

Effects of Acute and Chronic Hypoxia on the Radiosensitivity of Gastric and Esophageal Cancer Cells

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Abstract. *Aim: The aim of this study was to examine the effects of hypoxia on radiosensitivity and to analyze the mechanisms responsible for radiation resistance in gastric and esophageal cancer. Materials and Methods: A gastric cancer cell line, OCUM-12, and an esophageal cancer cell line, TE-6, were used. The effects of hypoxia with irradiation on the growth-activity, cell cycle distribution, and gene expression were examined. Results: Both acute and chronic hypoxia decreased radiosensitivity of cancer cells. The radiosensitivity of chronic hypoxic cells was significantly enhanced by reoxygenation. Acute and chronic all hypoxia reduced the percentage of cells in the G₂/M and S phases, respectively. In acute hypoxia, the mRNA expression of BRCA1 and BRCA2 was reduced in cancer cells. Reoxygenation increased the expression of BRCA1 and BRCA2. Conclusion: Hypoxia is associated with radiation resistance. Therefore, reoxygenation may enhance the radiosensitivity of hypoxic cells. BRCA1 and BRCA2 may be associated with factors for radiation resistance by regulation of cell cycle progression.*

Despite modern multimodality therapy, patients with gastric and esophageal cancers continue to have a poor prognosis (1, 2). Patients with these cancer types frequently experience recurrent tumors even after curative surgical resection. Because gastric and esophageal tumors are frequently diagnosed at an advanced stage, surgical treatment alone is an insufficient approach for patients with local and distal recurrences. Therefore, an additional therapeutic modality may be useful to prevent the recurrence of advanced carcinoma.

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The National Comprehensive Cancer Network guidelines on gastric cancer treatment recommend radiotherapy as a standard treatment for patients at a high risk for recurrence. This approach is also supported by the clinical trial INT0116 (3). In addition, one randomized study of esophageal cancer indicated that postoperative radiotherapy improved local control and survival rates for patients with positive lymph nodes (4). However, the development of resistance to radiotherapy in gastric and esophageal cancer patients is one of the obstacles to effective radiotherapy. Accordingly, analysis of the factors responsible for radiation resistance is necessary to maximize the effectiveness of this treatment approach.

Most solid tumors are hypovascular and possess microscopically heterogeneous hypoxic regions (5-7). Tumor hypoxia is often classified as chronic or acute: Cancer cells are frequently exposed not only to brief hypoxia (acute), but also to continuous hypoxia (chronic) because of their hypovascular nature and the limited diffusion of oxygen throughout the tissue. Cancer cells under hypoxic conditions typically exhibit malignant features, including resistance to anti cancer therapies (5, 8-11); however, the factors that may inhibit efficacy of irradiation remain unclear. It is, therefore, important to study the effects of acute and chronic hypoxia for comprehending the mechanisms of irradiation resistance (12). Although the effect of acute hypoxia on the phenotype of cancer cells has been studied extensively (13-15), there are only few reports on chronic hypoxia (16, 17). Because radiosensitivity is associated with the cell cycle (18-20), we examined the effects of chronic and acute hypoxia on radiosensitivity and cell cycle progression as well as the factors associated with the cell cycle under hypoxia in gastric and esophageal tumor cell lines.

Materials and Methods

Cell lines. A human gastric cancer cell line OCUM-12, and a human esophageal cancer cell line TE-6 were used. OCUM-12 cell line was established in our laboratory. TE-6 cell line was obtained from the Japanese Collection of Research Bioresources (JCRB) cell

