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Evaluation of Significant Biomarkers Associated with Progression Free Survival and Overall Survival in Thyroid Cancer Patients

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M. Sc, University of New Hampshire, 2001

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Rollins School of Public Health

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Advisor: Zhengjia (Nelson) Chen, Ph.D

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Abstract:

Thyroid cancer is the most prevalent endocrine cancer, and its incidence rate has increased over the last few decades. Although survival outcomes following surgery remain favorable, patients still have a lifelong risk of recurrence. The utility of cellular- and serum-based immunologic mediators as potential biomarkers of thyroid cancer recurrence was assessed in this study. Thirty five patients with differentiated thyroid cancer and twenty one healthy controls were enrolled in the study. Absolute counts of lymphocyte cell subsets and levels of immune regulatory cytokines were determined in peripheral blood samples using multiparameter flow cytometry and 51-panel multiplex ELISA (Luminex) assay. Functional activity of circulating B, T and NK lymphocytes was assessed. Differences in mean biomarker levels between recurrence and remission group, or between disease and normal groups were assessed by t-test or Wilcoxon test statistics at the significance level of 0.05. Significant correlations between biomarkers and disease progression were assessed by univariate and multivariate analyses with the COX proportional hazard model. Optimal cut-off values maximizing the sum of sensitivity and specificity of predicting recurrence and disease were established by receiver operating characteristics (ROC) analysis. The overall survival rate for thyroid cancer patients was 91% in this study. Fifteen out of 35 patients (43%) had recurrence. $CD4^+$ T cells, $CD8^+$ T cells, Tregs, and NK cells in the peripheral blood of thyroid cancer patients were higher than in controls. The most significant cytokines correlated with disease recurrence on multivariate analyses were sFAS Ligand (adjusted HR, 0.330; 95% CI (0.136 – 0.799); p = 0.014) and IFN-α (adjusted HR, 4.061; 95% CI (1.453 - 11.347); p = 0.008). These biologically relevant cytokines will be valuable in a risk-adapted surveillance strategy for thyroid cancer.

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Table of Contents

Chapter I

Introduction …… …1

Chapter II

Methods ……12

Chapter III

Results …….. 17

Chapter IV

Discussion …….....28

References ……34

Appendix ……...37

Appendix: List of Table and Figure

Table 1. Clinical characteristics of patients with remission (n=20) and recurrence (n=15) status…..37

Table 2. Statistics summary of biomarkers between disease patients and healthy controls……...38

Table 3. Statistic summary of biomarkers between remission and recurrence patients……...40

Table 4. Univariate progression free survival analysis of demographic and clinical characteristics in disease patients.…….43

Table 5. Univariate progression free survival analysis of biomarkers in disease patients..... 43

Table 6. Multivariate Cox proportional hazard model of progression-free survival time (PFS) in disease patients (n = 35) with biomarkers……...46

Table 7. Pearson correlation analysis of six significant biomarkers in the selected Cox PH model for PFS…….……46

Table 8. Multivariate Cox proportional hazard model of progression-free survival time (PFS) in disease patients (n = 35) with biomarkers and clinical characteristics ……..... 46

Table 9. Univariate overall survival analysis of demographic and clinical characteristics in disease patients ...……...…….. 47

Table 10. Univariate overall survival analysis of biomarkers in disease patients…….... 48

Table 11. ROC analysis of significant biomarkers between remission and recurrence patients……...50

Table 12. Summary of cutoff points for different sensitivities and specificities of significant biomarkers between remission and recurrence patients……..…… 50

Table 13. ROC analysis of significant biomarkers between disease patients and healthy controls…….. 51

Table 14. Summary of cutoff points for different sensitivities and specificities of significant biomarkers between disease patients and healthy controls……..52

Figure 1. Kaplan-Meier curve of overall survival (OS)…….54

Figure 2. Kaplan –Meier curve of progression free survival (PFS)……...55

Chapter I

Introduction

Types of thyroid cancer and treatment methods

Thyroid is an organ at the base of the throat that makes hormones to help control heart rate, blood pressure, body temperature, and weight. Four main categories of cancers arising from the thyroid are papillary, follicular, medullary, and anaplastic, which have been established by the International Classification of Disease for Oncology since the late 1970s (Enewold et al., 2009). Cell type is an important determinant of prognosis in thyroid cancer. The grading of thyroid carcinoma is based on increased mitotic activity, necrosis, nuclear polymorphism and invasiveness, which are of high clinical and prognostic significance. Thyroid carcinomas of follicular cell origin are a spectrum of tumors ranging from the indolent, well-differentiated papillary carcinomas and minimally-invasive follicular carcinomas to the almost universally lethal anaplastic carcinomas (Caruso, 2012). TNM staging systems is a standard way to sum up how large the cancer is and how far it has been spread (http://www.cancer.org/cancer/thyroidcancer/detailedguide/thyroid-cancer-staging). The diagnosis staging of thyroid cancer are based on physical exam, biopsy, imaging tests such as ultrasound, radioiodine scan, CT scan, MRI, chest X-ray, and /or PET scans. T indicates the size of the main (primary) tumor and whether it has grown into nearby areas. N describes the extent of spread to nearby (regional) lymph nodes. Cells from thyroid cancer can travel to lymph nodes in the neck and chest area. M indicates whether the cancer has spread (metastasized) to other organs of the body. The most common sites for spread of thyroid cancer are the lungs, the liver and bones. The number 0 through 4

