The MLH1 –93 promoter variant influences gene expression

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The –93 SNP of MLH1 gene is associated with MLH1 gene methylation in endometrial and colorectal cancers. We undertook luciferase reporter assay and electrophoretic mobility shift assay (EMSA) to test whether the –93 SNP affects the MLH1 gene expression. The luciferase activity for –93A plasmid is significantly lower than –93G plasmid. In EMSA experiments, the –93A and –93G probes have different binding affinity to nuclear proteins of JEG3 cells. Our data indicate that –93 SNP affects MLH1 gene expression by altering protein binding to the promoter of MLH1 gene.

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1. Introduction

Loss of DNA mismatch repair leading to microsatellite instability (MSI) is seen in a high proportion of gastrointestinal and endometrial cancers. Epigenetic silencing of the MLH1 gene is the most common cause of defective DNA mismatch repair [1–7]. In a large series of endometrial carcinomas, 20% have MLH1 gene promoter methylation [1]. However, the molecular mechanism behind the methylation is not clear. We have previously shown that MLH1 gene methylation is associated with the –93 SNP of the MLH1 gene [8]. In this study, we undertook luciferase reporter assay and EMSA to further test whether –93 SNP affects the MLH1 gene expression.

In the MLH1 gene promoter region, Arita et al. [9] identified a protein-binding site which contains –93 SNP, by in vivo footprinting analysis. It is conceivable that –93G to A transition may change the protein binding at that site, and regulate MLH1 gene expression by altering its epigenetic status.

2. Materials and methods

2.1. Cells and cell culture

The human choriocarcinoma cell line JEG3 was obtained from American Type Culture Collection (Rockville, MD, USA) and cultured in RPMI-1640 medium (Hyclone, USA) supplemented with 10% fetal bovine serum (Hyclone, USA). Cells were maintained in 5% CO2 in air in a humidified atmosphere at 37 °C. Exponentially growing cells were used for the following experiments.

2.2. Construction of –93G and –93A reporter plasmids

Arita et al. determined the minimal region, –301 to –76 relative to a translation start site, which is essential for transcription [9]. An insert, –301 to +101 of MLH1 5′-flanking region containing –93 SNP, was amplified by the polymerase chain reaction (PCR). PCR was performed in 0.5 ml tubes (20 μl volume, 0.3 U of Pyrobest DNA polymerase (Takara Shuzo Co., Kyoto), 0.8 μl of 4 μM forward and reverse primers, 0.8 μl of 2.5 mM each dNTP, 20 ng of genomic DNA and 1 × Pyrobest Buffer II). Sense primers were designed to contain XhoI site (underlined): 5′-TAATCCGCTCGAGCGAGCTCTAAAAACGAACC-3′. The anti-sense primer contained HindIII site (underlined): 5′-GATATCCTCTTTTGATAGCATTAGCTGGC-3′. PCR products were double digested with XhoI and HindIII. Gel purified digests were ligated in the XhoI and HindIII sites of pGL3-Basic (Promega Co., Madison, WI, USA) with T4 DNA ligase to give pGL3-MLH1–93G and pGL3-MLH1–93A. Plasmids were purified with a GFX Micro Plasmid Prep Kit (Amersham Pharmacia Biotech, Inc., Piscataway, NJ, USA) according to a manufacturer’s manual. DNA sequencing of constructs was performed using an RVprimer3 (sense, Promega) and a BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) as recommended by the supplier. The reaction mixture was analysed on an automated DNA sequencer, ABI PRISM 3700 DNA Analyzer (Applied Biosystems).
2.3. Luciferase reporter gene analysis

JEG3 cells were transfected with reporter plasmids and the luciferase activity was measured using Dual-Luciferase® Reporter (DLR) Assay kit (Promega) as instructed by the supplier. Firefly luciferase units were normalised with Renilla luciferase units, and the pGL3-Promoter (Promega) and the pGL3-Basic were used as a positive and negative control respectively. Each transfection was repeated 12 times.

2.4. Electrophoretic mobility shift assay (EMSA)

Nuclear extract of JEG3 cells was prepared using NE-PER Nuclear and Cytoplasmic Extraction kit (Pierce, Rockford, IL, USA) as described in the manual, and the protein content was determined by a Bradford method. Synthetic single strand oligonucleotide, −119 to −61 containing −93 SNP, was end-labelled with biotin using Biotin 3′ End DNA Labeling kit (Pierce) as instructed by the supplier. The end-labelled forward and reverse single strand oligonucleotides were annealed by heating at 95 °C for 3 min in the presence of 200 mM NaCl and slowly cooling to room temperature. As a competitor, unlabelled probe (−119 to −61) or control EBNA DNA (5′-GTA CCC GGG GAT CCT ATC TGG CTA GCA TAT GCT ATC CTA ATG GAT CCT CTA GAG TCG ACC-3′) from LightShift Chemiluminescent EMSA kit (Pierce) was used. The binding reaction was performed for 20 min at room temperature in 20 μl of binding buffer containing 20 fmol of labelled probe, 1 μg of poly dI-dC, and 6 μg of nuclear extract. Separation of free biotin-labelled DNA from DNA–protein complexes was carried out on a 6.5% non-denaturing polyacrylamide gel. Electrophoresis was carried out in 0.5 TBE buffer at 100 V at room temperature. Electrophoretic transfer was performed in 0.5× TBE buffer at 340 mA for 1 h at room temperature. UV-light cross-linking was performed for 15 min with the membrane face down on a transilluminator equipped with 302 nm bulbs. Detection of the biotin-labelled DNA was undertaken using chemiluminescent Nucleic Acid Detection kit (Pierce) according to the manufacturer’s instructions.

