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The Effect of Vincristine on Hypoxic, Apoptotic, and Autophagic Gene Expressions in Gastric Cancer Cells under Hypoxic Conditions

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Abstract
Background: Apoptosis and autophagy functions are known as death-inducing mechanisms. Vincristine (VCR) is a chemotherapeutic drug that binds with tubulin dimers and induces apoptosis in cancer cells. Furthermore, VCR protects cancer cells from apoptosis, allowing them to enter autophagy. This makes cancer even more aggressive in its latter stages. Herein, we aimed to investigate the effect of VCR on autophagy and apoptosis in gastric cancer AGS and MKN45 cells under hypoxic conditions.
Method: In this experimental approach, we performed cell culture, cell viability analysis, ribonucleic acid (RNA) isolation, cDNA synthesis, and observation of gene expression levels. Subsequently, the amount of acidic vesicular organelles was investigated with acridine orange staining. Through the use of quantitative real-time polymerase chain reaction, we studied the effect of hypoxia and VCR on mRNA levels of hypoxia-related *vascular endothelium growth factor(VEGF)* and *hypoxia-inducible factor-1α (HIF-1α)*, as well as *apoptosis-related Bcl-2-associated X protein (Bax)* and *B-cell lymphoma 2 (Bcl-2)*, and *autophagy-related Beclin-1* and *microtubule-associated protein 1A/1B-light chain 3 (LC3-II)* on AGS and MKN45 cell lines. The optimum VCR concentration was selected via cell viability analysis.
Results: The optimum VCR concentration was selected as 300 nM. Vascular endothelium growth factor, hypoxia-inducible factor-1α, Bax, Bcl-2, and LC3-II mRNA expressions increased by 7.63, 3.34, 2.07, 4.09, and 3.1 folds, respectively, after VCR application to AGS cells under hypoxic conditions compared to the control cells. In MKN45 cells, Bax and LC3-II gene expressions increased by 5.21 and 12.2 folds, respectively, under the same conditions. In addition, the increase in the expression of autophagic LC3-II gene was consistent with acidic vesicular organelle formation.
Conclusion: It was observed that VCR affects hypoxic, apoptotic, and autophagic gene expressions in AGS and MKN45 cells under hypoxic conditions.
Keywords: Gastric cancer, Vincristine, Autophagy, Apoptosis, Hypoxia

Introduction

Carcinogenesis is a multistage genetic disorder that involves the irregular division and proliferation of cells, as well as invasion and metastasis elsewhere without a growth factor or the need for receptor activation.[1] Gastric carcinoma is the fourth most frequent cause of cancer death worldwide, with approximately

one million cases identified and more than 700,000 fatalities per year.[2]

Apoptosis plays a vital role in the developmental processes and maintenance of homeostasis in eukaryotes.[3,4,5] Several oncogenes and tumour suppressor genes are involved in mediating apoptosis.[6] The B-cell lymphoma 2 (Bcl-2) family includes important apoptosis regulators, and the molecule is overexpressed in many types of cancer cells. It has been shown in gastric cancer studies that overexpression of Bcl-2-associated X protein (Bax) accelerates cell death, and overexpression of antiapoptotic proteins; for instance, Bcl-2 suppresses this function of Bax. Therefore, the Bcl-2/Bax ratio may be one of the critical factors of a cell's threshold to undergo apoptosis.

Autophagy is a cellular destruction pathway, which also helps digest organelles, such as intracellular parasites, endoplasmic reticulum, and mitochondria.[7] Although autophagic cell death may prevent metastasis in gastric cancer, most available findings support the idea that autophagy can affect tumour metastasis by influencing various factors, such as inflammation, hypoxia, anoikis, epithelial mesenchymal transition, invasion, angiogenesis, and starvation.[8] Beclin-1 is one of the autophagic regulatory and tumour suppressor genes, whose overexpression is a highly frequent event in carcinomas.[9] Microtubule-associated protein 1A/1B-light chain 3 (LC3) is another regulator gene that can firstly break down into LC3-I, and turn to a soluble form known as LC3-II afterwards. Highly expressed LC3 in many gastrointestinal cancers, including stomach carcinoma, triggers autophagy. This autophagic conditions are advantageous for tumour cells.[10]

Vincristine (VCR) is a vinca alkaloid chemotherapeutic that inhibits the formation of mitotic spindles required for cell division and proliferation. [11] VCR has effects on apoptosis and autophagy. Cells exposed to VCR lose their mitotic ability due to weakly formed mitotic components. [12] Afterwards, they are suspended as undivided damaged cells and will be sent to apoptosis.[11] By targeting tubulins, VCR takes part in the fusion of autophagosomes with lysosomes in the fusion phase of autophagy. It also causes a decrease in the effect of autophagy.[13] VCR is known to trigger apoptosis in stomach cancer,[14] and autophagy in oral cancer.[15]

Oxygen is the primary essential factor for the aerobic metabolism of most eukaryotic organisms.[16] Hypoxia, which creates an aggressive microenvironment, is observed in many solid tumours due to the rapid increase in cell proliferation and the rise in tumour mass. To overcome oxygen deficiency, cancer cells activate various survival pathways and tumour vasculature.[17] Hypoxia-inducible factor-1 alpha (HIF-1α) is the most important and central regulator in a range of regulatory responses to adapt tumour cells to the hypoxic environment.[18] Vascular endothelial growth factor (VEGF) is a strong stimulator of angiogenesis, as both in vivo and in vitro. The expression of VEGF is accepted to mediate HIF-1α during hypoxia.

In this study, we aimed to show, for the first time in the literature, the effect of VCR on apoptosis and autophagy under hypoxic conditions. We compared it with normal conditions in AGS and MKN45 stomach cancer cells. To this end, we analysed the mRNA levels of HIF-1α, VEGF, Bax, Bcl-2, Beclin-1, and LC3-II under different conditions.

Materials and Methods

Herein, we applied an experimental approach. We performed cell culture of AGS and MKN45 cells, cell viability analysis, RNA isolation, cDNA synthesis, and observation of gene expression levels. Subsequently, the amount of AVOs was observed with acridine orange staining. The effect of VCR and hypoxic conditions on the mRNA levels of VEGF, HIF-1α, Bax, Bcl-2, Beclin-1, and LC3-II was studied on stomach cancer cell lines of AGS and MKN45.

Cell lines

AGS cells were obtained from ATCC while MKN45 cells were obtained from DSMZ. AGS and MKN45 cells were grown in Dulbecco's modified Eagle's medium-F12 (DMEM-F12) with 10% fetal bovine serum and in Roswell Park Memorial Institute (RPMI) 1640 medium with 10%, respectively. These cells were kept under normal conditions at 37°C in a humidified atmosphere of 5% CO_2, considered as control conditions. Hypoxic conditions were set at a humidified atmosphere of 1% O_2 and 5% CO_2 balanced with N_2 in a at 37°C.[19]

Cell cytotoxicity

In this study, we used 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) analysis for depicting the effect of VCR on the viability of cell lines. The experiment was performed in accordance with the Cell Proliferation Kit I (MTT) from Sigma Aldrich. 4×10^3 cells/well was treated with 200 nM-1 mM concentrations of VCR. Following the VCR treatment for 24, 48, and 72h, 10 µl MTT reagent was added to each well. The wells were incubated for another 3-4h. We then added 100 µl of MTT solubilization solution to the wells and incubated them overnight at 37°C under 5% CO_2 to terminate the reaction. The synthesis of formazan, determined by the colour intensity at 570 nm, was directly related to the number of live cells.

