RESEARCH ARTICLE

Effect of MUC1 siRNA on Drug Resistance of Gastric Cancer Cells to Trastuzumab

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Abstract

Trastuzumab is the first molecular targeting drug to increase the overall survival rate in advanced gastric cancer. However, it has also been found that a high intrinsic or primary trastuzumab resistance exists in some proportion of gastric cancer patients. In order to explore the mechanism of resistance to trastuzumab, firstly we investigated the expression of MUC1 (membrane-type mucin 1) in gastric cancer cells and its relationship with drug-resistance. Then using gene-silencing, we transfected a siRNA of MUC1 into drug-resistant cells. The results showed the MKN45 gastric cell line to be resistant to trastuzumab, mRNA and protein expression of MUC1 being significantly upregulated. After transfection of MUC1 siRNA, protein expression of MUC1 in MKN45cells was significantly reduced. Compared with the junk transfection and blank control groups, the sensitivity to trastuzumab under MUC1 siRNA conditions was significantly increased. These results imply that HER2-positive gastric cancer cell MKN45 is resistant to trastuzumab and this resistance can be cancelled by silencing expression of the MUC1 gene.

Keywords: HER2 - mucin1 - trastuzumab - drug resistance - gastric cancer - siRNA

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Introduction

Gastric cancer is one of the most common malignant tumors. Molecular targeted therapy combined with chemotherapy for advanced gastric cancer could enhance the efficacy and increase the overall survival rate. Trastuzumab is a monoclonal antibody targeting against HER2/neu proto-oncogene product, which could specifically regulate the HER2 extracellular binding region and inhibit the growth, thereby induce apoptosis of tumor cells. An open-label, international, phase 3, randomised controlled trial named ToGA (Trastuzumab for Gastric Cancer) found that trastuzumab is the first molecular targeting drug to show overall survival advantage (Bang et al., 2010). Effects on increasing the survival rate of patients with breast cancer was obvious.

Unfortunately, many breast cancer patients do not respond to trastuzumab therapy, even though their tumors overexpress HER2. These cancers are deemed to be ''intrinsically resistant''. There are also some patients do not respond to trastuzumab therapy after 1 year of treatment ,enen though they are drug-sensitive originally. These cancers are deemed to be ''secondary resistant''. Previously studies focused on trastuzumab resistance concluded that trastuzumab resistance could be characterized by: (1) alterations in trastuzumab-HER2 interactions; (2) changes in the expression of regulators of cell cycle; or (3) up-regulations of other growth factor receptors, co-receptors or their ligands.

Recently, overexpression of MUC1 was reported to be involved in trastuzumab resistance. Published studies reported that breast cancer cell lines T47D and ZR-75-30 were resistant to Herceptin in vitro growth assays. Notably, both these cell lines express high levels of MUC1. MUC1 has been reported to have anti-apoptotic properties as well as progrowth effects. However, the relationship between MUC1 in gastric cancer cells and its effect on the resistance is also unknown.

Here, we specifically examined the role of MUC1 in intrinsic trastuzumab resistance in gastric cancer cells and the potential for using MUC1 specificlly siRNA to reverse it. We used different concentrations of trastuzumab to treat two gastric cancer cell lines MKN45 and NCI-N87, screened resistant cell line with Cell Counting Kit-8 (CCK8). Then, MUC1 gene-targeting siRNA was transfected to the resistant cell line to silence MUC1 gene expression, and we investigated the effects of MUC1 overexpression on the resistance of gastric cancer cell to trastuzumab, to explore the relevance between MUC1 expression and the resistance of trastuzumab.

The results showed that HER2-positive gastric cancer cell MKN45 are resistant to trastuzumab, but that the resistance could be cancelled by silencing the expression of MUC1 gene in it. It implies that overexpression of MUC1 promote gastric cancer cells resistant to trastuzumab.

