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Expression of Mitochondrial Transcripts in Gastric MGC803 Cell Line Subjected by Hypoxia

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OBJECTIVE To determine the transcriptional expression of mitochondrial genome (mtDNA) in MGC803 cell lines subjected by various time-phase hypoxic dispositions, and further to discuss the influence of mtDNA transcripts on hypoxic resistance to irradiation.

METHODS The MGC803 cells exposed to anoxic environment were divided into control group (0 h), hypoxic group (2 h, 8 h, 16 h, 24 h) and irradiated group after exposing the hypoxia. RT-PCR was applied to detect the transcripts of cytochrome oxidase subunit I (COI), NADH dehydrogenase subunit 4 (ND4), ND5, cytochrome b (cyt-b) and ATPase6 (ATP-6) in MGC803 cell lines at various time-phases of hypoxic, and after X-ray irradiation. Flow cytometry and colony formation assay were conducted to evaluate the cell cycle phase and survival fraction.

RESULTS COI and ND4 transcripts of MGC803 cell lines were influenced remarkably by hypoxia. COI transcripts were decreased remarkably with the elongation time of exposing the hypoxic, and reduced to one fourth of its original amount of pre-hypoxia 24 h after exposing the hypoxia. ND4 transcripts were increased initially, and elevated to two folds 8 h after exposing the hypoxia, and then reduced to one second 24 h after exposing the hypoxia. Hypoxia resulted in G_1 phase blockage, especially after hypoxia for 16 h. The survival fraction of MGC803 cells exposing the hypoxia in irradiated group showed that as the time of exposing the hypoxic before irradiation is prolonged, the survival fraction of MGC803 cells may have an elevated tendency.

CONCLUSION The tumor cells with lower expression levels of the COI and the ND4 after exposing the hypoxic have stronger resistance to the radiation, which indicates that increasing the expression levels of the COI and the ND4 might be advantageous to enhance the sensitivity of hypoxic tumor cells to the radiotherapy.

KEY WORDS: gastric carcinoma, DNA, mitochondrial, hypoxia, irradiation.

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Introduction

Low linear energy transfer (LET) irradiation can kill tumor cells by indirectly ionizing H_2O molecule into plenty of reactive oxygen species (ROS). Subsequentially destroying the macromolecular substance in the tumor cells leads to the fatal death of the tumor cells. This process can be influenced by the intracellular partial pressure of oxygen, and the hypoxic tumor cells prone to be resistant to the irradiation[1]. Mitochondria uptake more than 90% of oxygen among the whole cell

organs in a cell, and the hypoxia can obviously affect the mitochondrial function[2]. Does the stability of mitochondrial DNA (mtDNA) have any relationship with the radioresistance? In this study, we detected the changed levels of transcriptional expression of some representative mitochondrial genomes (COI, ND4, ND5, cyt-b and ATP-6) in MGC803 cell lines subjected by various time-phase hypoxic dispositions, and further discussed the relationship between these changes and hypoxic damages and the hypoxic resistance to the irradiation in order to provide a new idea on the radiosensitization treatment against the hypoxic tumor cells.

Materials and Methods

Cell culture

Human gastric cancer cell lines, MGC-803 reserved in Cancer Institute of China Medical University, were cultured in RPMI 1640 (Gibco) which was added with 10% fetal bovine serum, glutamine (300 µg/ml), penicillin (100 IU/ml) and streptomycin (100 µg/ml), and then incubated in a humidified incubator containing 5% CO_2 at 37°C. The tumor cells at exponential growth phase were collected for subsequent research.

Disposition of hypoxia and irradiation

The MGC803 cells exposed to the anoxic environment (mixed gas composing of 1% O_2, 5% CO_2, and 94% N_2, flow rate 0.1 L/min) were divided into five groups as follows, according to the different period of time that the cells were exposed to the hypoxic, 0 h, 2 h, 8 h, 16 h, and 24 h. Partial hypoxic cells after exposing the hypoxic condition received the irradiation with single fraction of 4 Gy of the irradiation by 6 MV X-ray (SIEMENS Linear accelerator), and the dose rate was 2 Gy/min.