Application of VCR

There are five groups of VCR application:

1. Cells incubated for 48h (AGS cells) and 24h (MKN45 cells) under normal conditions, as the control group;
2. Cells treated with 300 nM VCR for 48h (AGS cells) and 24h (MKN45 cells) under normal conditions;
3. Cells incubated for 48h (AGS cells) and 24h (MKN45 cells) under hypoxic conditions, as the hypoxic group;
4. Cells treated with 300 nM VCR under hypoxic conditions for 48h (AGS cells) or 24h (MKN45 cells);
5. Cells treated with 300 nM VCR for 24h (prolonged stress) after being incubated under hypoxic conditions for 24h.

Hoechst and acridine orange staining

Acridine orange staining was performed to observe autophagosome formation. We carried out the procedures in accordance with the acridine orange from Thermo Fisher. The cells were fixed for 15 minutes in 4% formaldehyde. Between each reaction, a thorough PBS wash (twice) was performed to eliminate any remaining solvent. The cells were stained with Hoechst and acridine orange for 15 minutes in the dark before being examined under fluorescence microscopy with UV filters, following which images were taken with 40X objective via Inverted Microscope.

Quantitative real time PCR polymerase chain reaction (qRT-PCR)

Total RNA was extracted using the Machery Nagel RNA Isolation Kit. We synthesized cDNA from 1000 ng total RNA for AGS cells and 500 ng total RNA for MKN45 cells using Bioline SensiFAST and Sigma-Aldrich cDNA Synthesis Kit, according to the manufacturer's instructions. Subsequently, the products were amplified using sequence-specific primers under the following condition: 95 °C for 2 min, 95 °C for 5 s, 55 °C for 10 s, and 72 °C for 15 s (40 cycles) using the $2^{-\Delta\Delta Ct}$ method, normalised with GAPDH housekeeping gene. The primers were designed utilising NCBI, Primerblast, and Primer3.

We carried out all the experiments through two biological and three technical repeats.

Statistical analysis

To assess MTT analysis, we used ANOVA test for MKN45 cells, and Kruskal Wallis test for AGS cells. Through the use of ANOVA test, we determined the significance of qRT-PCR analysis for both cell lines. The data were shown as means ± standard deviations. Additionally, $P < 0.005$ was deemed to be statistically significant.

Results

MTT analysis

AGS and MKN45 cell lines

A decrease in cell viability was observed at all concentrations of VCR between 200 nM-1000 nM tested at time points of 24, 48, and 72h. Statistically significant ($P < 0.005$) cell death was observed at the concentration of 300 nM at 48h as shown in figure 1 for AGS, and 24h as illustrated in figure 2.

Concerning the experimental conditions for AGS cells, 300 nM concentration and 48h of incubation were assigned in this work.

Meanwhile, regarding the experimental conditions for MKN45 cells, 300 nM concentration and 24h of incubation were considered.

qRT-PCR

Following application of 300 nM VCR to AGS cells under hypoxic conditions for 48h, the mRNA expression levels of VEGF and HIF-1α (hypoxic markers), Bax and Bcl-2 (apoptotic markers), as well as Beclin-1 and LC3-II (autophagic markers) were determined. Under hypoxic conditions with 300 nM VCR application for 48h, VEGF, HIF-1, Bax, Bcl-2, Beclin-1, and LC3-II mRNA expressions increased significantly compared to the control AGS cells (Figure 3). There was a statistically significant increase in HIF-1α mRNA level after incubation under hypoxic conditions for 48h compared to the control group (Figure 3). We also observed a significant decrease in Bax mRNA expression following incubation under hypoxic conditions for 48h compared to the control group (Figure 3).

As a result of 300 nM VCR and hypoxic treatment to MKN45 cells, the mRNA expression levels of VEGF and HIF-1α (hypoxic markers), Bax and Bcl-2 (apoptotic markers), along with Beclin-1 and LC3-II (autophagic markers) were measured, shown in figure 4. Under the prolonged stress conditions, VEGF, Beclin-1, and LC3-II mRNA expressions increased significantly compared to the control MKN45 cells (Figure 4). Moreover, under hypoxic conditions with 300 nM VCR application, Bax and LC3-II mRNA expressions rose significantly compared to the control MKN45 cells (Figure 4). VCR treatment also increased the expression levels of Bax, Bcl-2, Beclin-1, and LC3-II genes compared with normal conditions (Figure 4).

VEGF and Beclin-1 gene expressions increased in the AGS cells following 300 nM VCR treatment compared with normal conditions. Meanwhile, in MKN45 cells, VEGF, Bax, Bcl-2, Beclin-1, and LC3-II gene expression levels increased under the same conditions (Figure 5). Furthermore, following VCR application, VEGF, Bax, Bcl-2, and LC3-II mRNA levels rose in AGS cells compared with hypoxic conditions. Nonetheless, in MKN45 cells, Bax and LC3-II expressions increased (Figure 6). Beclin-1 and LC3-II gene expressions increased while the HIF-1α gene expression decreased under prolonged stress in comparison with hypoxic conditions in AGS cells. Meanwhile, in MKN45 cells, VEGF, HIF-1α, Bcl-2, Beclin-1, and LC3-II gene expressions experienced an increase (Figure 7). Following prolonged stress, compared with the treatment of VCR under hypoxic conditions, VEGF, HIF-1α, Bax, Bcl-2, and LC3-II gene expressions decreased in AGS cells. On the other hand, mRNA level of the VEGF gene increased whereas that of Bax and LC3-II decreased in the MKN45 cells (Figure 8).

Tables 1-4 summarise all the gene expression changes observed herein.

Analysis of autophagy

Figures 9 and 10 illustrate acridine orange staining of AGS and MKN45 cells for the formation of acidic vesicular organelles (AVOs). There was a decrease in the number of AVO in the VCR-treated AGS cells compared with the control non-treated cells grown under normal conditions. The AGS cells under stress in hypoxic conditions had fewer AVOs in comparison with the cells grown under hypoxic conditions in the presence of VCR; however, the AGS cells grown under prolonged stress conditions had more AVOs than the cells grown under hypoxia. They also

showed quite similar responses regarding AVO number compared with the cells under hypoxic conditions in the presence of VCR.
On the contrary, there was an increase in the number of AVO in the VCR-treated MKN45 cells compared with the control non-treated cells grown under normal conditions. Additionally, MKN45 cells under stress in hypoxic conditions had strikingly more AVOs than those grown under hypoxic conditions in the presence of VCR. MKN45 cells grown under prolonged stress conditions also had more AVOs than the cells grown under hypoxic conditions in the presence of VCR.
Herein, in the AGS cell line, compared with the control, AVO formation decreased by eight times as a result of VCR application and increased by two times after VCR application in MKN45 cell line. When hypoxia was applied, the number of AVO decreased by 6.4 folds in the AGS cells compared with the control. Nevertheless, once hypoxia and VCR were applied simultaneously, AVOs increased by two times compared to the control. After prolonged stress application, formation of AVO increased by 2.2 times compared to the control condition. Moreover, when the same procedures were applied to MKN45 cells, the increase in AVOs under hypoxic conditions were 2.3 times compared to that in the control. Following prolonged stress application, we observed a 1.2-time increase in AVOs.

