AGR2 – a Novel Predictor of Neoadjuvant Chemoradiotherapy Response in Esophageal Squamous Cell Carcinoma

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Research

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Abstract

Background

Esophageal squamous cell carcinoma (ESCC) still has a poor prognosis despite the use of multidisciplinary therapy. In the locally advanced stage, neoadjuvant chemoradiotherapy (nCRT) followed by surgery might provide survival benefits to some patients.

Methods

In *this study*, we *aimed to identify* biomarker to *predict* tumor response against *neoadjuvant* chemoradiotherapy (nCRT) by next-generation sequencing (NGS).

Results

Our data showed that 464 genes was differentially expressed ESCC specimens, in which AGR2 was 2.8 fold up-regulated in the patients with Non-complete response before nCRT than complete response group. *In vitro* study showed that, AGR2 was significantly reduced in AGR2 knockdown CE146T/VGH, TE2, and CE48T/VGH cells. MTT assay indicated that cell viability of *AGR2*-knockdown TE-2 cell line significantly decreased following 2.5μM cisplatin and 3μM 5-FU treatment. Furthermore, 6μM cisplatin and 20 μM 5-FU treatment greatly decreased *AGR2*-knockdown-CE48T/VGH, CE146T/VGH and TE-2 cells compared to control group. We also found in *AGR2*-knockdown cells, that protein level of p21 was increased in comparison with the control group.

Conclusions

This study suggest that AGR-2 as a promising and potential prediction gene marker dataset for response to neoadjuvant chemoradiation in ESCC.

1. Introduction

Esophageal squamous cell carcinoma (ESCC) is one of the most common human cancers and it is the sixth most common cancer worldwide (1). In Taiwan, it is the fifth leading cause of death among men, and most prevalent in South-East and Central Asia. Esophageal cancer is histologically classified as squamous cell carcinoma or adenocarcinoma (2). Surgical resection remains the main treatment of early-stage esophageal carcinoma. Although multidisciplinary therapies, including radiotherapy, chemotherapy, and surgery, have been developed to prolong the survival of patients with locally advanced esophageal carcinoma, the prognosis remains poor (3, 4). Previous studies have shown that neoadjuvant chemoradiotherapy (nCRT) followed by surgery is a common multidisciplinary treatment for resectable esophageal carcinoma (5–12). However, it is still disappointing, and > 50% of such patients still exhibit poor response to nCRT (13–16). As broad tumor profiling becomes a common component of cancer care, next-generation sequencing (NGS) is increasingly used in many areas of cancer research and in clinical settings. Furthermore, endoscopic biopsies are suitable for targeted NGS, which provides quality

sequencing data and accurate mutational information (17–19). NGS is also a tool that is widely available to gastroenterologists (17–19). In this study, we aimed to identify potential genes for the prediction of the response to nCRT by using NGS in ESCC patients. Based on function analysis results, we chose AGR2 to perform further investigation. Our results showed that silencing of AGR2 might enhance sensitive to the cytotoxicity effect of cisplatin and 5-fluorouracil (5-FU) and may be through up-regulating p21 expression. Collectively, our results showed that, AGR2 might be a potential gene marker for response to nCRT in ESCC.

2. Material And Methods

2.1. Patient selection

From January 1, 2016, to December 31, 2018, 32 patients with ESCC who underwent nCRT at Taichung Veterans General Hospital were retrospectively enrolled. Only patients with one specimen of pre-treatment endoscopic biopsy (treatment-naïve tissue) and another specimen of post-treatment tissue were included. Samples of surgically resected tumors after nCRT were available from the Biobank of Taichung Veterans General Hospital. We collected clinical information such as age, sex, surgery type, complete or incomplete resection, histologic subtype, tumor stage, clinical image data, and therapeutic response. Both the data collection and gene expression analysis of tumor tissues were approved by the Institutional Review Board of Taichung Veterans General Hospital (IRB TCVGH No: CE17279A).

2.2. RNA sequencing and gene expression analysis

RNA libraries were generated using TruSeq Stranded mRNA Library Prep Kit (Illumina, San Diego, CA, USA) with 1μg of total RNA from all samples following the manufacturer's instructions. The prepared library was sequenced with paired-end runs using Illumina HiSeq 2500 sequencers. RNA reads were mapped to the human reference genome GRCh37 usingHISAT2aligner tools (20), read counts were calculated using feature Counts (21) and gene expression profiles were identified using DESeq2 (22). DAVID (the Database for Annotation, Visualization and Integrated Discovery, https://david.ncifcrf.gov/) functional tool was used for functional annotation of differentially expressed genes.

