MCPH1/BRIT1 represses transcription of the human telomerase reverse transcriptase gene

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MCPH1, a repressor of human telomerase reverse transcriptase (hTERT) function, is implicated in cellular immortalization. But little is known about how MCPH1 represses telomerase activity. In this study, to determine the mechanism by which MCPH1 regulates hTERT gene expression, we examined the role of MCPH1 in regulating the hTERT promoter in vitro. Co-transfection of the hTERT promoter with MCPH1 in Hela cells could inhibit the hTERT promoter activity. The EMSA assay demonstrated that MCPH1 could bind to the proximal hTERT promoter. Overexpression of MCPH1 could repress telomerase activity, and the repression was abolished by knocking down the MCPH1 expression using siRNA in U2OS cells. We propose that MCPH1 functions as a transcriptional repressor of hTERT in vitro. Since the activation of telomerase, widely observed in human tumor cell lines, is a critical step in tumorigenesis, our findings provide new insights into delineating the tumor-suppressing function of MCPH1 through its down-regulation of hTERT/telomerase expression.

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1. Introduction

Human telomerase, a ribonucleoprotein enzyme, protects the ends of human chromosomes from degradation, fusion, rearrangement and recombination (Blasco, 2003; Maser and DePinho, 2002). Telomeres consist of tandem repeats of the sequence “TTAGGG” (Nakamura et al., 1997). At the eukaryotic chromosomal ends, TRF1, TRF2, Ku and Rap1 can form protein-DNA complexes containing the D and T loops that regulate telomere homeostasis (Blackburn, 2001). In humans, telomerase is a ribonucleoprotein complex composed of two core components, the human telomerase reverse transcriptase (hTERT) and the integral RNA (hTERC or hTR) (de Lange, 2005). The telomere length mainly depends on the activity of hTERT that keeps the telomere elongation (de Lange, 2005). The activity of hTERT is restricted to embryonic stem cells and cancer cells while the expression of hTR is universal (Cong and Shay, 2008; Shay and Wright, 2002; Shay and Wright, 2006). In contrast, the telomerase activity in most normal cells is suppressed. In most somatic cells, the expression of hTERT gene is very low. But in the immortal cell lines like Hela, hTERT is active in order to keep the telomere from shortening during DNA replication (Jackson et al., 2002; Trimborn et al., 2004). Hence, dissecting the regulatory mechanism of hTERT expression is highly informative in understanding telomerase activation in tumors.

MCPH1 (also called BRIT1, BRCT-repeat inhibitor of TERT expression) was firstly identified as one of the genes mutated in autosomal recessive primary microcephaly (Jackson et al., 2002). The symptoms of primary microcephaly include small head circumference (HC), mental retardation and PCC (premature chromosome condensation) syndrome (Trimborn et al., 2004). Three microcephaly-causing mutations have been identified for MCPH1, including two premature stop codon mutations (S25X and 427insA), and one missense mutation in the N terminal BRCT domain (T27R) (Trimborn et al., 2005). Functionally, MCPH1 has been implicated in DNA damage response, cell cycle regulation, chromosome condensation and so on (Alderton et al., 2006; Lin and Elledge, 2003; Lin et al., 2005; Rai et al., 2006; Xu et al., 2004). Evolutionary studies have suggested a rapid sequence change of MCPH1 in primates, which is associated with the brain enlargement during primate evolution and human origin (Evans et al., 2004; Wang and Su, 2004). MCPH1 contains three BRCA1- Carboxyl Terminal (BRCT) domains, including one at the N terminus and a tandem pair at the C terminus. Many studies have implicated that the BRCT domain of MCPH1 functions as a protein interaction domain. The interaction of the tandem BRCT domains of MCPH1 with proteins like E2F1 and r-H2AX is required for the activation of cell cycle checkpoint, DNA repair and apoptosis (Jeffers et al., 2008; Wood et al., 2007; Yang et al., 2008).

Abbreviations: aa, amino acid(s); hTERT, human telomerase reverse transcriptase; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; WT, wild type; PBS, phosphate-buffered saline; BRCT, BRCA1-Carboxyl Terminal domains; siRNA, small interfering RNA; GST, glutathione S-transferase; HA, hemagglutinin; qPCR, quantitative PCR; EMSA, electrophoretic mobility shift assay; TRAP, telomeric repeat amplification assay.