Discussion

We herein revealed that VEGF, HIF-1α, Bax, Bcl-2, and LC3-II mRNA expressions increased significantly by 7.63, 3.34, 2.07, 4.09, and 3.1 folds, respectively, after 300 nM VCR application to AGS cells under hypoxic conditions compared to non-treated control cells. In MKN45 cells on the other hand, Bax and LC3-II gene expressions increased by 5.21and 12.2 folds, respectively, under the same conditions. In addition, the increase in the expression of autophagic LC3-II gene in both cell lines was consistent with AVO formation. Our results concerning AVO formation are concordant with those of a study suggesting that VCR induces autophagy in oral cancer and gastric cancer BGC-823 and SGC 7901 cells. [15] In a previous paper however, it was reported that AVO formation is not significantly induced by VCR,[20] which contrasts with the MKN45-related results herein.
VCR is known to decrease the expression of both HIF-1α and VEGF genes. It has been shown that HIF-1α is highly expressed in glioblastomas and application of VCR to these cells under hypoxic conditions causes a decrease in VEGF and HIF-1α levels; these findings are opposed to ours.[21] HIF-1α and VEGF expressions have been found to increase in the presence of hypoxia in HUVEC cells,[22] in line with our findings. This shows that VCR-mediated increase in VEGF might cause angiogenesis in AGS cells. VCR possibly appears to induce angiogenesis in AGS cells under hypoxic conditions. Under aggressive conditions however, in which hypoxia and VCR are applied simultaneously, VEGF is further triggered compared with normal conditions, as revealed by the increased levels of VEGF. This confirms that high VEGF expression when oxygen levels are minimal and chemotherapeutic drug VCR is present further necessitates angiogenesis. In the presence of VCR under hypoxic conditions, a noticeable increase in the HIF-1α and VEGF gene expressions may reveal the activation of the glycolytic pathway, which can result in metabolic remodelling in AGS cells. Additionally, in more aggressive MKN45, cells apoptosis and autophagy can probably be induced once VCR is applied under hypoxic conditions compared with hypoxic conditions. Accordingly, VCR application to aggressive tumours under hypoxic conditions seems to be advantageous.
We showed that under hypoxic conditions, mRNA levels of Bax gene decreased by 0.9 folds in AGS cells whereas it increased by 1.55 folds in MKN45 cells. Moreover, gene expression levels of Bcl-2 increased by 1.88 folds after the treatment of 300 nM VCR in MKN45 cells. Overexpression of Bcl-2

inhibits autophagy, especially under hypoxic conditions; hypoxia has been found to selectively induce mitochondrial autophagy.[23] Under hypoxic conditions, silencing of HIF-1α can inhibit antiapoptotic Bcl-2 gene, but stimulate pro-apoptotic Bax gene.[24] The expression of Bax gene has been shown to decrease in colon cancers under hypoxic conditions.[25] Higher antiapoptotic Bcl-2 expression than pro-apoptotic Bax and increased LC3-II levels might reveal the likelihood of AGS cells to choose antiapoptotic and autophagic pathways in the presence of VCR under hypoxic conditions compared to only the presence of hypoxic conditions. This might depict the effect of chemotherapeutic drugs on the induction of antiapoptotic and autophagic signalling in AGS cells.

We found that the expression of Beclin-1 gene increased by 5.27 folds compared with the control cells, where VCR was applied to MKN45 cells. Moreover, VEGF, Beclin-1, and LC3-II gene expressions increased by 10.08, 2.89, and 8.06 folds, respectively, in MKN45 cells after prolonged stress conditions. In aggressive MKN45 cells, according to our results, VCR might possibly induce angiogenesis and autophagy under prolonged stress. This may give cancer cells a proliferative advantage. To avoid proliferative advantage, it would be better not to treat aggressive cancer cells which survive under prolonged stress conditions with VCR. The expression of angiogenic VEGF and HIF-1α, proapoptotic Bax and anti-apoptotic Bcl-2, as well as autophagic LC3-II genes appears to be reduced under prolonged stress compared with the combined treatment of hypoxia and VCR in AGS cells. AGS cells under prolonged stress, most probably neither enter apoptosis, nor autophagy, which may consequently cause senescence under long-term stress. As another view, cells might enter quiescent phase. As a result of long-term stress in aggressive MKN45 cells, the increase in vascularative gene expression, the decrease in apoptotic, along with autophagic gene expression might lead to higher vascularization and lower apoptotic and autophagic response; this suggests that VCR is less effective in late-stage use.

The strength of this study is being the first to show an increase in the expression of an apoptotic gene, Bax, and an autophagic gene, LC3-II, in more aggressive gastric cancer MKN45 cells subjected to VCR under hypoxic conditions. This finding could indicate that the application of VCR in cases where hypoxia conditions develop in advanced gastric cancers will be more beneficial than its application in early-stage cancers. Nonetheless, the lack of in vivo experiments is a limitation of this study. Moreover, apoptotic cell death needs to be further investigated.

Conclusion

In conclusion, further research should be conducted to understand the association of VCR use under hypoxic conditions in gastric cancer cells with cellular mechanisms involved in apoptosis, autophagy, angiogenesis, senescence, and metabolic remodelling at the molecular level in early- and late-stage cancers.

Acknowledgements

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Conflict of Interest

None declared.