appeared after T, N, and M indicate increasing severity. The letter X means a category can't be assessed because the information is not available. .

The stage of a cancer is one of the most important factors in choosing treatment options and predicting chance of cure. There are different kinds of treatment for patients with thyroid cancer depending on the cancer type and stage (National Cancer Institute, http://www.cancer.gov/cancertopics/types/thyroid). Surgical removal of all or part of the thyroid, and even removal of nearby lymph nodes if necessary, is the most common treatment. Radiation therapy, such as radioactive iodine (RAI) therapy, is a treatment that uses high-energy X-rays or other types of radiation to kill cancer cells or keep them from growing. RAI destroys thyroid tissue and thyroid cancer cells without harming other tissue since only thyroid tissue takes up iodine (I131). Chemotherapy uses drugs to stop the growth of cancer cells by killing them or stopping them from dividing. Tyrosine kinase inhibitor (TKI) therapy is a type of targeted therapy that blocks signals needed for tumors to grow. Vandetanib is a TKI used to treat thyroid cancer. For patients who can't make enough hormones after removal or destruction of thyroid tissue, hormone replacement pills are prescribed. Other types of hormone therapy are also given to prevent the body from making Thyroid Stimulating hormone (TSH).

The epidemiology of thyroid cancer

The incidence trend of papillary thyroid cancer (PTC) is increasing in the last 10-15 years around the world (Pacini, 2012). The trend of increasing may be explained by increased scrutiny of the thyroid gland, exposed to ionizing radiation and higher incidence of autoimmune thyroiditis. The increase is mainly seen in papillary carcinomas

of less than 1-2 cm, also called Micro-papillary thyroid carcinoma (mPTC). It is most commonly occurring PTC in the USA in patients older than 45 years. The NCI (National Cancer Institute, http://www.cancer.gov/cancertopics/types/thyroid, 2012) estimates there were 56,460 new cases and 1,780 deaths in the United States in 2012. The incidence of thyroid cancer in the US more than doubled in the last three decades, from 4.3 cases per 100,000 individuals in 1980 to 12.9 cases per 100,000 individuals in 2008 (Surveillance, 2011).

The number of cases of papillary thyroid cancer reported between 2003-2005 increased nearly 100% among white non-Hispanic and Black females but only 20% to 50% among white Hispanics, Asian/Pacific Islanders, and Black males, when compared with the figures from 1992-1995 (Enewold, 2009). The papillary thyroid carcinoma was the most common (65-88%), followed by follicular carcinoma (9-23%). The increases were the most rapid for localized stage and small tumors. Among females, the highest rates occurred among individuals age 40 to 58 years, but the steepest increases were observed among those aged 60 to 79 years. Among males, by contrast, both the highest rates and the largest increase over time tended to be among older individuals.

A survey by the Lukas group (Lukas, Drabek, Lukas, Dusek, & Gatek, 2012) showed that the incidence of thyroid cancer has been steadily growing in the Czech Republic. Since the beginning of the 1980s, it has increased 4-fold. The Czech Republic has a higher incidence than most other European countries, with papillary carcinoma (PTC) accounting for over 80% of cases. The incidence for follicular and medullar cancers has not increased and the incidence for anaplastic carcinoma has slightly

decreased. Women over 40 years of age constitute the highest risk group in the Czech Republic.

The increasing incidence of thyroid cancer may be linked to improved diagnostic accuracy and thyroid screening in the general population (Pacini, 2012). A multitude of diagnostic tests such as ultrasound, thyroid nuclear scan, and fine needle aspiration cytology (FNAC) are available to the clinician for evaluation of thyroid nodules since 1990s (M. Gupta, Gupta, & Gupta, 2010). Micro-nodules can be found during neck examination for non-nodular thyroid diseases (such as autoimmune thyroiditis, Graves' disease) and 2-5% of theses nodules eventually become malignant and represent the larger proportion of thyroid cancer diagnosed at this time.

