miR-20b downregulates polymerases κ and θ in XP-V tumor cells

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Abstract. XP-V is a subtype of Xeroderma pigmentosum diseases with typical pigmentation and cancers in sun-exposed regions. The present study investigated the role of microRNA-20b (miR-20b) in the imbalance of polymerase expression levels in XP-V tumor cells. Following software prediction results, certain miRNAs were chosen as candidate regulators for the observed imbalance in polymerases in XP-V tumor cells. Reverse transcription-quantitative polymerase chain reaction and western blot were used to test candidate miRNAs for their ability to reduce the expression of these polymerases. A luciferase reporter assay was used to further verify the western blot results. Polymerases κ and θ were expressed at lower levels in XP-V tumor cells compared to normal control cells. A positive correlation was demonstrated between miR-20b and polymerases κ and θ. It was also demonstrated that a proportion of miRNAs had no effect on polymerases κ and θ, despite the software predicting that these miRNAs would target these two polymerases. Therefore, miR-20b may be responsible for the low expression levels of polymerase κ and θ in XP-V tumor cells, which accelerated mismatch in DNA replication repairing.

Introduction

Xeroderma pigmentosum (XP) is a sun-toxicity disease. A total of 8 subtypes (from XP-A to XP-G and XP-V) of this disease have been identified by their different pathogenic genes (1,2). The pathogenic mechanisms of almost all these subtypes result from a defect in nucleotide excision repair (3), except XP-V subtype, which results from a translesion synthesis (TLS) defect (4). XP-V is a common subtype (21%) in XP disease, and has a similar phenotype to other subtypes (2), including sun sensitivity, photophobia, early onset of freckling, and subsequent neoplastic changes in sun-exposed skin (5,6). The majority of studies have demonstrated that XP-V disease is a result of mutations in the *POLH* gene (encoding DNA polymerase η). Polymerase η is the main DNA polymerase responsible for TLS, and its defect could apparently reduce TLS efficiency and increase mismatch in DNA replication. These phenomena result in genomic instability, leading to a high incidence of tumors in patients (7-15). It has been previously demonstrated that polymerase η has defective expression in XP-V cells and that certain other polymerases involving TLS are unusually expressed, such as polymerase κ and ζ (encoded by *POLK* and *REV3 l*, respectively) (16). An additional polymerase, polymerase θ (encoded by *POLQ*), also has low expression in XP-V cells and tumor tissue and has the same function as polymerase η, which is to generate A/T mutations during the somatic hypermutation of immunoglobulin (Ig) genes (16,17). Given that a number of polymerases change their expression in XP-V cells and tumor tissue, certain factors may co-regulate the expression of these polymerases.

MicroRNAs (miRNAs) are endogenous, small non-coding RNAs that regulate translation and degradation of mRNAs at the post-transcriptional level (18). Protein expression from hundreds of genes are directly suppressed, albeit relatively mildly, by a single miRNA (19). Dysregulated miRNAs are correlated with various cancers and may function as tumor suppressors or oncogenes, depending on the function of their targets and cellular context (20). Therefore, certain miRNAs with unusual expression may explain the changes in expression of these polymerases in XP-V tumor that accelerate DNA mismatch.

Previous studies have mainly verified *POLH* mutation as an etiological factor of developing XP-V tumors (7-9,14). In the present study, polymerase-suppressive miRNAs associated with XP-V tumor were identified by analyzing miRNAs that may directly regulate DNA polymerases with unusual expression in XP-V tumor cells. miR-20b-5p was identified to be a polymerase suppressor by directly targeting *POLK* and *POLQ*.

Materials and methods

Prediction of miRNA as co-suppressor of POLK, REV3 l, and POLQ. POLK, *REV3 l* and *POLQ* all demonstrate low expression in XP-V tumor cells (16). Accordingly, Targetscan (http://www.targetscan.org),miRDB(http://mirdb.org/miRDB),

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and miRanda (http://www.microrna.org) were used to predict miRNA co-targeting these three genes.

