Genetic and epigenetic alterations of \textit{RIZ1} and the correlation to clinicopathological parameters in liver fluke-related cholangiocarcinoma

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Abstract. The retinoblastoma interacting zinc finger (\textit{RIZ1}) gene is adjacent to D1S228 where microsatellite instability has been associated with poor patient survival in liver fluke-associated cholangiocarcinoma (CCA). An understanding of the molecular mechanisms underlying the carcinogenesis and pathogenesis of CCA is necessary to improve patient survival. Therefore, we determined the genetic and epigenetic alterations of \textit{RIZ1} in 81 CCA samples and 69 matched non-tumor tissues. Methylation was found in 31 of 81 (38\%) tumor samples and in 5 of 69 (7\%) matched non-tumor tissues. Frameshift mutations (2 of 81) and loss of heterozygosity (LOH) (14 of 81) were not common. Statistical analysis found no significant correlation between \textit{RIZ1} alterations and clinicopathological features, but RIZPro704 LOH was associated with patient survival in the multivariate analysis. \textit{RIZ1} hypermethylation may be one of the crucial molecular events contributing to cholangiocarcinogenesis, and RIZPro704 LOH may adversely impact patient survival. The biological function of \textit{RIZ1} in CCA should be further investigated in order to verify its potential role in regulating this cancer.

Introduction

Cholangiocarcinoma (CCA), a malignancy of the biliary epithelium, is the major type of liver cancer found in northeast Thailand (1). The high incidence of CCA in this region is strongly associated with a high prevalence of liver fluke (\textit{Opisthorchis viverrini}) infection. Chronic irritation and inflammation caused by liver fluke infection are major factors contributing to the carcinogenesis and pathogenesis of CCA (2). Surgical resection is currently the most successful and accessible therapeutic method for CCA patients but is associated with poor survival. Hence, insights into the molecular mechanisms of carcinogenesis and pathogenesis are necessary for coping with this disease.

Our previous study on fine mapping at 1p36-pter revealed a significant association of microsatellite instability (MSI) at D1S228 with poor survival in CCA patients (3). D1S228 is adjacent to the gene, \textit{retinoblastoma interacting zinc finger} (\textit{RIZ}) (4). There are two isoforms of \textit{RIZ}, \textit{RIZ1} and \textit{RIZ2}, which are encoded by different promoters (5). Their amino acid sequences are almost identical except for the presence of an N-terminal PR (PRDI-BF1 and RIZ) domain in \textit{RIZ1} resulting in a reduction in cell proliferation and an induction of apoptosis (12). Several studies have demonstrated that \textit{RIZ1} is a downstream effector of the estrogen receptor (ER) pathway (13,14),
and its expression is decreased after estradiol treatment (14,15). In the absence of estradiol (E2), biological active estrogen, RIZ1 was found to bind directly to the DNA adjacent to the promoter region of ER target genes and to inhibit the transcription of these genes by methylating lysine 9 of histone H3 (14). The presence of E2 changes the role of RIZ1 from being a histone methyltransferase to an ER coactivator thus enhancing the maximum response to E2 (14). In addition, the ER signaling pathway can be activated by either estrogen or the growth factor signaling pathway such as IGF-1 (16).

Alterations of RIZ1 through both genetic and epigenetic mechanisms have been reported (17,18). Epigenetic inactivation by promoter hypermethylation is the most common mechanism leading to decreased expression of this gene in many types of cancers (19,20). As for genetic alterations, the majority are frameshift mutations at polyadenosine tracts, A8 and A9, located at the PR binding domain (21). The second most common genetic defect of RIZ1 in many types of cancer is loss of heterozygosity (LOH) (17,18,22). Other types of mutations are rare (21,23). The purpose of this study was to investigate the genetic and epigenetic defects of RIZ1 in CCA samples. Associations between RIZ1 alterations and clinico-pathological data were analyzed. Univariate and multivariate Cox regression were used for survival analysis.