2.3. Cell line and culture condition

Two esophageal cancer cell lines (CE48T/VGH, and CE146T/VGH) were cultured in Dulbecco's modified Eagle's medium (Gibco) supplemented with 2mM L-glutamine (Gibco), 10 % fetal bovine serum, 100 U/mL penicillin-streptomycin (Gibco), and 10mM non-essential amino acids (Gibco). TE-2 cells were cultured in the same medium supplemented with 1mM sodium pyruvate (Gibco). All cells were cultured in a 5% CO_2 atmosphere at 37 °C.

2.4. RNA interference (small interfering RNA) analysis

RNA interference (RNAi) technology has revolutionized the biological discovery, target discovery, and validation processes. A smart pool of four siRNA sequences derived from the coding sequence of AGR2

and individual duplex and control siRNA were designed and purchased from Dharmacon. The following siRNAs were use: AGR2 siRNA no. 1 5'–GCUGAAGACUGAAUUGUAA-3', no. 2 5'-GCAACAAACCCUUGAUGAU-3', no.3 AGUCAAACCUGGAGCCAAA-3', and no.4 5'-UGAAGAAAGCUCUCAAGUU-3'. The control siRNA were non-targeting pool sequences and included the following: no. 1 5'-UGGUUUACAUGUCGACUAA-3', no. 2 5'-UGGUUUACAUGUUGUGUGA-3', no. 3 5'-UGGUUUACAUGUUUUCUGA-3', and no. 4 5'-UGGUUUACAUGUUUUCCUA-3'. Each freeze-dried siRNA was dissolved in RNase-free water.

Using siRNA, we knocked down AGR*2* gene expression in esophageal cancer cells. The TransIT-X2 Dynamic Delivery System reagent (Mirus Bio) procedure was used to forward-transfect siRNA into the esophageal cancer cells. The esophageal cancer cell cells were plated in 6-well culture plates at a density of 4.0–6.0 $\times 10^5$ cells/well and cultured in 2mL growth medium for 24 h. Cells were transfected with siRNA to a final concentration of 25nM by using TransIT-X2 transfection reagent. Subsequently, cells were incubated with 5% CO_2 at 37°C for 72 h. Finally, cells were harvested and assayed for the knockdown of target gene expression.

2.5. Reverse transcription and quantitative polymerase chain reaction

The total RNA was extracted using the AllPrep DNA/RNA Mini Kit followed the manufacturer's protocol (cat. 80204). Reverse transcription proceeded according to the SS4 (Invitrogen) protocol. Quantitative reverse- transcription polymerase chain reaction employed the Fast Start TaqMan Probes system (Roche, Cat.4913947001) and AGR2 specific primers, the analysis was performed on a StepOne Plus Real-Time PCR System (Applied Biosystems, Foster, CA). Glyceraldehyde 3-phosphate dehydrogenase was used as an endogenous control, to determine the relative expression levels of target genes using the method.

2.6. Reagents

Cisplatin (P4394) and 5-fluorouracil (F6627) were purchased from Sigma-Aldrich (Merk). Cisplatin was dissolved in double-distilled water. 5-FU was dissolved in dimethyl sulfoxide (DMSO). The solvent was also used in the control group of the experiment.

2.7. MTT assay

To determine the cytotoxicity of the combined effect of AGR2- knock down and chemotherapeutic agents, cells were first plated in 24-well culture plates for 24 h. Then, cells were transfected with siRNA. After 24 h, we treated the cells with cisplatin (2.0–6.0μM) and 5-FU (3.0–20.0μM) for 72h. Cell viability was evaluated using MTT assay followed by our previous study (23). The medium was removed and the cells were washed twice with 1× phosphate-buffer saline (PBS). Then, 500 μL MTT solutions (1mg/mL) were added to cells, and the solution was incubated at 37°Cfor 30 min. The MTT solution was removed and replaced with 200 μL DMSO, subsequently, the cells were incubated for 5 min to dissolve completely. We transferred 100 μL DMSO of dissolved cells into 96-well enzyme-linked immunosorbent assay (ELISA)

plates to measure the absorbance a t 570/670 nm by using ELISA reader. Each experimental data point represents the average value obtained from three replicates.

