CHAPTER III
RESEARCH METHODOLOGY

1. Chemicals

All chemicals used in the experiments are molecular grade. Names and sources of chemicals are shown as listed: ethylene tetraacetic acid (EDTA) and boric acid from BDH Chemicals Co.Ltd., (Poole, England), tris-HCl from Amresco, Solon Inc., (Ohio, USA), PCR buffer, $MgCl_2$, platinum and Taq polymerase from Invitrogen, (CA, USA), *Hin*fI, NEB buffer and 100 bp DNA ladder were purchased from Biolabs Inc., (New England, England). Oligo (dT) primers, 10 mM dNTP mix were purchased from Promega, (Madision, USA), bromphenol blue from Sigma Chemical Co., (St.Louis, MO, USA), sucrose from Biobasic Inc., (Toronto, Canada), Agarose was obtained from Research Organics, Inc., (Ohio, USA). Other chemicals were of analytical grade.

Instruments used in this study were: Electrophoresis apparatus and power supply (Model 200/2.0) (Biorad Laboratories, Hercules, CA, USA); Micro-centrifuge (H1500FR, Kokusan ensinki Co. Ltd., Tokyo, Japan); Temperature cycler (Px2 Thermal Cycler) (Bio-Active Co.,Ltd., CA, USA); UV-light source (Electronic Duallight TM-Transilluminator) (Ultra-lum Inc., Carson, California, USA); Vortex mixer (Scientific Industries Inc., Bohemia, NY, USA).

2. Research design

The present study was set to determine the NQO1 genotype in patients with cholangiocarcinoma (CCA) and control subjects (Figure 12).

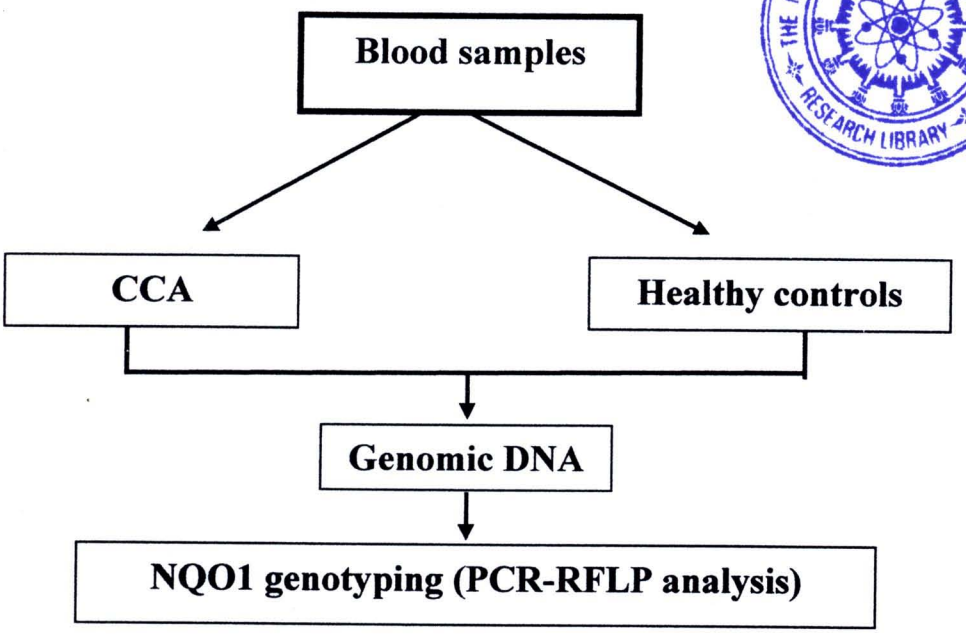

Figure 12 Experimental design of CCA and control subjects.

3. Subject population

Subjects in this study included 189 control subjects and 210 CCA patients. They are unrelated Thais who were native-born in the Northeast of Thailand. All subjects were interviewed for their ethnic backgrounds and only subjects with Thai origin for at least 2 generations were recruited in the study.

The control subjects were volunteers considered healthy by physical examination and with no current or history of cancer or other chronic diseases. Only individuals whose aged more than 30 year-olds were recruited. All CCA patients in the study were hospital based who were admitted and under gone operation at Srinagarind Hospital, Khon Kaen University. They had been confirmed the diagnosis of CCA by tissue histopathology. The CCA patients and control subjects who had previously received blood transfusion within 3 months were excluded.

The study protocols had been approved by the Khon Kaen University Ethics Committee for Human Research, Khon Kaen University (HE 521047; 2009). The blood samples were used for genomic DNA preparation and NQO1 genotyping was

examined by PCR-RFLP analysis. Survival status of patients was reviewed and survival time was recorded.

