Let-7b binding site polymorphism in the B-cell lymphoma-extra large 3'UTR is associated with fluorouracil resistance of hepatocellular carcinoma

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Abstract. B-cell lymphoma-extra large (Bcl-xl) is an anti-apoptotic member of the B-cell lymphoma 2 (Bcl-2) family that is often found to be overexpressed in human hepatocellular carcinoma (HCC), therefore conferring a survival advantage to tumor cells. microRNA (miRNA) let-7b is downregulated in HCC and its expression correlates with multidrug resistance. Using computational programs, it was predicted that the 3' untranslated region (UTR) of the Bcl-xl gene contains a potential miRNA binding site for let-7b, and that a single nucleotide polymorphism (SNP) site rs3208684 (A or C allele) resides within this binding site. Luciferase assays and western blot analysis demonstrated that let-7b targeted Bcl-xl gene expression and negatively regulated the amount of Bcl-xl protein. SNP rs3208684 (A>C) variation enhanced the expression of Bcl-xl by disrupting the binding of let-7b to the 3'UTR of Bcl-xl. The effects of the two polymorphic variants on chemotherapeutic drug sensitivity were determined by cell counting kit 8 assays. Overexpression of the Bcl-xl mutated (C) allele in BEL-7402 HCC cells significantly decreased fluorouracil (5-FU) sensitivity, as compared with mock transfection and overexpression of the wild-type allele. From this, it was concluded that let-7b increased 5-FU sensitivity by repressing Bcl-xl expression in HCC cells. These results suggest that SNP (rs3208684) may be a potential marker for personalized treatment.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most prevalent type of cancer worldwide and, with a five-year survival rate of <5%, HCC remains one of the most fatal cancers (1). HCCs are malignant tumors notorious for their resistance to conventional chemotherapy, and few treatments have proven to be effective. HCC exhibits multidrug resistance, mediated by the high expression of anti-apoptosis genes [including B-cell lymphoma 2 (Bcl-2) and B-cell lymphoma extra large (Bcl-xl)] that molecularly oppose the pro-apoptotic effects of chemotherapeutic agents (2-6).

The Bcl-2 gene family is involved in the regulation of cellular apoptosis and tumorigenesis. Bcl-xl, a member of the Bcl-2 family, plays a critical role in regulating cell survival and apoptosis, and is frequently overexpressed in various types of human cancer (7). The inhibition of this gene is associated with decreased tumorigenesis and resistance to conventional chemotherapy (8).

Research on the function of microRNAs (miRNAs) in various cancers has attracted significant attention due to the regulatory role of miRNAs in a broad range of biological processes, including embryogenesis, differentiation, proliferation, apoptosis and carcinogenesis (9). miRNAs are a class of small (19-23 nucleotides), single-stranded, endogenous non-coding RNAs that post-transcriptionally regulate gene expression by binding to the 3' untranslated region (UTR) of target mRNAs, thus inhibiting translation and/or inducing mRNA degradation (10). A previous study demonstrated that single nucleotide polymorphisms (SNPs) found in miRNA binding sites can affect the expression of miRNA target genes and may therefore contribute to the resistance of cancers to multiple drug treatments (11). Furthermore, our previous studies have shown that miRNAs can affect the sensitivity of HCC to drug treatment by regulating the expression of the Bcl-2 gene family (12,13). Therefore, we proposed that miRNA could additionally regulate Bcl-xl and that SNPs in miRNA binding sites could disrupt this regulation. Using various computational programs, a potential miRNA binding site was identified for the let-7 miRNA family in the 3'UTR of the Bcl-xl gene. The miRNAs of the let-7 family share a common seed sequence (GAUGGAG) and are poorly expressed in HCC as compared with normal tissues (14).

Materials and methods

Prediction of miRNA targets. Three computational programs were selected to predict miRNAs that could potentially bind to Bcl-xl mRNA: miRanda (http://www.microrna.org/), Pictar (http://pictar.mdcberlin.de/) and TargetScan (http://www.targetscan.org/).
Construction of the vector and dual-luciferase reporter assay. Full-length Bcl-x1 cDNA sequence containing the entire 3'UTR was synthesized and cloned into pcDNA3.1 plasmid and confirmed by DNA sequencing (Invitrogen Biotechnology Co., Ltd., Shanghai, China). Site-directed mutagenesis was achieved using the Takara MutanBEST kit (Takara Bio, Shiga, Japan) using the following primers: Forward primer, 5'-CCCTCAGGCAGGAAGGCAGGAAGAG'-3' and reverse primer: 5'-AGGGAAGACCTGGGCGCTCCC ATAGCTG-3'. The 3'UTR of Bcl-x1 was synthesized and inserted into the 3'-end of the Renilla luciferase gene in the dual-luciferase miRNA target expression vector psi-CHECK2 (Promega Corporation, Madison, WI, USA) between Xhol and NfI sites. Site-directed mutagenesis was performed in accordance with the manufacturer's instructions (Takara Bio, Inc.). The dual-luciferase reporter plasmids were co-transfected with let-7b mimics into HEK293 cells. At 48 h post-transfection, the cells were assayed for luciferase activity using the Dual-Glo Luciferase Assay System (Promega Corporation) according to the manufacturer's instructions. The Renilla luciferase activities were normalized to firefly luciferase activity. For each transfection, the luciferase activity was averaged from three replicates.