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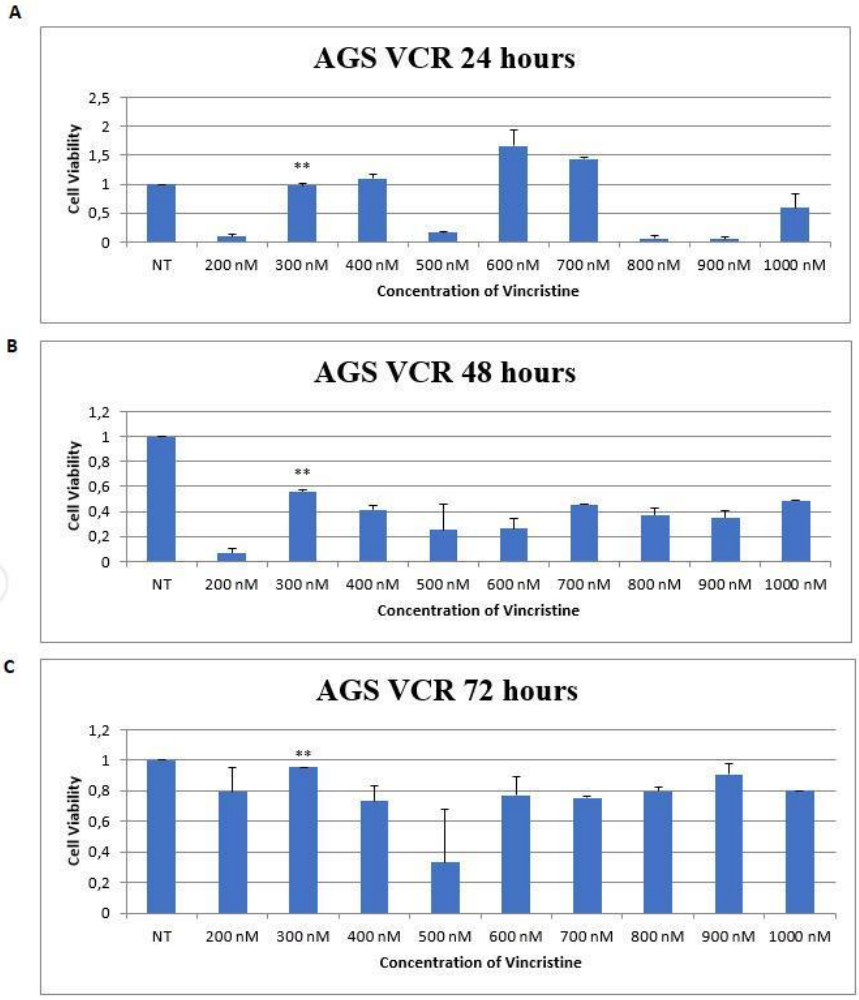


Figure 1. This figure shows the cell viability in AGS cell lines through MTT analysis after Vincristine treatment. A. Cell viability after 24 hours of VCR treatment to AGS cell line compared with non-treated conditions (** $P < 0.005$); B. Cell viability after 48 hours of VCR treatment to AGS cell line under non-treated conditions (** $P < 0.005$); C. Cell viability after 72 hours of VCR treatment to AGS cell line under non-treated conditions (** $P < 0.005$).

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; nM: Nanomolar; NT: Negative Control; VCR: Vincristine

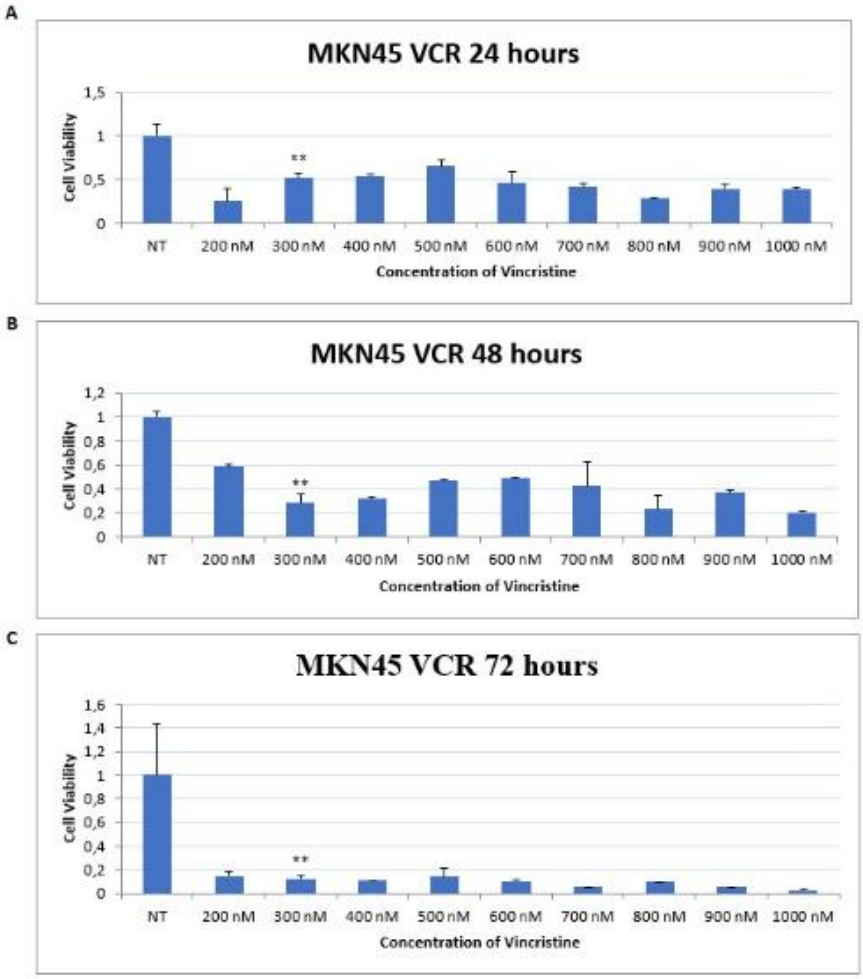


Figure 2. This figure shows the cell viability in MKN45 cell line via MTT analysis after Vincristine treatment. A. Cell viability after 24 hours of VCR treatment to MKN45 cell line compared with non-treated conditions (** $P <$ 0.005); B. Cell viability after 48 hours of VCR treatment to MKN45 cell line under non-treated conditions (** $P < 0.005$); C. Cell viability after 72 hours of VCR treatment to MKN45 cell lines under non-treated conditions (** $P < 0.005$).

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; nM: Nanomolar; NT: Negative Control; VCR: Vincristine

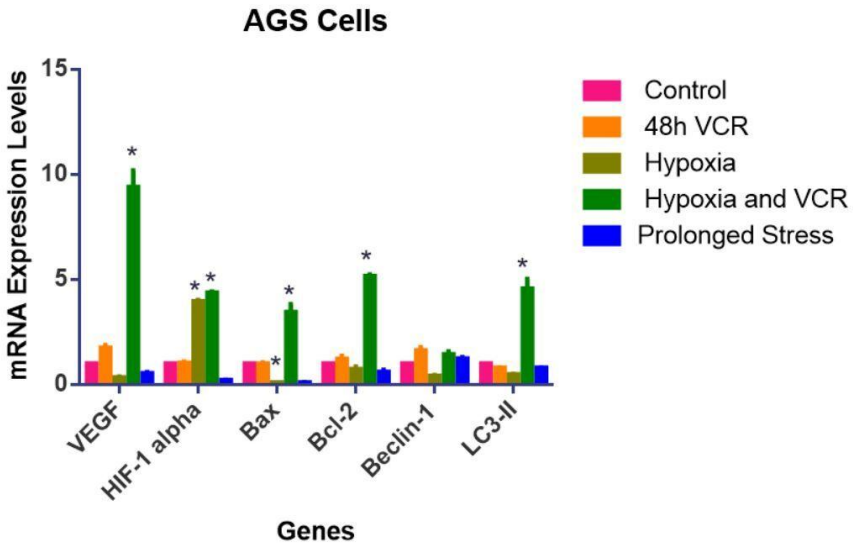


Figure 3. Changes in gene expression levels of hypoxic, autophagic, and apoptotic genes analysed with qRT-PCR in AGS cell lines compared with the non-treated controls. Change in VEGF, Bcl-2, and LC3-II expression levels as a result of 300 nM VCR and hypoxia application (*P < 0.001, *P < 0.001, and *P < 0.001, respectively). Changes in HIF-1α and Bax expression levels following hypoxic conditions (*P < 0.001 and *P < 0.05, respectively) and 300 nM VCR and hypoxia application (*P < 0.001).

