1 versus LOG FL4, in the siRNA/ALR-treated and control cells, is reported. A lower percentage of Annexin V⁺ cells in the culture of control cells (5.63 ± 1.55) compared with the percentage of Annexin V⁺ cells in the culture of siRNA/ALR-treated cells (40.72 ± 3.5) is observed (b). The data shown are the mean ± S.D. from three independent experiments

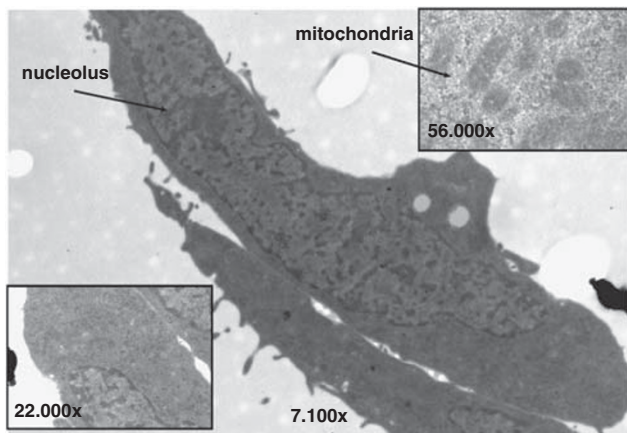


Figure 7 EM morphological analysis of control glioma cells. The cells (× 7.100) present peripheral cytoplasmic expansion and mitochondria of normal appearance (up-right insert; × 56 000) and the nuclei contain finely aggregated chromatin (left-down insert; × 22 000) and voluminous nucleoli (arrows)

located at the C-terminal domain of the protein and not at the N-terminal domain.^{23–26}

An additional finding that can help for the identification of the role of ALR in the cellular metabolism comes from *in vitro*

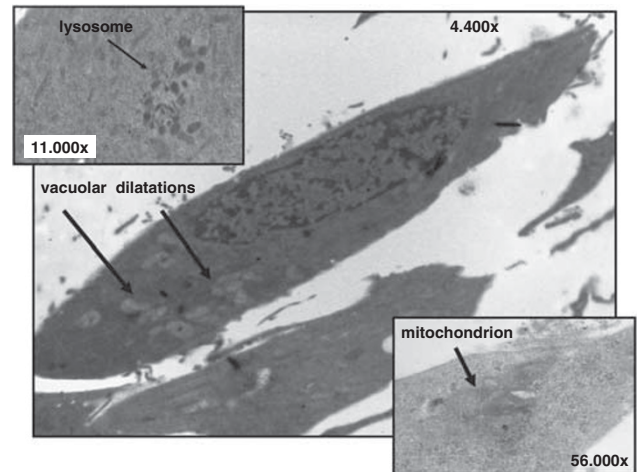


Figure 8 Effect of siRNA/ALR treatment on glioma cell morphology determined by EM. The siRNA/ALR-treated glioma cells present degenerated organules in the cytoplasm (× 4400), nuclei extremely irregular, mitochondria swollen or fragmented (right-down insert; × 56 000) and electron-dense granules resembling lysosome (up-left insert; × 11 000). Vacuolar dilatations of the endoplasmic reticulum are present (arrows)

studies. Analyzing the H₂O₂-induced apoptotic process in human-derived neuroblastoma cells, we demonstrated that the addition of rALR in the culture medium normalizes LPO, protein carbonylation and mitochondrial membrane permeability altered by the presence of H₂O₂.¹² All these parameters are typical of the mitochondrial apoptosis.

Furthermore, we demonstrated that ALR supports the OXPHOS and ATP production in mitochondria isolated from intact liver of rat intraperitoneally treated with rALR; these effects are achieved by (i) the induction of mtTFA, a mitochondrial DNA regulatory factor, and (ii) the upregulation of mitochondrial gene expression.³⁰

