The polymorphic terminal-loop of pre-miR-1307 binding with MBNL1 contributes to colorectal carcinogenesis via interference with Dicer1 recruitment

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Abstract

Colorectal cancer (CRC) is one of the most common malignancies in the world. Studies have demonstrated that single nucleotide polymorphisms (SNPs) in microRNA genes (miRSNPs) are involved in the occurrence of cancers. However, the relationship between the miRSNPs within the terminal-loops of microRNA precursors and the development of CRC is still largely unknown. In this study, we found that a miRSNP rs7911488 T>C in the terminal-loop of pre-miR-1307 was significantly associated with the occurrence of CRC. The C allele of rs7911488 is more prevalent in CRC patients than in healthy controls (P < 0.001), and this C allele prevalence is related to low level of miR-1307 expression. A RNA-binding protein MBNL1 binds with a 'UGCUGC' motif in the terminal-loop of the C-allelic pre-miR-1307 and blocks Dicer processing, resulting in downregulation of miR-1307 expression. Consequently, the antiapoptosis protein Bcl2, which is a direct target of miR-1307, is overexpressed in CRC. Furthermore, MBNL1 participates in processing of both C-allelic and T-allelic pre-miR-1307. In summary, our results show that rs7911488 C-allelic pre-miR-1307 binds to MBNL1 and interferes with Dicer processing, leading to reduced miR-1307 and increased Bcl2 expression, thus representing an important process in the initiation of CRC.

Introduction

MicroRNA (miRNA) is a small non-coding RNA with ~22 nucleotides. It is initially transcribed from the genomic DNA to long primary transcripts (pri-miRNA) and then cleaved by RNase Drosha into 60–70 nts hairpin-shaped precursor RNA (pre-miRNA). Pre-miRNA is transported from nucleus into the cytoplasm by Exportin-5 and further cleaved on the terminal-loop by Dicer to form a miRNA–miRNA duplex that is unwound by a helicase to release two mature miRNAs (1). miRNA negatively regulates gene expression by binding to the 3′-untranslated region (3′-UTR) of messenger RNA (mRNA) of target gene and specifying mRNA cleavage or translational repression. It is speculated one miRNA can regulate tens to hundreds of target mRNAs, and one mRNA can be targeted by multiple miRNAs. Through regulating multiple transcripts, miRNA takes part in almost all biological processes, such as development, differentiation, cell proliferation, apoptosis, as well as metabolism (2).
Abbreviations

CRC colorectal cancer
miRNA microRNA
mRNA messenger RNA
SNPs single nucleotide polymorphisms

Recent studies have demonstrated that single nucleotide polymorphisms (SNPs), the most common genetic variants, in miRNA genes that are associated with the risk of cancers (3), including lung cancer (4), thyroid cancer (5), breast cancer (6–8), renal cell cancer (9), gastric cancer (10) and leukemia (11) etc. These functional SNPs in miRNA genes (miR-SNPs), locating at pri-miRNA, mature miRNA or terminal-loop of pre-miRNA, affect the function of miRNA via transcription of pri-miRNA, processing of pri-miRNA and pre-miRNA or miRNA–mRNA interactions, respectively (3). Many studies focus on the analysis of miR-SNPs to the pri- and mature regions, as these are clearly defined. Emerging evidences suggest that the functional SNPs in pri-miRNAs are related to processing and levels of mature miRNAs (12). For instance, a germ-line mutation in pri-miR-16-1, through causing low levels of miR-16-1 expression, is associated with prognostic factors and disease progression in chronic lymphocytic leukemia (11). rs41275792 in let-7e leads to reduced levels of mature miRNA in vivo even though its secondary structure is not predicted to be changed (12). rs71428439 in the stem-loop of pri-miR-149 alters the structure of the precursor and affects miR-149 maturation and its ability to regulate the Puma protein in apoptosis (13).

