Original Paper

RECQL1 Plays an Important Role in the Development of Tongue Squamous Cell Carcinoma

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Key Words

RNAi • Human tongue squamous cell carcinoma • RECQL1

Abstract

Background/Aims: RECQL1, a member of the human RECQ helicase family, participates in DNA repair. Recent reports showed that RECQL1 silencing in cancer cells resulted in mitotic catastrophe, which prevented tumor growth in murine models. However, its therapeutic potential has never been examined in tongue squamous cell carcinoma (SCC). **Methods:** To explore the role of RECQL1 in the development of tongue SCC, we used RNA interference technology to silence RECQL1 in SCC-9 and SCC-15 human tongue SCC cell lines, and to subsequently evaluate its effects both *in vitro* and *in vivo*. **Results:** After RECQL1 was silenced in SCC cells by siRNA, we observed downregulation of RECQL1 mRNA and protein in cancer cells. RECQL1 is one of the predicted miR-203 targets, and we found that miR-203 downregulated the expression of RECQL1 at the post-transcriptional level. RECQL1-shRNA or miR-203 overexpression inhibited SCC-9 cell growth. In addition, there was accumulation of cells in the sub-G1 fraction and increased apoptosis 72 h post-transfection. In addition, knockdown of RECQL1 led to a strong anticancer effect, as the tumorigenicity of SCC-9 cells was inhibited *in vivo*. Moreover, we found that two immunosuppressive factors were also significantly downregulated upon RECQL1 knockdown or miR-203 overexpression *in vitro*. **Conclusion:** Collectively, these results indicate that RECQL1 plays an important regulatory role in cancer cell proliferation and tumor progression.

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Introduction

The most common type of tongue cancer is squamous cell carcinoma (SCC) [1]. There are other types of cancers of the tongue, but they are statistically uncommon. Finding an effective anticancer target would greatly reduce the pain of patients suffering from this disease. Studies have shown that RECQL1 is highly upregulated in SCC-9 cells, one of the human tongue SCC cell lines. Human RECQL1 DNA helicase (RECQL1), a member of the RECQ helicase family, participates in the maintenance of chromosome stability. It is highly upregulated in proliferating cells, especially in cancer cells and transformed cells [2-7]. RECQL1 unwinds specific DNA *in vitro* ATP-dependently, and increases base matching ATP-independently [8, 9]. Moreover, RECQL1 is a necessary enzyme for resolving Holliday junctions during cell proliferation [10, 11]. Therefore, it is assumed that RECQL1 executes mismatch repair function *in vivo* together with human EXO1 and the Msh2-Msh6 mismatch repair recognition complex [12]. Although RECQL1-deficient mice are indistinguishable from wild-type mice, their embryonic fibroblasts are sensitive to ionizing radiation [13].

Although RECQL1 maintains chromosomal stability by DNA repair, its function seems to be nonessential and complementary in other cellular repair systems, since no human disease is known to correlate with mutations in its gene. However, Li *et al.* showed that polymorphisms in RecQ1 genes involved in the repair of DNA double strand breaks, significantly affected the clinical outcome of patients with pancreatic cancer [14]. Furthermore, a single nucleotide polymorphism present in the RecQ1 gene is associated with the survival of pancreatic cancer patients [15]. One study reported that silencing of RECQL1 by RNA interference (RNAi) could induce mitotic catastrophe and death in a wide range of cancer cells that are deficient in the checkpoint system during the cell cycle [16]; however, in the noncancerous and normal cells tested, RECQL1 silencing did not result in mitotic death. In order to explore anticancer chemotherapy through such cancer-specific killing mechanisms, preliminary trials using model mice inoculated with various human cancer cells were performed [16].