Materials and methods

Patients. Informed consent was obtained from each patient according to the guidelines of the Ethics Committee of Khon Kaen University (HE500634). Blood and liver resection samples were obtained from 81 intrahepatic CCA patients undergoing surgery at Srinagarind Hospital, Faculty of Medicine, Khon Kaen University. DNA was extracted from leukocytes, frozen tissues and microdissected tissues as described previously (3,24). DNA samples obtained from frozen liver tissues were used for methylation analysis, and leukocyte and microdissected DNA samples were used for genetic studies including intragenic allelic alteration and frameshift mutation.

Primer sequences and annealing temperature (T_m) and product size.

<table>
<thead>
<tr>
<th>Methods</th>
<th>Sequences</th>
<th>T_m (˚C)</th>
<th>Product size (bp)</th>
<th>Reference no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylation</td>
<td>F: GGGTTGGTTATGCGGCCAAGCAGC        R: GCTATTTCCACACCGGCGCCAGG</td>
<td>68</td>
<td>177</td>
<td>32</td>
</tr>
<tr>
<td>Unmethylation</td>
<td>F: TGTTGGTATGCGGCCAAGCAGC        R: ACTATTTCCACACCGGCGCCAGG</td>
<td>64</td>
<td>175</td>
<td>32</td>
</tr>
<tr>
<td>LOH</td>
<td>RIZCA F: GGTGAAAACGTTGAAATCAGCACTG R: CAGAGCATAGTTGCTACTTTCGCTT</td>
<td>58</td>
<td>~207</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>RIZPro704 F: CCAAGAATAACTAUCTCCTT R: ACTCCATGGCTGGGACGTC</td>
<td>58</td>
<td>~266</td>
<td>22</td>
</tr>
<tr>
<td>Frameshift mutation</td>
<td>RIZA8 F: GAGCTCAGCAAAATGTGTCGTC R: CAAGTCGGCTTCTGCTTTTCGCTT</td>
<td>62</td>
<td>116</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>RIZA9 F: TCTCACATCCGCTTCATTCTGC R: GTGATGAGTCGCTCCACCTTTTC</td>
<td>62</td>
<td>144</td>
<td>23</td>
</tr>
</tbody>
</table>

Methylation-specific PCR (MSP). After bisulfite modification, DNA derived from 81 tumor and 69 matched non-tumor tissues of CCA patients were analyzed for RIZ1 promoter methylation using MSP as described previously (18,24,25). The concentration of MgCl2 used was 5 mM and the PCR reaction was hot-started at 95˚C for 5 min before addition of 1.5 units of Taq polymerase. Human placental DNA treated with SssI methylase (New England Biolabs, Ipswich, MA, USA) and human leukocyte DNA served as positive controls for the methylated and unmethylated reactions, respectively.

Intragenic allelic alteration and frameshift mutation analysis. LOH and MSI were determined as described previously (22,26). Markers included RIZCA and RIZPro704 located at the intron preceding exon 5 and amino acid residue 704 (Pro704) in exon 8, respectively. LOH was determined for both RIZCA and RIZPro704, and MSI was determined for RIZCA. Frameshift mutations were analyzed by PCR amplification of the repeated sequences in the coding regions (27). Primer sequences of A8 and A9 tracts were obtained from a previous report (23). Genetic alterations were analyzed using the GS-3000 gel scan fragment auto analyzer (Corbett Research, Australia).
DNA was extracted from undergoing surgery at Srinagarind Hospital, Faculty of Medicine, Khon Kaen University. DNA samples obtained from patients.

**Statistical analysis.** Clinicopathological features of the CCA patients including age, gender, tumor stages, histological types, blood vessel invasion, nerve invasion and lymphatic invasion were analyzed for correlations with RIZ1 alterations using the Chi-square test. Survival was assessed using the Kaplan-Meier log-rank method and Cox regression. All variables shown to be significant (P<0.150) in the univariate analyses were entered into a multivariate model using Cox's proportional hazards model in a backward stepwise manner and the log-likelihood ratio approach. Statistical analyses were performed using SPSS for Windows, version 15 (SPSS, Inc., Chicago, IL, USA). Two-sided values of P<0.05 were considered statistically significant.

**Results**

**RIZ1 promoter hypermethylation and intragenic alteration in CCA patients.** The frequency of RIZ1 promoter hypermethylation determined using MSP in 81 tumors and 69 matched non-tumor specimens from CCA patients was 38 (31 of 81) and 7% (5 of 69), respectively (P=0.006). DNA methylation was found in non-tumor samples only when its matched tumor sample also showed methylation. Representative results concerning the determination of RIZ1 methylation are shown in Fig. 1.