Case-control studies also provide evidences that the SNPs located in the mature miRNAs can work as regulators of miRNAs maturation and/or targets expression. For example, rs2910164 in miR-146a-3p reduces both pre- and mature miR-146a through interfering with the binding of a nuclear factor to pre-miR-146a and predisposes to papillary thyroid carcinoma (5). rs11614913 in miR-196a-3p, probably affecting both maturation and the repertoire of target mRNAs, contributes to the risk of developing lung (4) and breast cancers (6). rs2292832 in has-miR-149 is also proved to be involved in susceptibility with gastric cancer risk (10). Though the underlying biological mechanism of miR-499 in cancer has not been fully elucidated, rs3746444 in pre-miR-499 is found to be associated with susceptibility to breast cancer in a Chinese population (8).

As an SNP in the terminal-loop of pre-miR-27a, rs895819 confers a reduced risk of developing breast cancer (7). Because no changes of free energy or conformation of miR-27a/mRNA duplex are predicted in silico, this SNP may not affect miR-27a maturation or targeting (14). Up to now, the effects of the polymorphisms within the terminal-loop of pre-miRNAs on CRC susceptibility warrant in-depth investigations.

Given the important role of miRNA in gene regulation and carcinogenesis, we hypothesized that polymorphisms within the terminal-loop of pre-miRNAs might affect the maturation of miRNA and alter the expression level of miRNA, with consequences on the regulation of target genes thereby affecting individual’s risk in developing CRC. To test this hypothesis, we searched the polymorphisms residing in the terminal-loop of pre-mi-RNAs, and then genotyped seven putatively functional polymorphisms in 1026 CRC patients and 1026 healthy controls. In addition, we investigated the influence of these polymorphisms on the expression of miRNAs and their target genes.

Materials and methods

Subjects

The research protocol was approved by the institutional review board of Soochow University. A total of 1026 colorectal cancer (CRC) patients and 1026 health controls participated in this study after acquisition of informed consent. They were recruited from the First Affiliated Hospital of Soochow University (Suzhou, Jiangsu province) and Jiangsu Cancer Hospital (Nanjing, Jiangsu province) in recent 3 years. At recruitment, each participant was interviewed to collect detailed information on demographic characteristics. The patients were histologically confirmed by two pathologists. Those with previous or metastasizing cancer from other origins were excluded. The clinical–pathological characteristics were obtained from the medical records, such as tumor location, tumor size, histological type, differentiation grade, depth of tumor infiltration, lymph node metastasis, distant metastasis and tumor, nodes, metastasis stage (Supplementary Table 1, available at Carcinogenesis Online). The variables of depth of tumor infiltration, lymph node metastasis, distant metastasis and tumor, nodes, metastasis stage were examined and staged according to the American Joint Commission for Cancer Staging in 2002. The whole bloods and/or tissues of the patients were collected before radiotherapy or chemotherapy. The health controls, matched with the CRC patients by age (± 2 years) and gender, were selected from about 1700 individuals who participated in the routine health examinations. They have no gastrointestinal disorders or personal and familial history of cancers in more than three generations and second- and third-degree relatives. The whole bloods of the health controls were collected during the same period as the patients’.

Genomic DNA

The whole bloods from the patients and controls were collected and stored in Vacutainer® tubes (BD Franklin Lakes, NJ) containing anticoagulant of ethylene diamine tetraacetic acid. The genomic DNAs were extracted from the whole bloods by using phenol/chloroform method. The purities and concentrations of the extracted DNAs were determined by using UV–Vis spectrophotometer. The extracted DNA was stored at 4°C in TE buffer (10 mmol/l Tris–HCl, 1 mmol/l ethylene diamine tetraacetic acid, pH 8.0).

Genotyping

The SNPs in the terminal-loops of microRNA precursors were obtained from the published databases of miRNASNP 2.0 (www.bioguo.org/miR-NASNP2). The allele frequencies of the SNPs were obtained from NCBI dbSNP build 137. The SNPs with minor allele frequencies (MAF) of not less than 10% were investigated in this study. The SNPs were genotyped by using a MassARRAY SNP Genotyping platform (SEQUENOM) as described previously (15). Briefly, the DNA samples were amplified by polymerase chain reaction (PCR) with the primers listed in Supplementary Table 2, available at Carcinogenesis Online. The dNTP resided in the PCR products were digested with SAP enzyme. Then single-base amplification on the PCR products was performed using the primers listed in Supplementary Table 2, available at Carcinogenesis Online. Thereafter, the single-base amplificons were desalted by using resin and then transfected from the 384 well plates to the MassARRAY SpectroCHIP chips for the mass spectra analysis. The genotyping data were obtained based on the mass spectrums. The genotypes of 30 randomly selected DNA samples were confirmed by Sangier’s DNA sequencing method (GeneWiz, Suzhou, China). The association of genotypes with the occurrence of CRC and clinical–pathological characteristics were analyzed as described previously (15).