MicroRNAs (miRs) are a class of small non-coding RNAs that can regulate the expression of specific target genes by targeting the 3'-untranslated region (3'-UTR) of target mRNAs for translation repression, degradation, or both. They play important roles in a variety of cellular pathways, such as development, differentiation, cell proliferation, and apoptosis [17, 18], and their expression is deregulated in most human tumors [19-21]. Previous studies have reported the downregulation of miR-203 in diverse cancers, including SCC of the head and neck (SCCHN) [22] and esophageal squamous cell carcinoma (ESCC) [23, 24]. Subsequent studies have shown that the expression levels of miR-203 are inversely correlated with the cell proliferative capacity in human SCCHN [24], hepatocellular carcinoma [25], and chronic myelogenous leukemia [26]. In human tongue SCC, a global microRNA expression assay showed that miR-203 was significantly downregulated in tumor tissue compared to normal tongue tissue [27]. However, the effect of miR-203 on the proliferation of SCC of tongue cells, and its relationship with RECQL1 in human tongue SCC has not been reported.

The goal of this study was to investigate the effect of RECQL1 and miR-203 on the proliferation of tongue SCC, and to examine the regulation of RECQL1 expression by miR-203 in SCC-9 and SCC-15 cells. To this end, we evaluated the effects of RECQL1 knockdown and miR-203 overexpression on human tongue SCC. Firstly, we investigated the expression levels of RECQL1 mRNA and protein, and the relationships with miR-203 in SCC cells. Secondly, we studied the effect of RECQL1 silencing and miR-203 overexpression on cell proliferation *in vitro*. Thirdly, we evaluated the effect of RECQL1 knockdown and miR-203 overexpression on inhibition of tumorigenicity *in vivo*. Our data showed that two important immunosuppressive factors were significantly downregulated upon RECQL1 knockdown or miR-203 overexpression. These results demonstrate that RECQL1 may be an effective target in anticancer therapy.

Materials and Methods

Materials

Tongue SCC surgically resected paired tumor tissues and adjacent normal tissues used in real-time quantitative PCR (qRT-PCR) were collected from 26 tongue SCC patients. Surgically removed tissues were quickly frozen in liquid nitrogen until analysis. All samples were collected with the informed consent of patients, and experiments were approved by the ethics committee of Shanghai Ninth People's Hospital affiliated with Shanghai Jiaotong University. The experiments were conducted in accordance with the Declaration of Helsinki principles.

Cell culture

The human SCC cell lines, SCC-9 and SCC-15, and the normal cell line, HFL-a, (normal lung diploid fibroblasts from adult human) were used in our studies. SCC-9, SCC-15, and HFL-a cells were purchased from ATCC (Manassas, VA, USA). Cells were cultured according to the instructions of ATCC.

Microarray of RECQL1 in SCC-9, SCC-15, and normal cells

Total RNA was isolated from SCC-9, SCC-15, and HFL-a cells using Trizol reagent (Invitrogen; Carlsbad, CA, USA). Microarrays (Mingxin; Shanghai, China) were used to measure mRNA expression levels, and mRNA expression analysis was performed using Affymetrix GeneChips (Santa Clara, CA, USA), according to the manufacturer's protocol. Each array was repeated three times. Hybridization, washing, and staining were performed with GeneChip Hybridization Kit. Microarray images were processed using the Affymetrix 3000 scanner, and analyzed using the Affymetrix GeneChip Analysis Suite (GCOS v1.4) software. RECQL1 mRNA displaying ≥ 2 -fold changes in signal intensity between SCC-9, SCC-15, and normal cells was considered differentially expressed.

Plasmid construction and luciferase reporter assay

The 3'UTR of RECQL1 gene containing one putative miR-203 binding site and a mutation sequence containing the complementary sequence of miR-203 were amplified by PCR, and inserted into the Kpn I and Bgl II sites of the pGL3-control vector (Promega; Madison, WI, USA). The primer sequences for wild-type RECQL1-3'UTR were: 5'-GGGTACCCTATGAATGTTACTAAAT-3' (forward) and 5'-CCCCGGGGATTCTTTGTGAAATGTCAAAATATG-3' (reverse); the primers for the RECQL1-3'UTR mutant were: 5'-GGGTACCCTATGAATGTTACTAAAT-3' (forward) and 5'-CCCCGGGGATTCTTTGTCTTTAGTCAAAATATG-3' (reverse). For the luciferase assay, SCC-9 cells were cultured in 24-well plates and co-transfected using Lipofectamine 2000 (Invitrogen) with 100 ng/well of RECQL1 3'UTR wild-type plasmid or RECQL1 3'UTR mutant plasmid in the presence of either miR-203 or miR-control (Ambion; Carlsbad, CA, USA). The pRL-TK vector was as the internal control (Promega). After 48 h, luciferase activity was measured using the dual luciferase reporter assay system (Promega). Experiments were performed independently in triplicate.