RNA extraction

Cultured cells were treated by 0.25% trypsin. Subsequently, total RNA was extracted from the cells using RNAzol reagent according to the manufacturer's instructions. The extracted RNA was dissolved in DEPC-treated water, and stored at -80°C for use.

Reverse transcription PCR used to detect COI, ND4, ND5, cyt-b and ATPase6

Reverse transcription PCR (RT-PCR) was applied to detect the mitochondrial transcripts of COI, ND4, ND5, cyt-b and ATPase6 in MGC803 cell lines exposing the hypoxic at various time phases . Both of the software Oligo 2.0 and Primer Premier 5.0 were used to design the primers of mitochondrial genome (Table 1), and β-actin acted as standard internal control. Reverse transcription procedures were conducted following the manual of TaKaRa AMV RT-PCR kit. PCR reaction took place in a final volume of 50 µl of the Biometra Personal PCR system which requires an initial incubation at 94°C for 4 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 2 min, and a further extension at 72°C for 4 min.

PAGE and silver staining

The PCR products were detected by polyacrylamide gel electrophoresis (PAGE) and silver staining. The PAGE was performed with 3% of concentrated gel and 8% of separated gel. Five microliters of the mixture containing 3 µl of PCR products and 2 µl of sampling buffer, was running in the electrophoresis at 8mA for 20 min, and then at 15mA for 30 min. Subsequently, the gels were stained using the sliver staining. The gels were fixed in 10% ethanol for 5 min, pretreated with 1.13% of nitric acid for 5 min, and washed with sterile, deionized water for 1 min twice. And then the gels were stained using 12 mmol/L silver nitrate for 5 min twice, and then washed with sterile, deionized water for 1 min twice. Subsequently, the gels were developed in the developer containing 3% of sodium carbonate and 0.05% (v/v%) of formaldehyde, and then fixed in 10% acetic acid solution for 5 min, then in 5% glycerol for 5 min. Finally, the gels were sealed and stored. The sealed gels were scanned using ChemiImager™ system, and analyzed by ImageJ Software. The transcript levels of mtDNA were determined by normalization of β-actin marker.

Flow cytometry

Flow cytometry was performed to analyze the phases in

Table 1. Primer pairs used for PCR-amplified mtRNA.

Gene	Primer	Sequence	Length (mer)	Tm (°C)	GC%	Product size (bp)
COI	F6043	TCTAGGTAACGACCACATCTACAAC	25	62.4	44.0	614
	R6656	CGAAGCCTGGTAGGATAA	18	51.9	50.0	
ATPase6	F9000	CGCCTAACCGCTAACATTACTG	22	64.2	50.0	148
	R9147	AGGCGACAGCGATTTCTA	18	53.8	50.0	
ND4	F11581	ATCTGCCTACGACAAACA	18	48.3	44.4	443
	R12024	GTGGTGGGTGAGTGAGCCC	19	61.3	68.4	
ND5	F13028	CTGACTCCCCTCAGCCATAGA	21	57.2	57.1	276
	R13303	TGTGGTTGGTTGATGCCG	18	53.3	55.6	

the cell cycle. The procedures were introduced briefly as follows, digesting the cultured cells using 0.25% trypsin, rinsing the cells using phosphate buffer solution (PBS), fixing them in 70% ethanol at 4°C for 24 h, dyeing with propidium iodide for 30 min away from light, and then analyzing the cells using the flow cytometry (Becton Dickinson) at 488 nm of the excitation wavelength.