bank (Osaka, Japan). Cells were cultured at 37°C in 21% oxygen (normoxia) or 1% oxygen (hypoxia). Two hypoxia-resistant cell lines OCUM-12/Hypo and TE-6/Hypo were respectively established from the parent cell lines OCUM-12 and TE-6, as previously reported (21). Briefly, OCUM-12 cells and TE-6 cells were cultured in a humidified incubator at 37°C in an atmosphere of 5% carbon dioxide and 5% oxygen for 4 weeks. Although most OCUM-12 cells and TE-6 cells were dead under hypoxic conditions, some cancer cells were alive and grew gradually under 5% oxygen. The surviving cells were then cultured for 6 weeks in 1% oxygen. Most cells were dead at initial culture. Proliferative cancer cells under 1% oxygen were designated OCUM-12/Hypo (21) and TE-6/Hypo as hypoxia-resistant cell lines. Hypoxic conditions were maintained using a modular incubator chamber (Hirasawa Works, Tokyo, Japan) with 5% carbon dioxide and 1% oxygen balanced with N₂ gas. The culture medium consisted of Dulbecco's modified Eagle's medium (Nikken Bio., Osaka, Japan) with 10% fetal bovine serum (Life Technologies, Grand Island, NY, USA), 100 IU/ml penicillin (ICN Biomedicals, Costa Mesa, CA, USA), 100 µg/ml streptomycin (ICN Biomedicals) and 0.5 mM sodium pyruvate (Cambrex, Walkersville, MD, USA). Two parent cell lines, OCUM-12 and TE-6, were continuously cultured in 21% oxygen (normoxia), while the hypoxia-resistant cell lines OCUM-12/Hypo and TE-6/Hypo were continuously cultured in 1% oxygen (hypoxia). We defined that acute hypoxia is parental cancer cells (OCUM-12 and TE-6) exposed to 1% O₂ for 24 h and chronic hypoxia is hypoxia-resistant cancer cells (OCUM-12/Hypo and TE-6/Hypo) exposed to 1% O₂ for 24 h. And reoxygenation is hypoxia-resistant cancer cells (OCUM-12/Hypo and TE-6/Hypo) exposed to 21% O₂ for 24 h.

Irradiation. X-Ray irradiation was administered using an X-ray machine (MBR-1520R; Hitachi Medical Corporation, Tokyo, Japan), which operated at 150 kV and 20 mA with 0.5 mmol/l Al and 0.1 mmol/l Cu filtration. X-Ray irradiation was carried out 2, 4 and 6 Gy at a dose rate of approximately 1.4 Gy/min.

Colony-forming assay. Cancer cells were trypsinized to generate a single-cell suspension and a specified number of cells were seeded into each well of six-well tissue culture plates according to the respective irradiation dose. After cells were incubated in 21% and 1% oxygen for 24 h, the plates were irradiated. Ten to 12 days after seeding, colonies were stained with crystal violet, the number of colonies containing at least 50 cells was determined, and the surviving fractions were calculated. Survival curves were generated after normalizing for cytotoxicity as follows. The plating efficiency was defined as the total number of colonies in the vehicle control divided by total number of cells inoculated in the vehicle control. The surviving fraction of each irradiation dose was calculated as the total no. colonies divided by (total cells inoculated × plating efficiency).

Cell cycle assay. The cell cycle phase distribution was evaluated using flow cytometry. Cancer cells (2.0×10⁴ cells) were seeded into a 100-mm dish for the cell cycle assay. After incubation for 24 h at 21% or 1% oxygen, the cells were harvested and managed according to the Cycle Test Plus DNA reagent kit protocol (Becton Dickinson, Mountain View, CA, USA), then incubated with ribonuclease A for 10 min at room temperature, and with propidium iodide for 30 min in the dark on ice. The sub-G₀/G₁, S, and G₂/M

fractions of 2.0×10⁴ cells were determined by flow cytometry using a FACS caliber (Becton Dickinson). The results were analyzed using the Modofit software program (Becton Dickinson).

Quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR). Real-time PCR was performed to examine the mRNA expression of *breast cancer 1 (BRCA1)*, *BRCA2*, *p21*, *RAD51*, *retinoblastoma (RB) 1*, and *p53* genes which are associated with the cell cycle regulation. Cancer cells were incubated under normoxic or hypoxic conditions for 24 h. After incubation, the total cellular RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After the removal of genomic DNA by DNase, cDNA was prepared from 20 µg RNA with Maloney mouse leukaemia virus reverse transcriptase (Invitrogen) using random primers (Invitrogen). To determine fold changes for each gene, real time RT-PCR was performed on an ABI Prism 7000 instrument (Applied Biosystems, Foster City, CA, USA), using commercially available gene expression assays for *p53*, *p21*, *RAD51*, *BRCA1*, *BRCA2* and *RBI* (Hs01039246, Hs01121172, Hs00153418, Hs01556193, Hs00609073 and Hs01078066, respectively). PCR was performed at 95°C for 15 s and 60°C for 60 s for 40 cycles. As an internal standard to normalize mRNA levels for differences in sample concentration, amplification of *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* was used. The threshold cycle values were used to calculate the relative expression ratios between control and treated cells using the formula described by Pfaffl (22). Quantitative PCR was carried out in triplicate.