LOH was observed in 14 of 81 (17%) CCA cases, comprising 4 of 56 (7%) at RIZCA and 10 of 52 (19%) at RIZPro704 (representative results in Fig. 2). LOH at RIZPro704 and LOH at RIZCA were significantly independent (P=0.029). Frameshift mutations were found only at the A9 tract in 2 (2.5%) cases (Fig. 3). MSI at RIZCA was found in 8 (10%) cases.

As shown in the Venn-Euler diagram (Fig. 4), a RIZ1 alteration was found in 42 (52%) cases. A simultaneous alteration was found in 5 (6%) cases, 3 of which were methylated with LOH and 2 methylated with a frameshift mutation. RIZ methylation alone was found in 26 (32%) cases. LOH alone was found in 11 (14%) cases; 39 (48%) cases had no LOH or DNA methylation.

**Discussion**

DNA methylation was detected in matched non-tumor samples (7%) suggesting that methylation occurs early in carcinogenesis. This finding corroborates that of a previous study which found RIZ1 methylation in precancerous lesions (17). Since one study involving prostate cancer showed that RIZ1 methylation
is not associated with patient clinicopathological features but may be associated with carcinogenesis (28), it is likely that inactivation of RIZ1 by promoter hypermethylation may play a similar role in CCA. Moreover, the non-tumor cells used in our study, although having a normal appearance under gross and microscopic examination, may have already undergone genetic and/or epigenetic alterations. Nevertheless, the methylated bands found in most of the non-tumor samples were much less intense than those observed in the tumor specimens.

LOH at RIZPro704 was a significant independent predictor for postoperative survival (Cox regression, P=0.027). This finding corroborated previous studies involving colorectal
cancer (7) and parathyroid tumors (18) where RIZPro704 LOH was higher than and mostly independent of RIZCA LOH. Almost all RIZPro704 LOH+ samples (8 of 10) lost the smaller allele (Pro704+) which resulted from a deletion polymorphism. RIZPro704 is located in the RIZ1 coding region; however, its contribution to RIZ1 function in cancer is not much understood. Since RIZPro704 is close to the ER binding motif (amino acids 864-1,046 of RIZ1 protein) (13), this residue may be important for maintaining RIZ1 conformation. For this reason, interaction between ER and RIZ1 may occur only with the wild-type RIZ1 (Pro704+), which does not harbors a deletion polymorphism at Pro704. Loss of Pro704+ with remaining Pro704+ might be favorable for interaction between RIZ1 and ER. In a previous study, ER was up-regulated in 80% of CCA cases, while it was rarely expressed in normal liver tissues (29). IGF-1 and IGF-IR expression was found to be repressed by RIZ1 (12) while expression increased to approximately 60% in human intrahepatic CCA cases, whereas their expression was not detected in normal human liver tissues (29). Taken together, we postulated that the up-regulation of ER in CCA inhibits the tumor suppressive activity of RIZ1 and activates the expression of some target genes involved in cell proliferation such as IGF-1 resulting in poor prognosis of the patient. However, its response to estrogen and its association with bone mineral density in women remains controversial (30,31). Therefore, the biological role of RIZ1 and its response to ER signaling in CCA require further investigation.

The percentages of MSI at RIZCA (9.9%) and at D1S228 (11.2%) (3) are similar, indicating the defect of mismatch repair genes. Thus, we expected that the frequency of RIZ1 frameshift mutation in these samples might be similar to the MSI frequency found in both loci. Surprisingly, the frequency of frameshift mutations in RIZ was very low (2.5%) indicating that a frameshift mutation is not a common mechanism for RIZ inactivation in CCA, although its frequency is higher in other types of tumors (23). In conclusion, the present study showed that, in CCA, genetic alterations of RIZ1 such as LOH and frameshift mutations are not common compared to epigenetic alterations such as promoter hypermethylation. Epigenetic inactivation in RIZ1 may occur at an early step in the process of carcinogenesis. Pro704 LOH was correlated to poor patient survival; however, further study is needed to elucidate the mechanisms involved in CCA.

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