The intensity of the bands detected by EMSA was examined by using QUANTITY ONE 4.62 (Bio-Rad). Totally three experiments were done. Then the average of intensity was calculated.

In the antibody experiment, 1 μl of AP-4 antibody (2 μg/μl, sc-18593, Santa Cruz) was added and incubated for 20 min, and then the biotinlabelled probe was added and incubated for another 20 min before loading into the gel.

3. Statistical analysis

The difference between luciferase activity of pGL3-MLH1-93G and pGL3-MLH1-93A, and band intensity of EMSA was analysed by unpaired Student’s t-test (two tails).

4. Results

4.1. Luciferase activity comparison of MLH1-93G and MLH1-93A plasmids

In order to ascertain whether the −93 SNP of MLH1 gene affects gene expression, a DNA fragment, which covers −301 to +101 of MLH1 gene, was inserted into pGL3-Basic and transfected to human choriocarcinoma cell line, JEG3. The sequencing data showed that the cloned sequences and the junctions of inserts and vectors are correct. The luciferase activity of MLH1-93G plasmid was significantly higher than MLH1-93A plasmid (p = 0.002) (Fig. 1).

4.2. Binding proteins at the DNA fragment containing −93 SNP

In order to understand whether the −93 SNP affects protein binding to the MLH1 gene promoter, the nuclear extracts of JEG3 were incubated with biotin-labelled oligonucleotide containing −93 SNP in the DNA binding buffer containing poly dI-dC. The EMSA results showed that some proteins bound to −93G probe with higher intensity (arrows 1 and 2, Fig. 2; p < 0.0001 and p < 0.0001, Table 1), and some proteins bound to −93A probe with higher affinity (arrows 3–5, Fig. 2; p = 0.0001, p = 0.0048 and p < 0.0001, Table 1). These proteins specifically interacted with −93G and −93A probes, because specific competition could inhibit them; however, non-specific competition could not inhibit them (Fig. 2).

In a super gel shift assay with AP-4 antibody, no super-shifted bands were observed (data not shown).

5. Discussion

It is noteworthy that the −93A allele of MLH1 gene was previously associated with risk for squamous cell lung cancers in Korean patients [10]. The lung cancers were not, however, evaluated for MLH1 methylation and epigenetic silencing. Recent studies in Chinese and American non-small-cell lung cancers have shown that methylation of the MLH1 promoter and/or epigenetic silencing of MLH1 is a frequent event [11,12]. It is conceivable that the −93A allele is also associated with risk for MLH1 methylation in lung cancers and possibly in other malignancies. How variation in
the 5’ region of the MLH1 gene influences risk for epigenetic silencing is yet to be determined. Recent studies have suggested that site-specific repressors of transcription may recruit DNA methyltransferases, leading to de novo gene methylation and epigenetic silencing [13,14].

Based on footprinting experiments and reporter constructs, the associated variant (−93 SNP) is part of the 5’ MLH1 sequences that bind an unknown factor and are required for optimal expression [9]. Our data suggest that −93 SNP may alter protein binding to gene promoter to influence gene expression. The −93A plasmids showed significantly lower luciferase activity than −93G plasmids, possibly explained by the G to A transition, which could allow a repressor to replace an active transcription factor. And then the cloned MLH1 promoter of the −93A plasmids could be methylated. The EMSA data support the above speculation, because some proteins bind to the −93G probe with higher affinity and some to the −93A probe with higher affinity. With respect to −93G, it could possibly interact with a transcription factor (X1), which might recruit general transcription factors (GTFs) and RNA polymerase II [15,16], leading to MLH1 gene transcription. A previous study reported that there are 6 protein-binding sites within the MLH1 gene promoter [9]. Therefore, the MLH1 gene promoter may contain an additional transcription factor (X2) binding site, which could further enhance gene transcription (Fig. 3, left). With respect to −93A, it might interact with a repressor (Y), which could recruit co-repressors including DNA methyltransferase (DNMT), histone deacetylase (HDAC) etc. DNMT activity could be inhibited by the retinoblastoma (Rb) protein [17], resulting in negative methylation status of the MLH1 promoter. However, the X2 could still drive low-level transcription of the MLH1 gene (Fig. 3, right). The proposed model provides a good explanation of what we have seen in luciferase reporter assays. If DNMT activity is increased, or Rb function is lost, the DNMT could make the MLH1 gene promoter methylated, and gene transcription would be turned off.

Using software to search for transcription factors, we found that the −93G of the MLH1 gene could possibly bind to a transcription factor, Activating Enhancer Binding Protein 4 (AP-4). However, the super gel shift assay with AP-4 antibody did not show any super-shifted bands, indicating that transcription factor AP-4 does not interact with −93G of the MLH1 gene. Further studies to isolate those proteins binding to −93G and A probes, and analyse their functions associated with gene methylation and transcription, should help clarify that the −93 SNP participates in regulation of methylation and transcription of the MLH1 gene.

Conflict of interest

None declared.

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