In the present study, we tested the effect of ALR gene-silencing (*GFER*) on the cell morphology, mitochondrial apoptosis and cellular redox state of human-derived glioma cells, T98G, maintained in culture. The data obtained demonstrated a significant reduction of ALR protein in siRNA/ALR-treated cells (Figures 2a and b) and, as a consequence, (i) a decrease of clusterin expression (Figures 3a and b), an increase of (ii) ROS levels (Figure 4) and (iii) the apoptotic process, evaluated by bcl-2, bax (Figure 5a), caspase-9 (Figure 5b), activated caspase-3 protein expression (Figure 5c) and the number of the apoptotic cells (Figure 6). The induction of these biological events sufficiently justifies the cellular morphological alterations evidenced in the *GFER*-silenced glioma cells (Figure 8) compared with control cells (Figure 7). In addition, we demonstrated that the presence of increasing concentrations of rALR into the culture medium of siRNA/ALR-treated cells abolished, almost totally, the increased level of ROS induced by the siRNA/ALR treatment (Figure 4). As mentioned before, we already reported the beneficial effects of ALR on the cell redox state and on apoptosis.¹⁰

To efficiently collocate these data within the cell metabolism, some important considerations are necessary. The devastating effect on cell metabolism of ROS increase,

associated to the induction of apoptosis, has been unanimously reported under many experimental conditions.^{17,31} It is known that the 'oxidative stress' can cause DNA, protein, and/or lipid damage, leading to changes in chromosome stability, genetic mutation, and/or modulation of cell growth that may result in cancer.³² Recent data underlined the strong relationship between increased ROS levels and neoplastic transformation, attributing them the role of critical signalling molecules in cancer cells as well as in metastatic cells.³³ Indeed, such a state has been shown to regulate both genetic and epigenetic cascades, and mutation studies have suggested that chronic oxidative stress, particularly from chronic inflammation, is associated with carcinogenesis.³⁴ For example, ulcerative colitis has long been linked with a high incidence of colorectal cancer and chronic gastritis due to, for instance, the infection with *Helicobacter pylori*, with the gastric cancer,³⁴ as well as HCC in patients affected by CH. Most of these pathological conditions are associated with high ALR serum levels.⁸

Various reports reveal a defective or inefficient apoptosis of cancer cells,^{16,35,36} which renders almost ineffective any pharmacological treatment of human neoplasia.¹⁷ Thus, the identification of a mechanism that can weaken the apoptosis-resistance strategy of cancer cells is imperative to unravel novel drug targets for the design of more effective and target-selective therapeutic strategies. For instance, in an *in vivo* mouse xenograft study recently reported by Lin, co-administration of the anti-oxidant agent resveratrol, and conventional chemotherapy reduces brain tumour volumes by inducing cell apoptosis.³⁷

With this perspective, the increased apoptosis of glioma cells, reported here by us, related to the *GFER* decreased expression, could constitute an important and sufficient biological event to improve the effectiveness of the conventional, sometimes inefficient, chemotropic agents.

In addition, the present data, together with our previous observations^{10,12} and the data present in the literature,^{11,13–15} strongly persuade us that ALR not only supports cell survival by its anti-apoptotic effect but also holds the crucial function of an anti-oxidative agent sustaining the levels of reducing agents such as clusterin. It is not easy to determine the exact sequence of these two metabolic events induced by ALR; we can only state that, at least in partially hepatectomized rats, ALR increases in the first 6–12 h after surgery, favouring the anti-apoptotic event which occurs, in the same period of the regenerative process, in the liver. More data are necessary to resolve the aspects of the physiological role of ALR.

In conclusion, the present findings suggest that ALR could be considered as a potential new target for the chemotherapeutic treatment of gliomas and, considering the presence of ALR in all eukaryotic cells,^{6–14} and even in the viruses³⁸ we can hazard to say that it could be a possible support for the treatment of all human neoplasia. To our knowledge, with the data present in the literature, we cannot consider the ALR as an oncogene; we can only state that ALR is a factor overexpressed in all neoplasia so far studied^{8,14} and that for the exact definition of its role in the carcinogenesis, more studies are necessary. It is interesting to note that an ALR-KO mouse strain is not yet available, probably because the downregulation of *GFER* is not consistent with the cell survival.

