A let-7b binding site SNP in the 3'-UTR of the Bcl-xL gene enhances resistance to 5-fluorouracil and doxorubicin in breast cancer cells

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Abstract. The development of acquired resistance to chemotherapy is a major obstacle in the successful treatment of cancer. In breast cancer cells, B-cell lymphoma-extra large (Bcl-xL) is involved in the development of resistance to various chemotherapeutic agents; therefore, preliminary biological prediction was performed to identify a putative binding site for let-7b in the 3'-untranslated region (UTR) of the Bcl-xL gene and a single nucleotide polymorphism (SNP) within this binding region. The present study investigated the association between the SNP rs3208684 A>C and chemotherapeutic agent resistance in breast cancer cells. The data indicated that let-7b negatively regulates the expression of Bcl-xL and appears to sensitize MCF-7 cells to the chemotherapeutic agents 5-fluorouracil (5-FU) and doxorubicin. Furthermore, the SNP rs3208684 A>C was demonstrated to enhance Bcl-xL protein expression by disrupting the binding of let-7b to the 3'-UTR of Bcl-xL and, in MCF-7 cells, overexpression of let-7b in the presence of a mutant Bcl-xL 3'-UTR (C allele) significantly increased 5-FU and doxorubicin resistance. Thus, the results of the present study demonstrate that the SNP rs3208684 A>C may upregulate Bcl-xL protein expression and enhance the resistance of the MCF-7 cells to 5-FU and doxorubicin by decreasing the binding of let-7b to the 3'-UTR of Bcl-xL.

Introduction

Breast cancer is the leading cause of cancer-related mortality in females, worldwide (1). In the previous two decades, the incidence rate of breast cancer has increased at an average rate of 3.1% per year and the mortality rate has increased at an average rate of 1.8% per year (2). Chemotherapy is an important adjuvant systemic therapeutic approach for the successful treatment of breast cancer (3) and, during early-stage breast cancer, has been demonstrated to improve survival rate (4).

MicroRNAs (miRNAs) have previously been identified as important regulators of a number of key genes associated with chemoresistance (5,6). miRNAs are a class of endogenous, 18 to 25-nucleotide long, non-coding RNAs, which regulate gene expression at the post-transcription level (7,8). As a recognition mechanism, miRNAs complementarily pair to the 3'-untranslated region (UTR) of their target mRNAs, resulting in decreased translational efficiency and/or decreased mRNA expression levels (9-11). It has previously been reported that miRNAs commonly deregulate gene expression levels in specific types of human cancer, and may serve as oncogenes or tumor suppressors (12,13). However, dysregulated miRNAs appear to be associated with every aspect of the cancer-related biological process, including tumor progression, invasion and metastasis, as well as the acquisition of resistance to various chemotherapeutic agents (14,15). Previous studies have indicated that single nucleotide polymorphisms (SNPs) occurring in or near miRNA binding sites may be associated with tumor susceptibility and chemotherapeutic response in humans (6,17-19).

B-cell lymphoma-extra large (Bcl-xL) belongs to the Bcl-2 protein family and appears to confer resistance to apoptosis, thereby reducing the effectiveness of chemotherapy (20). It has previously been reported that overexpression of Bcl-2 and Bcl-xL increases resistance to taxol and etoposide administration in MCF-7 cells (21), whereas their downregulation sensitizes MCF-7 and MDA-MB-231 cells to doxorubicin, paclitaxel and cyclophosphamide administration (22). In the present study, we hypothesized that SNPs located in let-7b binding sites of the Bcl-xL gene 3'-UTR may regulate Bcl-xL expression, thus, increasing cellular resistance to chemotherapeutic agents in breast cancer cells. To investigate this hypothesis, bioinformatic analyses were performed to identify SNPs in the 3'-UTR of the Bcl-xL gene. We then functionally validated SNP rs3208684 A>C, which is located in the let-7b binding site in the 3'UTR of the Bcl-xL gene.
Materials and methods

**SNP selection.** To predict putative miRNA binding sites in the Bcl-xL 3’-UTR, mircrorna.org (http://www.mircrorna.org/mircrorna/home.do), PicTar (http://picTar.mdc-berlin.de/cgi-bin/PicTar_vertebrate.cgi) and TargetScan version 6.2 (http://www.targetscan.org/) were used. Furthermore, the National Center for Biotechnology Information SNP database (dbSNP; http://www.ncbi.nlm.nih.gov/SNP) was used to identify SNPs within putative miRNA target sites in the 3’-UTR of Bcl-xL. The search was focused on the miRNA seed region, as the seed sequence nucleates interaction between the miRNA and the complementary Bcl-xL mRNA target region, and is the predominant determinant for successful miRNA targeting.