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Moreover, the expression of MCPH1 in breast cancer cells is very low, implicating that it may function as a tumor suppressor gene (Rai et al., 2006). MCPH1 was initially identified as a BRCT-repeat inhibitor of hTERT expression, and it seemed to inhibit the hTERT promoter activity directly (Lin and Elledge, 2003), but the mechanism is yet to be revealed.

In the present study, in order to determine the MCPH1 suppressor function of telomerase, we analyzed the MCPH1 inhibition on the hTERT promoter. We showed that MCPH1 could repress the hTERT promoter activity by direct binding to the hTERT proximal promoter, leading to a reduced hTERT expression and telomerase activity. Through promoter mapping, we have identified the potential MCPH1 binding sites in the hTERT promoter. Our results suggest that the inhibition of TERT gene activity may be an important tumor repressor function for MCPH1.

2. Materials and methods

2.1. Cell culture

The HEK293T, Hela, and U2OS cell lines were obtained from ATCC. These cell lines were maintained in Dulbecco Modified Eagle Medium (Gibco, Rockville, MD) with 10% fetal bovine serum (HyClone, Logan, UT) at 37 °C in a humidified atmosphere containing 5% CO₂.

2.2. Plasmid construction and transient transfection

The cDNA of human MCPH1 was amplified by PCR and cloned into the pCGN-HAM and PM vector. The deletion mutants of MCPH1 were introduced by PCR and cloned into pCGN-HAM and PM vector. The point mutation of MCPH1 was introduced using the QuickChange II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) following the manufacturer’s instruction. The hTERT core promoter (−234 bp to +68 bp) was PCR amplified and inserted into pGL3-basic firefly luciferase reporter vector (Promega, Madison, WI). Series deletions of hTERT core promoter were generated by PCR and cloned into pGL3 vector. hTERT+4 promoter deletion mutant hTERT+4_del-GAAGCCT was introduced using the QuickChange II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) following the manufacturer’s instruction. All constructs were verified by sequencing. The transient transfection was carried out using Lipofectamine 2000 (Invitrogen) or Fugene HD (Roche, Basel, Swiss) for HEK293T cells, Hela cells and U2OS cells following the protocols provided by the manufacturer. Briefly, equal numbers of cells were plated in 24-well and 6-well plates and were grown by 80% confluence. The indicated amounts of vectors were mixed in OPTI-MEM medium (Invitrogen) with Lipofectamine 2000 (Invitrogen). The solution was incubated for about 30 min at room temperature, and then placed on the cultured cells. After 4–6 h, the medium was changed into Dulbecco Modified Eagle Medium (Gibco) with 10% fetal bovine serum (HyClone). The oligonucleotide primers used for the generation of deletion hTERT promoter constructs are shown in Table S1.

2.3. Luciferase reporter assay

Hela cells were grown in 24-well plates (Corning, NY, USA) and transfected with the indicated amounts of vectors by Lipofectamine 2000. pTK-Renilla was used as the internal control. Luciferase activity was assayed 16–28 h after transfection. The luciferase activity in cell extracts was determined by Dual-luciferase Reporter Assay System (Promega) according to the protocol supplied by the manufacturer. The relative light units were measured using a luminometer.

2.4. Western blotting

Protein from Hela cells was homogenized in RIPA lysis buffer (50 mM Tris–HCl, pH 7.4; 150 mM NaCl; 1 mM EDTA; 1% Triton-100; 1 mM Na3VO4) containing a cocktail of protease inhibitor (Sigma Chemical, MO, USA). Extracted protein (15–20 μg) was separated by SDS-polyacrylamide gel electrophoresis and transferred to a membrane by electrophoretic transfer. The membrane was incubated with anti-HA monoclonal antibody (Covance, NJ, USA). Immunoreactivity was detected with an enhanced chemiluminescence system (Pierce, IL, USA) with colored markers (Fermentas) as molecular size standards.

