A novel functional polymorphism in the Cdc6 promoter is associated with the risk for hepatocellular carcinoma

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\textbf{Abstract}

Cdc6 is essential for DNA replication and its deregulation is involved in carcinogenesis. To date, the biological significance of the polymorphism in Cdc6 promoter is still unknown. In this study, we aimed to evaluate the influence of the Cdc6 \textasciitilde 515A\textsuperscript{\textdagger}G polymorphism (rs4134994) on the individual’s susceptibility to cancer and on the function of Cdc6. The Cdc6 \textasciitilde 515A\textsuperscript{\textdagger}G polymorphism was genotyped in 387 hepatocellular carcinoma (HCC) and 389 age- and sex-matched healthy subjects. The association between the genotypes and the risk for HCC was then estimated by unconditional logistic regression analysis with adjustment for age, sex and HBV status. Compared with the AA homozygotes, the homozygous GG genotype (adjusted \textit{P} = 0.36, 95% confidence interval (CI) = 0.18–0.72, \textit{P} = 0.004) or the combined AG/GG genotypes (adjusted \textit{P} = 0.56, 95% CI = 0.36–0.86, \textit{P} = 0.008) were statistically significantly associated with the reduced risk for HCC. Moreover, the analysis using luciferase reporter system showed that the G-allelic Cdc6 promoter displayed a decreased transcriptional activity compared with the A-allelic one. These results indicate that the individuals with G allele may have reduced Cdc6 expression and are therefore in reduced risk for HCC. Further investigation using electrophoretic mobility shift assay (EMSA) revealed that the G allele had a stronger binding strength to nuclear protein(s) which might function as negative regulator(s) for Cdc6 transcription. Our findings suggest that the \textasciitilde 515A\textsuperscript{\textdagger}G polymorphism may affect the Cdc6 promoter binding affinity with nuclear protein(s) and in turn the Cdc6 expression, which consequently modulates the individual’s susceptibility to HCC.

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in turn affect gene expression [15,16]. Association of SNPs in the gene promoter with the risk of cancer has already been observed [15,17–19]. To date, the biological significance of the Cdc6 polymorphisms has not been reported. Considering the vital role of Cdc6 in the cell proliferation, we reason that some sequence variants in the regulation region may influence the expression level of Cdc6 and thereby modulate the individual's susceptibility to cancer.

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide. Its incidence rate displays striking racial and geographic differences. HCC is highly prevalent in China, especially in the southern region [20]. It has been shown that both genetic and environmental factors are involved in the etiology and its prevalence is much higher in Chinese population compared to other races [21]. Most cases of HCC are associated with the chronic infection of Hepatitis B virus (HBV) and/or Hepatitis C virus (HCV), which can induce hepatocarcinogenesis. It was observed that both HBV and HCV infection correlated with the development of HCC. However, only a small proportion of HBV or HCV infected patients develops HCC. Further investigation on the molecular mechanisms underlying the association revealed that the G-allelic Cdc6 promoter revealed a decreased transcriptional activity. Our results suggest that the Cdc6 –515A>G polymorphism is biological significant and may be employed as a biomarker for identifying the subgroup that is susceptible to HCC.

2. Materials and methods

2.1. Study subjects

This case–control study included 387 HCC patients and 389 cancer-free controls. All studied subjects were unrelated ethnic Han Chinese who lived in Guangzhou and its surrounding regions, where HCC is prevalent and HBV infection has been demonstrated as main attributable risk factor. Patients were recruited at the Cancer Center of Sun Yat-Sen University in Guangzhou, P.R. China from June 2000 to July 2005. All cases were diagnosed histopathologically. Control subjects were healthy checkup examinees without cancer history and were collected in the same period. HBV or HCV infection was diagnosed when HBV surface antigen (HBsAg) or HCV antibody (HCV-Ab) was detected by ELISA in the serum isolated from peripheral blood. In our study cohort, HBV and HCV infection were identified in 87.9% and 1.8%, respectively.

At recruitment, informed consent was obtained from each subject who was then interviewed to collect detailed information. This study was approved by the Institute Research Ethics Committee at the Cancer Center of Sun Yat-Sen University.