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Materials and Methods

Cell culture and regents

Gastric carcinoma cell line MKN45 (preserved in our laboratory), NCI-N87 and breast cancer cell line BT-474 (from Cell Bank of Shanghai Institutes of Biological Sciences, Shanghai, China) were cultured in RPMI 1640 medium (Sigma, St Louis,MI) supplemented with 10% fetal bovine serum (FBS) and cultured in a 5% CO_2 and 95% air at 37oC. DMSO and drugs in this work were purchased from sigma.

Screening of the resistant cell line to trastuzumab (CCK8)

Using different concentrations of trastuzumab (0 μg/ml, 0.01μg / ml, 0.03 μg / ml, 0.1 μg/ml, 0.3μg/ml, 1μg /ml) to treat two gastric cancer cell lines (MKN45, NCI-N87) for 24, 48 and 72 hours. Then they were continuing incubated for 4 h after 10 μl CCK 8 was added to each hole. The light absorption values of 450 nm wavelength was detected by enzyme-linked immunosorbent assay, and the growth inhibition rate was calculated, further screening the resistant cell line.

In vitro transfection

According to the MUC1 gene sequence provided by the U.S. National Center for Biotechnology and the GAPDH gene sequence in the NCBI database, MUC1-specific siRNA or control siRNA were synthesized by the Shanghai GenePharma company. Briefly, the day before transfection, 1×10^5 MKN45 cells per well were plated onto 24-well plates.And when the cells had reached 70% to 80% confluency,the siRNA was transfected with Lipofectamine 2000 to each well. The cells were incubated for another 72 hours before the experiments were conducted. Transfection was performed with Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. The experiment was divided into three groups: (1) control group: only liposomes without any siRNA; (2) negative control group: siRNA which is not directed against any target gene was transfected; (3) MUC1-siRNA group: siRNA targeted to MUC1 gene was transfected (siRNA: the liposomal = 1:6).

Growth of resistant cell line transfected with MUC1-specific siRNA in the presence of trastuzumab(CCK8)

After a 72 hour transfection, the old medium was replaced by medium containing different concentrations of trastuzumab (0 μg/ml, 0.01 μg / ml, 0.03 μg/ml, 0.1 μg/ml, 0.3 μg/ml, 1 μg /ml), which was replaced in turn with fresh medium after another 24 hour incubation. After 2 days, 10 μl CCK8 was added for another 4 hour incubation. The light absorption values of 450 nm wavelength was detected by enzyme-linked immunosorbent assay, and calculated the cell growth inhibition of the resistant cell line after 72 hours .

Western analysis

Protein extracts were equally loaded on 10% SDS–PAGE, electrophoresed, and transferred to nitrocellulose membrane (Amersham Bioscience, Buckinghamshire, UK). After blocking with 5% nonfat milk in PBS, the membranes were incubated with the indicated primary antibodies and followed by secondary antibodies. The signals were detected by chemiluminescence phototope-HRP kit (Pierce Biotechnology, Rockford, USA) according to manufacturer's instructions. The primary antibodies were as follows: HER2 from Santa Cruz Biotechnology (Santa Cruz, CA, USA) at a dilution of 1:100; MUC1 from Abcam (Abcam, Cambridge, MA) at a dilution of 1:2000, and β-actin from Biomart (Shanghai, China) at a dilution of 1:1000. Secondary antibodies included peroxidase-conjugated affinipure goat anti-mouse IgG from Jackson ImmunoResearch (West Baltimore Pike West Grove, PA, USA).

Primer design and synthesis

According to the MUC1 gene sequence provided by the U.S. National Center for Biotechnology and the GAPDH gene sequence in the NCBI database, the primers for quantitative PCR were designed using software Primer 5.0 according to the principle.The primers were synthesized and detected by Sangon biotechnology company (Shanghai). The specific primers for the PCR reaction were as follows: MUC1, 5-TATCTCATTGCCTTGGCTGTC-3 (forward) and 5-GGTACTCGCTCATAGGATGGTAG-3(reverse) with a product size of 100 bp; HER2, 5-CTGCCTCCACTTCAACCACA-3(forward) and 5-TCCCACGTCCGTAGAAAGGT-3(reverse) with a product size of 166 bp; GAPDH, 5-AACGGATTTGGTCGTATTG -3 (forward) and 5-GGAAGATGGTGATGGGATT -3(reverse) with a product size of 208 bp.