2.5. Quantitative RT-PCR

To determine the effect of human MCPH1 gene on the endogenous TERT expression, RNA was isolated from Hela cells transfected with 1.6 μg HA-human MCPH1 and 1.6 μg HA empty vector. These RNAs were reverse-transcribed with oligo-dt (20) primer and amplified by real time primers. Real time quantitative PCR (qPCR) reactions (15-μl total volume containing 0.5-μl 10 μM primer, 7.5-μl SYBR Green dye (Bio-Rad, CA, USA), and 2-μl of cDNA) were carried out with a DNA Engine Opticon Continuous Fluorescence Detection System (MJ Research Inc, Waltham, MA) for 40 cycles. The Ct values for each gene amplification were normalized by subtracting the Ct value calculated for GAPDH. The normalized gene expression values were taken as the relative quantity of TERT gene-specific messenger RNA (mRNA). The oligonucleotide primers used in the real-time quantitative PCR amplifications are shown in Table S2.

Semi-quantitative RT-PCR method involves reverse transcription using an oligo-dt (20) primer. The resulting cDNA thus represents
both house-keeping gene transcripts as well as specific transcripts to be semi-quantified. The RT reaction is then amplified in a pair of PCR series — one series is to amplify the house-keeping gene GAPDH cDNA, and the other is for the specific cDNA of interest (in separate PCR, using gene-specific primers). For each amplified product, the PCR conditions and cycle numbers were individually adjusted so that all reactions occurred within the linear range of product amplification. PCR products were analyzed by electrophoresis through 2% agarose gels containing ethidium bromide, and gels were photographed under UV light. The oligonucleotide primers used in the semi-quantitative PCR amplifications are shown in Table S2.

2.6. Electrophoretic mobility shift assay

The GST–MCPH1 fusion protein and GST protein were obtained commercially (Protein Tech, Chicago, USA). Briefly, a total of 1 μg recombinant proteins were used. The biotin-end-labeled double-strand synthetic oligonucleotides are listed in Table S3. The probe (10 pm) was incubated with synthetic proteins in the presence or absence of unlabeled MCPH1 competitive oligonucleotides on ice for 30 min and the reaction mixture was subjected to electrophoresis on a 10% polyacrylamide non-denaturing gel in 0.5× TBE buffer. The antibody against GST (GE Healthcare, London, UK) was added to the binding reactions for the supershift assay. The binding reactions were electrophoretically transferred to nylon membrane for 30 min at 180 mA. The transferred DNA was cross-linked to membrane at 120 mJ/cm² UV light and the biotin-labeled DNA was detected by chemiluminescence.

2.7. Telomeric repeat amplification (TRAP) assay

Telomerase enzymatic activity assay is a modification of the original telomeric repeat amplification protocol (TRAP) (Kim and Wu, 1997), which utilizes PCR to generate a ladder of products with six-base increments, starting at the position of 50 nucleotides. Briefly, the Hela cells were lysed in CHAPS lysis buffer (10 mM Tris-HCl, pH 7.5; 1 Mm MgCl₂, 1 Mm EGTA; 0.1 mM Benzamidine; 5 mM β-mercaptoethanol; 0.5% CHAPS; 10% Glycerol). After incubation on ice for 30 min, the lysates were centrifuged for 30 min at 12,000 g and the supernatant was stored at −80 °C. The protein concentrations were determined by the Bradford method using THERMO pierce protein dye reagent according to the manufacturer’s recommendations. The extracts were incubated with TS primer (5′-AAATCCGTCGAGCAGAGTTT-3′) and CX primer (5′-CCCTACCCTACCC-TACCCTAA-3′). The 25 μl reaction mixture also contained dNTP and 2.5 U Hotstar taq DNA polymerase (Takara, Tokyo, Japan). Following a 30 min incubation at 30 °C, samples were subjected to 31 cycles of PCR at 94 °C for 30s, 59 °C for 30s and 72 °C for 1 min. The PCR products were subjected to electrophoresis using 12.5%...
polyacrylamide gel and visualized with SYBR Green I nucleic acid gel stain (Invitrogen). Heat inactivated extracts were used as the negative control.