2.8. Western blotting

We employed western blot to determine the levels of AGR2 and associated proteins. Cells were washed with PBS and lysed in RIPA buffer (APOLO) supplemented with protease inhibitor cocktail. Protein concentrations were detected using a protein assay kit (Bio-Rad). Equal amounts of proteins (30μg) were subjected to sodium dodecyl sulfate 8% -12 % polyacrylamide gel electrophoresis. Fractionated proteins were transferred to Hybond-P membranes (Millipore). Membranes were blocked with PBS containing 5% nonfat milk and 0.2% Tween 20. For the detection of human anti-AGR2 (Invitrogen), anti-p21 (Proteintech), and anti β-actin (Sigma) the membranes were incubated overnight at 4°C, followed by the addition of anti-mouse or anti-rabbit IgG Horseradish peroxide -linked antibody (Jackson). Blots were then developed using an enhanced chemiluminescence reagent (Millipore) (24).

2.9. Statistical analysis

Statistical analysis of all results was performed using paired two-way analysis of variance with Tukey's test. All results reflect the mean ± standard error of the mean of data obtained from at least three independent experiments. Statistical significance was defined as $p < 0.05$.

3. Results

3.1. Clinical characteristic

The clinicopathological characteristics of ESCC patients (n = 32) is summarized in Table 1. The standard protocols for operable esophageal cancer patients were briefly described as followings. Chemotherapy was given concomitantly with cisplatin 20 mg/ml iv for 1 hour and fluorouracil 800 mg/ml iv for 24 hours daily(day 1 to day 4 (cycle1), and (day 29 to day 32 (cycle 2) of radiotherapy. Radiotherapy was performed 5 days per week, with a daily dose of 180 Gy for a total course of 5–6 weeks. Surgery was performed 4–6 weeks after complete nCRT. The surgical procedure included thoraco- scopic esophagectomy, at least 2-field lymph node dissection and esophagus reconstruction with gastric tube.

The patients' average age was 59.9 years (range 48–82 years). Patients were divided into two groups according to their response to nCRT; Complete response group (n = 13) and Non-complete response group (n = 19) (Table 2). Four patients in the Non-complete response group and two patients in the complete response group did not undergo operations after CRT. The response status of these patients was confirmed by clinical evaluation and endoscopic biopsies.

3.2. RNA expression differences between Complete response and Non-complete response groups of patients with ESCC

Sequencing reads were mapped against the human genome assembly (Ensembl Build 37) using Tophat (v2.1.1). We identified 464 differentially expressed genes (fold change > 2 or < 2, and a DESeq p value of

< 0.05), in which 240 genes were up-regulated and 224 were down-regulated among them. The values of fold change among top 20 up-regulated and top 20 down-regulated genes are shown in Table 3 and Supplemental Table 1, respectively. Unsupervised hierarchical clustering of 20 up-regulated and 20 down-regulated genes also revealed the differences in Complete response or Non-complete of nCRT (Fig. 1).The resulting heat map and HCL dendrogram showed a similar separation of the two nCRT types.

The Database for Annotation, Visualization, and Integrated Discovery (DAVID) was used to explore the potential functions of the differentially expressed genes and its biological function (25, 26). Up-regulated genes in Non-complete response group was associated with cellular protein metabolic process, glucose homeostasis, TGF-beta receptor signal response pathway, cholesterol homeostasis, cell differentiation and drug response (Fig. 2). We firstly assessed gene expression in up-regulated genes between Complete response and Non-complete response, and further selected four genes that are associated with cell proliferation and cell migration, namely AGR2 (27), PPP1R15A (28), GADD45B (29), and LRG1 (30). RNA sequencing results showed that, these four genes were significantly up-regulated in patients with Non-complete response group compared to complete response group (Fig. 3).

3.3 Knockdown of AGR2 in esophageal cancer cells is more sensitive to the cytotoxicity effect of cisplatin and 5-fluorouracil

Previous studies have indicated that AGR2 is associated with head and neck squamous cell carcinoma (HNSCC) by regulating cell transformation and epithelial mesenchymal transition (EMT) signaling pathways (31), in esophageal adenocarcinoma it promotes tumor growth (32). However, in this study we found that the AGR2 was up-regulated in the patients with Non-complete response before nCRT, thus we selected AGR2 for further investigation.

We then applied siRNA approach to knock down AGR2 expression in esophageal cancer cell lines (CE146T/VGH, TE2, and CE48T/VGH) and then performed MTT assay. Western blot analysis showed that the protein level of AGR2 was significantly reduced in AGR2 siRNA transfected CE146T/VGH, TE2, and CE48T/VGH cells. (Supplementary Fig. 1). MTT assay indicated that cell viability of the *AGR2*-knockdown TE2 cell line decreased significantly following treatment with 2.5μM cisplatin and 3μM 5-FU, whereas CE48T/VGH and CE146T/VGH cells showed no any change (Fig. 4). Whereas in *AGR2*-transfected cells (CE48T/VGH, CE146T/VGH and TE-2) increased concentration of (6μM) cisplatin and 20μM 5-FU significantly decreased cell viability compared to control group (Fig. 4). All these data indicated that *AGR2* down-regulated cells were more sensitive to cisplatin and 5-FU treatment.