RNA isolation and Real-time PCR analysis

Total RNA was isolated from cells using TRIzol (Invitrogen) according to the manufacturer's instructions. Following quantification, 2 μg of total RNA from each sample was subjected to cDNA synthesis. In order to quantify the transcripts of the interest genes, real-time PCR was performed using a SYBR Green Premix Ex Taq (Takara, Japan) on LightCycler 480 (Roche, Switzerland). Specifically, 1 μl of reverse transcription reaction mixture was utilized for a quantitative PCR reaction in a total volume of 20 μl. Relative expression levels of target genes= $2^{-\triangle\triangle Ct}$ ($\triangle\triangle Ct=\triangle Ct$ (experimental group) - $\triangle Ct$ (control group)).

Statistical Analysis

Values were shown as mean±SD. Using SPSS17.0 statistical software to analyze the data. Statistical differences were determined by a Student's t test or one-way ANOVA. Statistical significance is set as $P<0.05$.

Results

Gastric carcinoma cell MKN45 is resistent to trastuzumab

To investigate the difference of the sensitivity to trastuzumab between MKN45 and NCI-N87 cells, we dected the proliferation of both cells using CCK8 after treatment of trastuzumab for 24, 48 and 72 hours. The inhibition of gastric cancer cell MKN45 treated with trastuzumab from 0.01 μg/ml in vitro had no significant difference compared with the control group ($P = 0.076$),

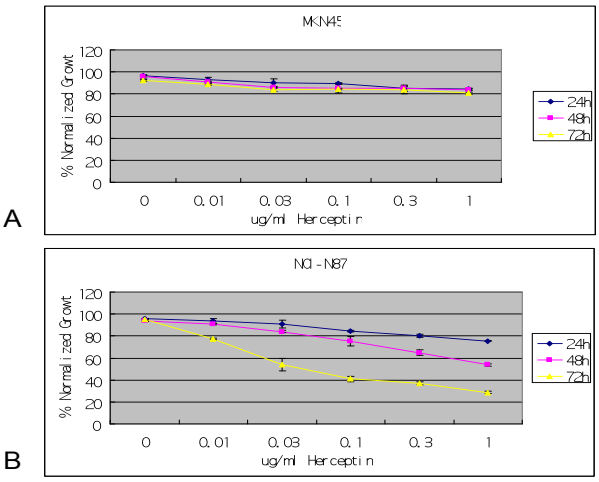

Figure 1. Determination of the Resistance of Gastric Cancer Cell Lines MKN45 and NCI-N87 to Trastuzumab. A. MKN45 is resistant to trastuzumab. B. The inhibition of NCI-N87 was dose-dependent

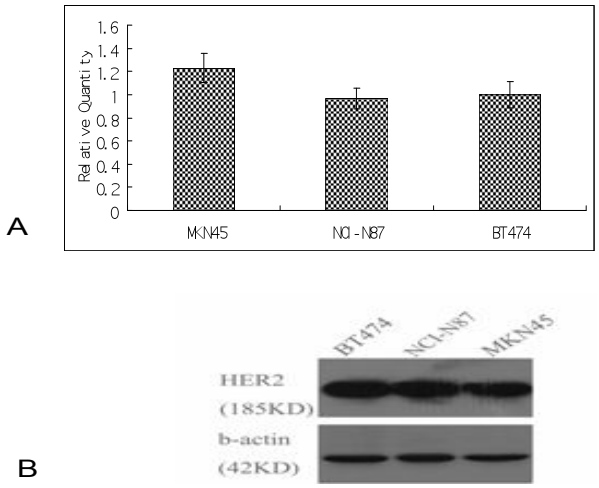

Figure 2. The mRNA and Protein Expression of HER2 in Three Cells. A. No significant difference in the expression of HER2 mRNA(P>0.05). B. No significant difference in the expression of HER2 protein (P> 0.05)

while NCI-N87 had a signåificant difference (P = 0.019), and the inhibitory effect was dose-dependent (Figure 1).

