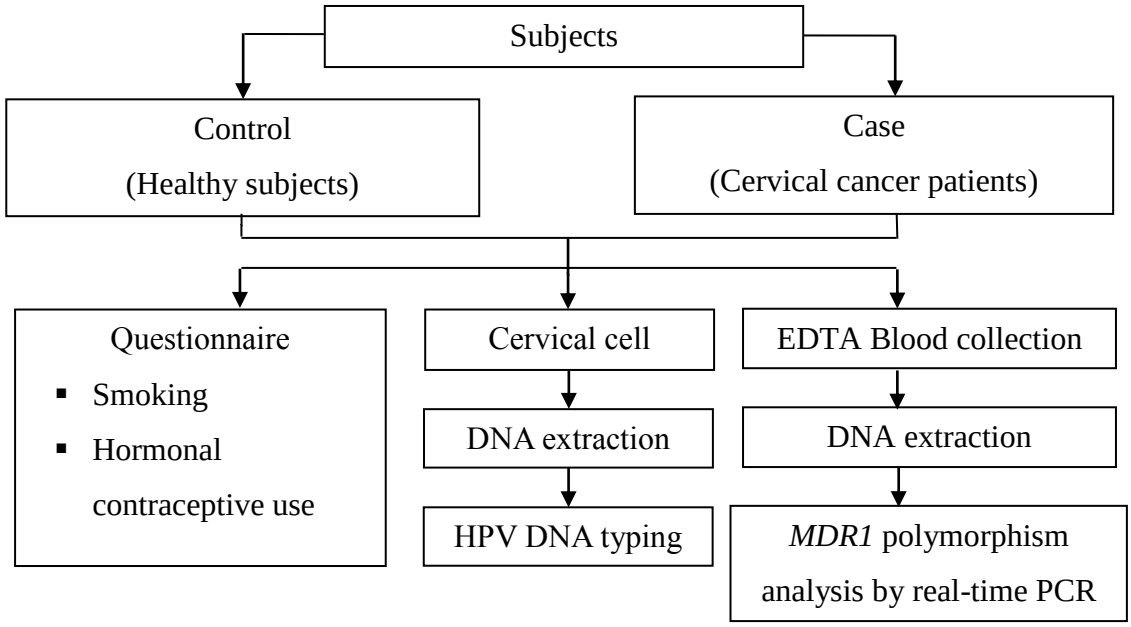
CHAPTER III
RESEARCH METHODOLOGY

1. Study plan



The questionnaire, HPV status and EDTA blood samples of this study were obtained from a previous study (Natphopsuk et al., 2013). This study was reviewed and approved by the Ethics Committee of both Khon Kaen University (HE 561382) and Khon Kaen Hospital (No. 03/02/2554), Khon Kaen province, Thailand.

2. Materials and Methods

2.1 Study subjects

The female volunteers were recruited from Srinagarind hospital, Khon Kaen University hospital and Khon Kaen hospital, Khon Kaen province, Thailand. The volunteers were divided into two groups, cervical cancer group and control group. Cervical cancer group was women with invasive squamous cell carcinoma of the cervix (SCCA) confirmed by histological examination whereas

control group was healthy women without cervical cancer and/or pre-invasive lesion of the cervix evaluated by cytology (Pap smear). Both groups were matched by age (5 years interval). The informed consent was obtained from all subjects and then peripheral blood leukocytes (EDTA blood) were collected for *MDR1* polymorphism analysis.

2.2 Sample size calculation

The numbers of subjects were calculated by sample size equation from EpiCalc 2000 version 1.02, the calculated data results in 198 controls and 198 cases as shown in Table 1.

Table 1 Sample size calculations

MDR1 **Genotype**	**OR**	**% control**	**Significance**	**Power**	**Case : control**	**Sample size**
TT	2.0	58	0.05	90	1 : 1	198 : 198

2.3 Subject criteria

2.3.1 Cervical cancer group

Inclusion criteria

(1) Subjects were diagnosed with cervical cancer by histological examination.

(2) Subjects with no evidence of other types of cancer.

Exclusion criteria

Subjects with the history of radiotherapy, hysterectomy and treatment with antiviral agents.