The 5′-UTR and Exon 3 flanking sequence of USMG5 gene were PCR amplified with the primers listed in Supplementary Table 3, available at Carcinogenesis Online, and were subsequently genotyped by using Sangier’s sequencing method.

Plasmids

The T allele pre-miR-1307 expression plasmids were purchased from GenePharma, Shanghai, China. The C-allelic pre-miR-1307 constructs were generated by using site-directed mutagenesis PCR method with the primer pairs listed in Supplementary Table 3, available at Carcinogenesis Online. Positive clones were confirmed by sequence-specific PCR and DNA sequencing method.

The MBNL1 protein expression plasmid was constructed as follows. A DNA fragment including the coding sequence of MBNL1 was generated by PCR with synthetic oligonucleotides listed in Supplementary Table 3, available at Carcinogenesis Online. The PCR-amplified fragments were digested by restriction endonucleases BamHI and XhoI (New England Biolabs), and cloned into pcDNA3.1 (+) vector (Invitrogen).
Luciferase reporter assays

The luciferase reporter assays were performed as described previously [15]. Briefly, scrambled miRNA or miR-1307-3p mimics were coinfected with the 3′-UTR/pGL3 constructs and pRL-TK plasmids (Promega) into CHO cells by using lipofectamine 2000 (Invitrogen). After incubating for 24 h, the activities of luciferase in cells were analyzed by using the dual-luciferase reporter assay system (Promega). Each transfection was performed in triplicate.

Results

The C allele of rs7911488 within the terminal-loop of pre-miR-1307 is a risk factor for the occurrence and development of colorectal cancer

A total of 121 SNPs in the terminal-loop of pre-miRNAs and their allele frequencies were obtained from the databases of NCBI dbSNP BUILED 129 and ENSEMBL v58. Among them, the MAF of 17 SNPs were more than 10% (Supplementary Table 4, available at Carcinogenesis Online). To investigate the correlation between these SNPs and the occurrence of CRC, we firstly determined the genotype distribution of these miRSNPs in 1026 CRC patients and 1026 healthy controls by using MassARRAY SNP Genotyping platform. In the 17 candidate miRSNPs, 7 miRSNPs were successfully detected. The other 10 miRSNPs were not genotyped by using this method, due to interaction among primers and/or unspecific amplification. The typical genotyping results of the 7 miRSNPs are shown in Supplementary Figure 1, available at Carcinogenesis Online. As shown in Table 1, the SNP rs7911488 in the terminal-loop of pre-miR-1307 was found to be significantly correlated to the occurrence of CRC.

Chi-square test results showed that the genotype distribution of rs7911488 in the healthy controls was in the Hardy–Weinberg equilibrium distribution pattern (P = 0.99). Bilateral chi-square test results showed that the CC homozygotes (18.23 versus 11.21%; OR = 1.82; P = 1.28 × 10⁻⁴; Table 1) and the C-allele carriers (60.72 versus 56.14%; OR = 1.21; P = 0.039; Table 1) were significantly more prevalent in the cancer patients as compared to the dominant TT homozygotes. Compared with the T-allele carriers, the CC homozygotes (18.23 versus 11.21%; OR = 1.77; P = 8.95 × 10⁻⁴; Table 1) were also significantly more common in the cancer patients. Furthermore, the frequency of C allele was apparently higher in the patients than that in the healthy controls as compared with the T allele (39.47 versus 33.67%; OR = 1.29; P = 1.30 × 10⁻⁴; Table 1). Similar statistical results were obtained in either Suzhou population (Supplementary Table 5, available at Carcinogenesis Online) or Nanjing population (Supplementary Table 6, available at Carcinogenesis Online). Stratified analyses by clinical–pathological features of CRC showed that the frequencies of either CC or C allele were significantly higher in the cancer patients than those in the health controls (Supplementary Table 7, available at Carcinogenesis Online). These findings demonstrate that the C allele of rs7911488 is a risk factor for the occurrence of CRC.