3.1 Sample size and study power

Since there is no previous report about NQO1 genotypes in Thai populations, this study calculated the number of subjects basing on our preliminary data. The frequency of the interested NQO1*2 allele found in 30 CCA patients is 0.47. The proportion of NQO1*2 allele in control subjects is, therefore, set as 0.32, power of test (β) as 0.8, alpha (α) as 0.05 and minimal expected difference in the proportion between control subjects and CCA patients is 0.15. Then, the estimated sample for two-sample comparison of proportions is 179 persons per group. Calculation was made by the programme Stata® version 8.2 (Stata Corporation., 2000). In this study, subjects for each group was therefore set at least 180 persons.

4. DNA preparation

4.1 Genomic DNA extraction method using guanidine thiocyanate

Genomic DNA was isolated from leukocyte rich fraction or buffy coat samples using guanidine thiocyanate. In brief, 0.5 ml of cell membrane lysis buffer (CMLB) (0.32 M sucrose, 1%(V/V) Triton X-100, 5 mM $MgCl_2$, 12 mM Tris-HCl (pH 7.6)) is added into 0.5 ml of buffy coat and mixed by hand inversion. The mixture was centrifuged at 10,000 g at 4°C for 2 min. After centrifugation, supernatant is removed and the pellet was washed with 0.5 ml of CMLB twice. After that, 0.35 ml of nuclear membrane lysis buffer (NMLB) (4 M guanidine thiocyanate (MW: 118.16), 12 mM Tris-HCl (pH 7.6), 12 mM EDTA (pH 8), 375 mM NaCl, 0.5% sodium N-Lauroyl sacosinate, 0.1 M β-mercaptoethanol) was added and the mixture was left for 5 min at room temperature for complete lysis. Then the mixture was centrifuged at 10,000 g at 4°C for 2 min. After centrifugation, the supernatant was transferred to a new tube. DNA was precipitated by adding 0.875 ml of cold absolute ethanol and gently mixed by hand conversion. Then the mixture was centrifuged at 10,000 g at 4°C for 10 min. After centrifugation, supernatant was discarded and DNA pellet is washed once with 1 ml of 70% ethanol and centrifuged again at 10,000 g at 4°C for 10 min. The pellet was dried briefly and dissolved by adding 100 ul of 1xTE buffer and stored at -70°C until used. The quantity and quality of the genomic DNA solution was

determined by using a UV-visible spectrophotometer at the wavelengths of 260 and 280 nm. In addition, quality is also checked by agarose gel elctrophoresis.

4.2 Measurement of DNA concentration

Genomic DNA solution was assessed for quantity by using a UV-visible spectrophotometer at the wavelengths of 260 and 280 nm. An acceptable DNA sample should have an A_{260}/A_{280} ratio of 1.5-2. The concentration of DNA sample was calculated as follows:

$$\text{DNA (ug/ul)} = A_{260} \times 50 \text{ ug/ml} \times \text{dilution factor}$$

5. NQO1 genotyping by using polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP)

PCR was carried out using the genomic DNA as a template (Seedhouse et al., 2002). The sequence of PCR primers are listed below.

Table 4 The sequence of PCR primers for human *NQO1* gene.

Genes		Primers	Product length (bp)
NQO1	Forward Reverse	5' -AAGCCCAGACCAACTTCT-3' 5' -TCTCCTCATCCTGTACCTCT-3'	304

NQO1 was amplified in a volume of 25 ul reaction and the mixture was incubated at 94°C for 3 min followed by 35 cycles of 94°C (50 s), 52°C (50 s), and 72°C (30 s) and a final 10-min extension at 72°C. The 304-bp PCR products were digested overnight at 37°C with 10 U of *Hinf* I [recognition site: 5 bp sequence (GAN*T*C)] in the manufacturer's buffer. All PCR products contain an internal *Hinf* I site and the Ser (NQO 609T) polymorphism also introduces an additional *Hinf* I site. The wild-type allele is cut into two fragments of 33 and 271 bp, while the mutant allele has three fragments of 33, 120 and 151 bp. The digested products were resolved on 2% agarose gels containing ethidium bromide. The PCR bands were visualized and pictures were captured with CCD-camera. The NQO1 genotypes are clarified into

3 groups, depend on pattern of digested band, NQO1*1/*1 genotype has two digested band whereas NQO1*1/*2 and NQO1*2/*2 have three digested band, with differential intensity pattern of the bands.

6. Statistical analysis

The observed allele and genotype frequencies of the NQO1 genes in CCA patients and control subjects were calculated, as prevalence and 95% confidence interval, according to the Hardy-Weinberg equilibrium ($q^2 + 2pq + p^2 = 1$; where p is the variant allele frequency). The distribution of genotypes in CCA population compared with the control population were assessed for significance by χ^2 testing. Risk associated with NQO1 genotype was calculated by logistic regression and odds ratio was adjusted with sex, age, smoking status and other relevant parameter. Association of survival time of CCA patients with NQO1 polymorphism was analyzed by log rank survival and Cox regreesion. *P* values of less than or equal to 0.05 were considered to represent significance. Analyses used the statistical package STATA version 8.2 and SigmaStat version 32.

7. Location of research condition

This experiment was performed at Department of Pharmacology, Faculty of Medicine, Khon Kaen University, Thailand.