Materials and Methods

Reagents. rALR expressed in transfected COS-1 cells has been produced by the Laboratory of Biochemistry and Molecular Biology (University of Georgia, Athens, GA, USA). In line with other investigators, in the present experiments, we used a rALR form of 15 kDa, containing the well-conserved, biologically active, C-terminal domain of the molecule.^{29,30,39} All the chemical reagents were purchased, if not specifically reported, from Sigma-Aldrich (Milan, Italy). The polyclonal antibody against ALR (MultiBind GmbH, Cologne, Germany; a gift from Dr. Thomas Lisowsky) has already been employed for specific identification of ALR.^{7,10,12} For raising the ALR antibody, a purified hexahistidinyl-tagged carboxyl-terminal fragment of ALR (residues 81–205) was used.⁷

Cell line. Human-derived T98G glioma cells (Interlab Cell Line Collection, Genoa, Italy) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich) supplemented with 10% (v/v) inactivated FBS (PAA Laboratories GmbH, Pasching, Austria), 2 mM L-glutamine (Sigma-Aldrich), 100 µg/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich), at 37°C in 5% CO₂. The cells, grown to 70% confluence and treated for 48 h, have been then trypsinized or treated as requested by the different protocol of the experiments and the biological parameters evaluated.

RNA interference. For the RNA interference experiments, the specific pre-designed siRNA (5'-GGAGUGUGCUGAAGACCUATT-3'; Applied Biosystems/Ambion, Austin, TX, USA), targeting the mRNA of the human *GFER* gene, was transfected, according to the manufacturer's protocol, in T98G glioma cells using siPORT NeoFX Transfection Agent (Applied Biosystems/Ambion). Silencer Negative Control 1 siRNA AM4635 (Applied Biosystems/Ambion) with no significant similarity to human, mouse or rat gene sequences was used as negative control.

H₂O₂-induced oxidative stress. Oxidative stress in human T98G glioma cells was caused by the addition of H₂O₂ (Sigma-Aldrich) to culture medium. Prior to each experiment, H₂O₂ was freshly prepared from 30% stock solution. To determine the most appropriate conditions for our experiments, different concentrations of H₂O₂ (50, 100 and 200 µM) were initially used (data not reported). Based on the data obtained using the different H₂O₂ concentrations in our previous experiments with neuroblastoma cells¹² and on the references reported in the literature,⁴⁰ in the present study, we decided to use H₂O₂ at a concentration of 100 µM. Culture cells maintained in DMEM supplemented with 10% (v/v) inactivated FBS and without H₂O₂ were used as control.

rALR treatment. With the purpose to test the ALR capacity to counteract the H₂O₂-induced apoptosis, rALR (from 1 to 100 ng/ml) was added to the culture medium of H₂O₂-treated glioma cells. The short form (15 kDa) of ALR was used. This molecule corresponds to the physiologically active protein that was originally identified and isolated by us⁵ and whose biological activity has been demonstrated in the majority of the experiments. Indeed, most independent scientific publications from a large number of research groups have used the 15-kDa ALR variant for their experiments^{4,10,12,14,15,29} and this is now the general standard, in ALR experiments, to have comparable data. T98G cells, maintained in DMEM supplemented with 10% (v/v) inactivated FBS were used as control. The rALR treatment was done, at least, three times for each experimental protocol.

ALR expression

Western blot analysis. Human T98G glioma cells were grown in 75-cm² flasks in DMEM supplemented with 10% FBS and in the presence (siRNA/ALR-treated cells) or absence (control cells) of *GFER*-specific siRNA. Cytosolic protein preparation and subsequent electrophoresis were done following a standard procedure.¹² Briefly, after 48 h of incubation, 2 × 10⁶ glioma cells were trypsinized (Trypsin 0.05%/EDTA 0.02%; M-Medical srl, Milan, Italy), added with 200 µl of RIPA buffer (Sigma-Aldrich), containing protease inhibitors (protease-inhibitor cocktail tablets 1 × , Roche Applied Science, Milan, Italy) and anti-phosphatases (sodium orthovanadate 2 mM; Sigma-Aldrich), gently stirred, centrifuged for 20 min at 14 000 × g at 4 °C and the supernatant collected. Protein concentration was evaluated using the Bradford assay (Bio-Rad Laboratories, Milan, Italy). Aliquots of 20 µg of total proteins were separated on 4–12% sodium dodecyl sulphate-polyacrylamide gels (Invitrogen srl), transferred onto a nitrocellulose membrane and then probed with primary antibody specific for ALR, diluted 1 : 200 in a blocking solution (5% of non-fat dry milk) (Bio-Rad Laboratories) in TBS-T (Tris Buffer Saline-Tween 20). The primary antibody was identified by an HRP-conjugated secondary