Statistical analysis. The quantitative ratios of different groups were compared using Student's *t*-test. Probability values of *p*<0.05 were considered statistically significant. All statistical tests were two-sided.

Results

Effects of acute and chronic hypoxia on radiosensitivity of gastric and esophageal cancer cells. Figure 1 shows the effects of hypoxia in combination with irradiation on the colony-forming rates of gastric and esophageal cancer cells. For the OCUM-12 cells, radiosensitivity at 1% O₂ (acute hypoxia) was significantly higher than that at 21% O₂ (normoxia). For OCUM-12/Hypo cells, radiosensitivity at 1% O₂ (chronic hypoxia) was significantly higher than that at 21%. Moreover, the radiosensitivity of OCUM-12/Hypo cells at 21% O₂ (reoxygenation) was significantly lower than that at 1% (chronic hypoxia). In esophageal TE-6 cancer cells, the colony-forming rate of irradiated cells at 1% O₂ (acute hypoxia) was significantly higher than that at 21% (normoxia). For irradiated TE-6/Hypo cells, the colony-forming rates at 1% O₂ was significantly higher than that at 21%. Moreover, the colony-forming rate of TE-6/Hypo cells at 21% O₂ was significantly lower than that at 1% O₂.

Hypoxia triggers cell cycle arrest at G₀-G₁. Figure 2 shows DNA histograms for cell cycle analysis at 24 h after hypoxia and normoxia. Parental cancer cells (OCUM-12 and TE-6) were exposed to 1% O₂ (acute hypoxia) for 24 h and then

