CHAPTER IV

DISCUSSION

A total of 82 cases of Thai ovarian cancer were analyzed. Histological subtypes of these cancer included 60 epithelial tumors, 5 sex cord stromal cell tumors, 5 germ cell tumors, and 12 metastatic tumors. The average age of this group of patients was 47.61 $\pm$ 12.10 years (ranging from 15 to 76 years). Clinicopathologic data of all cases in this study were shown in **Table 1**.

Several techniques have been used to analyze K-*ras* gene mutations in human tumors. In the past, reliable methods for K-*ras* mutation detection have been difficult to achieve. Among these are dot blot hybridization with oligonucleotide probe (Enomoto et al., 1990; Enomoto et al., 1991; Scambia et al., 1997), polymerase chain reaction with single strand conformation polymorphism (PCR-SSCP) (Ichikawa et al., 1994; Mandai et al., 1995; Mandai et al., 1998; Mok et al., 1993), polymerase chain reaction with restriction fragment length polymorphism (PCR-RFLP) (Caduff et al., 1999; Cuatrecasas et al., 1998; Cuatrecasas et al., 1997; Hogdall et al., 2003; Morita et al., 2000). The PCR-RFLP assays are normally limited to the detection, in each setting, of a base change at only one codon of a K-*ras* gene or even to the detection of only one specific K-*ras* mutation.

In order to detect mutations at codon 12 and codon 13 of K-*ras* gene in Thai ovarian cancer, we used the amplified created restriction site (ACRS) method with polymerase chain reaction- restriction fragment length polymorphism (PCR-RFLP) techniques. Because the lack of restriction enzyme sites at codon 12 and codon 13 of K-*ras* genes, we used the mismatched forward (codon 12) and reverse (codon 13) primers to introduce restriction sites for *Bst*NI (codon 12) and *Hea*III (codon 13) restriction endonuclease in the normal allele by amplified created restriction site with polymerase chain reaction. The methods have arisen for artificially introduce restriction sites to harness the ease of restriction enzyme mediated mutation analysis. This technique shows to be both fast and sensitive for detecting mutations at codon 12 and codon 13 of K-*ras* genes in tumor samples from ovarian cancer.

In this study, we have optimized various parameters of PCR condition to detect K-*ras* gene mutations at codon 12 and codon 13 in Thai ovarian cancer. To successful PCR analysis, the annealing temperature, the concentrations of the $MgCl_2$, dNTPs and primers were optimization. These parameters were optimal to provide the result without generated non-specific amplified products and high amplification efficiency.

For detection of K-*ras* gene mutations at codons 12, the annealing temperature was found to be one of the most important parameters. Variation of the annealing temperature from 56.0°C to 67.0°C had an alteration of the amplification efficiency. In this study, the most efficiency of annealing temperature was found at 60.0°C **(Figure 23 lane 3)**, which resulted the high amplification efficiency.

The dNTPs concentrations were varied from 50 μM to 250 μM respectively and the best result was 200 μM **(Figure 24 lane 4)**. The $MgCl_2$ concentration was increased stepwise from 1.5 mM to 3.0 mM. At the $MgCl_2$ concentration of 2.0 mM, the results showed an optimum of the PCR reaction with a satisfactory amount of product **(Figure 25 lane 3)**. The primer concentration in this study varied from 0.1 μM to 0.5 μM. A value of 0.3 μM of primers gave the high amplification efficiency, without visible dimer-primer formation and satisfactory amounts of PCR product **(Figure 26 lane 3)** for further experiment.

For amplification of codon 13 of K-*ras* gene, variation of the annealing temperature from 51.0°C to 67.0°C had an alteration of the amplification efficiency. In this study, the optimal annealing temperature was found at 63.0°C **(Figure 27 lane 5)**. The optimal dNTPs concentration was kept constant (200 μM) **(Figure 28 lane 4)**, while the $MgCl_2$ concentration was increased stepwise from 1.5 mM to 3.0 mM and the primer concentration in this study varied from 0.1 μM to 0.5 μM. At the $MgCl_2$ concentration of 2.0 mM, the results showed **(Figure 29 lane 3)**, and a value of 0.2 μM of primers gave the clear band and high amplification efficiency when compared with other concentration **(Figure 30 lane 2)**.