**Cell culture and transfections.** The human breast cancer cell line, MCF-7 (Cell Bank of the Chinese Academy of Sciences, Shanghai, China) was cultured in Dulbecco’s modified Eagle medium (HyClone Laboratories, Inc., Logan, UT, USA) with 10% fetal bovine serum (HyClone medium) with 10% fetal bovine serum (HyClone medium) with 1% penicillin and 1% streptomycin. Cells were seeded onto 24-well plates (2x10^4 cells per well). In each well, HEK293T cells were transfected with 0.5 μg Bcl-xL 3’-UTR luciferase reporter plasmids containing A or C alleles (Land Co., Ltd) and 100 nM let-7b mimics, let-7b inhibitor or NC inhibitor using Lipofectamine 2000 (Invitrogen Life Technologies, Shanghai, China) according to the manufacturer’s instructions.

**Luciferase reporter assay.** To conduct the luciferase reporter assay, HEK293T cells (Land Co., Ltd, Guangzhou, China) were seeded onto 24-well plates (2x10^4 cells per well). In each well, HEK293T cells were transfected with 0.5 μg Bcl-xL 3’-UTR luciferase reporter plasmids containing A or C alleles (Land Co., Ltd) and 100 nM let-7b mimics, let-7b inhibitor or NC inhibitor using Lipofectamine 2000 (Invitrogen Life Technologies), according to the manufacturer’s instructions. At 48 h post-transfection, the cell lysates were collected and a dual luciferase reporter assay system was used to measure luciferase activity using a Lumat LB953 luminometer (EG & G Berthold, Bad Wildbad, Germany). The Renilla luciferase activity was used as an internal control.

**Statistical analysis.** Statistical analyses were performed using GraphPad Prism software (version 5.0; GraphPad Software Inc., La Jolla, CA, USA) and Student’s t-test. Data are expressed as the mean ± standard deviation and P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Identification of let-7b SNP binding sites in the Bcl-xL 3’-UTR.** To identify possible miRNA binding sites in the 3’-UTR of the Bcl-xL gene, bioinformatic analysis was performed using three online prediction programs (PicTar, TargetScan and mircrorna.org). According to the putatively identified miRNA binding sites combined with information from the dbSNP database, it was identified that the Bcl-xL 3’-UTR SNP rs3208684 A>C is located within a predicted miRNA binding site for let-7b (Fig. 1). These results indicate that the C allele forms a non-perfect pairing with the let-7b mRNA seed and, thus, may escape let-7b-mediated regulation.

**Let-7b negatively regulates the protein expression levels of Bcl-xL and sensitizes MCF-7 cells to 5-FU and ADM.** Bioinformatic analysis identified a potential binding site of let-7b in the 3’-UTR of the Bcl-xL gene and a dual-luciferase reporter assay was performed to determine whether let-7b binds at this putative binding site. The full length Bcl-xL 3’-UTR was cloned into the psiCHECK-2 vector (Fig. 2A) and this psiCHECK2-WT-Bcl-xL 3’-UTR vector was subsequently cotransfected into HEK293T cells with let-7b mimics, NC, let-7b inhibitor or inhibitor NC. As indicated in Fig. 2B, luciferase activity was significantly suppressed in the presence of let-7b.
of let-7b mimics compared with the NC (P<0.01), whereas the luciferase activity displayed no significant difference in cotransfection rate between the let-7b inhibitor and NC inhibitor groups (P>0.05).