2.2. Genotyping

Genomic DNAs were extracted from blood samples of all subjects. Genotypes were determined by polymerase chain reaction based single strand conformation analysis (PCR-SSCA). The PCR primers, 5′-TGA GCC TGT TCA CTC TCA CCA A and 5′-GAC TGC AAG TGT CAA GAT GTG G, were used to amplify a 236-bp fragment covering the –515A>G polymorphism (rs4134994) in the Cdc6 promoter. The PCR was performed as follows: 20 ng genomic DNAs were amplified in a 20 μl reaction mixture containing 2 mM MgCl2, 0.1 mM dNTPs, 0.25 μM of each primer, and 0.5 unit of Taq polymerase (Promega, Madison, WI, USA). Thirty-five cycles of amplification were carried out at an annealing temperature of 59 °C.

The PCR product was mixed with a fourfold volume of loading buffer (95% formamide, 20 mM EDTA, 0.05% xylene cyanol and bromophenol blue), denatured 2.3. Cdc6 promoter-luciferase reporter constructs

To study the effect of the Cdc6 –515A>G polymorphism on the transcriptional activity, the promoter-luciferase reporter plasmids containing either A or G allele were constructed. The 724 bp Cdc6 promoter region from –649 to +75 relative to the transcription start site (vega transcription ID: OTTHUMT00000257129) was amplified from genomic DNA homozygous for the –515AA or GG, using primers 5′-GAT GGT ACC GCC GTT CAA ATT AGT GC (forward) and 5′-ATA CTC GAC AAC GGG GGA GGA AAT CTA C (reverse). The PCR fragments were inserted into the Kpn I and Xho I sites of the pGL3-basic vector (Promega). The pGL3-basic vector contained the firefly luciferase gene but lacked both promoter and enhancer. Therefore, the firefly luciferase was only expressed when an active promoter was inserted upstream of its coding gene.

The generated Cdc6 promoter-luciferase reporter construct that contained the –515AA genotype was designated as pGL3b-Cdc6-p515A and the one with the –515GG genotype was named pGL3b-Cdc6-p515G. The sequences of all constructs were verified by direct DNA sequencing.

2.4. Transient transfection and luciferase assays

The transcription activity of the Cdc6 promoter was measured using a dual-luciferase reporter assay system (Promega) in hepatocellular carcinoma cell lines (Hep3B, HepH2) and colon cancer cell line (HT29). The cells were seeded into 48-well culture plates one day before transfection. When the cells reached ~60% confluency, 40 ng of pRL-TK vector (Promega) and 200 ng of pGL3-basic or pGL3b-Cdc6-p515A or pGL3b-Cdc6-p515G plasmid were co-transfected by calcium phosphate precipitation. The transfected cells were harvested 24 h after transfection and the cell lysates were prepared and analyzed according to Promega’s instruction manual. Luciferase activity was determined using Infinite M200 multifunction microplate reader (TECAN, Männedorf, Switzerland). Transfections were done in duplicates and repeated at least three times in independent experiments.

The pRL-TK vector provided the constitutive expression of Renilla luciferase and was co-transfected as an internal control to correct the differences in both transfection and harvesting efficiency. Co-transfection of the pRL-TK and pGL3-basic vectors was used as a negative control. Firefly luciferase activity of each construct was normalized by Renilla luciferase activity. Relative luciferase activity that represented fold increase of the promoter transcription activity was calculated as a ratio of the normalized firefly luciferase activity of the –515A- or G-construct to the pGL3-basic vector. Difference between the Cdc6 –515A- and G-promoter activities was determined by Student’s t-test and a P-value of <0.05 was considered significant.