Cell culture. All cells including XP-V tumor fibroblast cell lines, human skin fibroblasts (HSFs), and HeLa cells were cultured in DMEM supplemented with 20% FBS (HyClone, Logan, UT, USA). HeLa cells and HSFs were purchased from the cell bank of the Chinese Academy Of Sciences (Beijing, China). XP-V tumor fibroblast cell lines (XP30RO, XP1CH, and XP1SF) were purchased from The Coriell Institute (Camden, NJ, USA). Cells were incubated at 37˚C in 5% CO_2.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) for candidate miRNAs in XP-V cells. QIAgen miScript miRNA PCR Arrays kit (QIAgen Inc., Hilden, Germany) was used to extract, reverse transcribe and amplify total miRNAs in XP-V cell lines and HSFs according to the manufacturer's protocol. U6 was used as an endogenous control to normalize the amount of total miRNA in each sample. ABI 7500 Real-time PCR System (Applied Biosystems, Carlsbad, CA, USA) was used to analyze the data. Primers were synthesized by GenePharma (Shanghai, China) and the sequences are presented in Table I. To identify differences in miRNA expression, samples of HSF cells were defined as reference samples, and the quantity of all tested miRNAs in the reference sample was defined as '1.0.' Student's *t*-test was used to compare relative expression levels between XP-V cell lines and HSF control cells.

Transfection. HeLa cells were transfected with 200 nM candidate miRNA, miR-NC mimics, or miRNA inhibitor (GenePharma, Shanghai, China) using Turbofect transfection reagent (Thermo Fisher Scientific, Waltham, MA, USA) when cells reached 70-80% confluence.

Western blot analysis. All cells were harvested using RIPA lysis buffer (Beyotime, Shanghai, China). Then, 1% PMSF (Bioprimacy Co., Ltd., Wuhan, China) was added directly prior to use. Protein concentration was measured using BCA protein assay (Thermo Fisher Scientific, Inc.). Protein was loaded onto 10% SDS-PAGE gel (Beyotime, Shanghai, China) and then transferred to PVDF membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The blot was blocked with 5% skim milk for 2 h and then probed with primary mouse monoclonal polymerase κ (dilution, 1:6,000; catalog no., ab57070), rabbit polyclonal polymerase θ (dilution, 1:3,000; catalog no., ab80906), and mouse monoclonal β-actin (dilution, 1:8,000; catalog no., ab8226) antibodies (Abcam, Cambridge, UK). After incubation at 4˚C overnight, the blot was washed with TBST and incubated in secondary goat anti-mouse IgG-horseradish peroxidase antibody (dilution, 1:10,000; catalog no., sc-2005; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and goat anti-rabbit IgG-horseradish peroxidase antibody (dilution, 1:10,000; catalog no., sc-2004; Santa Cruz Biotechnology, Inc.) for 1 h at 25˚C. The signal was developed with ECL reagent (Advansta, Inc., Menlo Park, CA, USA).

Luciferase reporter assay. The 3'-untranslated regions (UTRs) of *POLK* and *POLQ* were amplified using PCR from human genomic DNA and then ligated into pMIR-report (Ambion, Thermo Fisher Scientific, Inc.). Then, QuikChange Lightning site-directed mutagenesis kit (Stratagene Agilent Technologies, Santa Clara, CA, USA) was used to induce miR-20b-5p target sequences (complementary to the seed region for miR-20b-5p) to mutate TACTTT to GTGAAA in *POLK* and CACTTT to GTGAAA in *POLQ*. All constructs were confirmed by sequencing. Primers used are summarized in Table I. HeLa cells were co-transfected with wild-type or mutant 3'UTR luciferase reporter construct, the Renilla luciferase construct pRL-TK, and either miR-20b-5p or miR-NC mimics. Then, 48 h after transfection, luciferase activities were measured using the Dual Luciferase Reporter Assay System (Promega Corporation, Madison, WI, USA) and normalized by dividing the firefly luciferase activity with Renilla luciferase activity.

Statistical analyses. Values are expressed as mean ± standard deviation (SD) from triplicate experiments. Student's *t*-test was used to compare relative expression levels. Statistical analyses were performed by SPSS software, version 16.0 (SPSS Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Using three web software predictions, it was found that no miRNA was predicted to target all three genes. Certain miRNAs were predicted to co-target *POLQ* and *POLK* but not *REV3 L* (Fig. 1). To find miRNAs co-regulating polymerases in XP-V tumor cells, only miRNAs that matched both *POLK* and *POLQ* from more than two software prediction results were selected. All other miRNAs were predicted to match only one of three genes, which were removed from the subsequent analysis. miR-520b, miR-520e, miR-302a, miR-302b, miR-302c, miR-302d, miR-93, miR-373, miR-548k, miR-20a, miR-20b, miR-106a, and miR-106b were chosen as candidate miRNAs.

