

RESEARCH ARTICLE

An Interleukin-6 Receptor Polymorphism is Associated with Opisthorchiasis-Linked Cholangiocarcinoma Risk in Thailand

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Abstract

The cholangiocarcinoma (CCA) is a relatively rare cancer worldwide but it is highly prevalent in Thailand where the liver fluke, *Opisthorchis viverrini* is endemic. There are reports that interleukin 6 (IL-6) may play an important role in the pathogenesis of opisthorchiasis associated CCA. Functionally, IL-6 can act on target cells through its receptor, IL-6R, and IL-6R polymorphisms may affect the functional activity of IL-6 leading to susceptibility to cholangiocarcinogenesis. Therefore, we assessed the association of the 48892 A/C (Asp358Ala) polymorphism in exon 9 of the IL-6R gene in 79 CCA cases compared to 80 healthy controls using the PCR-RFLP technique. The results showed significant differences between CCA cases and controls in overall genotype ($p=0.001$) and allele frequencies ($p=0.0002$). Chi-square for trend test revealed a significant association between genotype and CCA susceptibility ($p=0.0002$). The odds ratios (ORs) for genotype were 0.283 (95% CI=0.131-0.605, AC vs. AA; $p=0.0003$) and 0.206 (95% CI=0.196-1.245, CC vs. AA; $p=0.0416$), the OR for alleles was 0.347 (95% CI=0.187-0.633, allele C vs. allele A; $p=0.0002$) and that for the carrier C variant was 0.272 (95% CI=0.130-0.564; $p=0.0001$). This study demonstrated a close association between an IL-6R polymorphism, specifically higher A allele, and cholangiocarcinoma.

Keywords: Interleukin 6 polymorphism - cholangiocarcinoma - opisthorchiasis - PCR-RFLP

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Introduction

Cholangiocarcinoma (CCA) is a rare malignancy worldwide but it is relatively prevalent in Asian countries (Shin et al., 2010). Several risk factors for this fatal primary liver cancer in the region have been documented such as inflammatory bowel disease (Huai et al., 2014), metabolic syndromes (Wu et al., 2012) and hepatitis virus (Matsumoto et al., 2014). However, the most known risk factor of CCA in Southeast Asia is the human liver fluke, *Opisthorchis viverrini* which is designated as Group 1 carcinogen by the World Health Organization (IARC, 1994; Bouvard et al., 2009). Thailand has reported the highest incidence of CCA in the world that is associated with *O. viverrini* infection (Sripa and Pairojkul, 2008; Manwong et al., 2013). Pathogenesis of the liver fluke-induced CCA may be from mechanical injury and inflammatory reaction to fluke metabolic products (Sripa et al., 2007; 2012a). The continuous irritation and chronic inflammation may bring about enhancing certain cytokine and growth factor expression leading to cholangiocarcinogenesis (Subrungruanga et al., 2013).

Among others, elevated *O. viverrini* specific interleukin-6 (IL-6) production has been reported from peripheral blood mononuclear cells from infected individuals with advanced periductal fibrosis and CCA (Sripa et al., 2009; Sripa et al., 2012b) as well as cholangiocytes cocultured with *O. viverrini* excretory-secretory products (Ninlawan et al., 2010). This pro-inflammatory cytokine may potentiate the proliferation and survival of cholangiocytes within local microenvironment (Sripa et al., 2012a).

A pleiotropic IL-6 cytokine plays a role of fibrogenic, mitogenic and survival factors as an autocrine and paracrine manner. These may be by activating the pro-survival p38 mitogen activated protein kinase and induces the expression of anti-apoptotic myeloid cell leukemia-1 (Mcl-1) through STAT3 and AKT signaling pathways (Park et al., 1999; Malhi and Gores, 2006; Blechacz and Gores, 2008; Wise et al., 2008). Functional activity of IL-6 on the target cells occurs by binding to IL-6-specific receptor (IL-6R) both a membrane-bound (mIL-6R) and soluble form (sIL-6R) together with gp130 (Heinrich et al., 1998; 2003). There are several studies describing the association between single nucleotide polymorphisms