We also analyzed the association of genotypes with the clinicopathological features of CRC. By statistical analysis, we found that rs7911488 was markedly associated with the maximum diameter of tumor (Table 2). Compared with the TT homozygotes, the patients with heterozygotes had significantly smaller size of tumors (OR = 0.58; P = 5.46 × 10⁻⁴), while the patients with CC homozygotes were more likely to burden larger tumors (OR = 1.67; P = 0.006). Similar results were obtained for the CC homozygotes (OR = 2.18; P = 5.17 × 10⁻⁴) and heterozygotes (OR = 0.49; P = 5.57 × 10⁻⁴) as compared to the T-allele carriers and
Table 1. The association of rs7911488 with the occurrence of CRC

<table>
<thead>
<tr>
<th>Model</th>
<th>Genotype</th>
<th>Cancer, n (%)</th>
<th>Control, n (%)</th>
<th>OR (95% CI)</th>
<th>P value&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Codominant</td>
<td>T/T</td>
<td>403 (39.28)</td>
<td>450 (43.86)</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T/C</td>
<td>436 (42.50)</td>
<td>461 (44.95)</td>
<td>1.06 (0.88–1.27)</td>
<td>0.599</td>
</tr>
<tr>
<td></td>
<td>C/C</td>
<td>187 (18.23)</td>
<td>115 (11.21)</td>
<td>1.82 (1.39–2.38)</td>
<td>1.28 × 10^-4</td>
</tr>
<tr>
<td>Dominant</td>
<td>T/T</td>
<td>403 (39.28)</td>
<td>450 (43.86)</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T/C-C/C</td>
<td>623 (60.72)</td>
<td>576 (56.14)</td>
<td>1.21 (1.01–1.44)</td>
<td>0.039</td>
</tr>
<tr>
<td>Recessive</td>
<td>T/T-C/C</td>
<td>839 (81.77)</td>
<td>911 (88.79)</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>Overdominant</td>
<td>T/T-C/C</td>
<td>590 (57.50)</td>
<td>565 (55.07)</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>Allelic</td>
<td>T/T</td>
<td>810 (81.77)</td>
<td>461 (44.93)</td>
<td>0.91 (0.76–1.08)</td>
<td>0.285</td>
</tr>
<tr>
<td></td>
<td>C/C</td>
<td>436 (42.50)</td>
<td>115 (11.21)</td>
<td>1.77 (1.37–2.27)</td>
<td>8.95 × 10^-4</td>
</tr>
</tbody>
</table>

<sup>a</sup>The P values less than 0.05 are presented in bold.

Table 2. The association of rs7911488 with the maximum diameter of tumor

<table>
<thead>
<tr>
<th>Model</th>
<th>Genotype</th>
<th>&gt;5 cm, n (%)</th>
<th>≤5 cm, n (%)</th>
<th>OR (95% CI)</th>
<th>P value&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Codominant</td>
<td>T/T</td>
<td>128 (42.67)</td>
<td>275 (37.88)</td>
<td>1.00</td>
<td></td>
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<td></td>
<td>T/C</td>
<td>91 (30.33)</td>
<td>345 (47.52)</td>
<td>0.57 (0.42–0.77)</td>
<td>3.90 × 10^-4</td>
</tr>
<tr>
<td></td>
<td>C/C</td>
<td>81 (27.00)</td>
<td>106 (14.60)</td>
<td>1.64 (1.15–2.35)</td>
<td>0.007</td>
</tr>
<tr>
<td>Dominant</td>
<td>T/T</td>
<td>128 (42.67)</td>
<td>275 (37.88)</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T/C-C/C</td>
<td>172 (57.33)</td>
<td>451 (62.12)</td>
<td>0.82 (0.62–1.08)</td>
<td>0.160</td>
</tr>
<tr>
<td>Recessive</td>
<td>T/T-C/C</td>
<td>219 (73.00)</td>
<td>620 (85.40)</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C/C</td>
<td>81 (27.00)</td>
<td>106 (14.60)</td>
<td>2.16 (1.56–3.00)</td>
<td>5.25 × 10^-4</td>
</tr>
<tr>
<td>Overdominant</td>
<td>T/T-C/C</td>
<td>209 (69.67)</td>
<td>381 (52.48)</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>Allelic</td>
<td>T/T</td>
<td>347 (57.83)</td>
<td>895 (61.64)</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C/C</td>
<td>253 (42.17)</td>
<td>557 (38.36)</td>
<td>1.17 (0.97–1.42)</td>
<td>0.112</td>
</tr>
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</table>

<sup>a</sup>The P values less than 0.05 are presented in bold.