2.5. Electrophoretic mobility shift assay (EMSA)

The binding affinity of the Cdc6 promoter with nuclear proteins was analyzed by EMSA. Nuclear extracts from Hep3B and HepH2 cells were prepared according to the manufacturer’s protocol (NucBuster Protein Extraction Kit, Novagen, Darmstadt, Germany). Complementary oligonucleotide pairs corresponding to the Cdc6 promoter sequence were synthesized by Invitrogen (Carlsbad, CA, USA). The oligonucleotide sequences for the Cdc6 promoter were as follows: (bold letters indicate polymorphism): –515A allele, 5′-ACC TTG TAT AGA GTC CTT TGT A-3′; –515G allele, 5′-ACC TTG TAT AGA GTC CTT TGT A-3′. The complementary oligonucleotide pairs were annealed at 95 °C for 10 min by mixing 2 μl of each of two complementary oligonucleotide stocks (17.5 μM) with 16 μl H2O. Then 2 μl of the annealed oligonucleotide was end-labeled with [γ-32P]ATP at 37 °C for 1 h using T4 polynucleotide kinase (MBI Fermentas, Hanover, MD, USA). EMSA was performed using a Gel Shift Assay System (Promega). Binding reaction with 5 μg of Hep3B or HepH2 nuclear extracts and 1 μl of 32P-labeled oligonucleotide was carried out according to the manufacturer’s instructions. For the competition assay, Five- and 50-fold molar excess of unlabeled oligonucleotide was added to the binding reaction mixture as a specific competitor. As a nespecific competitor, an unlabelled scrambled oligonucleotide (5′-TIC AGA TGT CTT CTA GCT CTA G 3′) was used to show the background signal. This experiment was performed 3 times, and the results were reproducible.

2.6. Statistical analysis

A statistical power analysis was performed using a program for power and sample size computations (known as PS program) [21]. Pearson’s χ2-test was used to examine the distribution difference of the genotypes between cases and controls. Association between the polymorphism and the risk for HCC was estimated by unconditional logistic regression analysis with adjustment for age, sex and HBV status. Hardy–Weinberg equilibrium was tested by a goodness-of-fit χ2-test. These statistical analyses were done with the SPSS software package (version 13.0, SPSS Inc., Chicago, IL, USA). All statistical tests were two-sided and a P-value of less than 0.05 was used as the criterion of statistical significance.

3. Results and discussion

We first performed a statistical power analysis using the PS program to verify whether the recruited samples could provide...
adequate power in identifying the association between the polymorphism and HCC. Under the population parameter settings of the effect size of odd ratios of 0.61 and the allelic frequency of 0.392, our samples with 387 HCC cases and 389 healthy controls can provide a statistical power of 90% at the nominal Type I error rate of 0.05. The power analysis indicates that our sample size is sufficient for statistical analysis.

Genotype for each studied subject was determined by PCR-SSCA. A 236-bp Cdc6 promoter fragment was generated by PCR from genomic DNA. Three patterns of electrophoretic mobility, representing −515AA, GG and AG genotypes, were detected (Fig. 1). The relevant characteristics of the studied subjects were shown in Table 1. There were no significant differences in age or sex distribution between the cases and the controls, which suggest adequate matching on the basis of these two variables (P = 0.206 and 0.199, respectively). The incidence of HBV infection in the HCC group (87.9%) was much higher than that in the control subjects (13.4%, respectively). The incidence of HBV infection in the HCC group (87.9%) was much higher than that in the control subjects (13.4%, respectively). The incidence of HBV infection in the HCC group (87.9%) was much higher than that in the control subjects (13.4%, respectively). The incidence of HBV infection in the HCC group (87.9%) was much higher than that in the control subjects (13.4%, respectively). The incidence of HBV infection in the HCC group (87.9%) was much higher than that in the control subjects (13.4%, respectively). The incidence of HBV infection in the HCC group (87.9%) was much higher than that in the control subjects (13.4%, respectively). The incidence of HBV infection in the HCC group (87.9%) was much higher than that in the control subjects (13.4%, respectively). The incidence of HBV infection in the HCC group (87.9%) was much higher than that in the control subjects (13.4%, respectively). The incidence of HBV infection in the HCC group (87.9%) was much higher than that in the control subjects (13.4%, respectively). The incidence of HBV infection in the HCC group (87.9%) was much higher than that in the control subjects (13.4%, respectively). The incidence of HBV infection in the HCC group (87.9%) was much higher than that in the control subjects (13.4%, respectively). The incidence of HBV infection in the HCC group (87.9%) was much higher than that in the control subjects (13.4%, respectively).