Colony formation

Colony formation assay was performed as follows. Trypsinized cells were calculated and diluted stepwise, then inoculated in the culture dish (diameter = 10 cm). One hundred cells were inoculated in the anoxic environment and 1,000 cells inoculated in the hypoxia followed by irradiation. Cell culture was terminated after incubated for 14 days in a humidified incubator containing 5% of CO_2 at 37°C. The cells were fixed in 95% ethanol, and stained in crystal violet, then the number of the cell colonies consisting of no less than 50 cells was counted. Three parallel samples were set up, and the experiment was repeated twice, then the average of the results was calculated. Plating efficiency (PE) was defined as the ratio between the number of colonies and the number of the inoculated cells. The survival fraction was defined as the ratio between the PE in the hypoxic group (or hypoxia plus irradiation group) and PE in control group.

Results

Influence of hypoxia on the transcriptional expression of mitochondrial genome in MGC803 cell lines

With the extended hours of exposing the hypoxic, the COI transcripts of MGC803 cell lines were decreased remarkably. After exposing the hypoxia for 24 h the COI was reduced to one fourth of the amount of the control group. ND4 transcripts were increased initially, and then elevated to two folds after hypoxia for 8 h, and then reduced to one half of the amount of the control group after hypoxia for 24 h. ATP-6 and cyt-b transcripts were not significantly influenced by hypoxia (Fig.1).

Varying trend of COI and ND4 transcripts under hypoxia, cell cycle blockage and survival rate under irradiation after exposing the hypoxia

The transcripts of ND4 were increased initially and gradually decreased with the prolonged hypoxic time. After hypoxia for 8 h, the transcripts of ND4 were decreased to one half of the amount presented before exposing the hypoxia. The expression levels of COI were decreased with the extended hours of exposing the hypoxia. The division of the cells in the irradiated group after exposing the hypoxic environment was blocked in G_1 phase initially. The G_1 arrest became more and more obvious with the extended hours of exposing the hypoxia. Interestingly, the percentage of G_1 phase began to decrease after hypoxia for 16 h, and lowered to the level of the control group after hypoxia for 24 h. The decreased transcripts of ND4 in hypoxic group appeared earlier than G_1 phase arrest of the cells in hypoxia plus irradiation group (Fig.2). The survival rate presented a downtrend in hypoxic group, and it was slightly increased after hypoxia for 24 h. The survival rate in the hypoxia plus irradiation group had an ascendant trend with the extended hours of exposing the hypoxia before irradiation, and it had a peak point after hypoxia for 2 h (Fig.3).

Discussion

Mitochondria not only produce plenty of energy, but also involve in oxidative stress, apoptosis, human evolution, some hereditary diseases and tumorigenesis[3]. These years, some researches had already probed into many respects of mitochondria such as mitochondrial permeability transition pore (mPTP)[4], mitochondrial membrane potential (MMP)[5], oxidative injury, apop-