2.8. Small interfering RNA

Double stranded small interfering RNA (siRNA) synthesized by Invitrogen was used to knock down the endogenous MCPH1 protein levels. The siRNA sequences are: #1: AGGAAGUUGAAGGGAUC-CAdTdT; #2: CUCUCUGUGAAGGACCAdTdT. Briefly, for transient transfections, U2OS cells were seeded at a density of 50 to 60% in 6-well plates. On the following day, transfections were performed by using Oligofectamine (Invitrogen). Cells were harvested 48 h after transfection. Western blot and TRAP assay were conducted as described above.

2.9. Statistical analysis

For statistical analysis, the data were analyzed by using the two-tailed Student’s t test. A p-value of <0.05 is considered statistically significant.

3. Results

3.1. Human MCPH1 functions as a transcriptional repressor

In order to test whether MCPH1 functions as a transcriptional repressor or activator, we constructed a fusion protein of MCPH1 and DBD (DNA-binding domain) of the yeast transcription factor Gal4. The results shown in Fig. 1 demonstrate that MCPH1 can significantly inhibit PFR’s activity, suggesting that it is a transcriptional repressor. To detect which part of MCPH1 protein harbors the transcriptional repressor function, we then constructed a series of fusion proteins containing different domain regions of MCPH1 (Fig. 1A). Surprisingly, all MCPH1 domain regions tested could significantly repress PFR’s activity (p<0.01, two-tailed Student’s t test). In comparison, the repressing effects of BRCT1 and BRCT23 are relatively weak compared with MCPH1△23, MCPH1△1 and MCPH1 (233–566) (Fig. 1B). This implies that all major domains of MCPH1 account for the repressing function.

3.2. Human MCPH1 inhibits hTERT gene promoter activity

It has been shown that MCPH1 can inhibit hTERT transcription (Lin and Elledge, 2003), but the underlying mechanism is unknown. To answer this question, we tested whether MCPH1 could repress the hTERT promoter activity. Hela cells were used in transfections with the MCPH1 construct and the hTERT core promoter. As expected, we found that MCPH1 could strongly repress the hTERT promoter activity. When 0.2 μg wild-type MCPH1 (wtMCPH1) vector together with 1 μg hTERT reporter plasmid were co-transfected, the hTERT promoter activity was reduced up to 50% compared to the control (Figs. 2A, B), demonstrating that the down-regulation of hTERT by MCPH1 occurs at the transcriptional level. To further evaluate the effect of MCPH1 on hTERT inhibition, we co-transfected increasing amounts of MCPH1 with hTERT promoter in Hela cells. Although we did not observe a dose dependent effect, we found that MCPH1 could repress the hTERT promoter activity when adding 400 ng (p<0.05) to 6400 ng MCPH1 expression vector (p<0.001) (Fig. 2C).

Next we sought to detect the responsible domains in MCPH1, and we constructed various fragments of HA fusion proteins. In contrast to the results of the transcriptional repressor assay (Fig. 1), HA–MCPH1△1, HA–MCPH1△23 and MCPH1 (233–566) did not inhibit the hTERT promoter activity while HA–BRCT23 and HA–BRCT1 exhibited repressing activity (Fig. 3A).

As the hTERT core promoter harbors multiple E2F1 (a known inhibitor) (Crowe et al., 2001) binding sites, there is a possibility that the inhibiting function of MCPH1 may involve the interaction with E2F1 (Yang et al., 2008). To test this, we co-transfected the hTERT promoter with wtMCPH1 and wtE2F1, and no significant changes were detected compared with the co-transfection with wtE2F1 only, suggesting that the repressing function of MCPH1 is not E2F1 dependent (Fig. 3B).
3.3. Identification of hTERT promoter elements responsible for MCPH1-mediated transcriptional repression

To further investigate whether MCPH1 represses hTERT expression at the transcriptional level, we conducted a luciferase (Luc) reporter gene experiment to determine which part of the hTERT core promoter is responsible for the expression inhibition. Various regions of the hTERT upstream, ranging from −234 bp to +68 bp, were fused to the Luc reporter, and the luciferase activities were measured in the transfected cells (Fig. 4A). The results showed

![Image](image-url)
that co-transfections of MCPH1 led to reduction for the reporter constructs carrying different promoter regions. Notably, the shortest promoter region ranging from +4 bp to +68 bp could still respond to the MCPH1 inhibition, indicating that the MCPH1 response element likely lies within the 63 bp DNA sequence (Fig. 4B).

