RESEARCH ARTICLE

Association of NRF2 Polymorphism with Cholangiocarcinoma Prognosis in Thai Patients

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Abstract

Cholangiocarcinoma (CCA), a malignancy of biliary duct with a very poor prognosis, is the leading cause of cancer death in countries of the Mekong subregion. Liver fluke infection is the main etiological factor, but genetic variation has been recognized as also important in conferring susceptibility to CCA risk. Nuclear factor (erythroid derived 2)-like 2 (NRF2) is a key transcription factor in detoxification and antioxidant defense. Emerging evidence has demonstrated that genetic polymorphisms in the NRF2 gene may be associated with cancer development. The objectives of this study were to investigate the association of NRF2 genetic polymorphism with CCA risk and to evaluate the influence of the NRF2 genotype on survival time of affected patients. Single nucleotide polymorphisms (SNPs) of the NRF2 gene, including rs6726395: A/G, rs2886161: C/T, rs1806649: C/T, and rs10183914: C/T, were analyzed using TaqMan® SNP genotyping assays. Among 158 healthy northeastern Thai subjects, the allele frequencies were 41, 62, 94, and 92%, respectively. The correlation of NRF2 SNPs and CCA risk was analyzed in the 158 healthy subjects and 198 CCA patients, using unconditional logistic regression. The results showed that whereas the NRF2 SNPs were not associated with CCA risk ($p>0.05$), Kaplan-Meier analysis of 88 intrahepatic CCA patients showed median survival time with rs6726395 genotypes of GG and AA/AG to be 344±138 (95%CI: 73-615) days and 172±37 (95%CI: 100-244) days, respectively, ($p<0.006$). On multivariate Cox proportional hazard analysis, the GG genotype of rs6726395 was found to be associated with longer survival with a hazard ratio of 0.54 (95%CI: 0.31-0.94). In addition, non-papillary adenocarcinoma was associated with poor survival with a hazard ratio of 2.09 (95%CI: 1.16-3.75). The results suggest that the NRF2 rs6726395 polymorphism can be a potential prognostic biomarker for CCA patients.

Keywords: NRF2 - polymorphism - cholangiocarcinoma - Thai population - prognosis

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Introduction

Nuclear factor (erythroid derived 2)-like 2 (NRF2) is a master transcriptional activator regulating the expression of various detoxifying and antioxidant genes in response to environmental and endogenously derived oxidative/electrophilic stressors. NRF2 regulates the expression of several genes encoding detoxifying and antioxidant proteins such as NAD(P)H: quinone oxidoreductase 1 (NQO1), glutathione-S-transferases (GSTs), superoxide dismutase (SOD), heme oxygenase 1 (HO1), glutathione peroxidase (GPx), and γ-glutamate cysteine ligase regulatory (GCLγ) subunit (Rushmore et al., 1991; Venugopal and Jaiswal, 1996; Taguchi et al., 2011). NRF2 functions as a critical molecule in the defense mechanism against oxidants. Consistently, many previous reports showed that disruption of the NRF2 gene resulted in an oxidant-antioxidant imbalance and development of severe oxidative stress. The loss of functional NRF2 is linked to amplification of inflammatory response and diverse inflammation related conditions by uncontrolled chronic oxidative stress. Recently, NRF2 have received increasing attention for its paradoxical roles in cancer promotion.

Accumulated evidence shows that constitutively high expression of NRF2 promotes cancer development and contributes to chemoresistance by creating an environment conducive for cancer survival (Taguchi et al., 2011; Ma et al., 2012). Defective NRF2 signaling pathway may increase cancer susceptibility. Targeting NRF2 is shown to effectively enhance chemotherapeutic agent in suppression of tumor growth in several animal models (Ren et al., 2011; Manandhar et al., 2012). As genetic polymorphism plays an important role in gene expression and apparent phenotypic characteristics, there is little information regarding the frequency distribution of NRF2 alleles among populations and its association of NRF2 functions with cancer risk. For example, frequencies of rs1806649 and rs10183914 in European population

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are lower than those in Asian population (von Otter et al., 2010). Recently, some of the SNPs are supposed to affect NRF2 transcriptional activity and may impact both transcription and translation of downstream detoxifying and antioxidant genes. Previous studies showed that NRF2 SNPs were correlated with lower lung function (Masuko et al., 2011), development of inflammatory disease (Siedlinski et al., 2009), and impaired regulation of vascular function (Marczak et al., 2012). Genetic polymorphism of NRF2 on several SNPs (including rs6721961 and rs2886162) were associated with breast cancer risk (Hartikainen et al., 2012). Moreover, a recent report showed that rs6721961 polymorphism is associated with female lung cancer (Okano et al., 2013). The role of SNPs in NRF2 as the modifier of NRF2 function or as a biomarker for diseases is needed further clarification.