The RT-qPCR results demonstrated that only miR-20a, miR-20b, miR-106a, miR-106b, and miR-548k were expressed at significantly different levels between XP-V cell lines and HSFs (Fig. 2).

The western blot analysis results verified that polymerase κ and θ were expressed at lower levels in XP-V tumor cell lines compared to the normal control cell line. Furthermore, when the above five miRNAs were transfected into HeLa cells, only miR-20b transfection resulted in reduced polymerase κ and θ levels (Fig. 3).

To determine whether such an inhibitory effect on translation was mediated by specific and direct interaction of miR-20b-5p with *POLK* and *POLQ* target site, luciferase reporter plasmids containing 3'UTR of both genes were constructed. The dual-luciferase assay demonstrated that the introduction of miR-20b-5p significantly reduced luciferase activity with respect to miR-NC, whereas such inhibitory effect was absent in cells transfected with reporter plasmids containing the mutant 3'UTR of both genes (Fig. 4).

Discussion

Low expression levels of polymerases in XP-V cells such as polymerase η, κ, and ζ may lead to a significant reduction in

Table I. Primers sequences.

Primer name	Primer sequence, 5'-3'
Primers for quantifying miRNA	
miR-520b	AAGTGCTTCCTTTTAGAGGGA
miR-520e	GGTGCTTCCTTTTTGAGGG
miR-302a-3p	TGCTTCCATGTTTTGGTGA
miR-302b-3p	GCGTGCTTCCATGTTTTAGTA
miR-302c-3p	TGCTTCCATGTTTCAGTGG
miR-302d-3p	AGTGCTTCCATGTTTGAGTGT
miR-93-5p	GTGCTGTTCGTGCAGGTAG
miR-373-3p	GCTTCGATTTTGGGGTGT
miR-548k	AAAGTACTTGCGGATTTTGCT
miR-20a-5p	CGTCAGGCCTAAAGTGCTTAT
miR-20b-5p	CAAAGTGCTCATAGTGCAGGTAG
miR-106a-5p	AGTCAGGCCAAAGTGCTTAC
miR-106b-5p	GTAAAGTGCTGACAGTGCAGA
Primers for mutagenesis	
Forward Primer for mutagenesis in *POLK UTR*	TTAAGCTAACTACTATTAAGCTGTCTTCTTTCACAAAT ATTAATATTTCACCTGATAGAAATGTAACTAAGATACA TAATGTGTTTTAATACACAT
Reverse Primer for mutagenesis in *POLK UTR*	ATGTGTATTAAAACACATTATGTATCTTAGTTACATTTC TATCAGGTGAAATATTAATATTTGTGAAAGAAGACAG CTTAATAGTAGTTAGCTTAA
Forward Primer for mutagenesis in *POLQ UTR*	CATGGTTTACCCAGACAGATGTGGAACCTTTCACCTA AGTGCATATTTCAAGCATCTGTTCT
Reverse Primer for mutagenesis in *POLQ UTR*	AGAACAGATGCTTGAAATATGCACTTAGGTGAAAGG TTCCACATCTGTCTGGGTAAACCATG

miRNA, microRNA; UTR, untranslated region; POLK, polymerase κ; POLQ, polymerase θ.