3.4. MCPH1 directly binds to the hTERT promoter in vitro

We next determined whether MCPH1 would bind to this 63 bp sequence directly because previous studies have reported that MCPH1 could bind to the p73 promoter in vivo (Yang et al., 2008). EMSA was performed using the 63 bp DNA sequence. The results indicated that the GST–MCPH1 fusion protein (but not GST alone) could bind to the end-labeled wild-type 63 bp oligos (Fig. 4C). Incubation of the GST–MCPH1 protein with up to a 2000-fold molar excess of unlabeled probe could effectively compete for the binding affinity (Fig. 4C). We also performed the super-shift experiments and found that inclusion of the anti-GST antibody in the binding reactions produced a slight but consistent super shift, indicating that GST–MCPH1 did bind to the 63 bp sequence (Fig. 4D). Further analysis using nuclear extract from MCPH1 positive cell line (Hela) confirmed the in vivo binding of MCPH1 to the hTERT promoter probe (Fig. 4E).

Then we conducted the deletion mutation assay, and we found that when the 10 bp sequence from the 3′ end of the hTERT sequence was deleted, the binding of GST–MCPH1 was abolished. This suggests that the DNA element “GAAGCCCT” at the 3′ end of the 63 bp DNA sequence is likely responsible for MCPH1 binding (Figs. 5A, B). In order to test whether the inferred binding motif “GAAGCCCT” is still responsive for MCPH1 suppression, we deleted “GAAGCCCT” in the hTERT+4 promoter. As expected, the mutant MCPH1 did not show the repression activity compared with the control (Fig. 5C).

3.5. Overexpression of wtMCPH1 inhibits telomerase enzymatic activity

Telomerase activity was measured in untransfected Hela cells and transient transfected Hela cells with HA empty vector and wtMCPH1
by using a modification of the TRAP assay. Hela cells are lack of functional wtMCPH1, and therefore have strong telomerase activity. As expected, transient expression of wtMCPH1 could inhibit telomerase activity in Hela cells compared with the empty vector or the untransfected control cells (Fig. 6).

It was reported that among the MCPH1-related microcephaly causing mutations, T27R is the only point mutation (Thr → Arg, located in the N terminal BRCT domain) that still produces a full length MCPH1 protein (Trimborn et al., 2005). Compared with the wtMCPH1, overexpression of the mutant MCPH1 (27) showed greater telomerase activity (Fig. 6). As the hydrophobic property of the mutant Arg (−4.5) is highly different from the wild type Thr (−0.7), it is possible that the point mutation may change the N terminal BRCT structure of MCPH1, therefore seriously damage the function of MCPH1, and eventually lead to primary microcephaly.

3.6. Knock-down of wtMCPH1 activates telomerase enzymatic activity

To test the effect of knocking down MCPH1, we used U2OS cells because they express weak telomerase enzymatic activity. We transfected U2OS cells with MCPH1 specific siRNA (50 nM) (18) and compared the effects of telomerase enzymatic activity with the scrambled-sequence siRNA. We found that the cells treated with MCPH1 siRNA showed increased telomerase enzymatic activity compared with the controls (Fig. 7A), again supporting that MCPH1 is a direct repressor of hTERT. Collectively, these results suggest that the lack of endogenous MCPH1 contributes to hTERT expression in cancer cells.

3.7. MCPH1 causes down-regulation of TERT mRNA expression

To investigate the mechanisms by which MCPH1 inhibits telomerase transcription, we determined its effect on expression of a subset component of the telomerase holoenzyme, including TERT and HSP90. The mRNA expression of HSP90 did not change significantly. However, the MCPH1 transfected cells showed decreased expression of TERT mRNA, and the pattern remained for three replications (Figs. 7B, C).