DNA amplified from each of the 82 Thai ovarian cancer specimens was analyzed for point mutations at codon 12 and codon 13 of the K-*ras* gene by the amplified created restriction site method with PCR-RFLP. We used 12% non-denaturing polyacrylamide gel to determine the products of restriction enzyme digestion. The resolving power of polyacrylamide gel is so great that can separate one base pair different in the PCR product. After K-*ras* gene was amplified, the

mutation at codon 12 was determined by restriction fragment length polymorphism (RFLP). The forward primer containing one base mismatch at 3' end created a restriction site for "*Bst*NI (CC↓TGG)" in the PCR product obtain from normal sequence at codon 12 (GGT), and produced a digested product of 133 bp and 29 bp in length, whereas the PCR product amplified from mutant sequence at codon 12 was absent of this restriction site, and generated a 162 bp undigested product. The reverse primer containing one base mismatch at 5' end created a restriction site for "*Hea*III (GG ↓ CC)" in the PCR product obtain from normal sequence at codon 13 (GGC), and produced a digested product of 85, 48 and 26 bp in length, while the mutant fragments abolish one position of restriction site so the PCR product have only two fragments of 85 and 74 base pairs.

Eighty-two ovarian cancers were screened for the presence of the mutations at codon 12 and codon 13 of K-*ras* gene using the ACRS with PCR-RFLP technique. We detected the K-*ras* mutation in 15 of 82 (18.29%) cases of Thai ovarian cancer. **Table 2** shown the summarization of the relevant data of PCR-RFLP analysis in all cases included in this study. Of the 15 cases presented K-*ras* mutation, the point mutation at codon 12 of the K-*ras* gene was detected in 17.1% (14/82) of Thai ovarian cancer specimens (cases OT003, OT011, OT023, OT036, OT038, OT041, OT047, OT049, OT057, OT060, OT063, OT065, OT069, and OT073) and only one case (1.2%) presented a mutation at codon 13 (case OT026). Thus, 93.33% of K-*ras* mutation in ovarian cancers examined in this study was found to present the mutation at codon 12 of the K-*ras* gene.

Mutations in the K-*ras* gene are frequently identified in ovarian cancer. In this study, K-*ras* mutations were found in a total of 18.29% (15 of 82) of Thai ovarian cancer samples. The frequency of K-*ras* gene mutation in our study (18.29%) is similar to the values found in ovarian cancers in Japan that reported by Tanimoto et al., 1997 (20.00%: 4 out of 20 cases), Morita et al., 2000 (20.83%: 10 out of 48). The frequency of K-*ras* gene mutation in our study is more than the values found in ovarian cancers in Denmark that reported by Hogdall et al., 2003 (9.70%: 16 out of 165 cases), and in USA which reported by Gemignani et al., 2003 (14.42%: 15 out of 104 cases) but our result below that described in ovarian cancers in Spain that reported by Cuatrecasas et al., 1998 (30.56%: 44 out of 144 cases). In the present study, the percentage of K-*ras* mutations in Thai ovarian cancers, which was the first reported in Thailand, was highly

comparable to the reported form Japan but difference from the reported form Europe and American.

The occurrence of K-*ras* mutations in Thai ovarian cancers correlated with histologic classification. The presence of point mutations at codon 12 of K-*ras* gene were found in 8 of 16 mucinous tumors of LMP (50%), 2 of 4 mucinous adenocarcinomas (50%), 2 of 5 serous tumors of LMP (40%), one endometrioid tumors of LMP, and one metastatic adenocarcinoma. All other epithelial tumors, mucinous cystadenoma, serous adenocarcinomas, clear cell adenocarcinomas, and endometrioid adenocarcimas presented the normal finding at codon 12 of K-*ras* gene. No mutations at codon 12 were detected in any of the five sex cord-stromal cell tumors and five germ cell tumors. Only one mutation at codon 13 of the K-*ras* gene was mixed epithelial tumors of LMP. The result of K-*ras* mutations and histologic classification data are summarized in **Table 3**.