Bax: Bcl-2-associated X protein; Bcl-2: B-cell lymphoma 2; HIF-1α: Hypoxia inducible factor 1 alpha; LC3-II: Microtubule-associated protein 1A/1B-light chain 3-II; nM: Nanomolar; qRT-PCR: Quantitative real-time polymerase chain reaction; VCR: Vincristine; VEGF: Vascular endothelial growth factor

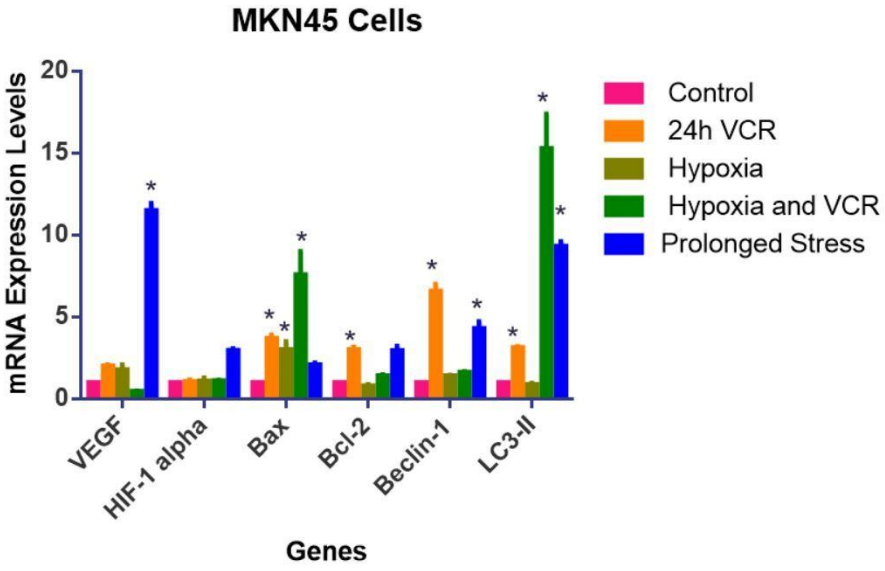


Figure 4. Changes in gene expression levels of hypoxic, autophagic, and apoptotic genes analysed with qRT-PCR in MKN45 cell lines compared with the non-treated controls. Changes in VEGF expression levels in the prolonged stress conditions (*$P < 0.001$). Changes in Bax expression levels as a result of 300 nM VCR (*$P < 0.01$), hypoxia application (*$P < 0.05$), and 300 nM VCR and hypoxic conditions (*$P < 0.001$). Changes in Bcl-2 expression levels as a result of 300 nM VCR (*$P < 0.05$). Changes in Beclin-1 expression levels as a result of 300 nM VCR application (*$P < 0.001$) and in the prolonged stress conditions (*$P < 0.001$). Changes in LC3-II expression levels as a result of 300 nM VCR treatment (*$P < 0.05$), hypoxia and VCR application (*$P < 0.001$), and in the prolonged stress conditions (*$P < 0.001$).

Bax: Bcl-2-associated X protein; Bcl-2: B-cell lymphoma 2; HIF-1α: Hypoxia inducible factor 1 alpha; LC3-II: Microtubule-associated protein 1A/1B-light chain 3-II; nM: Nanomolar; qRT-PCR: Quantitative real-time polymerase chain reaction; VCR: Vincristine; VEGF: Vascular endothelial growth factor

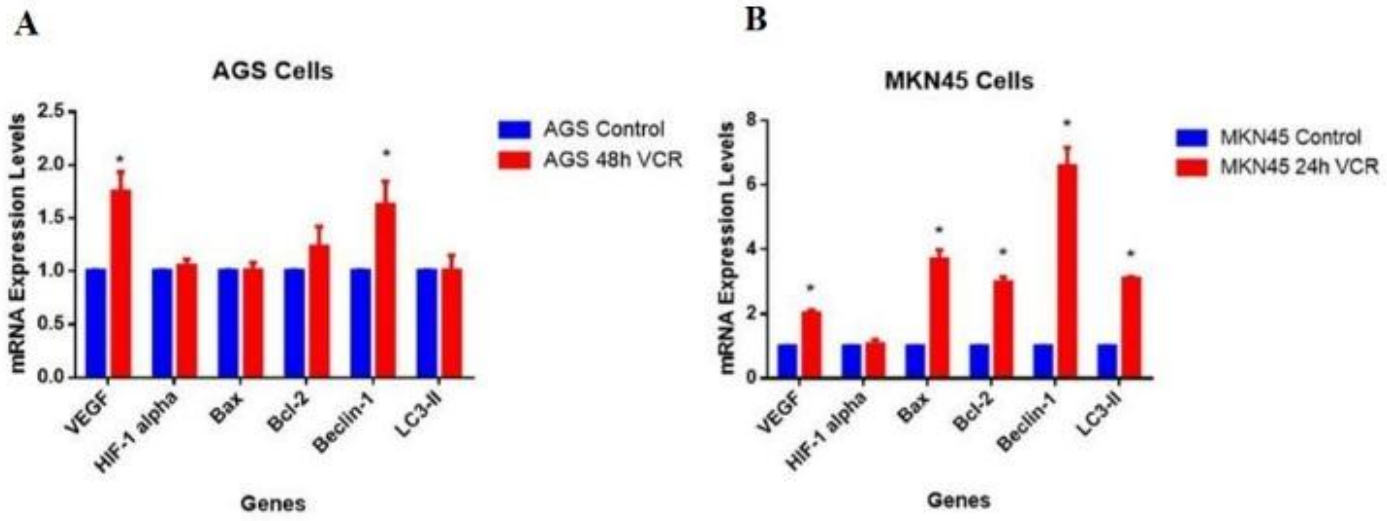


Figure 5. Changes in gene expression levels of hypoxic, autophagic, and apoptotic genes after treatment of 300 nM VCR for 24 and 48 hours analysed via qRT-PCR in cell lines compared with the non-treated controls. A. Changes in VEGF and Beclin-1 expression levels in AGS cells after 48 hours (*$P < 0.001$ and *$P < 0.001$, respectively); B. Changes in VEGF, Bax, Bcl-2, Beclin-1, and LC3-II gene expression levels in MKN45 cells after 24 hours (*$P < 0.05$, *$P < 0.01$, *$P < 0.01$, *$P < 0.001$, and *$P < 0.01$, respectively).