Transfection of miRNA mimics and vectors. The let-7b mimics were purchased from GenePharma (Shanghai, China). The let-7b mimic (20 µM) or control oligonucleotide was transfected into BEL-7402 cells using Lipofactamine™ 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Forty-eight hours after transfection, the cells were harvested and lysed. For transient co-transfection, control vector, pcDNA3.1-Bcl-x1 (A) or pcDNA3.1-Bcl-x1 (C) was co-transfected with let-7b mimic (20 µM) or control oligonucleotide into BEL-7402 cells using Lipofactamine™ 2000. Forty-eight hours after transfection, the cells were harvested and lysed.

Western blot analysis. At 48 h post-transfection, the cells were harvested and lysed in radioimmunoprecipitation assay buffer with protease inhibitors. Proteins were separated on 12% SDS-polyacrylamide gel and transferred to polyvinylidene fluoride membranes (Merck Millipore, Darmstadt, Germany). A PageRuler prestained protein ladder (Pierce Biotechnology Inc., Rockford, IL, USA) was used as a molecular marker. A single membrane was cut into three parts at the 43, 30 and 20 kD bands and incubated with anti-β-actin, -Bcl-x1 and -Bcl2-associated X protein (Bax) monoclonal primary antibodies (Cell Signaling Technology, Inc., Danvers, MA, USA), respectively. The protein was detected using a horseradish peroxidase-conjugated secondary antibody and a Chemilucent Enhanced Chemiluminescence Detection system (Millipore, Billerica, MA, USA).

Results

The 3'UTR of Bcl-x1 contains an miRNA binding site for let-7b, and SNP rs3208684 A>C disrupts the binding efficiency. To identify the miRNAs that regulate Bcl-x1 expression, three computational programs (TargetScan, Pictar and miRanda) were used to search for miRNA binding sites in the 3'UTR of the Bcl-x1 gene. Each program predicted different miRNA binding sites; however, one common binding site for the let-7b miRNA (Fig. 1A) was identified. Subsequent to searching the Ensembl genome browser (http://www.ensembl.org/index.html), an SNP (rs3208684) was identified in the let-7b binding site of the Bcl-x1 3'UTR. The SNP rs3208684 results in the production of two alleles, A and C (Fig. 1A and B).

To investigate the effect of SNP rs3208684 A>C variation on miRNA binding, the wild-type and mutant 3'UTR were cloned into the dual-luciferase reporter vector psi-CHECK2 and co-transfected with miRNA mimics into HEK293T cells. The A>C substitution in the miRNA binding site increased the luciferase activity of the Bcl-x1 3'UTR, indicating that the A>C substitution suppressed or disrupted the binding of let-7b to its target (Fig. 1C).

Let-7b downregulates endogenous Bcl-x1 expression and sensitizes BEL-7402 cells to 5-FU. To examine the effects of let-7b on endogenous Bcl-x1 expression, let-7b mimics and negative control were transfected into BEL-7402 cells, which are known to express high levels of Bcl-x1 protein. The enhanced expression of let-7b in BEL-7402 cells significantly decreased the amount of Bcl-x1 protein, compared with mock transfection (Fig. 2A and B), whereas the Bax protein expression was not significantly different.

To further investigate whether let-7b sensitizes BEL-7402 cells to 5-FU, let-7b mimics and negative control were transfected into BEL-7402 cells. CCK8 assay was performed to determine the cell survival rate. The results indicated that the transfection of let-7b mimics significantly reduced cell viability, as compared with the negative control and mock transfection (Fig. 2C). These results demonstrated that let-7b sensitizes BEL-7402 cells to 5-FU by negatively regulating Bcl-x1 expression.
SNP rs3208684 A>C variation in the Bcl-xl 3' UTR inhibits the binding of let-7b and enhances Bcl-xl expression. To determine the effects of SNP rs3208684 on the let-7b-mediated regulation of Bcl-xl expression constructs of Bcl-xl containing a wild-type (A allele) or mutant (C allele) Bcl-xl 3' UTR were inserted downstream of the Renilla luciferase gene of the psi-CHECK2 vector. (C) Inhibition of Renilla luciferase activity by the interaction between miRNA and the predicted miRNA binding site. Each luciferase construct was co-transfected with let-7b mimics into HEK293T cells. At 48 h post-transfection, the luciferase activity was examined. The Renilla luciferase activity was normalized to firefly luciferase activity. The Renilla luciferase activity of the cells that were transfected with miRNA mimics was presented as the percentage activity relative to that of the cells that were transfected with negative control miRNA mimics. Data are shown as the mean ± standard deviation of three independent experiments. **P<0.01. NC, negative control; Bcl-xl, B-cell lymphoma-extra large; 3' UTR, 3' untranslated region; miRNA, microRNA.