When grouping histology type with K-*ras* mutation, the resulted shown that 10 of 22 mucinous tumors (45.45%), 2 of 18 serous tumors (11.11%), 1 of 11 endometrioid tumors (9.09%), 1 of 3 mix epithelial tumor (33.33%), and 1of 7 metastatic tumors (14.28%). K-*ras* mutations were not found on neither 5 sex cord stromal cell tumors nor 5 germ cell tumors. The incidence of K-*ras* mutaion in Thai ovarian cancer was higher in mucinous ovarian tumors (45.45%: 10 of 22) than all other histologic types combined (8.33%: 5 of 60). Thus, K-*ras* mutations were differently distributed in relation to the histologic classification. In mucinous tumors, no difference of the incidence of K-*ras* mutation was found between mucinous tumors of LMP (8 of 16) and mucinous adenocarcinomas (2 of 4). The high incidence of K-*ras* mutations in ovarian mucinous tumors of low malignant potential and mucinous adenocarcinomas than in mucinous cystadenoma (0 of 2) is consistent with previous report (Morita et al., 2000). Interestingly, the mutations of K-*ras* gene are detected more frequently in tumors of low malignant potential (52.17%: 12 of 23) than in adenocarcinoma (7.69%: 3 of 39). This finding is consistent with previous studies that the mutation of K-*ras* gene was more frequently detected in mucinous carcinomas than in other tumors of the ovary and was detected at codon 12 (Cuatrecasas et al., 1998; Cuatrecasas et al., 1997; Enomoto et al., 1991; Garrett et al., 2001; Gemignani et al., 2003; Hogdall et al., 2003; Ichikawa et al., 1994; Mok et al., 1993; Pieretti et

al., 1995). In addition, mutations of K-*ras* gene occurred more frequently in tumors of low malignant potential than in malignant ovarian carcinomas (Haas et al., 1999).

In addition, the ovarian cancer specimens that shown the mutation at codon 12 and codon 13 of the K-*ras* gene were further characterized the specific base substitution type in both codons. The first exon of K-*ras* gene were amplified by PCR and sequenced out by the dideoxynucleotides chain termination method and was analyzed by using automate DNA sequencing.

For amplified DNA fragments covering codon 12 and codon 13 on the first exon of K-*ras* gene, the new pair of primer should design and optimization of the annealing temperatures and $MgCl_2$ concentration were experimated. The annealing temperatures and $MgCl_2$ concentration were varied from 56.0°C to 69.0°C and 1.0 to 3.0 mM. The optimal annealing temperature was found at 63.0°C **(Figure 44 lane 4)** and the optimal concentration of $MgCl_2$ was 2.0 mM **(Figure 45 lane 3)**, which showed the high amplification efficiency.

After the exon I of K-*ras* gene was amplified by using these appropriate conditions, the amplified PCR products were purified with low melting point agarose gel electrophoresis. The gel fragment (260 bp) by purified with gel extraction purification kit. After purification, direct sequencing was carried out with the dideoxynucleotides chain termination method and was analyzed by using automate DNA sequencer.

The mutations at codon 12 and codon 13 in exon I of of K-*ras* gene were found in the 15 cases of Thai ovarian cancers and the result shown in **Table 4**. Fourteen cases which had the mutation at codon 12 of K-*ras* gene, including 4 cases (26.67%) had the changing from GGT to GTT (glycine⟶valine) **(Figure 46)**, 2 cases (13.33%) had the changing from GGT to CGT (glycine⟶arginine) **(Figure 47),** 3 (20.00%) cases had changed from GGT to GCT (glycine⟶alanine) **(Figure 48)** and 5 (33.33%) cases had the nucleotide change from GGT to GAT (glycine⟶aspartate) **(Figure 49)**. Among 15 cases of mutation, only one cases (6.67%) had the mutations at codon 13 of K-ras gene. This case had a nucleotide change from GGC to GAC (glycine⟶aspartate) **(Figure 50)**. The type of the base substitution among the Thai ovarian cancer was shown in **Table 5**. It seem that the transversion nucleotide from GGT to GTT and GGT to CGT transition nucleotide at codon 12 of K-*ras* gene play an important role in ovarian carcinogenesis in Thai patients.