The rs7911488 C allele is related to low expression of miR-1307-3p

We detected the expression of miR-1307-3p in 20 pairs of CRC tissues and para-cancer tissues, and found that the expression of miR-1307-3p were downregulated in 18 (90%) cancer tissues as compared to the para-cancer tissues (Figure 1A). Moreover, we found that the expression of miR-1307-3p in rs7911488 CC homozygotes were lower than those in the heterozygotes or TT homozygotes (Figure 1B). To further confirm the effect of rs7911488 genotypes on the expression of miR-1307-3p, we transfected rs7911488 T-allelic or C-allelic pre-miR-1307 expression plasmid into HCT-116 cells and then determined the expression level of mature miR-1307-3p. We found that the level of miR-1307-3p in the cells transfected with C-allelic pre-miR-1307 expression plasmid was predominantly lower than that in the cells transfected with T-allelic pre-miR-1307 expression plasmid (Figure 1C), even though a comparable level of the binding intensity of MBNL1 with C-allelic pre-miR-1307 is stronger than that of T-allelic pre-miR-1307. MBNL1 can bind to either C-allelic or T-allelic pre-miR-1307, and both C-allelic pre-miR-1307 and T-allelic pre-miR-1307 were in the pull down of the MBNL1 coated beads (Figure 2C). The PCR products from both C-allelic pre-miR-1307 and T-allelic pre-miR-1307 were further confirmed by sequencing. These findings suggest that MBNL1 can bind to either C-allelic or T-allelic pre-miR-1307, and the binding intensity of MBNL1 with C-allelic pre-miR-1307 is stronger than that of T-allelic pre-miR-1307.

We then analyzed the expression of MBNL1 and DICER1 in the cancer cells, and found that the expression of miR-1307-3p in T-allele harboring cells were positive correlated to the expression of MBNL1 and DICER1 (Figure 2D). When we knock-downed DICER1 or MBNL1, we found that the expression of miR-1307-3p...
In this study, we investigated the relationship between miRSNPs and the occurrence of CRC. Our results reveal a significant correlation of rs7911488 T>C in the terminal-loop of pre-miR-1307 with the occurrence of CRC. This polymorphism to a certain extent controls individual expression of miR-1307 via affecting the binding of MBNL1 with pre-miR-1307, leads to over-expression of Bcl2 and increased susceptibility to CRC (Figure 4).

MBNL1, one of muscleblind like (MBNL) family of pre-mRNA alternative splicing factors, binds to (CUG)n and (CCUG)n expansion RNAs including both ssRNA and dsRNA elements (16). The MBNL proteins were first characterized as factors involved in the pathogenesis of myotonic dystrophy (DM) (17). Loss of MBNL1 activity in DM tissues would lead to persistence of fetal splicing patterns in the adult. In addition, MBNL1 can recognize premRNA with a binding site at the stem-loop in Tnnt3 intron eight upstream of the F exon (18). Recently, MBNL1 was proved to be able to bind to a UGC motif in the loop of pre-miR-1 and subsequently interfere with Dicer processing. As a consequence of miR-1 loss, expression of GJA1 and CACNA1C, which are targets of miR-1, is increased in both DM1- and DM2-affected hearts (19). In this study, we provide evidence that MBNL1 is a cancer-related splicing regulator which acts as a splicing repressor by recognizing a ‘UGCUGC’ motif in the stem-loop of C-allelic pre-miR-1307, resulting in interference with Dicer1 processing. Based on this observation, it is likely that MBNL1 recognizes rs7911488 C-allelic pre-miR-1307 stem-loop to inhibit Dicer1 recruitment, leading to low-expression of mature miR-1307. This conclusion is supported by: (i) the expression of miR-1307 was obviously lower in cells transfected with C-allelic pre-miR-1307 expression plasmid than those transfected with T-allelic pre-miR-1307 expression plasmid, even though the initial expression levels of pre-miR-1307 were comparable in these cells; (ii) the expression of miR-1307 in cells transfected with C-allelic pre-miR-1307 expression plasmid was markedly repressed by downregulating MBNL1, but not Dicer1; (iii) RNA immunoprecipitation results showed stronger binding of MBNL1 with C-allelic pre-miR-1307 than T-allelic pre-miR-1307; (iv) the expression levels of miR-1307 were evidently lower in cancer tissues, especially in CC homozygotes; and (v) the expression of BCL2 protein was reduced after transfection of miR-1307 mimics into CRC cells. We found that the expression of BCL2 mRNA were apparently decreased (**P<0.05**).