antibody diluted 1:20 000 (Bio-Rad Laboratories), subsequently detected by a chemiluminescent substrate of HRP (Pierce Biotechnology, Inc., Rockford, IL, USA). To detect the β -actin housekeeping protein, the membrane was washed with the Restore Western Blot Stripping Buffer (Pierce Biotechnology, Inc.) for 15 min and reprobed with the anti- β -actin primary antibody diluted 1:500 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), subsequently identified by an HRP-conjugated secondary antibody diluted 1:20 000 (Bio-Rad Laboratories) and detected by a chemiluminescent substrate of HRP (Pierce Biotechnology, Inc.) as for ALR detection. The densitometric analysis of each protein-related signal was obtained using the Molecular Imager Chemidoc (Bio-Rad Laboratories) and normalized against β -actin expression.

Confocal microscopy. Human T98G glioma cells were grown, for 48 h, onto cover glasses, maintained in petri dishes in DMEM added with 10% FBS in presence (siRNA/ALR-treated cells) or absence (control cells) of *GFER*-specific siRNA. The cover glasses were then pulled out from the dishes and fixed in 4% PFA (Sigma-Aldrich) in PBS for 15 min and washed with PBS. The cells were then permeabilized by 0.25% Triton X-100 (Sigma-Aldrich) for 20 min. After three washes with PBS, nonspecific protein binding was inhibited by a blocking solution (5% of FBS and 3% BSA) (Sigma-Aldrich) in PBS for 2 h. The cells were then incubated overnight at 4°C with the anti-ALR antibody diluted 1:200 in the blocking solution. A negative control was performed treating different cover glasses with the blocking solution short of the ALR-specific primary antibody. The cells were then washed with PBS and incubated with the Alexa Fluor 488 secondary antibody (Invitrogen srl) diluted 1:200 and with TO-PRO-3 (Invitrogen srl) diluted 1:7000 in PBS for 20 min for nuclear staining. The cover glasses were then mounted with Fluoromount K024, an anti-fading agent (Diagnostic BioSystems, Pleasanton, CA, USA), and analyzed with the confocal microscope Leica TCS SP2 (Leica Microsystems, Wetzlar, Germany). To verify the specificity of the immunoreaction, appropriate controls were performed incubating the cells with only the secondary antibody or using the pre-immune rabbit serum as primary antibody. We repeated the experiments three times. The green colour identifies ALR immunodetection and the blue colour identifies the nuclei.

Clusterin expression

Western blot analysis. Cytosolic protein preparation and subsequent protein electrophoresis were done as described for ALR western blot analysis. The nitrocellulose membrane, with the transferred proteins, were probed with a primary antibody specific for clusterin (Santa Cruz Biotechnology, Inc.) diluted 1:200 in blocking solution, subsequently identified by an HRP-conjugated secondary antibody (Bio-Rad Laboratories) and finally detected by a chemiluminescent substrate of HRP (Pierce Biotechnology, Inc.) and normalized to β -actin signal. Clusterin and β -actin densitometric expression was determined by the Molecular Imager Chemidoc (Bio-Rad Laboratories) as referred for ALR western blot analysis. The evaluation was done at least three times.

Confocal microscopy. Human T98G glioma-cell preparation for clusterin immunofluorescence detection was performed as described for ALR immunofluorescence detection. The cells, grown on cover glasses, were incubated with the anti-clusterin antibody (Santa Cruz Biotechnology, Inc.), diluted 1:200 in the blocking solution, overnight at 4°C. A negative control was performed treating different cover glasses with the blocking solution short of the clusterin-specific primary antibody. The cells were then washed with PBS and incubated with an Alexa Fluor secondary antibody (Invitrogen srl) diluted 1:200 and with TO-PRO-3 (Invitrogen srl) diluted 1:7000 in PBS for 20 min for nuclear staining. The cover glasses were then mounted with Fluoromount K024 and analyzed with the confocal microscope Leica TCS SP2 (Leica Microsystems, Wetzlar, Germany). To verify the specificity of the immunoreaction, appropriate controls were prepared, incubating the cells with only the secondary antibody or using the pre-immune rabbit serum as primary antibody. The green colour identifies clusterin immunodetection and the blue colour identifies the nuclei. We repeated the experiments at least three times.

