CHAPTER VI
DISCUSSION

The inhibitor of apoptosis proteins (IAPs) are a family of highly conserved cell apoptosis inhibitors that have been found in yeast, invertebrates and vertebrates. The basal level of apoptosis is tightly controlled by endogenous IAPs in mammalian cells. The dysregulation of IAPs expression results in tumorigenesis and chemoresistance. Up to now, eight IAP-family members have been identified in human cells: NAIP, c-IAP1 (MIHB, HIAP-2), c-IAP2 (HIAP-1, MIHC, API2), XIAP (hILP, MIHA, ILP-1), survivin, BRUCE (apollon), ILP-2 and livin (ML-IAP, KIAP) (43, 45). Among these IAP members, livin and survivin are highly expressed in cancer cells and transformed cells, but show little or no expression in normal differentiated tissues (43, 46, 49, 53). These anti-apoptotic genes are believed to play a major contribution to the development and progression of cancer. High levels of livin and survivin expression in non-small cell lung cancer (NSCLC) were previously reported using reverse transcription polymerase chain reaction (RT-PCR) and immunohistochemical detection (23, 58, 59, 78). In our study, we examined protein expression level of livin and survivin in tumor tissues compared with normal tissues of lung cancer patients by Western blot analysis and found that livin and survivin were undetectable in normal tissues, but overexpressed in 93% (62 of 67) and 97% (65 of 67) of tumor tissues, respectively. The high overexpression rate of

these two proteins in almost all of tumor tissues despite stages of the tumor indicates their crucial role in early malignant transformation and thus a valuable tumor marker in aiding diagnosis of cancer in which non-invasive body fluid can be readily obtained. A number of previous studies have investigated urinary survivin using both mRNA-based and protein-based method as a potential diagnostic marker for bladder cancer (79, 80). Weikert et al. (81) have reported that detection of survivin mRNA in urine yield a sensitivity of 68.8% and a specificity of 100% for identifying non-invasive bladder cancer, whereas void urine cytology gave only a sensitivity of 31.4% and a specificity of 97.1%. In addition, overexpression of survivin had also been detected in exhaled breath condensate (82), pleural effusion (83) and circulating cancer cells (84) of lung cancer patients suggesting a promising value of this protein as a diagnostic tumor marker for lung cancer.

A number of studies have demonstrated that cancer sera contain antibodies, which react with a unique group of autologous intracellular antigen known as tumor-associated antigens (TAAs). The production of autoantibodies can be induced by a variety of proteins associated with malignant transformation such as tumor suppressor protein p53, oncoprotein c-myc and inhibitor of apoptosis protein (IAP) family. These autoantibodies can be detected in readily available serum, thus obviating the need for an invasive protocol. The presences of serum livin and survivin autoantibodies have been reported in many types of human cancer, including gastrointestinal, renal cell carcinoma (RCC), colorectal and lung cancers (28, 29, 32, 34, 36). Therefore, in this study we tested whether autoantibodies to livin and survivin can be used as a marker for the diagnosis and prediction of lung cancer.

The enzyme-linked immunosorbent assay (ELISA) is the most common antibody detection assay used in the laboratory which involves coating the detection plate with the antigen of interest and allowing serum antibody to bind to protein for later labeling and detection. The patient sample is directly in contact with the coating antigen. In the principle, high purified 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 protein which is useful for antigen purification. In our study, we produced livin and survivin antigens in the form of fusion protein from the prokaryotically expression system (pET-15b vector) to develop livin and survivin autoantibodies detection kit. The pET-15b-livin and pET-15b-survivin DNA constructs were transformed into their expression hosts, *E.coli* BL21 (DE3) and BL21 (DE3) pLysS strains and subjected to produce recombinant proteins by IPTG induction. Western blot analysis showed that there was barely detection level of livin or survivin fusion protein in absence of IPTG in BL21(DE3) and BL21(DE3)pLysS expression host strains. However, IPTG can greatly increase the level of protein expression as the expected band was detected in cell lysate prepared from IPTG-induced bacterium. Altogether, the Western blotting results confirmed that we have successfully expressed the pET-15b-livin and pET-15b-survivin constructs in BL21 (DE3) and BL21 (DE3) pLysS cells, respectively.