3.4. Cisplatin and 5-FU induced mortality in *AGR2*-knockdown esophageal cancer cells by up-regulating p21 expression

Esophageal cancer cells were transfected with *AGR2-siRNA* for 24 h and then exposed to cisplatin and 5-FU for 48 h. In*AGR2*-knockdown cells, p21 protein level was increased in compared to control group (Fig. 5). These results suggest that the cytotoxicity sensitivity to cisplatin and 5-FU in esophageal cancer was associated with AGR2 expression and might be through the p21 pathway.

4. Discussion

In humans, Anterior gradient (AG) proteins are mainly distributed in endoderm-derived organs, such as the lungs, stomach, small intestine, colon, and prostate (27, 33). AGR2 was initially found in human breast cancer specimens (27), contrarily, decreased AGR2 expression was found in breast cancer (27, 34), esophagus (35), stomach (36), lung (37), pancreas(38), ovarian (39) and prostate cancer. (40). AGR2 is a member of the disulfide isomerase family of endoplasmic reticulum (ER) proteins, it catalyzes protein folding and thiol-disulfide interchange reactions (41).

DiMaioet al. retrospectively examined 116 specimens of esophageal carcinoma; they demonstrated that the presence of diffused AGR2 expression is highly sensitive to esophageal adenocarcinoma. However, focal expression of AGR2 was found in only approximately one-third (36.59%) of ESCC specimens (42). In this study, we firstly attempted to identify biomarker to predict nCRT responses in esophageal cancer patients (n = 32) by next-generation sequencing (NGS). We found 464 genes were differentially expressed in ESCC that were associated with nCRT response. We then assessed gene expression in up-regulated genes between Complete response and Non-complete response group, and further selected four genes that are associated with cell proliferation and cell migration, namely AGR2 (27), PPP1R15A (28), GADD45B (29), and LRG1 (30). Based on the gene fold change differences, we scrutinized AGR2 with 2.8 fold difference in the patients with Non-complete response than treatment group. Thus we decided to use AGR2 to do further investigation. AGR2 expression was significantly correlated with metastasis and poor prognosis in breast cancer and biomarker in prostate cancer (43–45).

Previous study showed that, AGR2 induces cancer cell proliferation, invasion and survival, chemotherapy resistance, metastasis and tumor growth. Therefore in this present study at first we knock-downed AGR2 expression in esophageal cancer cell lines (CE146T/VGH, TE2, and CE48T/VGH cells) and then analyzed for AGR2 expression using Western blotting. AGR2 protein expression was greatly decreased in all the three si-AGR2 transfected cancer cells. To further correlate the role of AGR2 with nCRT response, we knockdown AGR2 expression and treated cells with cisplatin and 5-FU and analysed for its cell viability. Cancer cells -CE48T/VGH and CE146T/VGH treated with low concentration of 2.5μM cisplatin and 3μM 5-FU showed no any change in the cell viability percentage compared with TE2 cell lines. This may be due to nature of cell type, contrarily when the treatment dose was increased all the three cells showed significant decrease in cell viability compared to control group. This data clearly explains us the significant role of *AGR2* down-regulation in cisplatin and 5-FU treatment in esophageal cancer cells.

Previous study showed that AGR2 expression and cell cycle proteins showed as good prognosis marker in epithelial ovarian cancer (46), in this study in *AGR2*-knockdown cells, cisplatin and 5-FU treatment increased p21 protein level compared to control group. These results suggest that the cytotoxicity sensitivity to cisplatin and 5-FU in esophageal cancer was associated with AGR2 expression and might be through the p21 pathway. This was a retrospective study; further prospective studies are required to verify the correlation and precise pathway. Although many studies have confirmed that endoscopic

biopsy results in a high concordance between biopsy and resected specimens (18, 47, 48), the bias of gastroscopic biopsy remains a concern.

5. Conclusions

Using specimen from gastroscopic biopsy and NGS, we obtained sufficient specimens confirm the overexpression of genes in patients with ESCC. The AGR2 gene might be one of the predictors of poor response to nCRT. Sensitivity to cytotoxicity may be associated with p21 expression. These findings might inform alternative personalized therapeutic plans or new directions for targeted therapy in the future.