We also examined the expression of two telomere repeat binding factors, TRF1 and TRF2 (also known as TERF1 and TERF2, respectively). Although TRF1 and TRF2 are not the components of the telomerase enzyme, these factors are involved in the regulation of telomeres. In Hela cells, overexpression of wtMCPH1 had no obvious effect on the TRF1 and TRF2 mRNA levels (Fig. 7B).
4. Discussion

Low activity of telomerase is a common phenomenon in vast majority of human somatic cells for prevention of cancer formation (Horikawa et al., 2005). Tumor suppressors like p53 and E2F1 are known to inhibit telomerase activity (Crowe et al., 2001; Xu et al., 2000), suggesting that the functioning of tumor suppressors may be linked to negative regulation of telomerase. Recently, one study has shown that MCPH1 could interact with TRF2 to regulate telomere length and telomere DNA damage response (Kim et al., 2009). In addition, MCPH1 could repress hTERT gene expression (Lin and Elledge, 2003). Therefore, it is a plausible hypothesis that MCPH1 represses hTERT gene expression through inhibiting the hTERT promoter activity.

In this study, we have demonstrated that MCPH1 is a novel transcriptional repressor. The repression was mediated by binding to the proximal portion of the hTERT promoter. Through domain mapping of the hTERT core promoter, we showed that the MCPH1 binding sequence was located in +4 to +68 of the hTERT core promoter, which was further confirmed by the EMSA assays. The deletion assay has identified an 8 bp element (GAAGCCCT), which is likely the MCPH1 binding sequence. Taken together, these observations suggested that MCPH1 could repress hTERT expression by directly binding to its promoter in vitro and our results provided evidence that MCPH1 functions as a novel tumor suppressor gene. One major limitation of our study is that we did not perform CHIP assay to confirm that MCPH1 could directly bind to this sequence in vivo.

The proposed tumor suppressor function of MCPH1 is consistent with the observation that the expression levels of MCPH1 are decreased in several types of human cancer including breast and ovarian cancers (Rai et al., 2006). We have demonstrated that inactivation of MCPH1 gene expression may promote activation of the telomerase, therefore promoting cell immortalization, one of the critical steps in tumorigenesis. More data is needed in order to confirm the tumor suppressor function of MCPH1, especially to reveal the potential interaction of MCPH1 with other telomerase regulators. The application of large scale expression analyses will likely reveal more genes regulated by MCPH1. Also, a MCPH1 overexpression model would be helpful in revealing the physiological functions of MCPH1 as a transcriptional repressor. Our characterization of MCPH1 as a transcriptional repressor of human telomerase gene expression represents a unique role for MCPH1. It would be informative to explore the regulatory role of MCPH1 on telomerase in human cancers because in some cancers, the telomerase activity is very high and it can be considered as a potential drug target.

In addition, previous data suggested that MCPH1 could interact with TRF2 and bind to telomere DNA to regulate telomere length (Kim et al., 2009). Therefore, the observed suppression of hTERT by MCPH1 suggests another regulatory pathway at the transcriptional level. Another direction that should be explored is the interaction between MCPH1 and BRCA1 since BRCA is also a repressor of hTERT (Lin et al., 2005; Xiong et al., 2003).

It was reported that a missense mutation (T27R) of MCPH1 could cause primary microcephaly (Trimborn et al., 2005). We tested whether this missense mutation has any effect on the hTERT gene activity. Our results indicated that overexpression of the mutant MCPH1 in Hela cells led to the loss of MCPH1’s inhibiting function, resulting in a higher hTERT activity. The missense mutation is located in the N terminal BRCT domain which was shown to inhibit the hTERT promoter activity. Hence, the dysfunction of the mutant MCPH1 is likely caused by the disruption of the N terminal BRCT domain in microcephaly patients, which needs to be confirmed by more experiments.

In summary, we show that MCPH1 directly binds to the promoter of hTERT and represses the telomerase activity, and knock-down of MCPH1 can activate telomerase. Additionally, an intact N terminal BRCT domain is essential for the proper inhibiting function of MCPH1. Given that hTERT/telomerase activation is crucial for cell immortalization and tumor development and progression, the proposed novel function of MCPH1 may suggest an important mechanism of tumor suppression mediated by MCPH1.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.gene.2011.12.053.

References