mRNA and protein expression of HER2 is high in MKN45 and NCI-N87 cells

The application of real-time PCR and Western blot were used to detect the HER2 mRNA and protein expression of gastric cancer cells MKN45, NCI-N87 and breast cancer cell BT-474. The results showed that the HER2 mRNA levels had no significant difference among the three cell lines (Figure 2A). The HER2 protein expression had also no significant difference in the three cell lines (Relative absorbance were as follows: 0.9945±0.0371, 1.1272±0.057, 1.5378±0.0113, P>0.05) (Figure 2B).

mRNA and protein expression levels of MUC1 in MKN45 cells is significantly higher than NCI-N87

The application of real-time PCR and Western blot were used to detect the MUC1 mRNA and protein expression of gastric cancer cells MKN45, NCI-N87. The

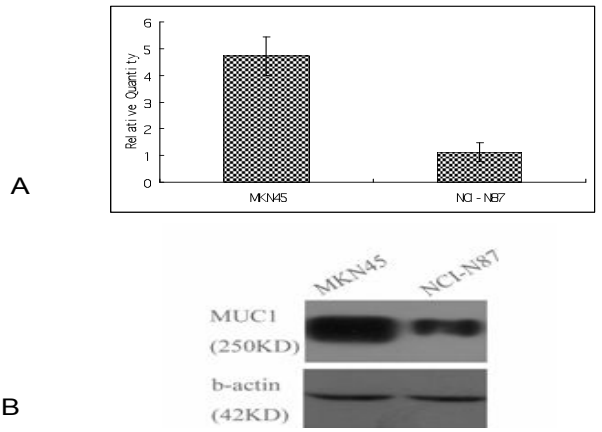

Figure 3. The mRNA and Protein Expression of MUC1 in Both Gastric Carcinoma Cells. The MUC1mRNA expression levels in gastric cancer cell MKN45 were significantly higher in NCI-N87 (P<0.05). B. The MUC1 protein expression level in MKN45 was also significantly higher than NCI-N87 (P<0.05)

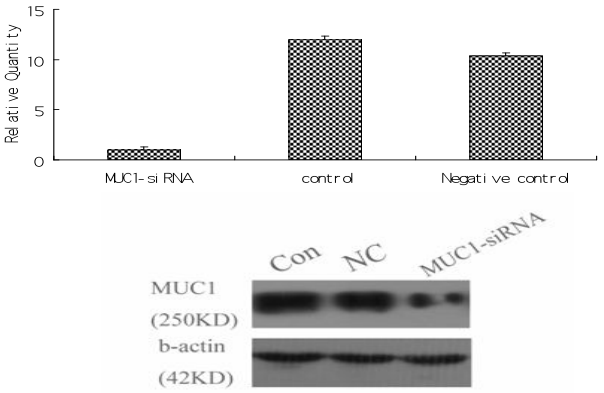

Figure 4. MUC1 mRNA and Protein Expression in Gastric Cancer Cells MKN45 after Transfection 72 Hours Later. After transfected with MUC1 siRNA, the MUC1 mRNA expression level was significantly lower than that of control group and negative control group(P<0.05). B. After transfected with MUC1 siRNA, the MUC1 protein expression level was significantly lower than that of control group and negative control group (P<0.05)

results showed that the MUC1mRNA expression level in gastric cancer cell MKN45 was significantly higher in NCI-N87 (P=0.0281) (Figure 3A); the MUC1 protein expression level in MKN45 was also significantly higher than NCI-N87 (Relative absorbance were as follows: 1.3864±0.0315 vs 0.3959±0.0242, P<0.05) (Figure 3B).

The MUC1 gene was specifically "silenced after transfected with MUC1 siRNA

The application of real-time PCR and Western blot were used to detect the MUC1 mRNA and protein expression of each transfection group (control group, negative control group, MUC1-siRNA group). After transfected with MUC1 siRNA, the MUC1 gene was specificlly "silenced"; and the expression of MUC1 protein was significantly inhibited (Figure 4).