Abbreviations

ESCC, Esophageal squamous cell carcinoma; nCRT, neoadjuvant chemoradiotherapy; NGS, next-generation sequencing; 5-FU, 5-fluorouracil; EMT, epithelial mesenchymal transition

Declarations

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Authors' contributions: C.H.L. and H.N.C. designed the research, performed the experiments and wrote the manuscript; T.H.H., V.B.K., and C.H.H. analyzed the data and interpreted data; C.Y.H., L.W.L., and C.L.M. contributed new reagents and analytical tool. All authors were involved in editing of the manuscript and approved its final submitted version.

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Availability of data and material: The data and material of this study is available for publishing in public.

Ethics approval and consent to participate: Both the data collection and gene expression analysis of tumor tissues were approved by the Institutional Review Board of Taichung Veterans General Hospital (IRB TCVGH No: CE17279A).

Consent for publication: **Written informed consent was obtained from all participants.**

Conflicts of interest/Competing interests: The authors have declared that they have no conflicts of interest.

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Tables

Due to technical limitations, table 1, 2 and 3 is only available as a download in the Supplemental Files section.

Figures

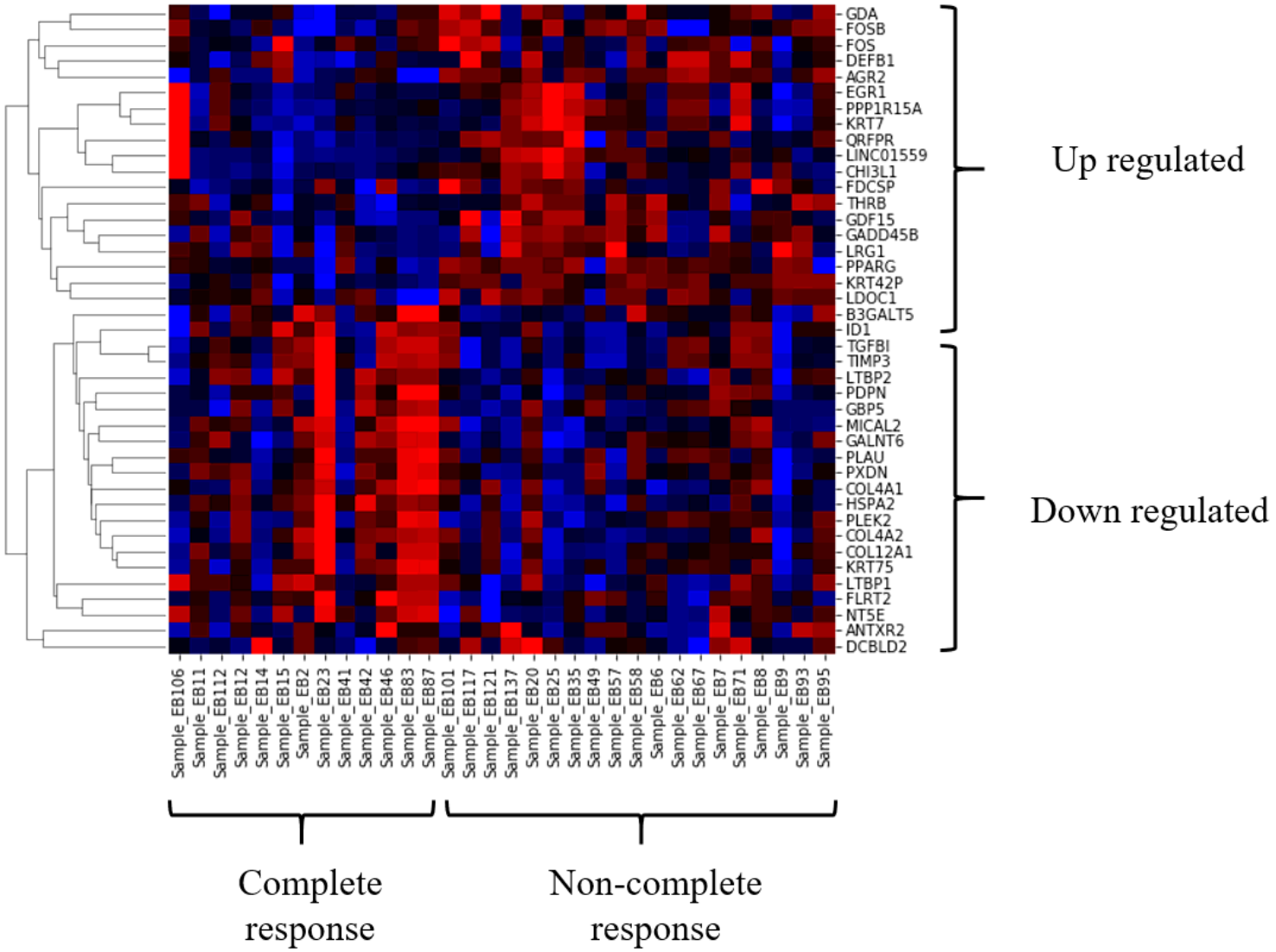

Figure 1

Hierachical cluster analysis (HCL) of 20 proteins identified in patients with ESCC before neoadjuvant chemoradiotherapy. HCL of the 20 differentially expressed genes identified between groups with Complete and Non-complete response to ESCC treatment was performed via unsupervised hierarchical classification and distance trees were constructed from all genes. High expression of genes is highlighted in red and low expression in blue; different levels of shade indicate the extent the expression level difference from the median.