When compared to the previous report, Ichikawa et al. (1994) studied in 104 Japanese ovarian cancers, eight cases shown the transitions from GGT⟶GAT, eight cases had GGT⟶GTT transversions and one case shown GGT⟶GCT transversions at codon 12. On the other hand, Mok et al. (1993) studied in 48 ovarian cancers, sixteen cases had GGT⟶GTT transversions, three case shown GGT⟶GAT transitions and one case was transversions from GGT⟶TGT at codon 12. Recently, Gemignani et al. (2003) analyzed K-*ras* mutations in formalin-fixed and paraffin-embedded tissue blocks from 104 American patients with ovarian cancer using PCR amplification followed by direct sequencing. Their results demonstrate that 15 of 104 cases had codon 12 mutations. Of the 15 mutations, 8 cases were GGT⟶GAT mutations, 5 cases were GGT⟶GTT mutations, and one each case was TGT and AGT mutation. These evidences suggest that the differences in nucleotide substitutions in K-*ras* gene may not correlate with ethnolography.

In our results, the occurrence of nucleotide substitutions at codon 12 and codon 13 of K-*ras* gene in Thai ovarian cancers correlated with histological classification. Eight cases were mucinous tumors of LMP presented the mutations at codon 12 of K-*ras* gene, including 3 cases were the nucleotide substitutions from GGT⟶GTT, 3 cases were GGT⟶GAT, 1 case was GGT⟶GCT, and one case was GGT⟶CGT. In two cases of mucinous adenocarcinomas had the nucleotide substitutions at codon 12, involving a GGT⟶GCT and GGT⟶CGT mutation. Two serous tumors of LMP showed a nucleotide substitution at codon 12 from GGT⟶GTT and GGT⟶GAT at codon 12. The case of endometrioid tumor of LMP and matastatic adenocarcinoma had point muatation from GGT⟶GAT and GGT⟶GCT, respectively. In addition, only one case of mixed epithelial tumors of LMP had the changing from GGC to GAC at codon 13 of K-*ras* gene. This suggests that the type of nucleotide substitution at codon 12 and codon 13 of K-*ras* gene in Thai ovarian cancers may not correlated with histology, which consistent with previous reported by Hogdall et al., 2003. The mechanism of nucleotide alterations at codon 12 and codon 13 of K-*ras* gene in ovarian cancers are still largely unknown but in animal model found that the G⟶A transition at codon 12 of the K-*ras* gene is a frequent mutation found in animals treated with nitrosamines and other alkylating agents, while the G⟶T transversion has been mostly seen in animals exposed to benzo pyrene or other polycylic aromatic hydrocarbon (PAH) carcinogens (Li et al., 2002).

The normal nucleotide sequence at codon 12 and codon 13 of K-*ras* gene were GGT and GGC, respectively, which encode glycine residue. The point mutation of K-*ras* gene at there positions arise the specific amino acid change from the smallest side chain glycine residue to the aliphatic hydrocarbon side chain alanine or valine residue, and to the negative charge aspartate or positive charge arginine residue. The amino acid alteration may influence of the protein structure and GTPase activity of K-*ras* proteins, which may influence downstream signals that contribute to tumorigenesis.

In this study, K-*ras* mutations were preferentially identified in mucinous tumors of low malignant potential and mucinous adenocarcinomas compared to mucinous cystadenomas. Cuatrecasas et al. (1997) reported the comparative frequencies of K-*ras* mutations in benign, low malignant potential and malignant tumors, which the mutations were detected in benign, low malignant potential and malignant tumors with increasing frequencies (Cuatrecasas et al., 1997). These results support the hypothesis that K-*ras* mutations play a role in the early events of ovarian mucinous tumorigenesis (Garrett et al., 2001).

On the other hand, a lower incidence of K-*ras* mutation in serous ovarian cancer compared to mucinous was observed in our study. K-*ras* gene mutations appearing in serous tumors low malignant potential and endometrioid tumors low malignant potential however was absent in malignant tumors, which consistent with the analysis of nonmucinous ovarian tumors that shown a low frequency of K-*ras* mutations in malignant tumors (Cuatrecasas et al., 1998). This suggests that K-*ras* mutations are not only more frequent in mucinous tumors but also tend to occur in ovarian tumors of low malignant potential.

Additionally, the genetic alterations involved in ovarian cancers are still not clearly understood. These results indicate possible pathways, other than K-*ras* gene activation, in the development of ovarian neoplasms. More detailed studies are required to define genetic alterations in ovarian carcinogenesis.