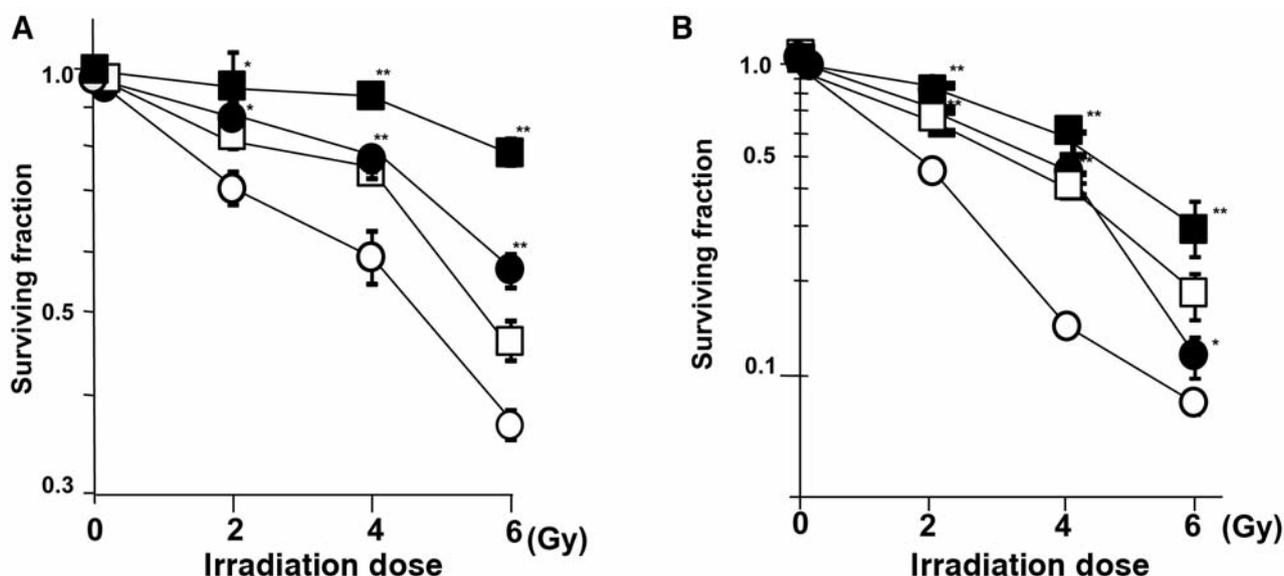


Figure 1. Effects of irradiation on colony formation in gastric cancer cells (A) and esophageal cancer cells (B) under acute or chronic hypoxia. Radiosensitivity of cancer cells at 1% O₂ (●) level was significantly higher than that of cells at 21% O₂ (○) level in both gastric cancer (OCUM-12) and esophageal cancer (TE-6) cells. Radiosensitivity of hypoxia-resistant cells (OCUM-12/Hypo and TE-6/Hypo) at a 1% O₂ (■) was significantly higher than that of parental cells (OCUM-12 and TE-6) at 21% O₂ (○). Moreover, radiosensitivity of hypoxia-resistant cells was significantly increased by reoxygenation with 21% O₂ (□) as compared to that at 1% O₂ (■). The survival fraction of each irradiation dose was calculated as the total number of colonies (total cells inoculated × plating efficiency). Three independent experiments were carried out. The mean of the results is presented, and the bar indicates SD. **p*<0.05, ***p*<0.01 vs. control (0 Gy).

examined by flow cytometry. Hypoxia-resistant cancer cells (OCUM-12/Hypo and TE-6/Hypo) were exposed to 21% O₂ for 24 h (reoxygenation) and then examined by flow cytometry. In OCUM-12 cells, acute hypoxia increased the percentage of cells in the G₀-G₁ phase (80.4%) and reduced it in the S (13.0%) and G₂-M phases (6.7%) as compared to the control cells (G₀-G₁, 41.4%; S, 41.3%; and G₂-M, 17.4%). In OCUM-12/Hypo cells, reoxygenation increased the percentage of cells in the G₂-M phase (7.9%) and reduced it in the G₀-G₁ phase (75.1%) as compared with the control cells (G₂-M, 5.2%; G₀-G₁, 83.2%) (Figure 2A). In TE-6 cells, acute hypoxia increased the percentage of cells in the G₀-G₁ phase (59.0%) and reduced it in the S and G₂-M phases (12.7%) as compared with the control cells (G₀-G₁, 47.4%; G₂-M, 23.5%). In TE-6/Hypo cells, the introduction of O₂ to the chronic all hypoxic cells increased the percentage of cells in the G₂-M phase (11.0%) and reduced it in the G₀-G₁ phase (57.6%) as compared with the control cells (G₂-M, 5.0%; G₀-G₁, 75.2%) (Figure 2B). Statistical analysis was not analyzed in this assay.

Effects of acute and chronic hypoxia on gene expression in gastric and esophageal cancer cells. Parental cancer cells were exposed to 1% O₂ for 24 h (acute hypoxia). Figure 3A shows the effects of acute hypoxia on the mRNA expression levels of *BRCA1*, *BRCA2*, *p21*, *RAD51*, *RBI*, and *p53* in

OCUM-12 and TE-6 cells. Acute hypoxia significantly reduced the expression levels of *BRCA1*, *BRCA2*, *p21*, *RAD51*, and *RBI* by more than 2-fold. In contrast, the *p53* level was significantly increased under acute hypoxia by more than 3-fold. Figure 3B shows the effects of reoxygenation on the mRNA expression level in hypoxia-resistant cancer cells. The expression levels of *BRCA1* and *BRCA2* in OCUM-12/Hypo and TE-6/Hypo cells were significantly increased by more than 3-fold by reoxygenation with 21% O₂ as compared with those at the chronic hypoxia level (1% O₂). The *RAD51* expression levels in OCUM-12/Hypo and TE-6/Hypo cells were significantly increased by oxygenation with 21% O₂ as compared with those at the chronic hypoxia level (1% O₂). The expression levels by oxygenation of other genes including, *p21*, *RBI*, and *p53* were different between the two cell lines OCUM-12/Hypo and TE-6/Hypo.

Discussion

Cell cycle kinetics have been considered to be of major importance in examining the effectiveness of radiosensitivity (23, 24). Therefore, in this study, we examined the effects of chronic and acute hypoxia on the cell cycle. In this study, the radiosensitivity of tumor cells was significantly reduced under both acute and chronic hypoxia. In addition, the