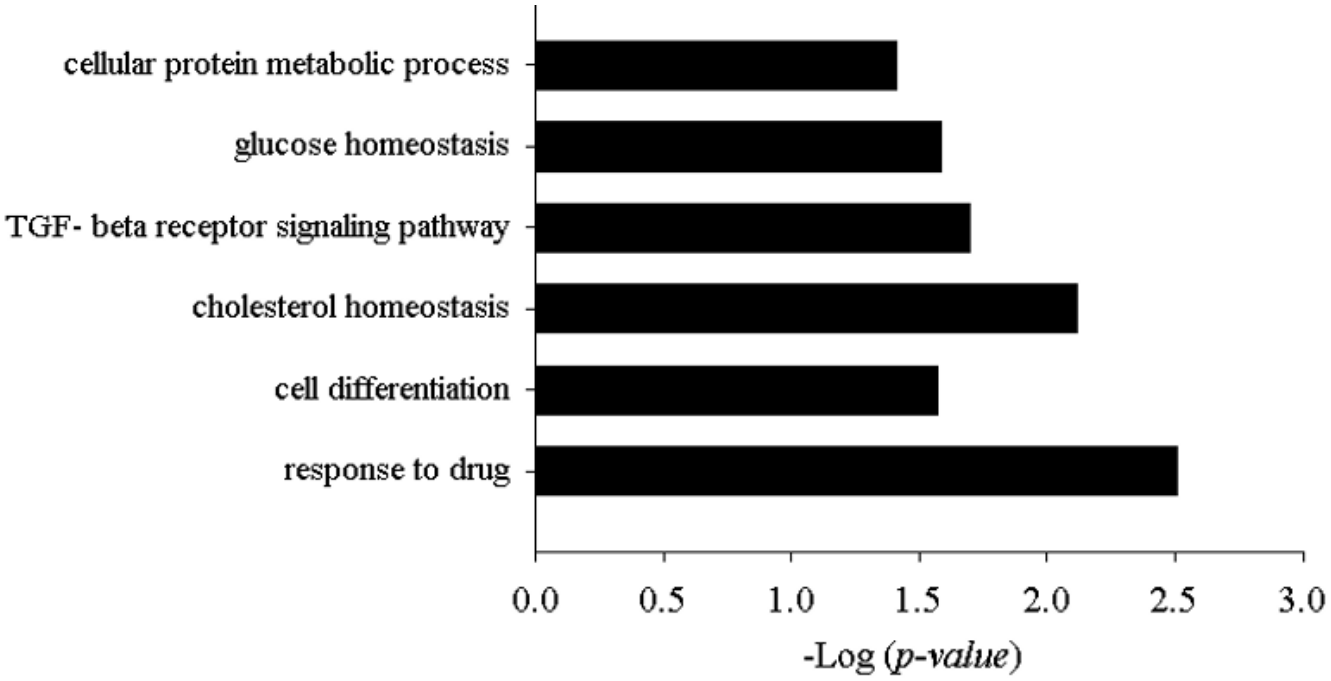

Figure 2

Up-regulated gene expression pattern according to functional analysis. The Database for Annotation, Visualization, and Integrated Discovery was used to predict the potential functions of the differentially up-regulated expressed genes. Clusters with $p < 0,05$ are presented.