Sensitivity to trastuzumab in MUC1 siRNA condition was significantly increased

The growth and inhibition of gastric cancer cells before

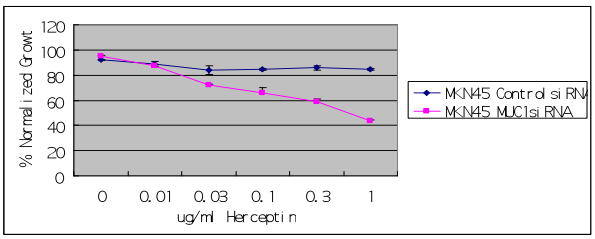

Figure 5. Inhibitory Effect of Different Concentrations of Trastuzumab to Gastric Cancer Cells MKN45 after MUC1-siRNA Transfected. The inhibition of trastuzumab to gastric cancer cells MKN45 after transfection with MUC1-siRNA was significant when compared with the control group (P<0.05)

and after transfected with MUC1 siRNA were observed with CCK8 method. After treatment with different concentrations of trastuzumab (0.01 ug/ml, 0.1 ug/ml, 1 ug/ml, 10 ug/ml, 100 ug/ml, 1000 ug/ml), the sensitivity to trastuzumab in MUC1 siRNA condition was significantly increased compared with the junk transfection condition and blank control group (P=0.034) (Figure 5).

Discussion

Trastuzumab (Herceptin) is a monoclonal antibody targeting against HER2/neu proto-oncogene product, which could specifically regulate the HER2 extracellular binding region and inhibit the growth, thereby induce apoptosis of tumor cells. Since trastuzumab was listed, its effect on increasing the survival rate of patients with breast cancer was obvious, but some patients with HER2-positive breast cancer had primary drug resistance and some acquired secondary resistance after one year treatment. Similarly, in the ToGA trial,the addition of trastuzumab to chemotherapy for patients with HER2-positive gastric cancer led to an additional absolute increase in response rate of only 12% (Bang et al., 2010). This indicates the existence of a high intrinsic or primary trastuzumab resistance in this subpopulation.Furthermore, the majority of patients who had initially responded to treatment developed acquired or secondary resistance.

Part of the studies suggest that intratumoral heterogeneity of gastric cancer may contribute to trastuzumab resistance (Gerlinger et al., 2012; Yang et al., 2012). In addition, some studies found that the molecular mechanisms promoting trastuzumab resistance of gastric cancer cells may include: (1) excessive activation of the downstream PI3K/Akt signaling pathway (Velho et al., 2005) (2) cell surface proteins such as HSP90 (Citri et al., 2004; Lang et al., 2007) and mucins (Price, Schiavi et al., 2002) decrease the interaction between trastuzumab and the HER2 receptor, thereby blocking the inhibitory actions of the drug. However, recent studies have shown upregulation of mucin-1 (MUC1), a protein with growth factor receptor-like activity (Fessler et al., 2009), mediated the growth of tumor cells, especially the trastuzumab-resistant HER2-positive breast cancer cells. The relationship between MUC1 in gastric cancer cells and its effect on the resistance is unknown.

Mucin1(MUC1), the main components of the gastric mucus gel, is a high molecular weight glycoproteins expressed by specialized epithelial cells lining the luminal surface of different organs. It is located on the luminal surface, such as respiratory, gastrointestinal tract, reproductive tract, etc. MUC1 is overexpressed and under-glycosylated in almost all human epithelial cells of adenocarcinoma leading to the exposure of new peptide epitopes and oligosaccharides, that serve as a novel target molecule, making MUC1 an attractive and broadly applicable target molecule for cancer therapy. The overexpression of MUC1 and distribution on cell surface are assumed to influence the biological behavior of the tumor cells during malignant transformation and tumor progression such as pancreatic, breast, myeloma cells. Many efforts that have been made in recent years to improve MUC1 promote trastuzumab resistance on breast cancer, but its possible mechanism is not very clear. Preliminary studies have shown that the ways MUC1 promote tumor cell resistance may include: inhibition of endogenous apoptosis pathway (Yin et al., 2003; Ren et al., 2004) and continuing activation of PI3K/Akt12 and other signal transduction pathways (Raina et al., 2004). However, the researches about the relationship between MUC1 and the resistance of gastric cancer cells and the possible mechanism have been in blank.