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(SNPs) of the IL-6R and IL-6 activity such as Kim et al. (2003). Among these, the non-synonymous 48892A/C SNP in exon 9 (rs8192284) resulting in an amino-acid substitution from Asparagine to Alanine (D358A) at the proteolytic site (Mullberg et al., 1993; 1994) have shown to be associated with inflammatory diseases, i.e. rheumatoid arthritis (Lamas et al., 2010; Marinou et al., 2010) and periodontitis (Galicia et al., 2006); metabolic syndrome i.e. obesity (Esteve et al., 2006; Jiang et al., 2010) and diabetes (Wang et al., 2005), as well as malignancy, i.e. melanoma (Gu et al., 2008). However, there is no study of this IL-6R variant in CCA. In this study, therefore we aimed to explore the IL-6R polymorphism and its association with the risk of CCA in Thai patients.

Materials and Methods

Study subjects

This work was performed using age-sex matched case-control study. Seventy nine histologic proven CCA cases were patients who underwent surgery at Srinagarind Hospital, Faculty of Medicine, Khon Kaen University, Khon Kaen Province, Thailand. These patients were referred from opisthorchiasis endemic areas of Khon Kaen or neighboring provinces. The controls (n=80) were recruited from individuals living in the Northeast Thailand who showed no hepatobiliary abnormalities, i.e. portal vein radical echoes, echoes in liver parenchyma, indistinct gall bladder wall, gallbladder sludge or liver mass (Mairiang et al., 2012). Demographic data of the cases and controls is shown in Table 1. All cases and controls gave informed consent for their participation in the study which was approved by the Khon Kaen University Ethic Committee for Human Research, Khon Kaen University, Thailand (HE561089 and HE551323).

Analysis of the 48892A/C SNP in exon 9 of IL-6R gene polymorphism

Genetic polymorphism of the non-synonymous 48892A/C SNP in exon 9 of IL-6R gene (rs8192284) was done by polymerase chain reaction with restrict fragment length polymorphism (PCR-RFLP) as previously described by Bustamante et al. (2007). Briefly, genomic DNA was extracted from peripheral blood samples from controls and CCA cases using Genra PureGene® Kit (QIAGEN®). A 286 bp fragment was amplified by polymerase chain reaction technique using two specific primers covering the Asp358Ala (A/C) polymorphism of IL-6 receptor gene: forward primer (5'-AAG CTT GTC AAA TGG CCT GT-3') and reverse primer (5'-GGA CCC ATC TCA CCT CAG AA-3'). The PCR mixture in a final volume of 25 µl reaction mixture containing of about 20 ng genomic DNA and 12.5 µl of 2X GoTaq® colorless

master mix with final concentration of 20 µM of each dNTP, 1.5 mM MgCl₂, 0.75 mM primers, and 1.25U Taq DNA polymerase. PCR condition consisted of an initial denaturing step at 95°C for 5 min, followed by 35 cycles of denaturing at 95°C for 40s, annealing at 60°C for 40s and polymerization at 72°C for 40s, with a final elongation step at 72°C for 5 min. PCR products were digested with the restriction enzyme HinfI (Fermentus) overnight at 37°C, subjected to electrophoresed, stained with ethidium bromide and photographed under UV illumination. The homozygote CC samples remained uncut, while the AA homozygotes gave two DNA fragments of 101 and 185 bp, respectively (Figure 1).

Statistical analysis

Statistical analyses were carried out with STATA Software (Stata Corp LP, College Station, TX, USA). Differences in the genotypic and allelic distribution between groups of cases and control subjects were evaluated by using Chi-squares test. Deviation from Hardy-Weinberg equilibrium (HWE) was evaluated by comparing observed and expected genotype frequencies by an exact goodness-of-fit test separately in cases and controls. The odds ratio (OR) and associated 95% confidence intervals (CIs) were calculated as estimates of the strength association between Asp358Ala (A/C) (rs8192284) in exon 9 of IL-6R gene polymorphisms and the risk of development of CCA using unconditional logistic regression. All statistical tests were two sided and p values <0.05 were considered as statistically significance.