Let-7b miRNA binding site polymorphism in the Bcl-xl 3' UTR desensitizes human BEL-7402 cells to 5-FU. Since SNP rs3208684 A>C variation enhances the expression of Bcl-xl protein, and Bcl-xl overexpression has been shown to decrease chemosensitivity in tumor cells (15,16), we analyzed whether SNP rs3208684 affected the response to 5-FU treatment. BEL-7402 cells were co-transfected with let-7b mimics and wild-type (A allele) or mutated (C allele) Bcl-xl cDNA constructs, and treated with various concentrations of 5-FU (0, 5, 50, 500, 5000, 50,000 µM) were added and the cells were incubated for 24 h and subjected to cell counting kit 8 assay. The data are presented as the mean ± SD (n=3). *P<0.05 vs. the negative control group. (C) The let-7b mimics and negative control were transfected into BEL-7402 cells, respectively. At 48 h after transfection, various concentrations of 5-FU (0, 5, 50, 500, 5000, 50,000 µM) were added and the cells were incubated for 24 h and subjected to cell counting kit 8 assay. The data are presented as the mean ± SD (n=3). *P<0.05. NC, negative control; Bcl-xl, B-cell lymphoma-extra large; Bax, Bcl2-associated X protein; 5-FU, fluorouracil; SD, standard deviation.
Discussion

Let-7, first identified in 2001 (17), is a precursor of human miRNA and has been shown to be downregulated in several malignancies. Numerous target genes have been identified for let-7 miRNA, including Ras (18), Myc (19), HMGA2 (20), CDC25A and CDK6 (21). The major function of these genes is the promotion of cell proliferation. These genes have been shown to exhibit oncogenic function in tumor cells; therefore, let-7 miRNA is considered to function as a tumor suppressor (22). In the present study, we present evidence that let-7b miRNAs are negative regulators of Bcl-xl, which is consistent with findings of Shimizu et al (23) and Qin et al (24). Using computational programs, it was predicted that the 3'UTR of Bcl-xl contained a potential binding site for let-7b. The binding of let-7b to the 3'UTR of Bcl-xl was then verified using the luciferase assay. Finally, the effects of let-7b on the expression of endogenous Bcl-xl were investigated. Ectopic expression of let-7b suppressed the expression of Bcl-xl in BEL-7402 cells.

The let-7 family is expressed at lower levels in HCC than in normal liver tissues, and its low expression is correlated with 5-FU resistance (25). In this study, it was shown that Bcl-xl overexpression in HCC BEL-7402 cells also decreased sensitivity of cancer cells to 5-FU. Cells that were transfected with wild-type Bcl-xl, which contains a 3'UTR that can bind to let-7b, had significantly higher sensitivity to 5-FU than cells with mutant Bcl-xl. These results suggest that overexpression of let-7b induced 5-FU resistance by repressing Bcl-xl expression in BEL-7402 cells.

SNPs are the most common form of genetic variation that occurs in the human genome. SNPs that are located at or near an miRNA binding site of a functional gene can affect gene expression by altering the interaction between miRNA and mRNA (26,27). Previous studies have shown that miRNA binding site polymorphisms are associated with tumor susceptibility and the chemotherapeutic response (11,28-30). For example, the miR-200b/200c/429 binding site polymorphism in the 3'UTR of the AP-2α gene is associated with cisplatin resistance (31). Identifying SNPs that are associated with cancer and chemosensitivity may therefore be useful in the generation of personalized cancer diagnostic and therapeutic approaches. Numerous studies have shown that Bcl-xl status is associated with the chemosensitivity of cancer cells (32,33). In the present study, it was found that the SNP (rs3208684) A>C variation in the Bcl-xl 3'UTR disrupted the interaction between the let-7 precursor miRNA and Bcl-xl, thus upregulating Bcl-xl expression and Bcl-xl-mediated 5-FU resistance. These results suggest that SNP rs3208684 may be a potential prognostic marker for 5-FU treatment and that patients with the A allele of SNP rs3208684 may be more sensitive to chemotherapy than those with the mutant C allele of SNP rs3208684.

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MicroRNA let-7a: The let-7 family