Overexpression of miR-203

The pre-miR-203 sequence was amplified from genomic DNA and cloned into the Ad Shuttle Vector 1.0-CMV (Ambion). Adenoviral packaging and infection were performed according to standard protocols as recommended by the manufacturer. Empty adenoviral vector was used as a control. The primers used were as follows: 5'-GCGCCTCGAGGTGCGCGACCAGCGGGGATC-3' (forward) and 5'-GCGGCGACTAGTCCCTGGATTGGTCGCCGGCG-3' (reverse).

siRNA and RNAi

siRNAs (21 bp) targeting RECQL1 mRNA (RECQL1-siRNA) and negative control siRNA (NS-siRNA) were chemically synthesized (GenePharma, China). The siRNA sequences used in this study were selected and used as described by Elbashir et al. [28] RECQL1 mRNA-specific siRNA (RECQL1-siRNA) contained the sense sequence: 5'-GUU CAG ACC ACU UCA GCU UdTdT-3' corresponding to position 273-291 in RECQL1 mRNA. Firefly luciferase gene sequence GL3 (Promega): 5'-CUU ACG CUG AGU ACU UCG AdTdT-3' (sense strand) and 5'-UCG AAG UAC UCA GCG UAA GdTdT-3' (antisense strand) was used as non-silencing siRNA (NS-siRNA) that represented a negative control for the RNAi. Transfection of siRNA duplexes was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. SCC-9 and SCC-15 cells were transfected with RECQL1-siRNA and NS-siRNA, respectively.

Quantitative analysis of RECQL1 mRNA and miR-203 by qRT-PCR

Thirty hours post-transfection, total RNA was extracted from transfected cells using the RNeasy Mini Kit (Qiagen; Hilden, Germany) according to the manufacturer's protocol. Analysis of qRT-PCR was carried out using the ABI PRISM 7000 Sequence Detection System with TaqMan probes and primers (ABI; Foster City, CA, USA) to estimate RecQL1 mRNA. The β -actin gene was used as the internal standard (TaqMan probe ID; 431088E; ABI). To determine the expression level of miR-203, Taqman miRNA reverse transcription assays (ABI) and appropriate primers were used according to the manufacturer's protocol. RNU66 (ABI) was used as an internal control.

Cell proliferation assays

Cell proliferation was determined by the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were seeded in 96-well culture plates at an optimal density of 7×10^3 cells/well. Proliferation was analyzed according to an average absorbance value. Twenty microliters of 5 mg/ml MTT substrate was added to the cells and incubated at 37°C for 4 h, and the resulting colored product was made soluble in 200 μ l dimethyl sulfoxide (DMSO). Cell proliferation was determined at days 1, 2, 3, and 4. Each assay was performed in triplicate and the experiment was repeated on at least three separate occasions.

For cell colony formation assays, cells in the logarithmic phase were digested and plated at a density of 1×10^3 per well in 35 mm dishes. Cell culture growth was stopped when the colonies were clearly visible by the naked eye. Following methanol fixation, cells were stained with hematoxylin. The number of colonies containing more than 50 cells was counted in three parallel dishes.

Flow cytometric analysis

Trysin-treated cells were washed with phosphate-buffered saline (PBS) and fixed in ice-cold methanol for 2 h. Cells were treated with pancreatic Rnase A and stained with propidium iodide (Sigma; Switzerland) for 30 min. Cells were then analyzed using flow cytometry. Fluorescence was measured using Epics XL (Beckman; Tokyo, Japan). For each sample, 10,000 events were analyzed.

