CHAPTER IV

I. DISCUSSION

The p53 protein is a phosphoprotein barely detectable in the nucleus of normal cells. Upon cellular stress, particularly that induced by DNA damage, p53 can arrest cell cycle progression, thus allowing the DNA to be repaired or it can lead to apoptosis if the damage was too severe. The most common changes of p53 in human cancers are missense point mutations within the coding sequences of gene. Patients with point mutations had single amino acid substitutions, which resulted in production of proteins with altered functions. These altered proteins had increased stability in contrast to wild type p53 which is rapidly degraded. Mutations of p53 gene can be found in approximately 50–75% of cancer patients, however, the positivity of p53 Abs in these patients is reported to be 30–50% or less. The mechanism of the development of autoantibody against p53 protein in cancer patients is intriguing because not all the p53 gene mutation carrying cancer patients elicit immune response against p53. Labrecque and colleagues (Labrecque *et al.*, 1993) suggest that the generation of p53 Abs is possibly due to an auto-immunization mechanism resulting from p53 protein accumulation in tumor cells. Vojtesek and Stephen (Vojtesek *et al.*, 1992; Stephen *et al.*, 1995) reported that most of the epitopes recognized by p53 Abs are reported to locate at N- or C-terminus of p53 proteins, which are located outside the domains containing mutational hot spots (Seleh *et al.*, 2004; Yewdell *et al.*, 1986). In addition to mutated p53 proteins, these elicited p53 Abs can also recognize wild-type p53 proteins (Roman *et al.*, 1989; Gire *et al.*, 1998).

Therefore, it is suggested that the development of p53 Abs, rather than the alternated molecular conformations of p53 protein resulted from genetic mutation, is due to the increased level of p53 protein. The presence of serum p53 antibodies have been found in many types of human cancer, including esophageal, oral, colon, gastric, hepatic, prostate, thyroid and bladder cancers (Crawford *et al.*, 1982; Ralhan *et al.*, 1998; Shimada *et al.*, 2003). Although, it is unclear what initiate the production of p53 autoantibody, many studies have shown that detection of p53 autoantibodies in cancer patients may potentially have clinical implications in early diagnosis and monitoring of tumor development or therapy response as well as prediction of prognosis.

The enzyme-linked immunosorbent assays are the most common antibody detection assay used in the laboratory which involves coating the detection plate with the antigen of interest and allowing serum antibodies to bind to protein for later labeling and detection. The patient sample is directly in contact with the coating antigen so the protein used for coating must be purified to avoid non-specific binding. Thus, highly purified p53 recombinant protein is necessary for such an assay. Furthermore, the sensitivity and specificity of the autoantibody detection kit is very depending on the quality of antigen used in the system. Therefore, there is a need for generation of recombinant proteins which is useful for antigen purification. In our study, we produced p53 antigen in the form of fusion protein from two prokaryotically expression systems (pET-15b vector and pAK400 vector) in order to develop a p53 autoantibody detection kit. The pET-15b-p53 DNA constructs was transformed into its expression hosts and subjected to induction. Western blot analysis showed that there was basal expression of p53 fusion protein even in absence of IPTG (Figure14, 15), especially with BL21(DE3) expression host strain. Although, basal expression of p53 fusion protein in BL21(DE3)pLysS host strain was also detected by Western blotting, it was less in comparison to BL21(DE3). This may due