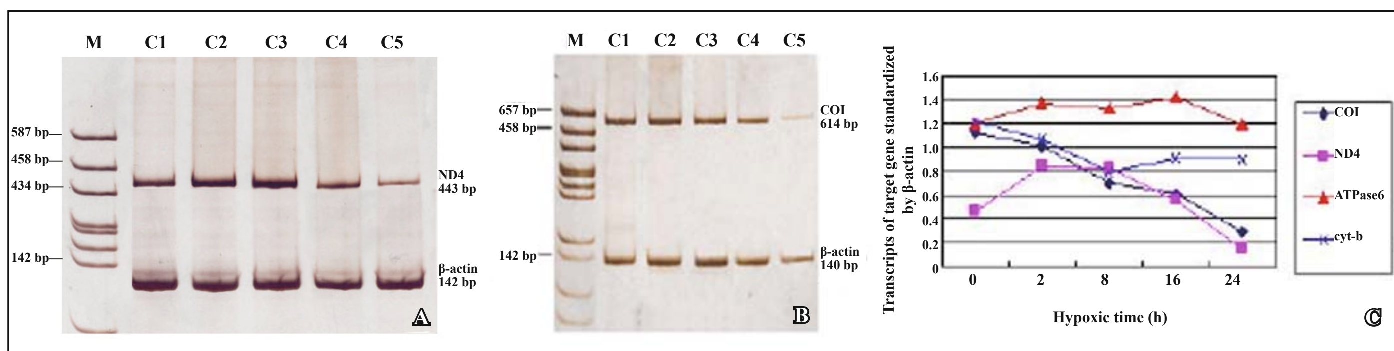

Fig.1. A, PAGE results of RT-PCR amplifying ND4 in hypoxic MGC803 cell lines. C1-C5: represent hypoxic time of 0, 2, 8, 16 and 24 h respectively M: pGEM-7Zf Marker (HuaMei Corporation). **B, PAGE results of RT-PCR amplifying COI in hypoxic MGC803 cell lines. C, Trendgram of influence of hypoxia on mitochondrial transcripts in MGC803 cell lines.** COI: cytochrome oxidase subunit I; ND4: NADH dehydrogenase subunit 4; ATPase6: ATPase subunit 6; cyt-b: cytochrome b.

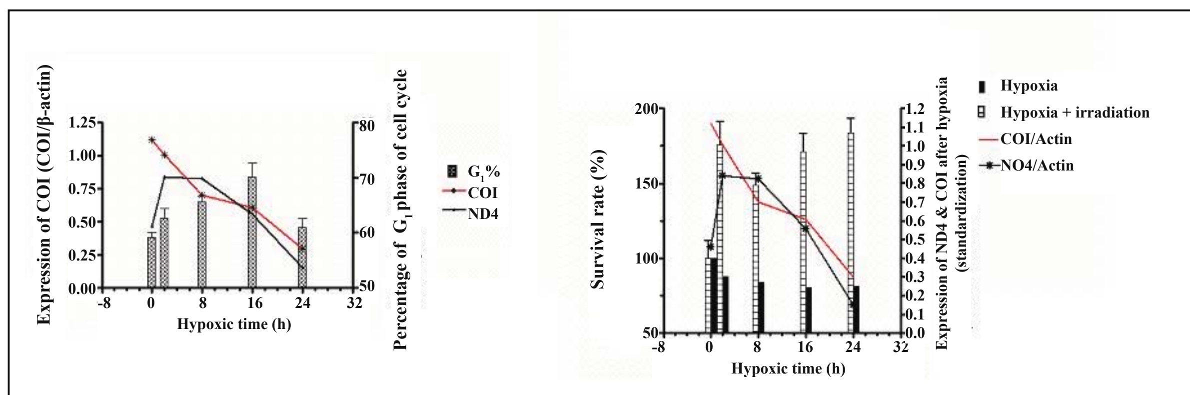

Fig.2. Trendgram of COI and ND4 expression under hypoxia and cell cycle blockage under radiation after hypoxia in MGC803 cell lines.
Fig.3. Trendgram of COI and ND4 expression under hypoxia and survival rate under radiation after hypoxia in MGC803 cell lines.

tosis precursor molecule, the stability of mitochondrial DNA[6], and cross talk between mitochondria and the nucleus[7,8].

Electron transport chain (ETC) and oxidative phosphorylation include 5 respiratory enzyme complexes encoded by 87 genes of nucleus and mitochondria. Thirteen subunits encoded by mtDNA involved in the formation of Complex I, III, IV and V[9]. The transcriptional expression levels (mtRNA) of mitochondrial genomes usually respond to the external stimulations such as oxidative stress, hypoxia, calcium load, ceramide and so on, so as to maintain survival or to induce apoptosis. Wang et al.[10] reported that the decreased transcripts of mtDNA were always accompanied with the increased cell apoptosis, which might be associated with aging. On the contrary, the increased transcripts frequently accompanied with the decreased apoptosis might be associated with tumorigenesis.