Bax: Bcl-2-associated X protein; Bcl-2: B-cell lymphoma 2; HIF-1α: Hypoxia inducible factor 1 alpha; LC3-II: Microtubule-associated protein 1A/1B-light chain 3-II; nM: Nanomolar; qRT-PCR: Quantitative real-time polymerase chain reaction; VCR: Vincristine; VEGF: Vascular endothelial growth factor

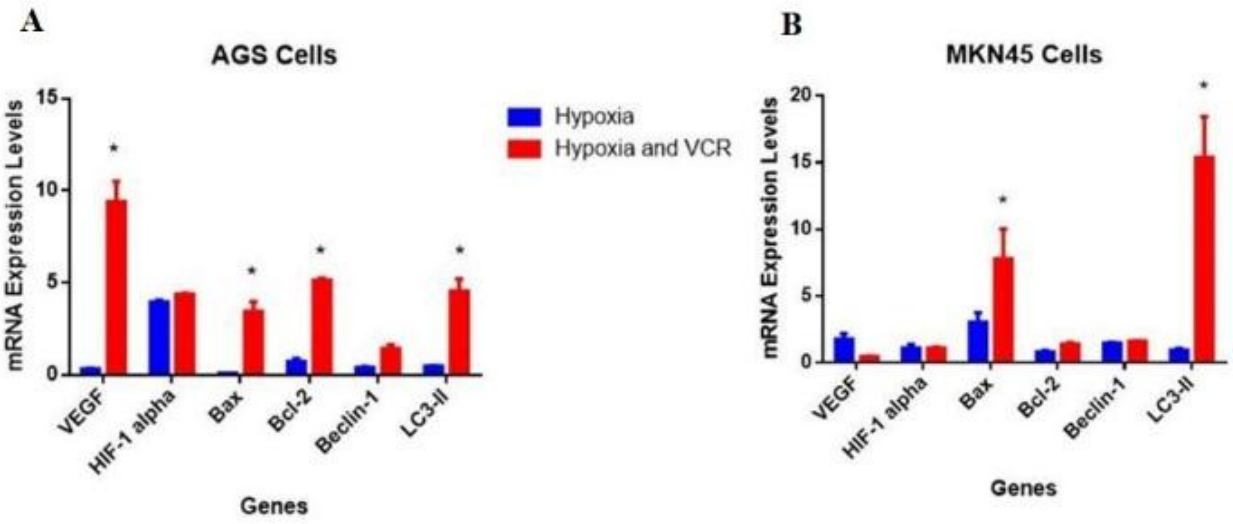


Figure 6. A. Hypoxia, autophagy, and apoptosis gene expression level changes in AGS cell lines in hypoxic conditions versus treatment of 300 nM VCR under hypoxic conditions via qRT-PCR. VEGF, Bax, Bcl-2, and LC3-II gene expression levels increased (*P < 0.001, *P < 0.001, *P < 0.001, and *P < 0.001, respectively); B. Hypoxia, autophagy, and apoptosis gene expression level changes in MKN45 cell lines in hypoxic conditions versus treatment of 300 nM VCR under hypoxic conditions via qRT-PCR. Bax and LC3-II gene expression levels increased (*P < 0.001 and *P < 0.001, respectively).

Bax: Bcl-2-associated X protein; Bcl-2: B-cell lymphoma 2; HIF-1α: Hypoxia inducible factor 1 alpha; LC3-II: Microtubule-associated protein 1A/1B-light chain 3-II; nM: Nanomolar; qRT-PCR: Quantitative real-time polymerase chain reaction; VCR: Vincristine; VEGF: Vascular endothelial growth factor

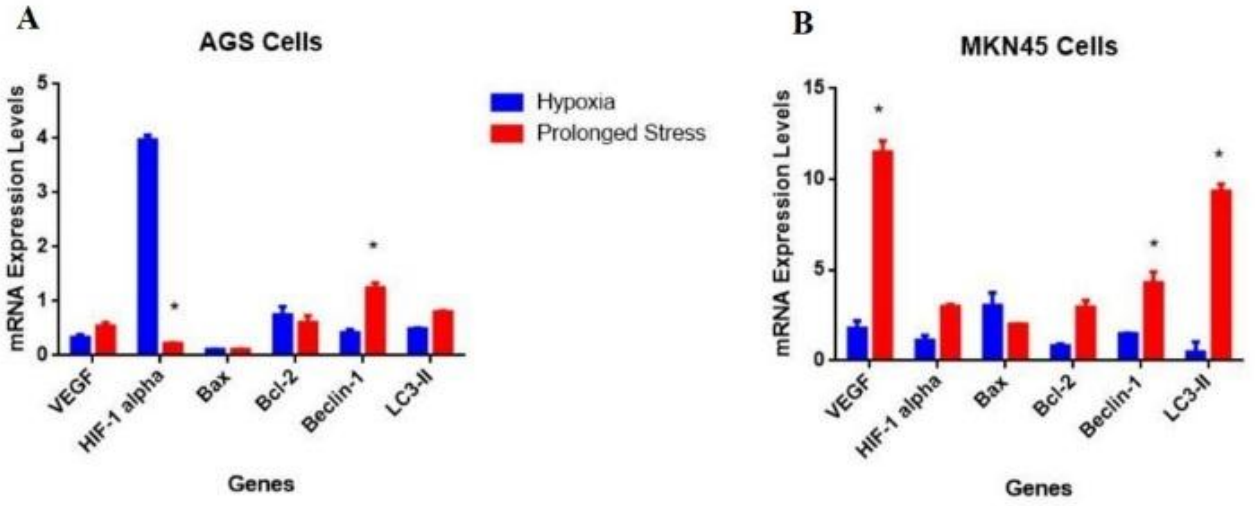


Figure 7. A. Hypoxia, autophagy, and apoptosis gene expression level changes in AGS cell lines in hypoxic conditions versus those in the prolonged stress conditions via qRT-PCR. Beclin-1 and LC3-II gene expression levels increased while HIF-1α gene expression levels decreased (*P < 0.001, *P < 0.001, and *P < 0.001, respectively); B. Hypoxia, autophagy, and apoptosis gene expression level changes in MKN45 cell lines in hypoxic conditions versus those in the prolonged stress conditions via qRT-PCR. VEGF, HIF-1α, Bcl-2, Beclin-1, and LC3-II gene expression levels increased (*P < 0.001, *P < 0.001 *P < 0.001, *P < 0.001, and *P < 0.001, respectively).