To exert their function, miRNAs suppress the expression of their target genes (23). To verify whether Bcl-xL is a target of the miRNA let-7b, Bcl-xL protein expression levels were analyzed in response to enforced expression of let-7b in MCF-7 cells. Following transfection with let-7b mimics or NC, MCF-7 cells were analyzed by performing western blot analysis and it was determined that overexpression of let-7b significantly inhibited endogenous Bcl‑xL protein expression levels. However, overexpression of let-7b had no effect on Bax protein expression levels (Fig. 2C). These biochemical findings indicate that let-7b is a posttranscriptional regulator of Bcl-xL expression in breast cancer cells.

To determine the effect of the SNP rs3208684 A>C on let-7b-mediated regulation of Bcl-xL expression, Bcl-xL gene expression constructs containing WT-Bcl-xL and Mut-Bcl-xL...
were generated and co-transfected into MCF-7 cells with let-7b mimics. As indicated in Fig. 3B, overexpression of let-7b caused a decrease in Bcl-xL gene expression in the presence of WT-Bcl-xL compared with Mut-Bcl-xL; however, the SNP rs3208684 A>C did not change Bax protein expression levels. Thus, the present study proposes that variation in the SNP rs3208684 A>C may mediate the upregulation of Bcl-xL protein expression by interfering with the binding of let-7b to the 3'-UTR of Bcl-xL in breast cancer cells.

Discussion

Bcl-xL is one of two protein products of the Bcl2l1 gene (24) and is a primary antiapoptotic factor, which has been recognized to mediate chemotherapeutic agent resistance. Previously, Bcl-xL and Bax were identified as key factors in the regulation of apoptosis by homodimerization and heterodimerization (25). The human let-7 family is classified as a tumor suppressor family in human cancer (26) and a number of previous studies have indicated that the expression of members of the let-7 family are significantly downregulated in various types of cancer (27), including breast cancer; furthermore, this downregulation is associated with a poorer clinical outcome (28). In the present study, let-7b was demonstrated to target Bcl-xL, resulting in its
downregulation in MCF-7 cells. In addition, the present study indicated that let-7b enhances the sensitivity of MCF-7 cells to ADM and 5-FU. These results indicate that let-7b overexpression may enhance cellular sensitivity to 5-FU and ADM via the repression Bcl-xL expression in MCF-7 cells.

In a number of critical genes, SNPs at or adjacent to miRNA binding sites are associated with the chemotherapeutic response of a tumor via the disturbance or obstruction of miRNA binding (29-32). The possible causes of this are the SNPs located in the 'seed' regions at the 3'-UTRs of human genes involved in multiple pathways such as cell proliferation, cell death, stress resistance which are likely to affect miRNA-target interaction and target expression accordingly (31). The dual-luciferase reporter assay conducted in the current study revealed that the presence of rs3208684 A-3'-UTR in the let-7b binding site of the Bcl-xL 3'-UTR significantly reduces the expression of luciferase compared with the presence of rs3208684 C-3'-UTR. This is consistent with the initial bioinformatic analysis, which indicated a functional interaction between let-7b and Bcl-xL mRNA. Additionally, the transfection of MCF-7 cells with rs3208684 A-3'-UTR and let-7b mimic demonstrated significant inhibition of Bcl-xL expression, and the SNP rs3208684 A>C was identified to cause 5-FU and ADM resistance in MCF-7 cells. Thus, these results indicate that the occurrence of an SNP in rs3208684 A-3'-UTR of Bcl-xL may contribute to the alteration of cellular resistance to 5-FU and ADM.

In conclusion, the present study, demonstrated that let-7b may enhance the sensitivity of MCF-7 cells to 5-FU and ADM by regulating Bcl-xL expression. The SNP rs3208684 A to C may inhibit the interaction between let-7b and Bcl-xL 3'-UTR, resulting in higher Bcl-xL expression, as well as cellular resistance to 5-FU and ADM. Thus, we propose that the SNP rs3208684 A>C may be a potential marker for personalized therapeutic approaches. Furthermore, these results provide insight into a potential novel chemotherapeutic strategy for breast cancer by combining let-7b with currently used chemotherapeutic agents.

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