Cholangiocarcinoma (CCA) is a devastating malignancy of the biliary duct system with poor prognosis. The global prevalence of CCA is markedly variable with the very high incidence found in northeastern Thailand and neighboring countries. Chronic inflammation of epithelial bile duct caused by liver fluke infestation is the major risk factor of CCA observed in the Greater Mekong subregion countries, while primary sclerosing cholangitis and hepatolithiasis are risk factors in developed countries (Srivatanakul et al., 1991; Ohshima et al., 1994). Apart from environmental factors, genetic variations have been recognized as important factors conferred susceptibility to CCA risk. In previous studies, an association of the polymorphism of detoxifying/metabolizing genes, CYP1A2, NAT1, NAT2, NQO1, GSTM1, and GSTO1 with the risk of CCA has been demonstrated (Honjo et al., 2005; Prawan et al., 2005; Kukongviriyapan, 2012). This study was conducted to determine the allele frequency of some NRF2 SNPs, in Thai population where frequencies of the SNPs have been reported in some populations and shown an association with pathological conditions (Hartikainen et al., 2012; Okano et al., 2013). The analysis of the SNPs in healthy subjects and CCA patients was conducted to estimate the risk for development of CCA. Furthermore, the association of the SNPs with prognosis (as a survival time) of CCA patients was also determined.

Materials and Methods

Study populations

A total of 198 CCA patients and 158 healthy subjects as controls were recruited in this study. All participants were unrelated Thais who were native-born in northeastern Thailand. CCA patients admitted to the Srinagarind Hospital, Khon Kaen University between 1999 and 2001 were recruited in this study. The diagnosis of CCA of the patients was confirmed by histopathology. Healthy subjects were healthy individuals by physical examination and normal routine laboratory tests, and have no history of any chronic diseases. The average age of CCA patients was 55±10 (mean±SD) years, with 129 males and 69 females, while the average age of healthy subjects was 47±10 years, with 117 males and 41 females. Smoking status of the participants in this study was defined as the number of cigarette packs/year. A current smoker was defined as an individual who had been smoking at least 20 packs/year of cigarette in their lives. The non-smoker was defined as a subject who smoked less than 20 packs/year or did not smoke at all. In this study, 16.9% of CCA patients and 17.1% of healthy subjects were non-smokers. Among 198 CCA patients, 88 were intrahepatic CCA from surgical pathology findings. This study was conducted using leftover genomic DNA specimens from the study of genetic polymorphism of N-acetyltransferases in association with the risk of cholangiocarcinoma (Prawan et al., 2005). The study protocol has been approved by the Khon Kaen University Ethics Committee for Human Research.

Genotyping

Four NRF2 SNPs, including rs6726395: A/G, rs2886161: C/T, rs1806649: C/T, and rs10183914: C/T, were genotyped using genomic DNA extracted from blood. TaqMan® Pre-Designed SNP genotyping assays (Applied Biosystems, Foster City, CA, USA) were used according to the TaqMan Allelic Discrimination technology on the Applied Biosystems® 7500 Real-Time PCR Systems (Applied Biosystems).

Statistical analysis

The NRF2 SNPs were analyzed for deviation from Hardy-Weinberg equilibrium using χ^2-test. Single marker associations were performed using logistic regression under dominant and recessive models (DD+Dd *vs* dd and DD *vs* Dd+dd where D=wild-type allele and d=mutant allele).

The Kaplan-Meier survival curves were generated for patients having DD/Dd and dd NRF2 genotypes, histological classification, and others. Hazard ratios and p-values for comparisons of patients having DD/Dd and dd NRF2 genotype were calculated based on multivariate Cox proportional hazards model, adjusting for their age at diagnosis, gender, smoking status, histological classification, status of surgical margin, and metastases at presentation. The p-value threshold for statistical significance used in this study was p=0.05. The analyses were conducted using Stata software version 10.1 (Stata Corp, College Station, TX, USA).