Low expression of miR-1307 results in upregulation of BCL2 protein in cancer cells

To investigate the influence of miR-1307 on the occurrence of CRC, we used online software miRanda and TargetScan to predict the potential targets of miR-1307. We found that miR-1307 might bind to the 3’-UTR of BCL2, an antiapoptosis molecule (Figure 3A). Then we detected the expression of miR-1307 and Bcl2 protein in five CRC cells, and found that the levels of Bcl2 protein were inversely correlated to the expression of miR-1307 (Figure 3B). After transfecting miR-1307 mimics into CRC cells, we found that the expression of BCL2 mRNA were apparently decreased (Figure 3C). Moreover, we found that the expression of BCL2 protein was reduced after transfection of miR-1307 mimics (Figure 3D). To further evaluate the binding of miR-1307 with BCL2, we constructed a BCL2/3’-UTR/pGL3 vector and cotransfected it with miR-1307 mimics into CHO cells. We found that the expression of BCL2/3’-UTR/pGL3 vector was significantly suppressed by miR-1307 (Figure 3E), indicating that miR-1307 can inhibit the expression of Bcl2 by binding with the 3’-UTR of BCL2 gene.

Discussion

In this study, we investigated the relationship between miRSNPs within the terminal-loops of pre-miRNAs and the occurrence of CRC.
both the CC homozygotes and C allele were significantly abundant in cancer patients.

Bcl2, a key antiapoptotic protein, has been studied intensively for the past decade because of its important role in apoptosis, tumorigenesis and the cellular response to antitumor therapies. Over-expression of Bcl2 protein has been detected in human leukemias, lymphomas and various carcinomas including CRC. Since BCL2 gene contains a 5.2-kb 3′-UTR, a number of potential miRNA-binding sites are predicted. miR-15 and miR-16 were firstly observed to be able to regulate Bcl2 expression in chronic lymphocytic leukemia (20). Thereafter, miR-181b (21), miR-497 (22), miR-125b and miR-155 (23), miR-195, miR-24-2 and miR-365-2 (24), miR-503 (25), miR-210 (26), miR-184 (27) and miR-30b (28), have been revealed to be involved in the regulation of human Bcl2 protein under various conditions. It is also interesting to observe that miR-125b, miR-195 and miR-184 were downregulated in CRC, but miR-15, miR-16, miR-181b, miR-155, miR-210 and miR-30b were upregulated in CRC (29), indicating that the regulation of Bcl2 protein could be mediated by varied miRNAs under different conditions.

miR-1307 is a conserved miRNA in mammals, including Homo sapiens, Canis familiaris, Pan troglodytes, Bos Taurus, Sus scrofa, Pongo pygmaeus etc (Supplementary Figure 3A, available at Carcinogenesis Online). By using software miRanda and TargetScan, we found a conserved binding site in the 3′-UTR of BCL2 gene (Supplementary Figure 3B, available at Carcinogenesis Online).
Our results suggest BCL2 is a direct target of miR-1307 based on the following observations: (i) the expression levels of Bcl2 protein in cancer cells were positively correlated to the expression levels of miR-1307; (ii) the expression of both BCL2 mRNA and BCL2 protein in cancer cells were suppressed by miR-1307 mimics; and (iii) the luciferase report assay results indicated that miR-1307 inhibited the expression of Bcl2 by binding with 3′-UTR of BCL2.