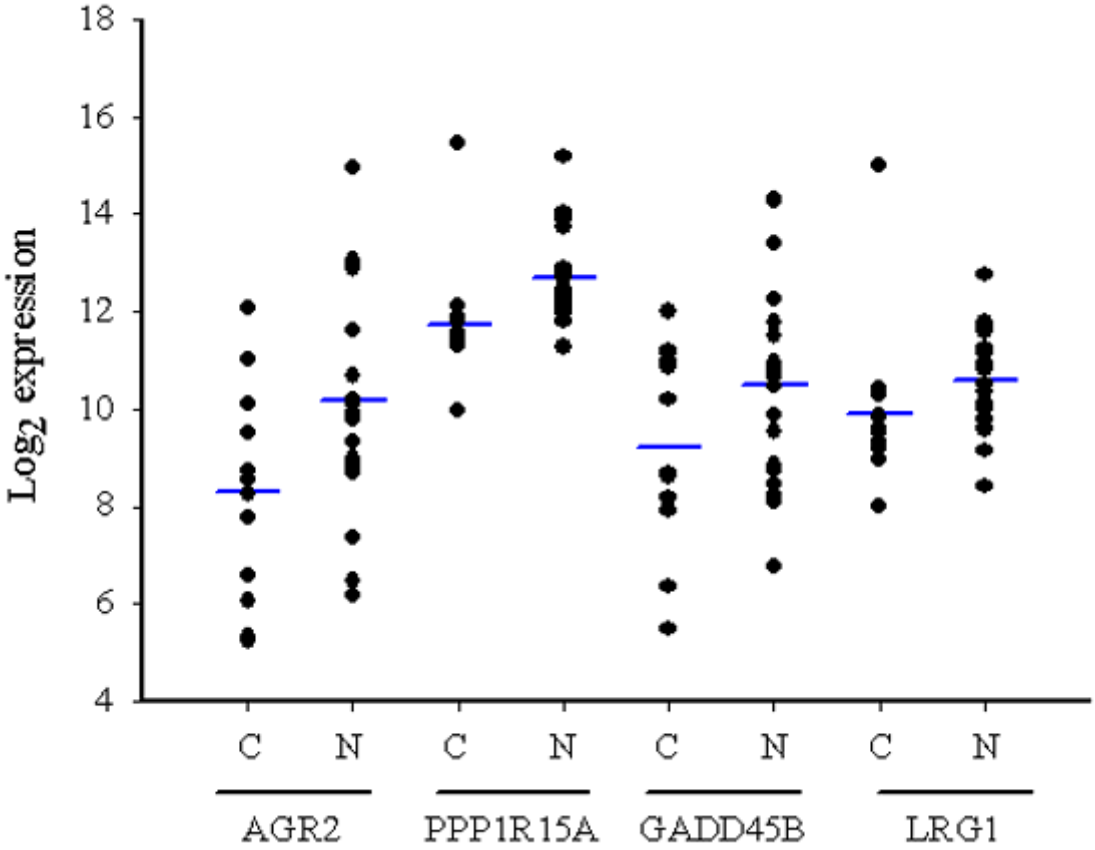

Figure 3

The log2 gene expression of AGR2, PPP1R15A, GADD45B and LRG1 in two groups of patients with ESCC before neoadjuvant chemoradiotherapy. RNA prepared from two groups of the patients with ESCC before neoadjuvant chemoradiotherapy were subjected to RNA sequencing, and quantitative analysis of gene expression levels was acquired among them, C = Complete response group (n= 13); N = Non-complete response group (n = 19).

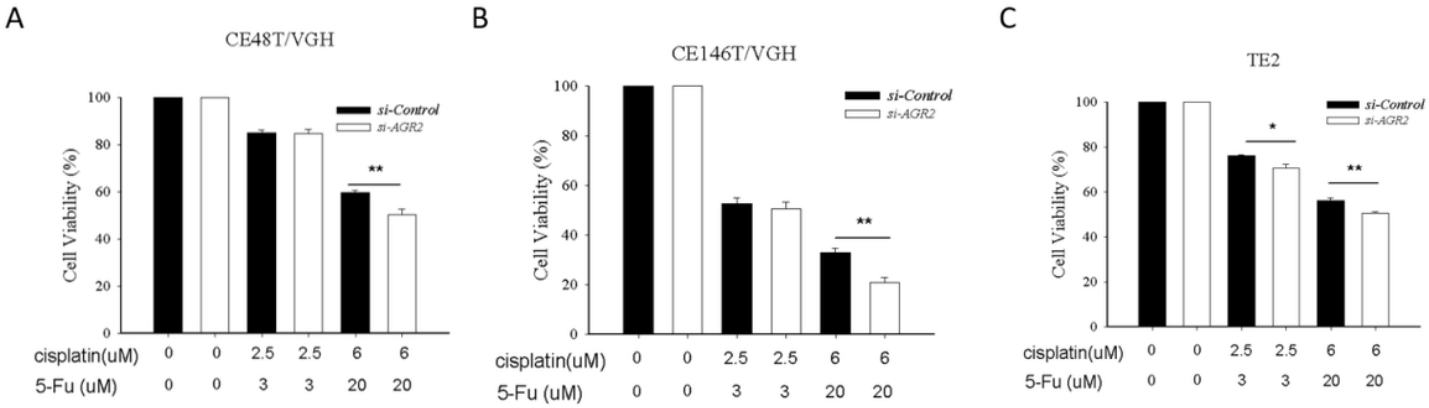

Figure 4

Inhibition of AGR2 enhanced the cytotoxicity of cisplatin and 5-fluorouracil treatment in esophageal cancer cell lines. CE48T/VGH, CE146T/VGH, and TE2 cells were transfected with control siRNA (si-control) and siRNA-AGR2 (si-AGR2) as indicated. After knock down for 24 h, the combinational treatment of cisplatin and 5-FU was added to the cells for 24 h. The cell viability were determined among these three cell lines by MTT assay. (* $p < 0.05$, ** $p < 0.01$).