to the fact that the BL21(DE3)pLysS strain carries a lysozyme encoding gene (pLysS) to digest basal level of T7 RNA polymerase produced in the absence of IPTG induction. Nevertheless, IPTG induction can greatly increase the level of expression and the expected band was absent in the negative control. Interestingly, the results showed that expression of the fusion protein represented overnight induction sample displayed a lesser intensity band in comparison to bands obtained from 1, 2, 3 and 6 hours induction, indicating that part of the recombinant protein may be accumulated in the inclusion body. However, the obtained band represent the overnight induction culture was clear from smaller fragment of recombinant protein. Therefore, for large scale preparation of $(His)_6$-p53 fusion protein, bacterial culture of BL21(DE3)pLysS carrying pET-15b-p53 was induced with 1 mM IPTG overnight (24 hours) as, this gave the highest percent yield and clear from the smaller fragment of fusion protein. For p53-BCCP expression, although 6 hours induction time represented a lesser band in western blotting in comparison to bands obtained from 4 or 5 hours induction, it gave the highest p53 production percent yield of total expression. Taken all together, the Western blotting results confirmed that we have successfully expressed the pAK400-p53 construct in *E. coli* Origami B and pET-15b-p53 construct in BL21(DE3) and BL21(DE3)pLysS cells, respectively.

After expression and purification step, the produced p53 was used to determine level of p53 antibody in an ELISA format. Although both p53 antigens from 2 different expression systems bind to the commercial p53 antibody in a dose dependent manner, but strong background were seen when the biotinylated p53-BCCP fusion protein was used as antigen to react with patient's serum. One possible reason for explaining why human sera react strongly to avidin coated plate is that human may have developed antibody against avidin. In consistent with these suspicions, it was previously reported that human serum contained natural antibodies to the egg-white glycoprotein avidin (Bubb *et al.*, 1993). Of

270 samples tested, all contained antibodies to different extents, mainly IgG and IgM classes, and were capable of activating complement system. Although biotinylated p53-BCCP protein can be selectively immobilized from crude cell lysate directly onto the avidin coated microplate, without having to go through the purification step first, the obtained strong background and the failure to differentiate weakly positive serum from those that were negative suggested that this system may not be suitable for preparing antigen to detect autoantibody in human serum. In contrast, the His_6-tagged p53 protein which was purified using Ni^{2+} affinity chromatography, although patient's serum reacted nonspecifically with microwell (0 µg), this was insignificant in comparison to the reactivity with microwell coated with p53 antigen. It appeared that microwell coated with 5 µg/ml of purified His_6-p53 protein was the optimal concentration in differentiating p53 antibody positive sera from those that were negative. Our data suggested that $(His)_6$-p53 fusion protein from pET-15b vector is more suitable to be used as antigen in order to develop p53 autoantibody detection kit than the biotinylated p53-BCCP fusion protein from pAK400 vector. In this study we used 1:200 diluted sera to perform ELISA in order to reduce non specific background, as it was widely used in the previous studies (Nielson *et al.*, 2003; Young *et al.*, 1980). Serum contains a very complex mixture of high titer antibodies. Therefore, it is necessary to dilute the serum to reduce nonspecific background from irrelevant antibodies in the serum.

The immunoreactivity of 26 lung cancer patient's sera to the purified $(His)_6$-p53 recombinant protein using an ELISA format was investigated and compared their obtained OD_{450} with the Western blot result. Of 26 patient's sera investigated, 16 patients were found to develop autoantibody against p53 as seen by the present of a reactivity band at around 53 kDa in Western blotting results. When we calculated the cut off value by combining mean OD_{450} of negative sera proven by Western blot analysis with its 3 SD

which was around 0.336, it was found that ELISA only able to discriminate 6 strongly positive cases out of total 16 positive sera from those that were negative. Although further investigations are needed, we initially proposed that Western blot analysis may be needed in combination with an ELISA results. Cut off value of ELISA should be designed to cover the minimum OD_{450} of positive sera proven by Western blot analysis which is around 0.200 and sera given reactivity range between 0.200-0.336 needed to be confirmed by Western blot analysis. However, it still need to be further investigated the clinical implications of developing of p53 autoantibodies in cancer patients and whether identifying the weakly positive cases by Western blot analysis will further improve their clinical usefulness.