Results

The genotypic distribution of Asp358Ala (A/C) (rs8192284) in exon 9 of IL-6R gene polymorphisms in all groups did not deviate from Hardy-Weinberg equilibrium (HWE) (p>0.05). The comparison of genotype and allele frequencies between CCA cases and controls is shown in Table 2 and Table 3. There was a significant different in genotype frequencies between CCA cases and controls (p<0.001), and allele frequencies (p=0.0002). Moreover, Chi-squares for trend test demonstrated a significant association between genetic doses and CCA susceptibility (p=0.0002).

Table 1. Characteristics of Subjects

Characteristics	Total patients (n=159)	CCA (n=79)	Healthy controls (n=80)
Mean age (years)	52±6.5 (33=65)	54±7.1 (33=65)	51±5.9 (33=60)
Gender (%)	159 (100%)	79 (100%)	80 (100%)
Male	101 (64%)	50 (63%)	51 (64%)
Female	58 (36%)	29 (37%)	29 (36%)

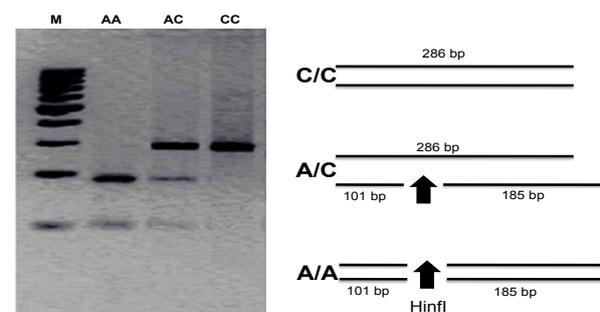


Figure 1. Analysis of IL-6R Genetic Polymorphism. The human Asp358Ala (A/C) (rs2228145) of exon 9 of IL-6R gene polymorphism was determined using PCR-RFLP. A/A: Genotype Asp/Asp in 101/185 bp; A/C: Genotype Asp/Ala in 101/185/286 bp; C/C: Genotype Ala/Ala in uncut 286 bp. M=Marker

Table 2. Association Test between Cases and Controls

Test for association	χ^2	P
Genotype frequency difference test	14.86	0.001
Allele frequency difference test	13.91	0.0002
Chi-square for trend test	13.77	0.0002

Table 3. Distribution Frequencies of the IL-6R A358D (A/C) Polymorphism among Cases and Controls, and Risk Analysis Results for Each Genotype, Allele and Carrier Rates

	Samples (n=80)		P	OR	95% CI
	Controls No. (%)	CCA No. (%)			
Genotype					
AA	37 (46%)	60 (73%)	-	1.00	Reference
AC	37 (46%)	17 (24%)	0.0003	0.28	0.13-0.61
CC	6 (8%)	2 (3%)	0.0416	0.21	0.20-1.25
Allele					
A	111 (69%)	137 (87%)	-	1.00	Reference
C	49 (31%)	21 (13%)	0.0002	0.35	0.19-0.63
Carrier rate					
A (+)	74 (46%)	77 (49%)	0.15	3.12	0.53-32.36
A (-)	6 (4%)	2 (1%)	-	1.00	Reference
C (+)	43 (27%)	19 (12%)	0.0001	0.27	0.13-0.56
C (-)	37 (23%)	60 (38%)	-	1.00	Reference

The risk assessment of genotype or allele among cases and controls is shown in Table 2. There was a significant association between genotype or allele between CCA cases and controls. The odds ratios (ORs) of genotype were 0.283 (95% CI=0.131-0.605, AC vs. AA; p=0.0003) and 0.206 (95% CI=0.196-1.245, CC vs. AA; p=0.0416), the ORs of allele was 0.347 (95% CI=0.187-0.633, allele C vs. allele A; p=0.0002) and the ORs of carrier C variant was 0.272 (95% CI=0.130-0.564; p=0.0001).