Immunoblotting

RECQL1 protein levels were further detected by immunoblot analysis. Cells were washed with ice-cold PBS, pelleted, and then lysed in sodium dodecyl sulfate (SDS) buffer containing 1% SDS, 2% β -mercaptoethanol, 20% glycerol, 30 mM Tris-HCL (pH 6.8), and 0.2 M dithiothreitol. Cell lysate was boiled for 10 min and electrophoresed on 5-20% gradient SDS-polyacrylamide gels. Proteins were then electrophoretically transferred to polyvinylidene difluoride membranes (Millipore; Danvers, MA, USA), which were blocked overnight in 5% skim milk in PBS. Membranes were then incubated with either anti-RECQL1 monoclonal antibody or anti- β -actin monoclonal antibody (ICN Biomedicals; Solon, OH, USA) for 1 h at room temperature. The membranes were washed with 0.05% Tween-20 in PBS, incubated with anti-mouse IgG conjugated with horseradish peroxidase (DakoCytomation; Carpinteria, CA, USA), and washed three times for 5 min each. Proteins were visualized using an enhanced ECL chemiluminescence reagent (Amersham Biosciences, UK).

Quantification of VEGF and IL-10 expression levels

IL-10 and VEGF levels in the supernatant of SCC-9 cells were measured after RECQL1-siRNA transfection or miR-203 overexpression using two commercial human IL-10 and VEGF ELISA kits (Invitrogen) according to the manufacturer's instructions.

Animal experiments

Five-week-old male BALB/c mice were purchased from Shanghai SLAC Laboratory Animal Co. Ltd (Shanghai, China), fed a standard laboratory diet, and given water *ad libitum*. They received human care in compliance with the guidelines for the care and use of experimental animals in research. Mice were divided into 4 groups with 10 mice in each group. The groups were as follows: control, NS-siRNA, RECQL1-siRNA, and miR-203 overexpression groups. Mice were injected with untransfected SCC-9 cells, NS-siRNA-transfected SCC-9 cells, RECQL1-siRNA-transfected SCC-9 cells, and miR-203 overexpressing SCC-9 cells, respectively. For injection, 5×10^6 cells were suspended in Dulbecco's Modified Eagle's Medium (DMEM), and injected subcutaneously into the right flank of each mouse in the appropriate group. Mice were humanely sacrificed

Fig. 1. RECQL1 mRNA and protein expression levels in SCC-9, SCC-15, and HFL-a cells. (A) Twenty-six paired clinical human tongue SCC tissues and adjacent normal tissues were analyzed by qRT-PCR. (B) Microarray analysis of RECQL1 mRNA expression levels in SCC-9, SCC-15, and HFL-a cells; (C) Cell lysates (10 μ g) were applied to each lane. Proteins underwent Western blot analysis by using individual antibodies; (D) Quantitative analysis of gene expression by determination of individual mRNAs with qRT-PCR.

on day 24, and tumors were dissected, photographed, weighed and analyzed by immunohistochemistry and RT-PCR.

This work was approved by the ethical committee of Shanghai Ninth People's Hospital affiliated with Shanghai Jiaotong University. The animals involved in this study were cared for according to the Institutional Animal Care & Use Committee.

Statistical analysis

Statistical analyses were carried out with the Student's *t*-test using SPSS software version 12.0 (SPSS Inc; Chicago, IL, USA). Statistical significance was set at $p < 0.05$.

Results

RECQL1 mRNA is highly upregulated in tongue SCC

Total RNA was extracted from tongue SCC tissues and cells, followed by qRT-PCR, which showed significant upregulation of RECQL1 mRNA in tumors compared to adjacent normal tissues (Fig. 1A). Microarray analysis showed significant upregulation of RECQL1 mRNA in SCC-9 and SCC-15 cells compared to normal cells. Specifically, the mRNA level of RECQL1 increased 4.1-fold ($p < 0.001$) in SCC-9 cells compared to HFL-a cells; β -actin mRNA levels were equal in both cell lines (Fig. 1B).

RECQL1 helicase expression levels in different cell lines

Mitotic cell death in cancer cells induced by RECQL1 silencing depends primarily on the progression of the cell cycle (i.e. cell proliferation), and secondarily on the highly required function of RECQL1 in cancer cells, which appears to correlate with RECQL1 expression levels [16]. A third essential point is that failure of cancer cells to arrest the cell cycle due to a defect in the G2 checkpoint is shown in most cancer cells to be due to mutations in p53 [3, 6]. In this study, RECQL1 expression in SCC-9 and SCC-15 human cell lines was investigated. As a control, normal HFL-a cells were similarly analyzed. Cancer cells expressed higher levels of RECQL1 than normal cells (Fig. 1C, 1D).