Growing studies have shown that SNPs in the gene promoter can alter the transcription and expression of the corresponding protein and thereby influence the individual susceptibility to cancer [15,17–19]. Therefore, to explore the molecular mechanism underlying the association of Cdc6 −515G allele with the reduced risk of HCC, we first investigated the potential effect of the −515G polymorphism on the Cdc6 transcription, by examining the luciferase activity of the reporter constructs driven by human Cdc6 promoter that contained the −515A or −515G allele. As shown in Fig. 2, the relative luciferase activities driven by the variant G-allelic Cdc6 promoter were 40%, 47% and 53% of those driven by the wild-type A-allelic promoter in Hep3B, HuH7 and HCT116 cell lines, respectively (P = 0.005, 0.002 and 0.001, respectively). As a key regulator of DNA replication, tightly regulation of the Cdc6 expression is necessary for ensuring genetic stability and for guarding against runaway proliferation [22,23]. Previous studies have shown that transcription factor EZF can transactivate human Cdc6 promoter through two recognition sites (nt −43 to −36 and −8 to −1 relative to the transcription start site) [23–25]. Recent reports document the overexpression of Cdc6 protein in different types of malignancy including cervix cancer, non-small-cell lung carcinoma and brain tumor [11–13]. Furthermore, growing evidences have suggested that increased Cdc6 expression can promote re-replication, cause genetic instability, and promote cellular immortalization and neoplastic transformation [11,26]. Therefore, Cdc6 may function as an oncopogene in the development of cancer. We found that the G-allelic promoter of Cdc6 displayed obviously lower transcription activity compared with the A-allelic one, indicating that the individuals

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**Fig. 1.** Genotyping of the Cdc6 −515A–G polymorphism. (A) SSCA analysis. Three patterns of electrophoretic mobility were detected. (B) Direct DNA sequencing. The samples representing different patterns of electrophoretic mobility were further confirmed by direct sequencing. The polymorphic site is underlined.

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**Table 1**

The characteristics of HCC cases and normal controls

<table>
<thead>
<tr>
<th>Variable</th>
<th>Cases (n = 387)</th>
<th>Controls (n = 389)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (year)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;40</td>
<td>120 (31.0)</td>
<td>148 (38.0)</td>
<td>0.206</td>
</tr>
<tr>
<td>40–49</td>
<td>93 (24.0)</td>
<td>87 (22.4)</td>
<td></td>
</tr>
<tr>
<td>50–59</td>
<td>96 (24.8)</td>
<td>89 (22.9)</td>
<td></td>
</tr>
<tr>
<td>≥60</td>
<td>78 (20.2)</td>
<td>65 (16.7)</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>343 (88.6)</td>
<td>330 (84.8)</td>
<td>0.119</td>
</tr>
<tr>
<td>Female</td>
<td>44 (11.4)</td>
<td>59 (15.2)</td>
<td></td>
</tr>
<tr>
<td>HBV[b]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>340 (87.9)</td>
<td>52 (13.4)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Negative</td>
<td>47 (12.1)</td>
<td>337 (86.6)</td>
<td></td>
</tr>
</tbody>
</table>

* Two-sided χ2-test.

**Table 2**

Association between the Cdc6 −515A–G polymorphism and the risk of HCC

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Cases (n = 387)</th>
<th>Controls (n = 389)</th>
<th>Adjusted OR (95% CI)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>204 (52.7)</td>
<td>152 (39.1)</td>
<td>1.00 (reference)</td>
<td></td>
</tr>
<tr>
<td>AG</td>
<td>151 (39.0)</td>
<td>169 (43.4)</td>
<td>0.64 (0.40–1.01)</td>
<td>0.055</td>
</tr>
<tr>
<td>GG</td>
<td>32 (8.3)</td>
<td>68 (17.5)</td>
<td>0.36 (0.18–0.72)</td>
<td>0.004</td>
</tr>
<tr>
<td>AG + GG</td>
<td>183 (47.3)</td>
<td>237 (60.9)</td>
<td>0.56 (0.36–0.86)</td>
<td>0.008</td>
</tr>
</tbody>
</table>

* Data were calculated by unconditional logistic regression with adjustment for age, sex and HBV status. OR, odds ratio; CI, confidence interval.

