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The role of Her2-Nrf2 axis in induction of oxaliplatin resistance in colon cancer cells

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ABSTRACT

Nuclear factor erythroid 2-related factor 2 (Nrf2) plays a pivotal role in promoting chemoresistance by regulation of antioxidants and detoxification enzymes. Her2 is a member of tyrosine kinase receptor family with a key function in resistance of cancer cells to chemotherapeutics. The aim of this study was to investigate the possible cross talk between Nrf2 and Her2 mediated signaling pathways in development of oxaliplatin resistance in colon cancer cells. We first generated oxaliplatin-resistant LS174T and SW480 colon cancer cells with different Her2 expression levels by employing IC50 concentrations followed by a resting period. We evaluated the viability and apoptosis of the cells by MTT and flow cytometry assays, respectively. Nrf2 and Her2 gene expression levels were examined by qRT-PCR. The morphology analysis and combination index calculation were performed using the ImagJ and CompuSyn softwares, respectively. Development of resistant cells revealed a marked increase in half maximal inhibitory concentration (IC50) value from 3.95 ± 0.92 μM to 29.27 ± 3.13 μM in SW480 cells and 377 ± 46 nM to 9.59 ± 0.76 μM in LS174T cells with a significant change in morphology of the cells from elongated to small round shape ($p < 0.05$). Her2 expression level was increased in both types of resistant cells, but the Nrf2 expression was increased in LS174T resistant (LS174T/Res) cells and decreased in SW480/Res cells which were consistent with the level of resistance in these cells (25 fold increase in IC50 value in LS174T/Res cells versus 7 fold increase in this value in SW480/Res cells). Inhibition of either Nrf2 or Her2 alone and in combination caused a significant increase in oxaliplatin-induced cytotoxicity and apoptosis with maximum effects in SW480/Res cells with low Her2 and Nrf2 expression levels. Altogether, our results suggest that inhibition of Nrf2 signaling in colon cancer patients with Her2 overexpression can be considered as an important strategy to overcome oxaliplatin resistance.

1. Introduction

Colon cancer is the third most prevalent cancer in men and women worldwide. Oxaliplatin is commonly used as the first line treatment in metastatic colon cancer patients after colorectal surgery. However, development of oxaliplatin resistance is a common phenomenon in these patients [1]. Therefore, understanding the signaling mechanisms underlying the development of oxaliplatin resistance and targeting the key molecules can be helpful to develop novel strategies to overcome chemoresistance. Her2 is a member of Her receptor network 'Her1, Her2, Her3, and Her4' that belong to the family of receptor tyrosine kinases (RTKs) [2]. From this family, Her2 is more attractive for target therapy with auto-kinase activity without any known ligand [3]. The overexpression of Her2 has been reported in several human tumors including breast and colon cancers [4–6]. Her2 signaling induces 5-fluorouracil, doxorubicin, paclitaxel, etoposide and camptothecin resistance in breast cancer in a phosphoinositide-3-kinase/AKT (PI3K/AKT)-dependent manner [7]. Moreover, Her2 somatic mutations in colorectal cancer are similar to those in breast cancer which are important for resistance to cetuximab and panitumumab [8].

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Nuclear factor erythroid 2-related factor 2 (Nrf2) is a master transcriptional regulator for both the genes coding battery of phases I, II, and III detoxification enzymes, and the genes involved in the production of antioxidant enzymes and related signal transduction pathways [9,10]. Several studies indicated that Nrf2 has important role in resistance to oxaliplatin and other chemotherapeutics [11–16]. Nrf2 functions by binding to the antioxidant response elements (ARE) or electrophile response elements (EpREs) within the promoters of its target genes [17,18]. Under normal conditions, keap1, as a major repressor of Nrf2, holds free Nrf2 in a low level in the cytoplasm. Keap1 bridges Nrf2 to Cul3-based E3 ligase and facilitates constantly a degradation of Nrf2 via the ubiquitin/proteasome pathway. Under stress conditions or presence of Nrf2 activator, some of Keap1 cysteine residues become oxidized by reactive oxygen species (ROS) or electrophiles. Then, conformation changes occur in the Keap1 structure. In this condition, keap1 is not able to repress Nrf2. Therefore, Nrf2 accumulates in the nucleus for transcriptional induction of its target genes to cellular redox homeostasis [18–20].

Most of common chemotherapeutics function through the production of ROS [3,21]. ROS acts as the key regulator of Nrf2, via the Keap1 dependent and independent pathways; moreover, Nrf2 as a smart regulator of cellular redox hemostasis, plays an important role in controlling the level of ROS in the cells [18,22,23]. It has been reported that ROS regulates many key targets of the RTK signaling pathways including Her2 receptors. Furthermore, Her2 receptors regulates Nrf2 DNA binding activity through direct interaction with Nrf2. PI3K and mitogen-activated protein kinase (MAPK) signaling pathways as the downstream effectors of Her2 can also promote Nrf2 binding to DNA which induces transcriptional activation of target genes [24,25].

In this study, we first developed two oxaliplatin-resistant colon cancer cell lines. After characterization of both cell lines for Nrf2 and Her2 expressions, we investigated the role of Her2-Nrf2 axis in promoting oxaliplatin resistance in both high and low Her2 expression colon cancer cell lines. Our results supported the idea that identifying Her2 positive colon cancer patients with high Nrf2 expression and then suppression of this signaling pathway can be applied as an important idea to prevent oxaliplatin resistance in these patients.