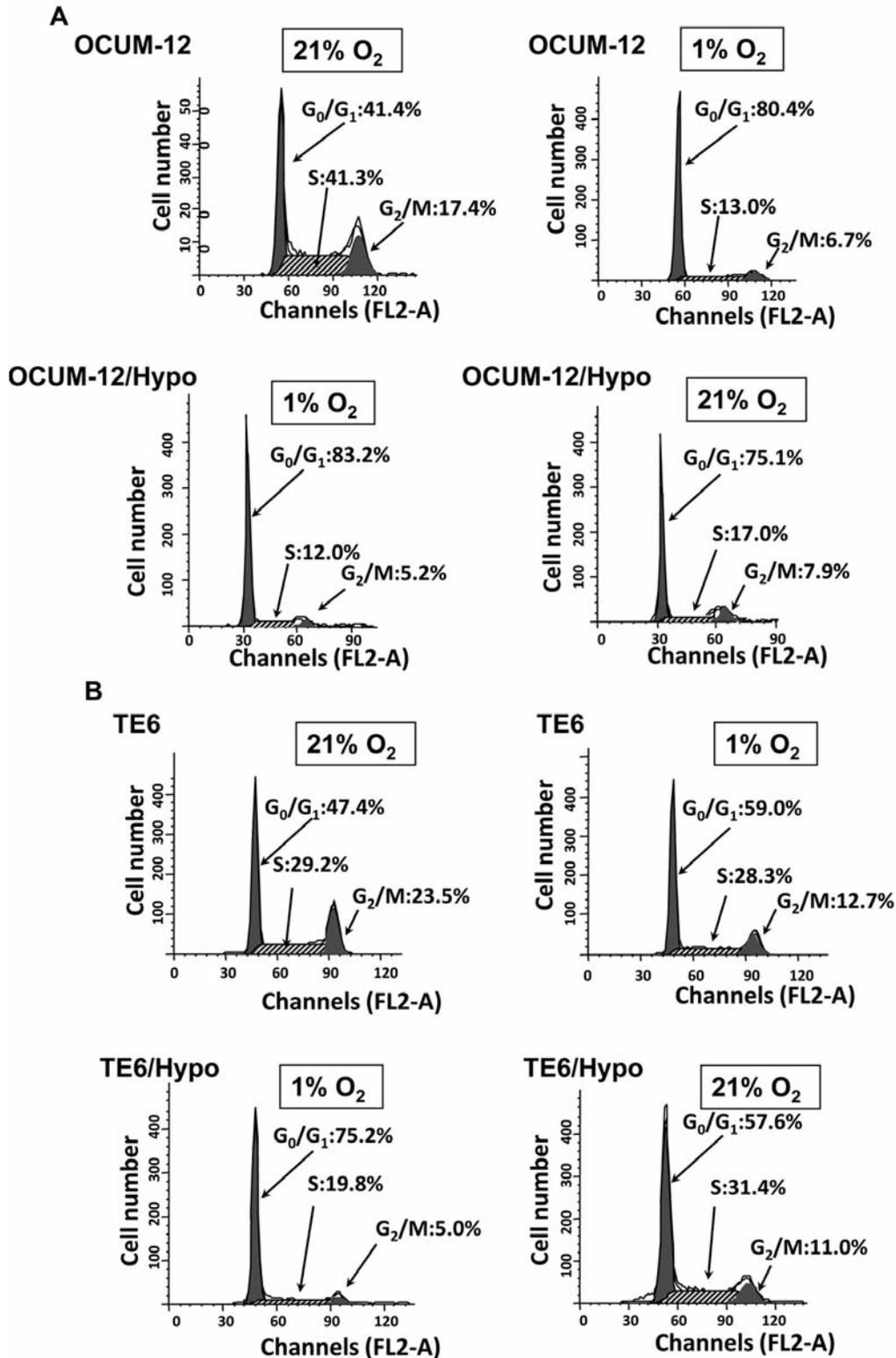


Figure 2. Effects of hypoxia on the cell-cycle distribution in gastric cancer cells (A) and esophageal cancer cells (B). Acute hypoxia increased the percentage of parental cancer cells in the G₀-G₁ phase and reduced it in the G₂/M phase in both OCUM-12 (A) and TE-6 cells (B). The percentage of cells in the G₂/M phase in OCUM-12/Hypo (A) and TE-6/Hypo cells (B) under chronic hypoxia (1% O₂) was lower than that of the parental cells under normoxia (21% O₂). An oxygenation level of 21% O₂ increased the percentage of parental cancer cells in the G₂/M and S phase and reduced it in the G₀-G₁ phase in OCUM-12/Hypo (A) and TE-6/Hypo cells (B).