Downregulation of RECQL1 expression by miR-203 targeting 3'-UTR of RECQL1

RECQL1 is one of the predicted miR-203 targets that was identified using TargetScan software, because it contains a putative miR-203 target site in its 3'-UTR (Fig. 2A). To confirm

Fig. 2. Restoration of miR-203 expression in SCC-9 cells downregulates RECQL1. (A) Diagram of RECQL1 3'-UTR containing the reporter construct. Predicted seed region and mutant miR-203 target sites in 3'UTR of RECQL1 are shown. (B) SCC-9 cells were co-transfected with 100 ng/well Luc-RECQL1 3'UTR wild-type or mutant plasmid and miR-203 or control miR-NC precursors, respectively. The luciferase reporter assay was performed 48 h post-transfection. A significant decrease of relative luciferase activity induced by miR-203 was reversed after a point mutation was made in the seed region. (C) SCC-9 and SCC-15 cells were transfected with a miR-203 overexpression vector or empty adenoviral vector. The expression of miR-203 mRNA levels was quantified by qRT-PCR. (D) RECQL1 protein levels and (E) RECQL1 mRNA levels were detected by immunoblotting and RT-PCR in SCC-9 and SCC-15 cells transfected with a miR-203 overexpression or empty adenoviral vector. Actin was used as an internal control.

this, we evaluated the reporter activity in SCC-9 cells co-transfected with miR-203 precursors (or control miR-NC precursors) and Luc-RECQL1 3'UTR wild-type plasmid (or Luc-RECQL1 3'UTR mutant plasmid). The cells co-transfected with Luc-RECQL1 3'UTR wild-type plasmid showed a significant decrease in relative luciferase activity in the presence of miR-203, but in the presence of miR-203 or miR-NC, cells co-transfected with the corresponding mutant plasmid and miR-203 showed no significant difference in relative luciferase activity (Fig. 2B).

We further detected the expression levels of RECQL1 protein and mRNA by immunoblotting and qRT-PCR, respectively, in SCC-9 and SCC-15 cells transfected with the miR-203 overexpression adenoviral vector or the empty vector. As shown in Figure 2C, miR-203 levels increased by 8.6- to 9.8-fold in cells infected with the miR-203 overexpression

Fig. 3. (A) Detection of RECQL1 repression after miR-203 overexpression cell by the colony formation assay. (B) Time course analysis of cancer and normal cells viability. Both SCC-9 and normal cells(HFL-a) were transfected with miR-203(stellate), RECQL1-siRNA (square) or NS-siRNA (rhombus). Cellviability is shown as relative growth to that of untreated cells; (C) Levels of IL-10 and VEGF in the supernatant of SCC-9 cells after RECQL1 silencing. Levels of IL-10 and VEGF are shown as miR-203 overexpression (stellate), RECQL1-siRNA (square), NS-siRNA (rhombus), control (triangle), respectively.

adenoviral vector compared to the control. miR-203 overexpression significantly repressed RECQL1 protein expression (Fig. 2D) with subsequent effects on RECQL1 mRNA levels (Fig. 2E) in SCC-9 and SCC-15 cells. These results indicate that miR-203 post-transcriptionally downregulates RECEL1 expression by targeting the 3'UTR of RECQL1 in SCC of the tongue.

Induction of mitotic catastrophe in the SCC-9 cell line by suppressing RECQL1

Results from the cell colony formation assay showed that the number of colonies of SCC-9 transfected with miR-203 or RECQL1-siRNA was significantly smaller than that of colonies from cells transfected with control and NS-siRNA ($p < 0.05$) (Fig. 3A). These findings suggest that miR-203 overexpression and RECQL1-siRNA decrease cell colony formation ability. We investigated the effect of RECQL1 silencing on SCC-9 cell growth. Our data showed an approximate 42% decrease in cell growth upon RECQL1 silencing with RECQL1-siRNA or miR-203 overexpression after 48 h (Fig. 3B). Moreover, RECQL1-siRNA efficaciously induced mitotic catastrophe, leading to a large amount of proliferating cancer cell deaths. Figure 4

Fig. 4. Growth inhibitory effect of RECQL1-siRNA in cancer cells. SCC-9 cells treated with RECQL1-siRNA. Microscopic features of cells were monitored at A, 24 h; B, 36 h; C, 48 h; and D, 72 h.