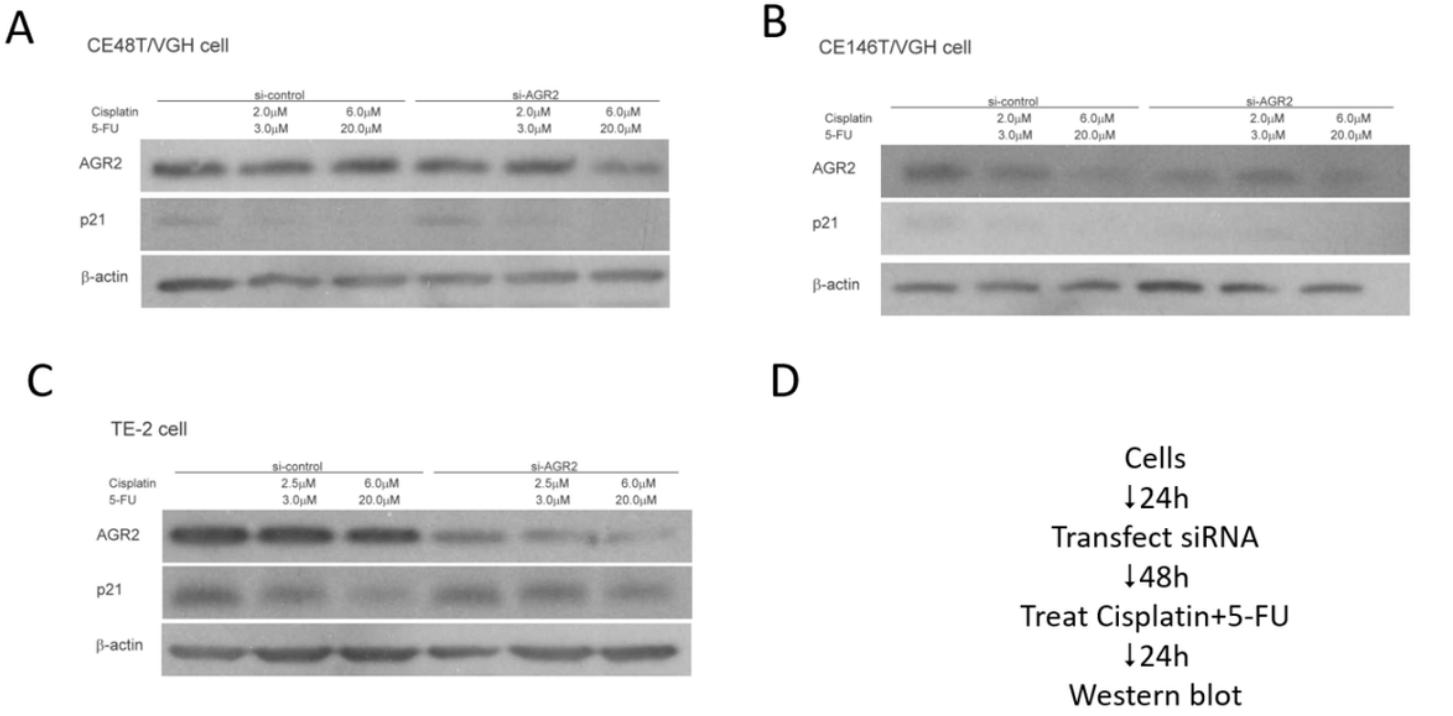

Figure 5

Inhibition of AGR2 (si-AGR2) enhanced the protein expression of p21. (A) CE48T/VGH, (B)CE146T/VGH, and (C)TE-2 cells were transfected with control siRNA (si-control) and siRNA-AGR2 (si-AGR2) as indicated. After transfection for 24h and treat cisplatin and 5-FU for 48h, the cells were harvested for protein expression (AGR2 and p21) was detected by western blot anlaysis. β-action was used as a loading control.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table.xlsx
- SupplementaryFigure1.tif