Figure 3. The regulation of miR-1307-3p on BCL2 expression. (A) The schematic of BCL2 3′-UTR harbors putative binding-site of miR-1307-3p. (B) The negative correlation between miR-1307-3p expression and the level of Bcl2 protein in CRC cell lines. (C) The inhibitory role of miR-1307-3p in BCL2 mRNA expression in CRC cell lines. (D) The repression of Bcl2 protein expression in LoVo cells mediated by miR-1307-3p. (E) The luciferase report assay results indicated that the expression of BCL2/3′-UTR/pGL3 construct was significantly suppressed by miR-1307-3p. *P < 0.05.

Figure 4. The mechanism of rs7911488-mediated CRC occurrence. This polymorphism recruits MBNL1 to participate in the processing of pre-miR-1307, which abandons the function of DICER1, resulting in loss of miR-1307 and consequent Bcl2 over-expression and CRC occurrence.

Online). Our results suggest BCL2 is a direct target of miR-1307 based on the following observations: (i) the expression levels of Bcl2 protein in cancer cells were positively correlated to the expression levels of miR-1307; (ii) the expression of both BCL2 mRNA and BCL2 protein in cancer cells were suppressed by miR-1307 mimics; and (iii) the luciferase report assay results indicated that miR-1307 inhibited the expression of Bcl2 by binding with 3′-UTR of BCL2. In addition, the online software predicted...
that FOXP3, PDCD4, RHOA, SOX2, STAT3 and WNT4 might be the target of miR-1307 (Supplementary Figure 4A, available at Carcinogenesis Online). While these molecules were reported to be over-expressed in CRC and were closely related to the development of CRC, the present luciferase report results showed that none of these genes was regulated by miR-1307 (Supplementary Figure 4B, available at Carcinogenesis Online).

Base variation on RNA not only affects the binding of protein, but also affects the stability or secondary structure of RNA. Therefore, we used online software mfold (http://mfold.rna.albany.edu/) to predict the secondary structures of rs7911488 C-allelic and T-allelic pre-miR-1307. We found that there was no difference on secondary structures between these two types of pre-miR-1307 (Supplementary Figure 5A, available at Carcinogenesis Online). Furthermore, we investigated the effect of rs7911488 on the stability of pre-miR-1307. We added actinomycin to terminate the transcription in cells transfected with C-allelic or T-allelic pre-miR-1307 expression plasmids, and then detected the expression level of pre-miR-1307 and miR-1307-3p at different time after transfection. As showed in Supplementary Figure 5B is available at Carcinogenesis Online, the expression levels of both pre-miR-1307 and miR-1307-3p were sharply decreased in the cells transfected with either C-allelic or T-allelic miR-1307 expression plasmids, indicating that both the C-allelic and T-allelic pre-miR-1307 were degraded by nucleases in cells. These data suggest that rs7911488 had no effect on the stability of pre-miR-1307.

Pre-miR-1307 locates in the exon 3 of USMG5 (Homo sapiens upregulated during skeletal muscle growth 5 homolog) gene containing six exons (Supplementary Figure 6, available at Carcinogenesis Online). This gene has three splicing transcript variants: the dominant transcript variant 1 (NM_001206426.1), which is the shortest one without exon 2 and exon 3, and the transcript variant 2 (NM_032747.3) and transcript variant 3 (NM_001206427.1) which does not contain the exon 2 and exon 3, respectively. We first analyzed the USMG5 mRNA sequences in 20 CRC tissues to determine whether or not the dysregulation of miR-1307-3p is on account of the splicing variants. We found that the USMG5 transcripts were the dominant transcript of pre-miR-1307 and the occurrence and development of CRC. To our knowledge, this study is the first to report on the association of this polymorphism with the individual risk of CRC. This polymorphism recruits MBNL1 to take part in the maturation process of pre-miR-1307, and interfere with the processing by DICER1, leading to a lower level of miR-1307 expression, elevated expression of Bcl2 and occurrence of CRC. The present findings are of clinical relevance in understanding the role of polymorphisms in the terminal-loops of pre-miRNAs in carcinogenesis.

**Supplementary material**

**Supplementary Tables 1–7** and **Figures 1–6** can be found at http://carcin.oxfordjournals.org/

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**References**