Results

Allele frequencies and genotype distribution of NRF2 polymorphism in Thai population

The allele frequencies of four SNPs in NRF2 gene in 158 healthy control Thai population are as follows; A allele of SNP rs6726395 (A/G), C of SNP rs2886161 (C/T), C of SNP rs1806649 (C/T), and C allele of SNP rs10183914 (C/T) were 41%, 62%, 94%, and 92% respectively. The frequency distributions of the all SNPs were consistent with Hardy-Weinberg equilibrium (χ^2-test, p-value>0.05). Among 158 healthy northeastern Thai subjects, the frequencies of AA genotype of rs6726395, CC genotype of rs2886161, CC genotype of rs1806649, and CC genotype of rs10183914 were 15, 37, 88 and 84%, respectively. However, TT genotypes of NRF2 SNP rs10183914 and rs1806649 were not found in this group. The frequencies

Table 1. The Association between NRF2 Genotype and CCA Risk.

		Control (n=158)	CCA (n=198)	Crude OR (95%CI)	Adjusted OR* (95%CI)	p-value
Genotype of NRF2 (dominant model)						
rs6726395 - n (%)	A carrier (AA/AG)	104 (65.8)	129 (65.2)	1	1	
	mutant (GG)	54 (34.2)	69 (34.8)	1.03	1.05	0.852
		(0.66-1.60)	(0.64-1.71)			
rs2886161 - n (%)	C carrier (CC/CT)	137 (86.7)	164 (82.8)	1	1	
	mutant (TT)	21 (13.3)	34 (17.2)	1.35	1.07	0.852
		(0.75-2.42)	(0.54-2.09)			
Genotype of NRF2 (recessive model)						
rs6726395 - n (%)	Wild-type (AA)	24 (15.2)	33 (16.7)	1	1	
	G carrier (AG/GG)	134 (84.8)	165 (83.3)	0.9	1.15	0.679
		(0.51-1.58)	(0.59-2.22)			
rs2886161 - n (%)	Wild-type (CC)	58 (36.7)	60 (30.3)	1	1	
	T carrier (CT/TT)	100 (63.3)	138 (69.7)	1.33	1.04	0.867
		(0.86-2.08)	(0.64-1.70)			
rs1806649 - n (%)	Wild-type (CC)	139 (88.0)	172 (86.9)	1	1	
	T carrier (CT/TT)	19 (12.0)	26 (13.1)	1.11	1.23	0.561
		(0.59-2.07)	(0.61-2.48)			
rs10183914 - n (%)	Wild-type (CC)	133 (84.2)	163 (82.3)	1	1	
	T carrier (CT/TT)	25 (15.8)	35 (17.7)	1.14	1.08	0.811
		(0.65-1.99)	(0.58-2.02)			

*Odds ratios were adjusted for age at diagnosis, gender and smoking status.

Table 2. The Association between NRF2 Genotype and Survival Time of Intrahepatic CCA under the Dominant Model

Genotype of NRF2		n	Median survival time±SD (day)	95%CI	p value
rs6726395	A carrier (AA/AG)	54	172±37	100-244	
	mutant (GG)	34	344±138	73-615	0.006
rs2886161	C carrier (CC/CT)	72	208±36	138-278	
	mutant (TT)	16	203±73	60-346	0.814
rs1806649	C carrier (CC/CT)	87	222±28	166-278	
	mutant (TT)	1	172	152-264	0.553
rs10183914	C carrier (CC/CT)	86	208±78	153-263	
	mutant (TT)	2	172	152-264	0.932

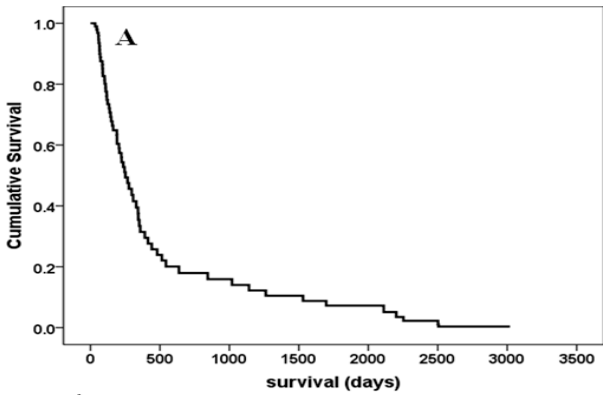
of T variant alleles of rs10183914 and rs1806649 of Thai population were significantly lower than those in Europeans ($p<0.05$). However, the frequencies of SNP rs6726395 and SNP rs2886161 in Thai population were not significantly different from those in Europeans and Japanese ($p>0.05$).

Association of NRF2 polymorphism with risk of CCA

The analysis of NRF2 polymorphism by simple allelic tests revealed no association between CCA patients and controls (Chi-square tests, $p>0.05$) (data not shown). Further analysis of genetic models of inheritance for association studies with dominant and recessive models were performed using logistic regression. The dominant model was tested with rs6726395 and rs2886161 only, because the other SNPs have too few frequencies precluding an analysis. The results showed that in both models, none of the SNPs were significantly associated with CCA risk (Table 1).