It has been postulated that detection of p53 autoantibodies possessed as high as 95% specificity for cancer, however it was compromised by the lack of sensitivity of only 20-40% (reviewed in Lubin *et al.*, 1995). From this study, Western blot analysis has identified 16 out of 26 (61.2%) cancer patients to be positive for producing p53 autoantibody. However, when the reactivity was assayed by an ELISA and cut off was designed by using mean values of negative sera plus 3SD, 6 out of 26 (23.0%) cancer patients were found positive. A higher sensitivity of Western blot analysis in detecting p53 autoantibody in comparison to an ELISA may due to several reasons. (1) It is possible that the denatured conformation of p53 protein antigen presented in Western blot analysis exhibited more recognizable epitopes than that the tertiary structure presented in ELISA did. (2) The use of enhanced chemiluminescence-based detection system in Western blot analysis could provide better sensitivity than the colorimetric-based detection system used in an ELISA. (3) It was shown in Western blot result that there was still some bacterial proteins contaminated with the purified $(His)_6$-p53 fusion protein, which might be responsible for the non-specific reactivity background observed in the

negative sera. This non specific background in negative sera can be differentiated from true weakly p53 autoantibody carrying sera using Western blot analysis, but not ELISA (by examining the size of reactive band). Therefore, further improvement of $(His)_6$-p53 purification processes may increase sensitivity of the ELISA. We haven't compared our established ELISA with the commercially available ELISA kits, but it is possible that these kits may designed cut off value base on mean +3SD of the negative sera thus caused the lack of sensitivity of the assay reported in previous studies. Nevertheless, some of cancer patients were negative for producing p53 autoantibodies. The reason why some cancer patients did not develop p53 autoantibodies may be partly explained by the report by Davidoff *et al.*(1992). It was proposed that development of an immune response to mutant p53 protein is depended on the type of p53 mutant being expressed by tumor cells and the ability of these proteins to bind a 70 kDa heat shock proteins (HSP70). Heat shock proteins (HSP) are a group of molecular chaperone supporting folding and transporting of a great variety of polypeptides and protein under normal physiological conditions and following stress stimuli. There are strong evidences to suggest the role of HSP70 in antigen processing and presentation (Anne *et al.*, 1997; Davidoff *et al.*, 1992; Trichillis *et al.*, 2004). Davidoff and colleagues demonstrated that p53 autoantibody negative tumors had mutation exclusively in exon 7 and 8, whereas p53 autoantibodies positive tumors had mutation primarily in exon 5 and 6. Interestingly, all antibodies eliciting tumors contained complexes of p53 binding to HSP70, whereas none of the p53 autoantibodies negative tumors contained this complex. Taken all together, the authors suggested that only mutant p53 proteins with the ability to form complex with HSP70 could induce a p53-specific immune response.

II. CONCLUSION

In this study, the expression vectors pAK400 and pET-15b harboring p53 encoding DNA were constructed and used to produce biotinylated p53-BCCP fusion protein and $(His)_6$-p53 fusion protein in respective successfully. The $(His)_6$-p53 containing bacterial cell lysate was purified through Ni^{2+}-coated resin column and subsequently subjected to Western blot analysis with serum from lung cancer patients in order to screen for p53 autoantibody possessing serum. An ELISA for p53 autoantibody detection was optimized and set up using the produced p53 fusion protein as an antigen. Our results showed that although the biotinylated p53-BCCP fusion protein can be directly and selectively immobilized onto avidin-coated microtiter plate, it produced high background and failed to discriminate weakly positive serum from those that were negative. Therefore, the purified $(His)_6$-p53 which produced very low non-specific background is more suitable. After obtaining optimal ELISA conditions, a set of human sera from 26 lung cancer patients were assayed and the results were subjected to comparison with Western blot analysis result. Our data suggest that Western blot analysis should be used in combination in order to confirm and improve sensitivity of the ELISA result. Our preliminary results indicated that Thai lung cancer patients may develop p53 autoantibody as high as 61.5% of total patients tested, however, it remains to be further investigated their clinical implications and in a large group of samples.