Bax: Bcl-2-associated X protein; Bcl-2: B-cell lymphoma 2; HIF-1α: Hypoxia inducible factor 1 alpha; LC3-II: Microtubule-associated protein 1A/1B-light chain 3-II; nM: Nanomolar; qRT-PCR: Quantitative real-time polymerase chain reaction; VCR: Vincristine; VEGF: Vascular endothelial growth factor

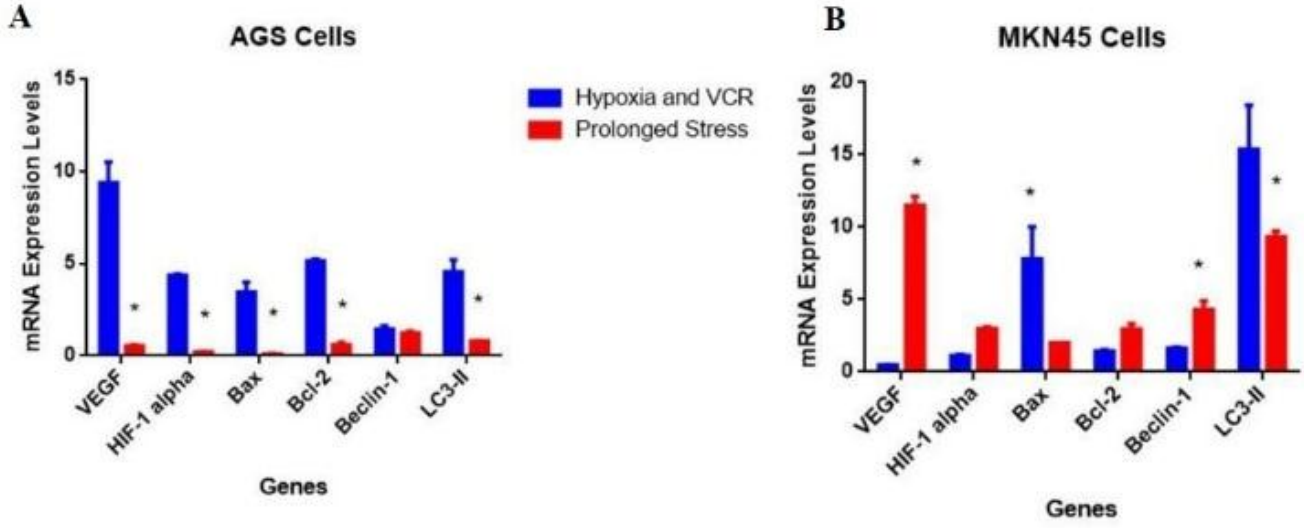


Figure 8. A. Hypoxia, autophagy, and apoptosis gene expression level changes in AGS cell lines in hypoxic conditions after treatment of 300 nM VCR versus those in the prolonged stress conditions via qRT-PCR. VEGF, HIF-1α, Bax, Bcl-2, and LC3-II gene expression levels decreased (*$P < 0.001$, *$P < 0.001$, *$P < 0.001$, *$P < 0.001$, and *$P < 0.001$, respectively); B. Hypoxia, autophagy, and apoptosis gene expression level changes in MKN45 cell lines in hypoxic conditions after treatment of 300 nM VCR versus those in the prolonged stress condition via qRT-PCR. VEGF gene expression levels increased while Bax and LC3-II gene expression levels decreased (*$P < 0.001$, *$P < 0.001$, and *$P < 0.001$, respectively).

Bax: Bcl-2-associated X protein; Bcl-2: B-cell lymphoma 2; HIF-1α: Hypoxia inducible factor 1 alpha; LC3-II: Microtubule-associated protein 1A/1B-light chain 3-II; nM: Nanomolar; qRT-PCR: Quantitative real-time polymerase chain reaction; VCR: Vincristine; VEGF: Vascular endothelial growth factor

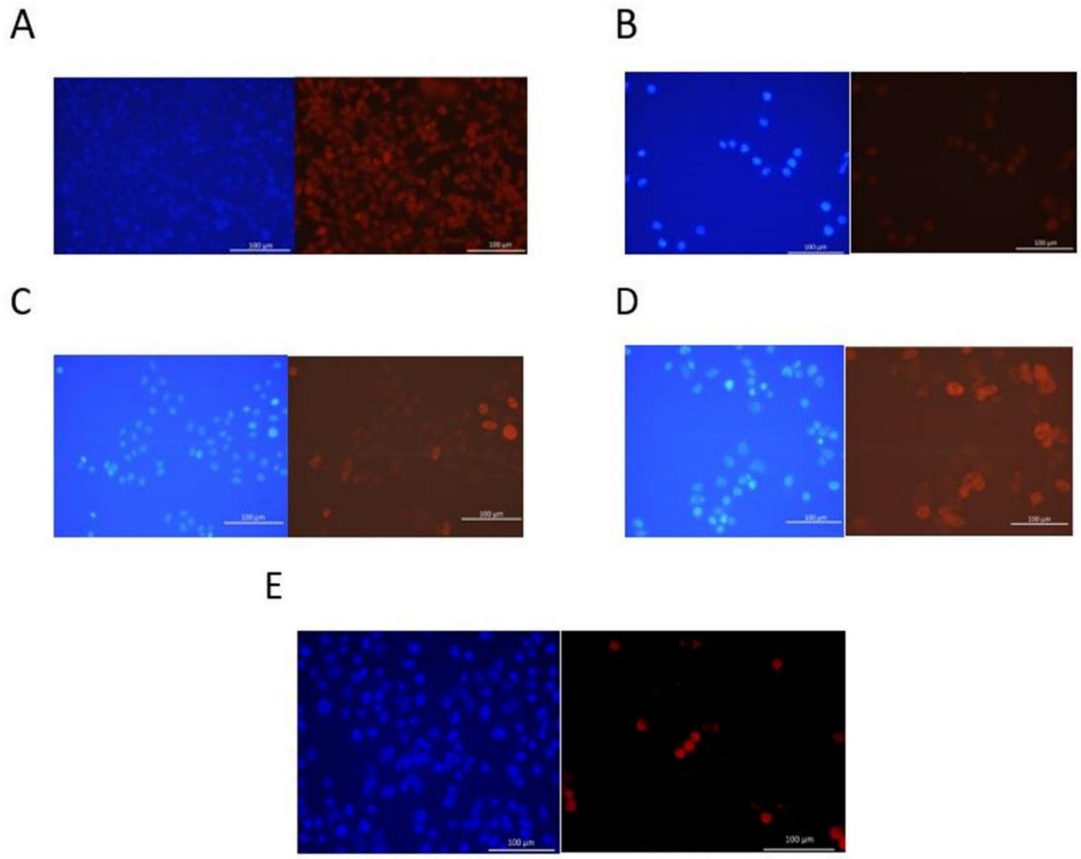


Figure 9. Microscope images of AGS cells Hoechst and acridine orange staining. A. The fluorescence microscope image of 48-h control of AGS cells of Hoechst staining and that of 48-h control of AGS cells of acridine orange staining; B. The fluorescence microscope image of 48h of 300 nM VCR treatment of AGS cells of Hoechst staining and that of 48h of VCR treatment of AGS cells of acridine orange staining; C. The fluorescence microscope image of 24-h hypoxia control of AGS cells of Hoechst staining and that of 24-h hypoxia control of AGS cells of acridine orange staining; D. The fluorescence microscope image of 300 nM VCR treatment for 24h under hypoxic conditions of AGS cells of Hoechst staining and that of 300 nM VCR treatment for 24h under hypoxic conditions of AGS cells of acridine orange staining; E. The fluorescence microscopy image of prolonged stress treatment of AGS cells of Hoechst staining and that of prolonged stress treatment of AGS cells of acridine orange staining (400×).
nM: Nanomolar; VCR: Vincristine