Fig. 5. Growth inhibitory effect of RECQL1 silencing on SCC-9 and SCC-15 cells. (A) RECQL1 protein levels after silencing in SCC-9 and SCC-15 cells, compared with the positive and negative control, respectively. (B) Flow cytometric analysis of SCC-9 cells and SCC-15 cells 72 h after RECQL1-siRNA or miR-203 overexpression.

shows the typical features of mitotic catastrophe observed in SCC-9 cells. To define this cell death effect by RECQL1 silencing, time course experiments were performed with cancer cells and normal cells. The cells were incubated with 40 nM siRNA or miR-203 overexpression adenoviral vector, and were cultured in siRNA-free and miRNA-free medium for several days. SCC-9 cell death began after a lag-time of about 24 h (Fig. 3B). In contrast, the growth of HFL-a cells was almost unaffected by RECQL1-siRNA or miR-203 overexpression. Only a slight retardation (10-15%) in growth was noted in this normal cell line when compared to non-silencing siRNA-treated cells.

Flow cytometric analysis indicated that SCC-9 cells underwent mitotic death with an accumulation of cells in the sub-G1 fraction (Fig. 5B). In contrast, normal HFL-a cells

Fig. 6. Suppression of RECQL1 dramatically inhibited tumorigenicity of cancer cells *in vivo*. Photographs (A) and weights (B) of tumors dissected from nude mice 24 days after injection with four differentially treated SCC-9 cells (control group, NS-siRNA, RECQL1-siRNA, miR-203 overexpression). The weight of most tumors in the RECQL1-siRNA and miR-203 overexpression group was half the weight of tumors in the control and NS-siRNA groups ($p < 0.05$).

showed no mitotic catastrophe because of premature arrest and repair in both the G1 and G2 phases. Non-silencing siRNA treatment under the same conditions did not affect cell cycle progression of SCC-9 and SCC-15 cells, indicating that mitotic catastrophe of such SCC cells are closely related to RECQL1 silencing.

IL-10 and VEGF levels are reduced after RECQL1 silencing

Cancer cells always secrete a variety of immunosuppressive factors to inhibit and modulate cytokine secretion, and downregulate the activity of immune cells, protecting tumor cells from specific cytotoxic T lymphocytes. IL-10 and vascular endothelial growth factor (VEGF) are two important immunosuppressive factors that negatively regulate the immune response and promote tumor cell proliferation. In this study, the effect of RECQL1-siRNA and miR-203 overexpression on IL-10 and VEGF levels was analyzed. We found that both immunosuppressive factors were downregulated upon RECQL1-siRNA transfection and miR-203 overexpression (Fig. 3C). IL-10 expression levels in the RECQL1-siRNA group (or miR-203 overexpression group) were decreased by 73.9% (or 71%) and 17.8% (or 19%) compared to the control after 16 h and 24 h, respectively. VEGF levels in the RECQL1-siRNA group (or miR-203 overexpression group) decreased by 66% (or 64%) and 12.8% (or 15%) compared to the control after 16 h and 24 h, respectively. There were virtually no differences in VEGF and IL-10 levels between the control and NS-siRNA transfection groups.

RECQL1 silencing inhibits the tumorigenicity of SCC-9 cells

Tumor assays were carried out using nude mice to evaluate the tumorigenicity of SCC-9 cells *in vivo*. Four differentially treated SCC-9 cells were inoculated subcutaneously into nude mice. All mice in the NS-siRNA group displayed steadily and progressively growing tumors, showing minimal difference with mice in the control group (Fig. 6A). The dissected tumors reached a weight of about 2.5 g within 24 days after inoculation. In contrast, mice in the RECQL1-siRNA and miR-203 overexpression groups developed significantly smaller tumors, and most weighed less than 1.2 g ($p < 0.05$, Fig. 6B). The tumors were analyzed by immunohistochemistry and qRT-PCR, and the results showed that RECQL1 protein expression

Fig. 7. (A) Immunohistochemical staining of RECQL1 protein from tumors in control, NS-siRNA, RECQL1-siRNA, and miR-203 overexpression groups (100 $\times$). (B) Relative RECQL1 mRNA expression levels were analyzed by qRT-PCR.

levels (Fig. 7A) and mRNA (Fig. 7B), respectively, were both downregulated after miRNA-203 overexpression and RECQL1 interference compared to the control and NS-siRNA groups.