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with GG genotype may have a lower expression level of Cdc6 and therefore are in reduced risk for HCC. The dose-dependent protective effect of the G allele displayed in our case–control study further support this contention.

SNPs in the promoter region of gene can potentially alter the affinity of DNA–nuclear protein interactions and in turn affect the efficiency of transcription [15,16]. We next performed EMSA to evaluate the influence of −515A>G polymorphism on the binding affinity of the Cdc6 promoter with putative transcription factor(s). When the radiolabeled −515A or −515G probe was incubated in the presence of nuclear proteins extracted from Hep3B cell line, a DNA–protein complex was formed (Fig. 3, indicated by an arrow) and the −515G probe showed stronger DNA–protein binding activity compared with the −515A probe (lanes 2 and 9). To further assess the binding specificity and the differences in binding affinity between the G and A allele, competition assay was performed with unlabeled −515A or −515G oligonucleotide. The results revealed that both unlabeled −515A and −515G oligonucleotides with 50-fold molar excess fully blocked the binding of the radiolabeled −515A probe or −515G probe with nuclear protein(s) (Fig. 3, lanes 4 and 6; lanes 11 and 13). However, 50-fold molar excess of non-specific competitor did not compete for binding (Fig. 3, lanes 7 and 14). Furthermore, fivefold molar excess of unlabeled −515A oligonucleotide was not as effective as the same amount of unlabeled −515G probe in disrupting the binding of the radiolabeled −515A probe or −515G probe with nuclear factor(s) (Fig. 3, lanes 3 and 5; lanes 10 and 12). The results were reproducible when the nuclear extracts from Huh7 were used (data not shown). These data suggest that the −515A→G polymorphism can affect the binding affinity of the Cdc6 promoter with transcription factor(s) and the variant G allele has a stronger binding strength compared with the A allele. Considering that the G-allele displays lower promoter transcription activity, we infer that the nuclear factor(s) bound to the region covering the −515A>G polymorphism may function as negative regulator(s) for Cdc6 transcription. Therefore, the −515A>G polymorphism may affect the Cdc6 promoter binding affinity with nuclear proteins and in turn the Cdc6 expression, which consequently modulates the individual’s susceptibility to HCC.

In summary, we have provided the evidence for a novel functional −515A>G polymorphism in the Cdc6 promoter that is

![Fig. 2. Transcription activity of the Cdc6 promoter with the sequence of −515A or −515G. The promoter transcription activity was measured by a dual luciferase reporter system (for details, please see Section 2). Firefly luciferase activity of each construct was normalized by Renilla luciferase activity. Relative luciferase activity that represented fold increase of the promoter transcription activity was calculated as a ratio of the normalized firefly luciferase activity of the −515A- or G-construct to the pGL3-basic vector. Values represent the mean ± S.D. of at least three independent experiments done in duplicates.](image)

![Fig. 3. Electrophoretic mobility shift assay (EMSA) of the Cdc6 promoter region containing the −515A-G site. The analysis was performed in the presence (+) or absence (−) of Hep3B nuclear extract. Each binding reaction contained γ-32P-labeled −515A (lanes 1–7) or −515G (lanes 8–14) probe. Five- or 50-fold (as indicated) molar excess of unlabeled/cold −515A or −515G oligonucleotides (lanes 3–6 or 10–13) were included in the binding reactions as specific competitors. Fifty-fold molar excess of unlabeled non-specific oligonucleotides was used (lanes 7 and 14). Labeled oligonucleotides incubated without the nuclear extracts were included as negative controls (lanes 1 and 8). The Arrow indicates the DNA–protein complexes.](image)
associated with the risk of HCC. Clearly, additional investigations are warranted to confirm our findings before we can employ this polymorphism as a biomarker for identifying the subgroup that is susceptible to HCC.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgements

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References