Hypoxia plays an important role in infiltration and metastasis of tumor cells. It's been known that hypoxia can increase the expression of hypoxia-inducible factor-1 (HIF-1), which can further induce activation of its target genes, such as angiogenetic factor (e.g. VEGF, VEGFR), matrix degradation enzyme (e.g. MMP and u-PA), oncogenic protein (e.g. MET), and chemokines (e.g. CXCR4)[11], and subsequently promote the ability of angiogenesis, degradation of matrix, migration and metastasis of tumor cells[12]. Meanwhile, it has been recognized that hypoxia can result in resistance to chemoradiotherapy of solid tumor[13]. The high oxygen pressure is often used in combination with the low LET radiation to treat tumors because the high oxygen pressure can consolidate the damage caused by ROS to the macromolecular substance[14]. Researchers began to doubt whether the anti-angiogenesis therapy might promote the infiltration and metastasis of the tumors on account of the increased anoxia in tumor matrix, thus leading to a poor prognosis[15]. The traditional radiotherapy and chemotherapy, combined with the drug tirapazamine (TPZ) which can kill the anoxic tumor cells selectively, could kill not only the perivascular tumor cells with proliferative activity but also the anoxic tumor cells away from blood vessels[16].

The influence of hypoxia on tumor cells involves many aspects. Hypoxia can also affect the mitochondrial metabolism and transcriptional expression of mtDNA. Vijayasarathy et al.[17] reported a coordinated down regulation of mitochondrial genome-encoded cytochrome oxidase I and II and nuclear genome-coded cytochrome oxidase IV and Vb mRNAs during hypoxia in vitro. Bae et al.[18] indicated the down-regulated COII expression in the human hepatocellular carcinoma (HCC) cells under hypoxia in vitro. But some other studies showed that the transcripts of cyt-c and 12Sr RNA were increased in brain and muscle tissues under hypoxia[19].

In this study, we found the transcriptional expressions of the ND4 and COI varied remarkably under hypoxia, and the ND4 and COI transcripts were reduced to one fourth and one half, respectively after hypoxia for 24h, however, transcripts of ATP-6 and cyt-b were not significantly influenced by the hypoxia. Wang et al.[10] found plenty of apoptotic cells in mTFA (tissue-specific mitochondrial transcriptional factor A) knockout animals with severe respiratory chain deficiency in heart. Furthermore, the author indicated that the ρ0 cell line with deficient mtDNA was susceptible to the apoptosis when stimulated, suggesting the respiratory chain deficiency induce cell apoptosis. However, Dey et al.[20] obtained a contrary outcome in the vitro experiment that the osteosarcoma cell lines without mitochondrial DNA and the murine embryos cells devoid of cytochrome c had an anti-apoptotic effect on death signals such as staurosporin (STP) and activating factor of death receptor. Tang et al.[21] reported that cell lines with deficient mtDNA (ρ0 cell) showed a stronger resistance to radiation than the cell lines with mutated mtDNA (syn-cell) and cell line with normal mtDNA (ρ+cell) by colony formation assay, and the lower percentage of radiation-induced apoptosis

was observed in ρ0 cells.

Our research indicated that, with extended time of exposing the hypoxia, the transcriptional levels of ND4 and COI in MGC803 gastric cells showed the decreased trend accompanied with the increased survival fraction and stronger resistance to radiation, suggesting that the anoxic tumor cells with lower expression of ND4 and COI had stronger resistance to the radiation.

It had been confirmed that the functional deficiency of respiratory chain plays an important role in the pathophysiological processes of neurodegenerative disorder, heart failure, diabetes mellitus and aging[22]. The inhibited apoptosis is the critical reason in the tumorigenesis and the excessive hyperplasia. One of main goals of radiotherapy and chemotherapy is to induce the apoptosis of tumor cells. Our research provides a very promising way in tumor treatment, that is, enhancement or inhibition of cell apoptosis by adjusting transcriptional expression of some specific mitochondrial genomes. Certainly it still needs a further and intensive study, especially to explore the applicative value of adjustment of mitochondrial transcripts by means of RNA interference and gene recombination.

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