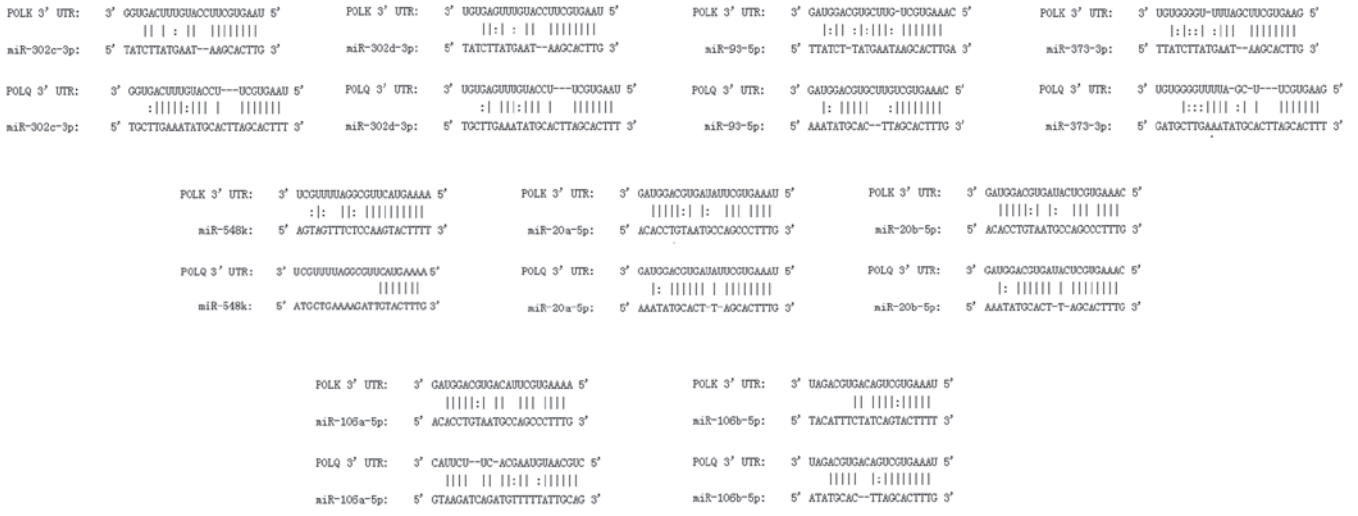

Figure 1. Prediction results of miRanda software for candidate miRNAs. Match sequences are listed between seed sequence in miRNAs and UTR sequence in genes. 'l' denotes complementary base pairing; ':' denotes G-U match. miRNA, microRNA; UTR, untranslated region.

the accuracy of TLS in XP-V cells (21). Polymerase θ has also been indicated to serve a role in base excision repair, and lower expression of polymerase θ may also seem unfavorable for DNA replication repair (22). In XP-V tumor cells, polymerases ζ, κ, and θ are indeed expressed at low levels, in addition to the dysfunction of polymerase η that disrupts DNA lesion replication and promotes genetic instability (16,21). In the present study, no miRNA was predicted to co-regulate

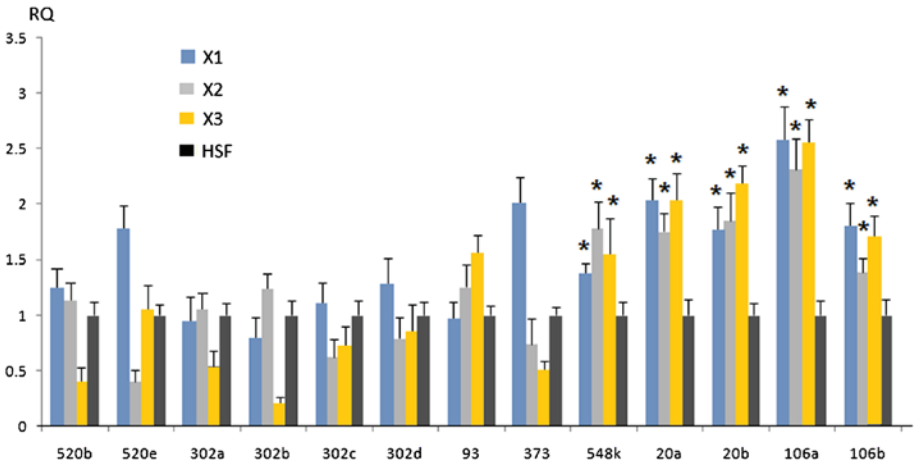

Figure 2. Results of quantitative polymerase chain reaction analyses of miRNAs in control and XP-V cells. Sample X1, X2, and X3 are XP-V tumor cell lines XP30RO, XP1CH, and XP1SF, respectively. *P<0.05 between samples X1, X2, X3 and HSFs. RQ denotes mRNA relative quantity.