Discussion

Interleukin-6 is a multifunctional cytokine and has been reported to play pivotal roles in pathogenesis of various cancers including pancreatic (Okada et al., 1998), gastric (Wu et al., 1996) and renal cell carcinomas (Dosquet et al., 1994) as well as CCA (Goydos et al., 1998). For opisthorchiasis, Sripta et al. (2009) recently reported a positive relationship between *O. viverrini*-specific IL-6 and advanced periductal fibrosis (APF). Moreover, significantly high elevation of serum IL-6 was found in opisthorchiasis-associated CCA and slightly elevation in opisthorchiasis-associated APF compare to controls (Sripta et al., 2012b). These data suggest that IL-6 may be involved in the pathogenesis of opisthorchiasis associated CCA. Since IL-6 exerts its biological effects by binding to IL-6R and genetic polymorphisms of this gene have been reported in several diseases including malignant tumors (Gu et al., 2008). Our study adds on the information of polymorphism of IL-6R at Asp358Ala (A/C) in exon 9 has a relative risk of CCA development.

Significant association between IL-6R variants, especially rs8192284 SNP and plasma IL-6 levels has been documented (Qi et al., 2007; Reich et al., 2007).

The IL-6R rs2228145 (also called rs8192284) is one of the novel seven single nucleotide polymorphisms (SNPs) of IL-6R gene previously reported in Korean population (Kim et al., 2003). This gene variation encodes a functional Ala358Asp non-synonymous amino acid substitution located at the site where the mIL-6R is cleaved to form sIL-6R. In our present study, we found that polymorphism of IL-6R gene associated with the risk of CCA in Northeast Thai population. Specifically, IL-6R +48892 AA genotype significantly increased CCA risk but not advanced periductal fibrosis (unpublished). As a surrogate susceptibility gene, several studies suggested that this polymorphic variant is associated with many diseases including metabolic syndromes (Esteve et al., 2006; Jiang et al., 2010), inflammatory diseases (Lamas et al., 2010; Marinou et al., 2010) as well as malignancy (Gu et al., 2008). All these reports speculate a high risk is associated with the lower allele frequency of allele C or the higher allele frequency of allele A. Similarly, our results showed that the allele C carrier reduced risk of CCA (OR=0.27; 0.13-0.56 p=0.0001) and it is in a genetic dosage-dependent manner (Chi-squares for trend, p=0.0002).

Functional activity within individuals who carry this IL-6R polymorphism (rs8192284) has not been reported in CCA. According to previous studies (Mullberg et al., 1994; Galicia et al., 2004; Hull et al., 2007; Reich et al., 2007; Marinou et al., 2010), possible mechanism(s) may be involved with the production of soluble IL-6R levels. The soluble IL-6R is the product resulted from differential proteolytic cleavage and/or differential alternative splicing. Specifically, the differential proteolytic cleavage caused by rs8192284 SNP minor allele (allele C carriers) yield a cleavage site which is susceptible to several metalloproteinases (ADAM). Mullberg et al. (1994) demonstrated that point mutation at the cleavage site within IL-6R Gln357/Asp358 and substituted with Gly, Phe, Leu or Arg reduce the shedding around 22-54% of the wild-type sIL-6R. Double point mutations, Gln357-Asp358 to Ala357-Ala358 reduced the sIL-6R shedding to 34%. Other studies support the rs8192284 SNP minor allele (allele C carriers) significantly increase serum sIL-6R (Galicia et al., 2004; Reich et al., 2007; Marinou et al., 2010). Moreover, this variant is also associated with systemic IL-6 and CRP levels (Reich et al., 2007). Another possible mechanism is that rs8192284 SNP minor (allele C) may be associated with alternative splicing of IL-6R pre-mRNA, leading to shedding soluble IL-6R form more than membrane-bound form (mIL-6R) (Hull et al., 2007). Therefore, this allele C carrier produces mutant amino acid codon of IL-6R may have less pro-inflammatory function upon IL-6 activation. On the other hand, more soluble IL-6R produced from this SNP may neutralize IL-6 activity and leading to less proinflammatory downstream signaling and less pathology than that of A allele.

In conclusion, we demonstrated that the IL-6R SNP, especially A allele is significantly associated with higher risk of cholangiocarcinoma. Further study should be carried out to clarify biological functions of this IL-6R polymorphism in details.

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