ROS generation. ROS generation was monitored using an oxidation-sensitive fluorescent probe DCFH-DA (Sigma-Aldrich). Non-ionic, non-polar DCFH-DA crosses cell membranes and is hydrolyzed by intracellular esterases to the nonfluorescent (DCFH), which can be rapidly oxidized to the highly fluorescent 2,7-dichlorofluorescein in the presence of ROS. In all, 5×10^5 siRNA/ALR-treated or control T98G cells per well were plated into sterile black Culture Plate 96-F wells (PerkinElmer, Life and Analytical Sciences, Inc, Waltham, MA, USA). After 48 h, the

cells were incubated (or not) with a final 100 μ M H₂O₂ per well in DMEM and incubated for 10 min at 37°C in 5% CO₂. Then the cells were washed twice with PBS and incubated for 4 h at 37°C in 5% CO₂ with different concentrations of rALR (1–100 ng/ml). Subsequently, the culture medium was removed and cells were incubated with 0.1 mM DCFH-DA in DMEM at 37°C in 5% CO₂ in the dark for an additional 30 min and then washed thrice with PBS. Fluorescence intensity was measured directly in each well at an excitation wavelength of 485 \pm 20 nm and an emission wavelength of 530 \pm 25 nm using the multiwell fluorescence plate reader Wallac Victor mod.1421 (PerkinElmer).

Apoptotic gene expression. Bax, bcl-2, caspase-9 and activated caspase-3 protein expression were evaluated using western blot analysis on nitrocellulose membranes prepared as reported for ALR western blot analysis. Each nitrocellulose membrane was probed with the primary antibody specific for bax or bcl-2 (Santa Cruz Biotechnology, Inc.), or for caspase-9 (Santa Cruz Biotechnology, Inc.) or activated caspase-3 (Abcam Ltd, Cambridge, UK), all diluted to 1:200 in the blocking solution. The primary antibody was then identified by an HRP-conjugated secondary antibody (Bio-Rad Laboratories) diluted 1:20 000, which was subsequently detected by a chemiluminescent substrate of HRP (Pierce Biotechnology, Inc.). Each protein-related electrophoretic band was evaluated as referred for ALR western blot analysis and the densitometric value normalized with β -actin protein signal. Each analysis was done five times.

Flow-cytometric detection of apoptotic cells. Annexin V-FITC/7-AAD Kit PN IM3614 (Beckman Coulter, Milan, Italy) was used to detect apoptosis on siRNA/ALR-treated or control T98G cells, grown in 75-cm² flasks in complete medium (DMEM + 10% FBS). After 48 h, the cells were trypsinized (trypsin 0.05%/EDTA 0.02%) (M-Medical srl), washed with cold PBS and then centrifuged and resuspended in cold $1 \times$ binding buffer to 5×10^6 cells/ml. Ten μ l of annexin V-FITC solution and 20 μ l of AAD viability dye were then added to 100 μ l of the cell suspensions and the mixture was kept on ice for 15 min in the dark. Subsequently, 400 μ l of $1 \times$ binding buffer was added and gently mixed. The cell preparation was then analyzed by Cytomics FC500 (Beckman Coulter) flow cytometry within 30 min. The average of the different cell populations was determined using siRNA/ALR and control cells from, at least, three different experiments.

EM. siRNA/ALR-treated or control T98G cells, maintained in 75-cm² flasks in complete culture medium (DMEM + 10% FBS) for 48 h, were trypsinized (trypsin 0.05%/EDTA 0.02%; M-Medical srl) and then fixed in a mixture of 3% PFA and 1% glutaraldehyde in 0.1 M PBS at pH 7.4 for 5 h at 4°C. Subsequently, the cells were postfixed in 1% OsO₄ in PBS for 30 min at 4°C, washed in several changes of PBS, dehydrated in graded alcohols and embedded in Epon-Araldite (TAAB, Reading, UK). Semi-thin sections (1 μ m thick) were heat-stained with toluidine blue borate. Ultra-thin sections for EM were mounted on formvar-coated nickel grids and stained routinely with uranyl acetate and lead citrate. The grids were observed under a Morgagni 268 electron microscope (FEI, Hillsboro, OR, USA).

Statistical analysis. The results obtained are expressed as mean \pm S.D. Statistical comparison among groups was determined using analysis of variance. Where indicated, individual comparisons were performed using Student's *t*-test. Statistical significance was ascribed to the data when $P < 0.05$. If not specifically reported, each datum is representative of at least three different and separated experiments.

Conflict of Interest

The authors declare no conflict of interest.

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