2. Materials and methods

2.1. Materials

Oxaliplatin (O9512), Thiazolyl Blue Tetrazolium Blue (MTT) (M2128) powder and phosphate Buffer saline (PBS) (SLBJ2117V) tablets were purchased from Sigma-Aldrich (St. Louis, MO, USA). Brusatol (FB300161501) was obtained from Carbosynth (Compton, Berkshire, UK) and mubritinib (S2216) was purchased from SelleckChem (Houston, USA). The oxaliplatin was dissolved in the sterile deionized water and the mubritinib and brusatol were dissolved in dimetylsulfoxide (DMSO) (K44917952; Merck, Germany). Roswell Park Memorial Institute 1640 (RPMI1640) (1775866) and Dulbecco's Modified Eagle's medium (DMEM) (1791923) high glucose media, Trypsin/EDTA 0.25% (1726653) and Fetal Bovine Serum (FBS) (42Q7363K) were obtained from Gibco (Maryland, USA). RNA isolation kit and primer sequences were purchased from CinnaGen (9561071; Tehran, Iran) and Macrogen (Seoul, South Korea), respectively. The cDNA (AK5601) synthesis kit and Prime Script RT Master Mix (A9104-1) were provided from TaKaRa (Tokyo, Japan). Annexin V/Propidium iodide (Annexin V/PI) was obtained from e-Biosciences (88-8005-72; San Diego, CA, USA).

2.2. Cell culture

The colon cancer cells (SW480 and LS174T) were obtained from Iran National Cell Line Bank (Pasteur Institute Cell Culture Collection Tehran, Iran). We cultured SW480 cells in RPMI-1640 medium and LS174T cells in DMEM high glucose medium supplemented with 10% (v/v) FBS without antibiotics and incubated at 37 °C in a humidified atmosphere containing 5% CO2.

2.3. Generation of clinically relevant oxaliplatin-resistant colon cancer cells

Resistant SW480 and LS174T cells were developed as described before [26,27]. Briefly, these cells were exposed to half maximal inhibitory concentration (IC50) concentrations of oxaliplatin for 72 h. Then, the drug was removed and the cells were cultured in a drug free media for recovery. After that, the cells were again exposed to the same concentration of oxaliplatin for another 72 h. Resistant cells were achieved by repeating this cycle 15 times during 7 months.

2.4. Assessment of cell viability using MTT assay

MTT assay was applied to evaluate cell viability. First, the cells (10^4 cells/well) were seeded in 96-well plate in triplicate. After incubation of the cells with different concentrations of oxaliplatin, the media was replaced with fresh media containing 2 mg/ml MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide) solution and incubated for an additional 4 h at 37 °C. Then, the media was removed and 200 µl DMSO plus 25 µl Sorenson glycine buffer were added. After gently shaking (15 min), the optical density (OD) of the wells were measured in 570 nm by 96 well-Plates Reader (Labsystems, Finland) [28].

2.5. Assessment of mRNA expression via Real-time RT-PCR

For extraction of total RNA, we applied RNA X Plus solution according manufacture protocol. The total amount of extracted RNA was measured by Nano Drop 1000 Spectrophotometer (Wilmington, USA) and qualified by agarose gel electrophoresis. Revers transcription of extracted RNA into cDNA was performed by cDNA synthesis kit (Takara, Japan). Quantitative reverse transcription PCR (RT-qPCR) was implemented using SYBR green (Prime Script RT Master Mix, Takara) and MIC PCR (Bio Molecular System, Australia). The following primer sequences were used: Nrf2: F: 5′-CCAAAACCACCCTGAAAGCAC-3′ and R: 5′-GTGATGCCACACTGGGACT-3′, Her2: F: 5′-AGACCCGCTGAACAA TACCAC-3′ and R: 5′-CCTTCCACAAAATCGTGTCC-3′, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH): F: 5′- CAAGATCATC ACCAATGCCT-3′; R: 5′- CCCATCACGCCACAGTTTCC-3′. The analysis of raw data was performed by Pffafl method and GAPDH gene was considered as an internal control [29].

2.6. Flow cytometry assay

The sensitive and resistant cells (10^5 cells/well) were seeded in six well plates. After 72 h incubation with desired concentrations of oxaliplatin in the presence and absence of Her2 and Nrf2 inhibitors, the cells were harvested and washed with PBS, stained with Annexin V–FITC/PI and analyzed for apoptotic cells using MACS Quant flow cytometer (Miltenyi Biotec, Bergisch Gladbach, Germany) and FlowJo (7.6.1) software for acquisition and analysis as described before [30,31].

2.7. Analysis of drug interactions by sequential combination index

Resistant colon cancer cells were incubated with sequential treatment of brusatol and mubritinib alone and in combination for 2 h, followed by incubation with oxaliplatin concentrations for 72 h. Inhibition of cell proliferation was detected applying MTT assay and combination index (CI) values were calculated using CompuSyn v.1 software. CI value of 1 shows an additive effect; while a CI $<$ 1 exhibits synergism and CI $>$ 1 reveals an antagonism [32,33].