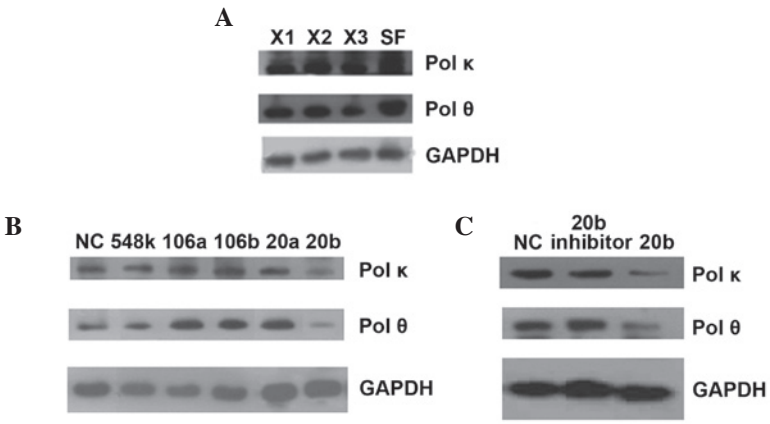

Figure 3. Western blot results of endogenous Pol κ and θ protein in different cell lines. (A) Compared with HSF cells, three XP-V cell lines had lower expression of Pol κ and θ. X1, XP30RO; X2, XP1CH; X3, XP1SF. (B) When HeLa cells were transfected with candidate miRNA mimics, only miR-20b-5p in candidate miRNAs could decrease Pol κ and θ expression. NC, miR-negative control; 548k, miR-548k; 106a, miR-106a-5p; 106b, miR-106b-5p; 20a, miR-20a-5p; 20b, miR-20b-5p. (C) Verification of miR-20b inhibition for Pol κ and θ expression in HeLa cells transfected by miR-20b-5p mimics. 20b inhibitor, the inhibitor of miR-20b-5p. GAPDH was used as endogenous control to normalize each sample. pol κ and θ, polymerase κ and θ; HSF, human skin fibroblasts.

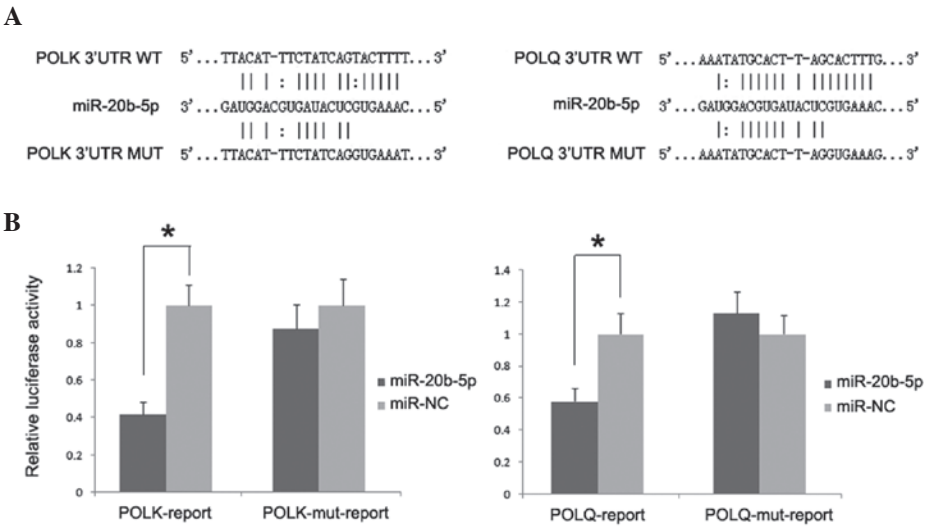

Figure 4. Analysis of luciferase activity. (A) HeLa cells were co-transfected with firefly luciferase reporter containing either wild-type (POLK/POLQ-report) or mutant (POLK/POLQ-mut-report) 3'UTR, Renilla luciferase reporter pRL-TK (as internal control), and either miR-20b-5p or miR-NC mimics. (B) Relative luciferase activity was measured and normalized by Renilla luciferase activity. Normalized luciferase activity for miR-NC transfected cells was set as 1. Data shown are the mean ± standard deviation from three independent experiments. *P<0.01 vs. miR-NC; unpaired Student's *t*-test. POLK, polymerase κ; POLQ, polymerase θ; UTR, untranslated region.

POLK and *REV3 L* expression, although these two genes both belonged to the Y-family of DNA polymerases (23). However, miR-20-5p was verified to function as co-suppressor of *POLK* and *POLQ* depending on its targets. The high expression of miR-20-5p in XP-V tumor cells could obviously decrease the expression of *POLK* and *POLQ*. Moreover, in XP-V tumor cells, these two polymerases with low expression may explain abnormal DNA replication repair apart from polymerase η dysfunction (21). Therefore, miR-20-5p may also serve an important role in XP-V tumors, accelerating DNA instability by down-regulating *POLK* and *POLQ*.

In summary, the current study demonstrated miR-20b-5p may co-regulate *POLK* and *POLQ*. Furthermore, miRNA may also be a novel factor that affect error-prone DNA replication in XP-V tumor cells.

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