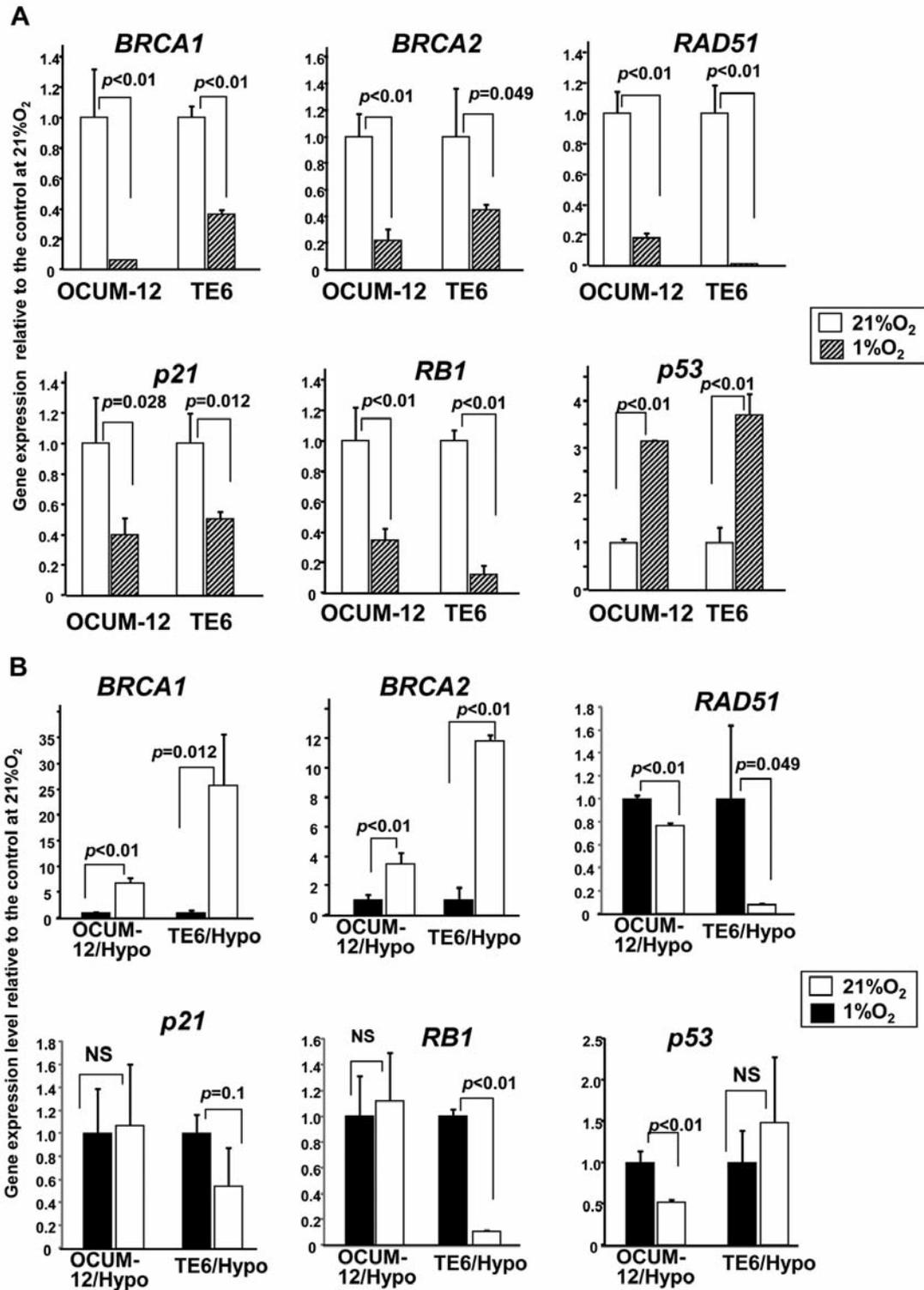


Figure 3. Effects of hypoxia and reoxygenation on gene expression of cancer cells. A: Parental OCUM-12 and TE-6 cancer cells were exposed to an acute hypoxia (1% O₂) for 24 h. The expression levels of BRCA1, BRCA2, RAD51, p21, and RB1 were significantly reduced by acute hypoxia, while that of p53 was significantly increased in both OCUM-12 and TE-6 cells. B: Hypoxia-resistant cancer cells, OCUM-12/Hypo and TE-6/Hypo, were reoxygenated with 21% O₂ for 24 h. The mRNA expression levels of BRCA1 and BRCA2 at the 21% level were significantly higher in comparison with those at the 1% level in OCUM-12/Hypo and TE-6/Hypo cells. In contrast, the mRNA expression level of RAD51 at 21% O₂ level was lower as compared to that at 1%. The p21, RB, and p53 expressions were different between the two cell lines.