The study showed that gastric cell cline MKN45 was primary resistant to trastuzumab by comparing the growth inhibition of trastuzumab on MKN45 and NCI-N87. Simultaneous, we detected the mRNA and protein expression of HER2 in three cells (MKN45, NCI-N87, and breast cancer cell line BT474 which is of high expression of HER2) and found that there was no significant difference between them. We further analyzed the mRNA and protein expression of MUC1 in those two gastric carcinoma cells, and found that the resistant gastric cancer cell line MKN45 was of high expression of MUC1. This was consistent with the conclusions that the breast cancer cell lines resistant to trastuzumab were of the high expression of MUC1. However, whether there was correlation or not between MUC1 overexpression and the resistance is unclear. We further investigated the effects of MUC1 overexpression on the resistance of gastric cancer cell to trastuzumab by silencing MUC1 in gastric cancer cell line MKN45. Three siRNAs were designed and synthesized against three different sites of the MUC1 gene, and then were transfected to interfere its expression. The growth and inhibition of gastric cancer cells before and after transfected with MUC1 siRNA was observed with CCK8 method. We showed that this intrinsic resistance to trastuzumab could be overcomed by using MUC1 specific siRNA. Our results showed that the trastuzumab resistance was accompanied by a dramatic increase in the expression of MUC1 but the resistance was reversed by treating the cells using MUC1-specific siRNA. These findings suggest that therapies that include a MUC1-specific siRNA could rescue the large percentage of patients whose gastric cancers to resistance to Herceptin (Nahta et al., 2007; Valabrega et al., 2007). In addition, our results imply that a much broader subset of cancers, previously thought to be resistant to trastuzumab (Vogel et al., 2001), could be successfully treated with trastuzumab if combined with a MUC1-targeting drug.

MUC1 can exert oncogenic effects through pro-growth as well as anti-apoptotic properties, and therefore it is not surprising that it plays a role in drug resistance. Of the 1.3 million tumors, diagnosed in the US each year, over 60% show tumor-associated aberrant overexpression of MUC1 (Ren et al., 2004). Reaserches previously showed that ligand-induced dimerization of the extracellular domain of MUC1 induced ERK2 phosphorylation, cell proliferation and survival (Hikita et al., 2008; Mahanta et al., 2008). Thus, in trastuzumab resistant cells, MUC1 could function independently by homodimerization to increase cell growth and inhibit apoptosis, thereby countering the growth inhibitory effects of trastuzumab.

MUC1 overexpression has been noted in other contexts of resistance and recurrence (Ren et al., 2004; Siragusa et al., 2007). Similar to our study, it has been reported that the ovarian cancer cell line SKOV-3 and the breast cancer cell line BT474 also made refractory to trastuzumab in vitro, showed MUC1 upregulation. In another study, the only gene out of 26,000 tested, that corresponded to prostate cancer recurrence was MUC1. An increase in the amount of MUC1 mRNA was shown to increase the probability of prostate cancer recurrence (Lapointe et al., 2004). A study of patient matched ovarian tumors, showed that MUC1 was one of 121 genes, out of 21,000 tested, which was upregulated in the post chemotherapy tumor compared with the primary tumor (L'Esperance et al., 2006).

Our results suggested that the development of MUC1-targeting molecules could lead to a promising new class of therapeutics to treat an even broader spectrum of cancers. Next step, we should further explore the mechanism about its upregulation and its association with clinical trastuzumab resistance. And finally, cell biological studies are needed to further elucidate potential interactions between MUC1 and erbB2.

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