Survival analysis

Although NRF2 polymorphism was not associated with CCA cancer risk, NRF2 may play a role in cancer progression and prognosis of patients. We analyzed whether these NRF2 SNPs were associated with the survival time in intrahepatic CCA patients, of which incidence has increased significantly worldwide in recent years.



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NRF2 SNP rs6726395	n	Survival rate (%)				
		1-yr	2-yr	3-yr	4-yr	5-yr
All	88	31.82	21.59	17.05	14.77	12.50
A carrier (AA/AG)	54	22.22	12.96	9.26	7.41	5.56
mutant (GG)	34	47.06	35.29	29.41	26.47	23.53

Figure 1. Cumulative Survival Curves for Intrahepatic Cholangiocarcinoma. A) The survival function at mean of covariates; **B)** Survival function stratified by the genotype of NRF2 SNP rs6726395; **C)** Survival rate of intrahepatic CCA stratified by NRF2 SNP rs6726395 under the dominant model

The median survival time of the patients in this series was 208±29 (95%CI: 152-264) days. Using Kaplan-Meier and log rank analysis, the median survival time of intrahepatic CCA patients by NRF2 SNPs using the

Table 3. Multivariate Analysis by COX Proportional Hazards

Variable		Hazard ratio	95%CI	p-value
Age (years)	≤57	1		
	>57	1.23	0.72-2.13	0.449
Gender	Male	1		
	Female	0.78	0.41-1.48	0.447
Smoking status	Never-smokers	1		
	Smoker	1.53	0.67-3.51	0.312
Metastasis	No evidence	1		
	Presence	2.45	1.26-4.75	0.008
Surgical margin	R0	1		
	Not R0	1.63	0.93-2.88	0.089
Histological types	Papillary	1		
	Non-papillary	2.09	1.16-3.75	0.014
NRF2 SNP rs6726395	A carrier (AA/AG)	1		
	mutant (GG)	0.54	0.31-0.94	0.03

genetic dominant model (DD/Dd vs dd) revealed that the patients with GG genotype of NRF2 SNP rs6726395 had a significantly longer survival time than those with AA/AG genotype (p=0.006; median survival time: GG genotype were 344±138 (95%CI: 73-615) days) whereas the AA/AG genotype were 172±37 (95%CI: 100-244) days. Other SNPs (rs2886161, rs10183914, rs1806649) were not significantly associated with the survival time (Table 2). However, all of four NRF2 SNPs in this study were not significantly associated with survival time when the analysis was performed using the genetic recessive model (DD *vs* Dd/dd) (data not shown). Further analysis was performed to determine the impact of other parameters on survival time. The results show that CCA patients with papillary adenocarcinoma have longer survival time than other histological types: 344±117 (95%CI: 115-573) days *vs* 172±28 (95%CI: 117-227) days, respectively. The 5 year survival rate of overall patients was 12.5%, while the 5-year survival rate of AA/AG genotype and GG genotype were 5.6% and 23.5%, respectively (Figure 1). The patients with the tumor metastasis had shorter survival than those who without metastasis (149±20 (95%CI: 110-188) days *vs* 351±47 (95%CI: 260-442) days).

Then, we analysed the influence of predictors using multivariate Cox proportional hazards analysis. CCA patients with rs6726395 genotype of GG have longer survival than those who have genotype AA or AG after adjusted for age, gender, metastasis, histological type, smoking, and tumor residue at surgical margin, with the hazard ratio of 0.54 (95%CI: 0.31-0.94) (Table 3). The patients with papillary adenocarcinoma have a significantly longer survival time than those with other histological types with the hazard ratio of 2.09 (95%CI: 1.16-3.75). The patients with metastasis were at risk with the hazard ratio of 2.45 (95%CI: 1.26-4.75) when compared with those who have no metastasis.

Discussion

The NRF2 gene polymorphisms is associated with oxidative stress-related and inflammation-related diseases, and also with some cancers (Marzec et al., 2007; Cordova et al., 2010; Bouligand et al., 2011; Hartikainen et al., 2012; Okano et al., 2013). However, the impact of NRF2 polymorphisms on CCA has not been studied to date. In this study, we examined the effects of four polymorphisms (rs6726395, rs2886161, rs10183914 and rs1806649) within the NRF2 gene on the risk of CCA and the survival time of intrahepatic CCA in Thai subjects who were native-born and living in northeastern Thailand. The results showed that, all these NRF2 SNPs were not associated with the risk of CCA. On the other hand, rs6726395 was found to be associated with prognosis of intrahepatic CCA patients, in that those having the GG genotype have longer survival than the AA or AG genotypes.