Discussion

Squamous cell carcinoma (SCC) is one of the most common types of tongue cancer and it presents a serious threat to human health. The identification of squamous cell carcinoma associated genes is essential for SCC diagnosis and treatment. Interestingly, we found that RECQL1, a key DNA helicase, is highly expressed in SCC cells compared to normal cells, suggesting its potential role in SCC proliferation.

DNA helicases play important roles in cellular processes, including DNA replication, recombination, repair and transcription, by unwinding the duplex genome strands. Among the many kinds of DNA helicases, the RECQ helicase family has unique properties that are conserved in all organisms that participate in various repair functions to maintain genomic integrity. There are five helicases in the RECQ helicase family; namely, RECQL1, BLM, WRN, RTS, and RECQ5. Current studies have proven that mutated BLM, WRN, and RTS helicases cause Bloom syndrome, Werner syndrome, and a subset of Rothmund-Thomson syndrome, respectively, all of which are recessive genetic disorders that show genomic instability and a high risk of cancer [6]. It seems that the relationship between the RECQ helicase family and human disease is unclear [4]. In this study, we investigated RECQL1 expression in SCC-9 and SCC-15 cells, and found that its expression was almost 4-fold greater than in normal cells (Fig. 1B). These data verify that RECQL1 expression is closely related to human tongue squamous cell carcinoma.

RECQL1 helicase is always highly upregulated in rapidly proliferating cancer cells and other transformed cells [29], suggesting that the high level of RECQL1 helicase is necessary in rapidly proliferating cells. In this study, we showed that RECQL1 silencing induced mitotic catastrophe, leading to cell death in the SCC-9 cells. However, the normal cells, HFL-a fibroblastic cells (Fig. 3B), showed high tolerance to RECQL1 silencing. The effect of RECQL1 silencing is dependent on: (i) Cell Proliferation: DNA damage forms endogenously

in association with DNA replication, which results in long-term arrest in the M-phase and mitotic cell death after activation of the spindle checkpoint system; (ii) Defective Checkpoint System: Cancer cells were sensitive to RECQL1 silencing because they failed to arrest the cell cycle in order to correct the DNA damage caused by the absence of RECQL1. The results obtained from this study are indeed consistent with the above mechanism underlying the selective mitotic catastrophe in cancer cells. In cancer cells, mitotic catastrophe due to DNA damage could probably be avoided by highly upregulated repair enzymes, such as RECQL1, that coordinate well with DNA replication for repair of DNA damage. Cancer cell death caused by silencing the RECQL1 helicase is thus consistent with the findings of Daniel et al. [30].

Upon suppression of RECQL1, the expression of immunosuppressive factors, IL-10 and VEGF, was significantly reduced in the supernatant of SCC-9 compared to the control, while there was less difference in their expression levels between the control and Ns-siRNA group (Fig. 3C). These data indicate that RECQL1 silencing downregulates the expression level of immunosuppressive factors that are necessary for regulating the migration of tumor cells. We found that IL-10 and VEGF were downregulated by RECQL1-siRNA or miR-203 overexpression, but the mechanism and relationship between these two important factors remain unclear and thus need further study. Our *in vivo* experiments showed that the tumorigenicity of SCC-9 cells was effectively repressed by RECQL1 silencing. Mice inoculated with RECQL1-siRNA or miR-203 overexpressing SCC-9 cells displayed significant differences compared to mice in the control group. ($p < 0.05$)

Our results demonstrate that RECQL1 silencing can prevent the proliferation of SCCs cells, because they highly express RECQL1, which is necessary for DNA repair. In addition, RECQL1 mediates the expression of immunosuppressive factors to ensure the rapid proliferation and tumorigenicity in cancer cells, although the signaling pathway involved is still unknown.

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