After the expression step, the produced $(His)_6$-livin and $(His)_6$-survivin were used to determine the level of livin and survivin autoantibodies in an ELISA format. In this study, $(His)_6$-livin and $(His)_6$-survivin were selectively immobilized from crude cell lysate directly onto the Ni^{2+} coated plate, without having to go

through the purification step. It was found that both livin and survivin antigens specifically bound to anti-livin and anti-survivin mAbs, respectively, in a dose dependent manner. However, non-specific reactivity with an empty Ni^{2+}-coated plate was seen when patient's serum was tested (data not shown). Therefore, in order to detect the reactivity specific for livin and survivin antigens, each serum was subjected to react with plate coated with and with-out protein antigen, simultaneously. The specific reactivity of a particular serum was obtained by subtracting total reactivity with its own non-specific reactivity to an empty Ni^{2+}-coated plate. Furthermore, $(His)_6$-survivin purified by Ni^{2+} affinity chromatography, was used to pre-absorb the commercial anti-survivin mAb and anti-survivin autoantibody in serum of lung cancer patients, to determine the specificity of the established ELISA. The results showed that the reactivity of both anti-survivin mAb and serum was decreased after pre-absorption with high concentration of purified recombinant survivin.

After confirming the specificity of the established ELISA, the immunoreactivity of sera from 250 lung cancer patients and 55 healthy volunteers to the $(His)_6$-livin and $(His)_6$-survivin recombinant proteins were investigated. By using the cut off value calculated by combining the mean OD_{450} of healthy volunteer's sera with its 2SD, it was found that 27 patients developed autoantibody against livin (10.8%) and 44 patients developed autoantibody against survivin (17.6%). Despite high frequency of protein overexpression in tumor tissues, the prevalence of livin and survivin autoantibodies in sera was low. The positivity rate of anti-survivin in lung cancer reported in our study is in the middle range compared

to previous studies. A number of previous studies reported a higher positivity rate include a

study by Rohayem (21.6%) and by Yagihashi (58.1%). However, a lower positivity rate has also been reported by other studies including a study by Karanikas (7.7%) and Zhang et al (10.7%). In the case of livin, previous studies have reported the positivity rate of lung cancer ranged between 47-51.3% (28, 34, 37, 85).

Nevertheless, the differences in positivity rate reported by different studies may due to the combination of variation in tumor stage, histology type and ethnical of the studied population. Although autoantibody to either one of these two antigens do not reach levels of sensitivity which could become routinely useful in diagnosis, it suggests that if more number of tumor antigens were investigated, a better sensitivity should be accomplished.

Up to now, little is known about the mechanism by which livin and survivin are presented to the immune system. Recently, livin-derived HLA-A2 and -A3 restricted epitopes were found to be presented on surfaces of various cancer cells as were, survivin-derived HLA-A1, -A2, -A3, -A11, -A24 and -B35 restricted epitopes. Spontaneous immune responses against these antigen-derived peptides were detected in patients with a variety of cancers. These reports suggested that livin and survivin acted as major cancer antigens and could induce both B- and T-cells response in cancer patients (66, 86, 87).

It is generally recognized that smoking is the major lung cancer causative. In concordance, tobacco component nicotine and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) has been reported to increase transcription of the

survivin gene in non-small cell lung cancer cells (88). Although, in our study, overexpression of survivin and livin was detected in almost all of tumor tissue of

patients both with and without a history of smoking, it appeared that only sera from smoking patients contained autoantibodies against these two anti-apoptotic proteins. Therefore, it is possible that some component in the tobacco may some how enhance the activation of B and T-lymphocytes and thus result in a better autoantibody response in smoking patients.

Our efforts to correlate the present of livin and survivin autoantibodies with the clinical parameters of the patient or to identify the characteristics of those appearing with elevated levels at diagnosis, showed that anti-livin antibody was found in all clinical states, interestingly anti-survivin antibody was statistic significantly found only in the stage I tumors (p=0.005). As almost all of tumor tissues overexpressed survivin but only tumor at the early stage developed anti-survivin autoantibody; therefore, we hypothesize that the overexpression of survivin may triggers autoantibody response only at the early stage of malignant transformation. Afterward, although survivin is still being overexpressed by tumor tissues, the patient's immune system may some how stop responding or become resistant to this alteration. Nevertheless, the induction of survivin autoantibody response at the early stage of tumor development makes it potentially an ideal early tumor marker for identifying lung cancer patients, although the absence of this response at the advance stage of the disease may compromise its clinical usefulness. To this end, an investigation of autoantibody response against a panel of tumor antigens in order to identify lung cancer patients with all stage of tumor is needed

and this may lead to a better understanding of tumor immunology and a discovery of ideal tumor marker.