2.3.2 Control group

Inclusion criteria

(1) Subjects without cervical cancer and/or lesion of cervix confirmed by cytological and pathological examination.

(2) Subjects with no evidence of other types of cancer.

Exclusion criteria

Subjects with the history of radiotherapy, hysterectomy and treatment with antiviral agents.

2.4 Questionnaire

The variables of interest (i.e. hormonal contraceptive use and information regarding the details of smoking of subjects in both groups and their partners) were obtained from a previous study (Natphopsuk et al., 2013).

2.5 DNA extraction

Venous blood (3 ml.) was collected from all subjects and kept in vacuum tube containing EDTA. Centrifugation at 3500 rpm/min. was used for separation of red blood cell and plasma. The plasma and buffy coat (white blood cell and platelet) was kept at -80°C. Genomic DNA (gDNA) was extracted from buffy coat using GF-1 Blood DNA Extraction Kit (Vivantis, USA) and stored at -20°C. The DNA quality was verified by 1.5% agarose gel electrophoresis and was visualized under UV light.

2.6 The *MDR1* polymorphism analysis

Real-time PCR was used for *MDR1* polymorphism (C1236T, G2677T/A and C3435T) detection. The real-time PCR reaction was performed using total volume 20 µl/well, containing 7.5 µl double distilled water, 10 µl Taqman genotyping master mix, 2 µl DNA template and 0.5 µl of Taqman probe with primers, which were designed by Applied Biosystems (Applied Biosystems, USA). The reactions were set under the following conditions:

- C1236T and C3435T, holding stage at 95 ^{o}C for 10 minutes, follow by 40 cycles each of denaturation at 95 ^{o}C for 15 seconds, annealing at 60 ^{o}C for 60 seconds and extension at 60 ^{o}C for 30 seconds.

- G2677T/A holding stage at 95 ^{o}C for 10 minutes, follow by 50 cycles each of denaturation at 95 ^{o}C for 15 seconds, annealing at 60 ^{o}C for 60 seconds and extension at 60 ^{o}C for 30 seconds.

The specific sequences for SNPs of *MDR1* were detected with the StepOnePlus™ Real Time PCR Systems (Applied Biosystems, USA).

A Taqman probe consist of 5’ fluorescence conjugated oligonucleotide (A reporter dye) and 3’ quencher conjugated oligonucleotide, which were designed to hybridize within the target sequence. Taqman probe act as 5’-3’ exonuclease activity of the thermostable enzyme *Thermus aquaticus* DNA polymerase.

Taqman probe was designed for specific SNP following:

- C1236T SNP in exon 12 (Assay: C_7586662_10) and C3435T SNP in exon 26 (Assay: C_7586657_20) (wild-type of Taqman probe is specific with allele C and mutant-type of Taqman probe is specific with allele T), CC genotype and TT genotype bound only with FAM and VIC fluorescence dye's probe, respectively, while CT genotype bound with both VIC and FAM fluorescence dye's probe.

- G2677T/A SNP in exon 21 which provided 6 genotypes were performed in 2 assays. In the first assay, detection of G and A allele (Assay: C_11711720D_40) (wild-type of Taqman probe is specific with allele G and mutant type of Taqman probe is specific with allele A), GG and AA genotype bound only with VIC and FAM fluorescence dye's probe, respectively, while GA genotype bound with both VIC and FAM fluorescence dye's probe. For the second assay, detection of G and T allele (Assay: C_11711720C_30) (wild-type of Taqman probe is specific with allele G and mutant-type of Taqman probe is specific with allele T), GG and TT genotype bound only with VIC and FAM fluorescence dye's probe, respectively, while GT genotype bound with both VIC and FAM fluorescence dye's probe and TA genotype could not bound with both VIC and FAM fluorescence dye's probe. Double distilled water was used for negative control.

2.7 Statistical analyses

Statistical analyses were performed using the STATA software. The difference in genotype frequency between controls and cervical cancer patients was determined by using the Chi-square test and Fisher's exact test. The odds ratio (OR) and 95 % confidence interval (95%CI) was calculated to estimate the strength of the association between *MDR1* polymorphism and cervical cancer risk. A *p*-value less than 0.05 was considered to be statistically significant.