radiosensitivity of chronic hypoxic cells was significantly enhanced by reoxygenation with 21% O₂. Both acute and chronic hypoxia reduced the percentage of cells in the G₂/M and S phases and increased it in the G₀-G₁ phase in OCUM-12 and TE-6 cells. This suggests that cells exposed to hypoxia were arrested at the G₁/S phase boundary. Because cells in the G₂/M phase are most sensitive to irradiation (18), arrest at the G₀-G₁ phase by hypoxia is arguably a mechanism responsible for radiation resistance in both gastric and esophageal cancer cells.

It has been reported that hypoxia induces proteome changes in neoplastic cells and affects neoplastic growth (23, 25, 26). On this basis, we analyzed the factors associated with the cell cycle distribution under acute and chronic hypoxia. In acute hypoxia, the mRNA expression levels of *BRCA1*, *BRCA2*, *RAD51*, *p21*, *RB1*, and *p53* decreased in both OCUM-12 and TE-6 cells. *BRCA1*, *BRCA2*, and *RAD51* are DNA repair proteins that contribute to homologous recombination (27, 28). Previous studies also reported that hypoxia down regulated homologous recombination proteins in other types of tumor cells (29-31). DNA damage is repaired through such proteins operating in the S and G₂ phases of the cell cycle (27, 32). These findings suggest that the downregulation of *BRCA1*, *BRCA2*, and *RAD51* may be one of the reasons for the G₀-G₁ arrest under acute hypoxia.

RB1, p21, and p53 also regulate the cell cycle; in particular, p21 and RB1 are associated with a major G₁ checkpoint blocking S-phase entry. In addition, RB1 and p21 may be involved in the G₀-G₁ arrest under acute hypoxia. In contrast, p53 expression may not be associated with the cell cycles of OCUM-12 and TE-6 cells under hypoxia because these two cell lines show p53 mutation (21, 33). Although p21 and p53 regulate apoptosis and the cellular response to DNA damage in either a dependent or independent manner (34-37), p21 may regulate the cell cycle progression through a p53-independent pathway of OCUM-12 and TE-6 cells under acute hypoxia.

In chronic hypoxia, the percentage of cells in the G₂/M and S phases was increased on reoxygenation for 24 h in both the hypoxia-resistant cancer cells as compared with that of cells cultured under continuous hypoxia. The reoxygenation increased the expression levels of *BRCA1* and *BRCA2* but reduced that of *RAD51*. Therefore, *BRCA1* and *BRCA2* may be associated with radiosensitivity in both chronic and acute hypoxia. The reoxygenation induces expression of homologous recombination proteins with the consequence of increased sensitivity to ionizing radiation through up regulation of *BRCA1* and *BRCA2* in hypoxia-resistant cancer cells. In contrast, *RAD51* expression differed under chronic and acute hypoxia. Several studies have demonstrated an association between down regulation of *RAD51* expression and increased radiosensitivity in several

cell types (38). *RAD51* may have different effects on radiosensitivity under chronic and acute hypoxia; whereas, p21 and RB1 may not be associated with radiosensitivity under chronic hypoxia.

Although it has also been reported that hypoxic cells are more resistant to irradiation than their well-oxygenated counterparts (8), the majority of this scientific literature refers to an experimental setup in which the supply of oxygen is at the acute phase level. In this study, we found that the hypoxia-resistant cancer cells experiencing chronic hypoxia were also resistant to irradiation; thus, reoxygenation may increase the radiosensitivity of hypoxia-resistant cancer cells.

In conclusion, both acute and chronic hypoxia were associated with radiation resistance. Reoxygenation may well enhance the radiosensitivity of chronic all hypoxic cells. Hypoxia caused the cell cycle to be arrested at the G₁-S phase boundary. Reduction of *BRCA1* and *BRCA2* expression may account for the G₀-G₁ arrest under hypoxia and is likely be associated with factors leading to radiation resistance.

Competing Interests

The Authors declare that they have no competing interests.

Acknowledgements

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