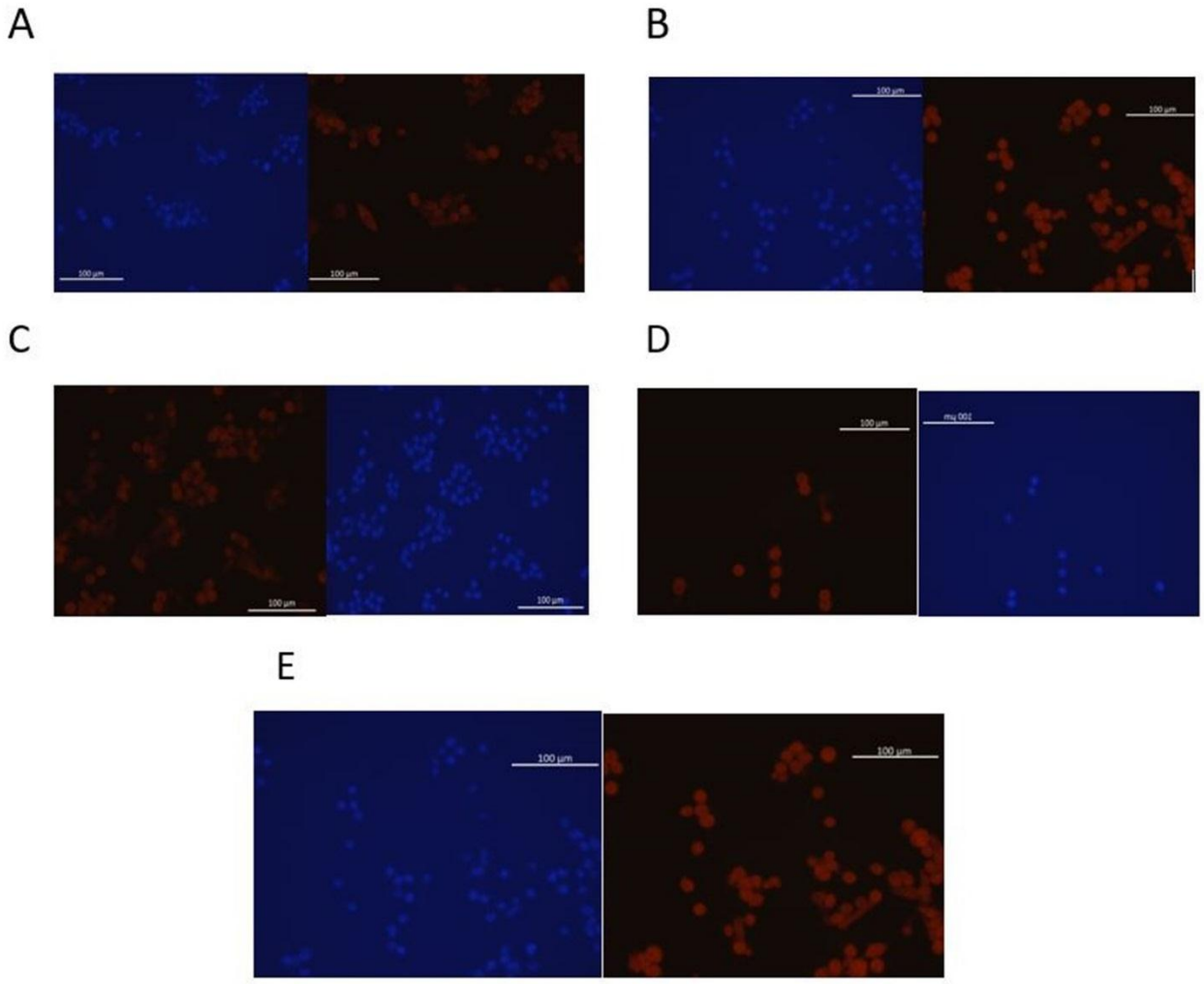


Figure 10. This figure shows the microscope images of MKN45 cells of Hoechst and acridine orange staining. A. The fluorescence microscope image of 24-h control of MKN45 cells of Hoechst staining and that of 24-h control of MKN45 cells of acridine orange staining; B. The fluorescence microscope image of 24h of 300 nM VCR treatment of MKN45 cells of Hoechst staining and that of 24h of VCR treatment of MKN45 cells of acridine orange staining; C. The fluorescence microscope image of 24-h hypoxia control of MKN45 cells of Hoechst staining and that of 24-h hypoxia control of MKN45 cells of acridine orange staining; D. The fluorescence microscope image of 300 nM VCR treatment in 24h under hypoxic conditions of MKN45 cells of Hoechst staining and that of 300 nM VCR treatment in 24h under hypoxic conditions of MKN45 cells of acridine orange staining; E. The fluorescence microscopy image of prolonged stress treatment of MKN45 cells of Hoechst staining and that of prolonged stress treatment of MKN45 cells; acridine orange staining was examined at 400× magnification (400×).

nM: Nanomolar; VCR: Vincristine

Table 1. Fold changes in gene expression levels after application of 300 nM VCR

	Cells	
Genes	AGS	MKN45
VEGF	0,62-fold increase	0,97-fold increase
HIF-1α	0,1-fold increase	0,13-fold decrease
Bax	0,14-fold decrease	2,51-fold increase
Bcl-2	0,9-fold increase	1,88-fold increase
Beclin-1	0,47-fold increase	5,27-fold increase
LC3-II	0,19-fold decrease	2,07-fold increase

Table 2. Fold changes in gene expression levels under hypoxic conditions

	Cells	
Genes	AGS	MKN45
VEGF	0,71-fold decrease	0,5-fold increase
HIF-1α	2,91-fold increase	0,1-fold decrease
Bax	0,9-fold decrease	1,55-fold increase
Bcl-2	0,36-fold decrease	0,27-fold decrease
Beclin-1	0,62-fold decrease	0,46-fold increase
LC3-II	0,52-fold decrease	0,13-fold decrease

Table 3. Fold changes in gene expression levels after 300 nM VCR application under hypoxic conditions

	Cells	
Genes	AGS	MKN45
VEGF	7,63-fold increase	0,55-fold decrease
HIF-1α	3,34-fold increase	0,10-fold increase
Bax	2,07-fold increase	5,21-fold increase
Bcl-2	4,09-fold increase	0,35-fold increase
Beclin-1	0,30-fold increase	0,60-fold increase
LC3-II	3,1-fold increase	12,2-fold increase

Table 4. Fold changes in gene expression levels under prolonged stress conditions

	Cells	
Genes	AGS	MKN45
VEGF	0,51-fold decrease	10,08-fold increase
HIF-1α	0,79-fold decrease	1,89-fold increase
Bax	0,89-fold decrease	0,99-fold increase
Bcl-2	0,48-fold decrease	1,68-fold increase
Beclin-1	0,17-fold increase	2,89-fold increase
LC3-II	0,21-fold decrease	8,06-fold increase

nM: Nanomolar; VCR: Vincristine, VEGF: Vascular endothelial growth factor; HIF-1α: Hypoxia inducible factor 1 alpha