NRF2 gene polymorphisms are not widely studied and their association with disease conditions remains unclear. In this study, we reported allele frequencies of 4 SNPs in NRF2 gene in Thai population. The allele frequencies of rs6726395 and rs2886161 in Thais are comparable to that of other Asian and Caucasian populations (Siedlinski et al., 2009; von Otter et al., 2010; Masuko et al., 2011). Moreover, the frequencies of rs10183914 and rs1806649 in Asian population were much lower than that in Caucasian populations. Until our study, there was no report over these alleles in Asian population. Further study with larger sample numbers is required to evaluate whether these alleles are really distinct between Asian and Caucasian populations.

In this study, we could not detect significant association of SNPs in NRF2 with the CCA cancer risk, as allele frequencies of the SNPs in CCA patients and healthy subjects were of similar. In other studies, rs1806649 is not associated with breast cancer (Hong et al., 2007). However, in further analysis in the intrahepatic CCA, only SNP rs6726395, but not others, was associated with the prognosis of CCA. CCA is a collection of intrahepatic and extrahepatic CCA, and these two types are suggested to be biologically different cancers (Cardinale et al., 2010). Several epidemiological studies indicate that the incidence and mortality of intrahepatic CCA is progressively increasing worldwide, whereas those of extrahepatic CCA seem to be stable or decreasing (Khan et al., 2008; Singal et al., 2011). In this study we revealed that the NRF2 SNP was correlated with the prognosis of the patients having intrahepatic CCA.

To our knowledge, the present study is the first such study to demonstrate that intrahepatic CCA patients with GG genotype of rs6726395 have a longer survival time compared to those having AA/AG genotype. Such an association was not seen in other SNPs (rs2886161, rs10183914 and rs1806649). The association of genotype and phenotype of NRF2 polymorphism has been reported in rs6726395. The SNP is located in the first intron of the NRF2 gene, was associated with NRF2 mRNA levels according to the GENEVAR database studying in three cell types; fibroblasts, lymphoblastoid cell line and T-cells (Masuko et al., 2011). It is possible that SNP rs6726395 may has some influence on NRF2 expression in CCA patients. On the other hand, there is still no report regarding the association in the other SNPs. However, it should be noted that there is no non-synonymous SNP with frequency ≥ 5% within NRF2 either in HapMap or in the NCBI SNP database (dbSNP). It still needs to elucidate the effect of the polymorphism of rs6726395 on NRF2

phenotype. The previous study showed that rs6726395 has clinical implications i.e. association with the decline of the mean annual forced expiratory volume in one second (FEV1), where GG genotype is associated with worst outcome (Masuko et al., 2011). Alternatively, this SNP may be in linkage disequilibrium with causative genes.

Apart from NRF2 SNP rs6726395, the present results revealed that histological classification and metastases at presentation could be a significant and independent predictor associated with prognosis of the intrahepatic CCA patients; i.e. the patients having non-papillary adenocarcinoma have worse prognosis than those having papillary adenocarcinoma. Our result is in agreement with the previous report in CCA patients of Thailand (Subimerb et al., 2010; Buranrat et al., 2012; Wirasorn et al., 2013). The patients having metastasis at presentation have poorer prognosis than those having no-metastasis at presentation. Residual tumor after surgical operation is usually regarded as the predictor of poor survival after surgery. The complete excision of tumor with surgical margin negative (R0) is associated with long-term survival (Guglielmi et al., 2009). Our study is consistent with the concept of patients without R0 excision are at higher risk than those with R0 operation.

Present study did not assess the degree of liver fluke infection. The burden of liver fluke infection may affect the risk of CCA and confound the observed genetic association with cancer in present study. Liver fluke presented risk is clearly observed when study is performed across populations in endemic and non-endemic areas of liver fluke infection. However our study was carried out in the endemic areas of liver fluke infection, where people in this area are infected with liver fluke at relatively high rate. The univariate analysis in this study could still be useful as a simple indicative of the strength of the genetic association. On the other hand, polymorphism of NRF2 of rs6726395 is clearly associated with survival time of the patients.

In conclusion, although four NRF2 SNPs examined in this study were not significantly associated with the risk of CCA, NRF2 SNP rs6726395 was associated with the prognosis (survival time) of intrahepatic CCA patients. The findings of this study have important implications for estimation of prognosis of the intrahepatic CCA patients. SNP rs6726395 in NRF2 may be used as an independent prognostic biomarker for the intrahepatic CCA..

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