Other factors affecting epidemiology of thyroid cancer may be social economics status (SES) or education level. Many reports suggest that SES is highly associated with access to health care. In the SEER database in US, the majority of thyroid cancers are papillary type (82%) and only 2% are of medullary type. In high SES counties, thyroid cancer incidence increased moderately before late 1990s and more pronounced after late 1990s (Li, Du, Reitzel, Xu, & Sturgis, 2013). In low SES counties, incidence increased steadily with an APC of 3.5 ($P<0.05$) during entire study period (1998-2008). For tumors smaller than 4.0 cm, incidence was higher in high SES counties, and APC was higher for high versus low SES counties after late 1990s. For tumors larger than 4.0 cm, high and low SES counties had similar increasing incidence trends. Similar pattern exists for tumor<=2.0 cm. The incidence trends differed between counties that are in or adjacent to metropolitan areas and counties that are in rural areas, whereas for tumor > 2.0 cm, all counties regardless of area of residence had similar increasing trends. A study conducted

in Switzerland found that patients with thyroid cancer tended to be better educated (odds ration =2.1, 95% I 1.1-4.1 for >=14 vs. <= 8 years of educations (Levi et al., 1991).

Recurrence and survival in thyroid cancer patients

Age greater than 45 years, male sex, African American or minority race, multifocality, lymph node metastases, extrathyroidal invasion, and distant metastases are significant risk factors for overall survival (X. M. Yu, Wan, Sippel, & Chen, 2011). In a large retrospective study including 18,445 papillary thyroid microcarcinoma (PTM) patients with surgery from 1988 to 2007 from the Surveillance Epidemiology and End Results Cancer Database of the National Cancer Institute, the disease-specific survival was 99.5% and 99.3% at 10 and 15 years, respectively. The study to demonstrate the changing pattern of thyroid carcinomas with follicular phenotype in the period of 1973-2003 from the same database showed that 10-year relative survival rate was greater than 90% for blacks and whites with the exception of follicular carcinoma in blacks (Albores-Saavedra, Henson, Glazer, & Schwartz, 2007). The 10-year survival rate for anaplastic carcinoma in patients over 40 years of age was 4.7%. Thyroid cancer was more common in whites than in blacks and in females more than in males. Papillary carcinomas rapidly increased during adolescence and reached a peak around age 52-56, then declined. Follicular carcinomas increased steadily, but at a lower rate until age 80.

Relapses of the disease and cause-specific deaths in children with DTC are possible even after 2 to 3 decades from the time of initial diagnosis. The follow-up for young patients should be maintained throughout patients' life to detect and effectively treat late relapse. Children with DTC present with more advanced disease; however, they

have a more favorable outcome (Kiratli et al., 2013). In the retrospective study of 50 pediatric patients, who received radioiodine treatment (RAI) between 1976 and 2010, 13 (26%) showed recurrence with a median of 16 months. Metastatic disease was more common in boys and below 15 years of age. Patients with local disease had longer disease-free survival compared with patients with distant metastasis. These finding are in concordance with general knowledge on pediatric DTC patients.

In a series follow up study of 900 PTM patients after surgery or radioiodineremnant ablation (RRA) during 1945 – 2004 by Mayo Clinic, recurrence rates were 6% and 8% for twenty-year and forty-year, respectively. Higher recurrence rates were seen with multifocal tumors and node-positive patients. More than 99% of PTM patients are not at risk of distant spread or cancer mortality and the overall survival did not differ from expected for an age and gender matched control group (Hay et al., 2008). A clinical trial in China showed that the age of onset, tumor size at initial visit, and rate of early metastasis were significantly different between the mortality group and the survival group, and between the recurrence group and the recurrence-free group (Zhao, Zhang, Liu, & Shi, 2012). The follow-up period for their study ranged from 4.2 to 31 years. During the period, 140 (93.3%) patients survived, 30 (20.0%) patients relapsed, and 10 (6.7%) patients died of differentiated thyroid cancer (DTC).

The timing of surgery was not critical for survival. A clinical trial in Japan provided patients with the choice to have surgery and followed them for up for 15 years (Y. Ito et al., 2003) (Y. Ito et al., 2010). For the 340 out of 910 patients who did not have surgery, the size of their tumors increased by 3mm or more in 6.4% and 15.9% at 5 and 10 years, respectively. The novel appearance of lymph node metastatses was

documented in 1.4% and 3.4% of the cases at 5 and 10 years, respectively. Fifty-six patients were referred to surgery during follow-up. The TNM features of these patients were not different from that of the patients who were referred for immediate surgery at the time of diagnosis, suggesting that delaying the time of surgery dose not harm the final outcome.

The effect of local lymph node metastasis on survival in PTC remains controversial (Eskander, Merdad, Freeman, & Witterick, 2012). There is ample data in the literature associating lymph node metastasis with decreased disease-specific survival and increased risk of local recurrence. Outcome data from large institutional cohorts from the US, Canada and Germany have shown a significant and independent negative impact on survival with lymph node metastasis. Lateral neck metastasis is currently considered a significant prognosticator according to the latest thyroid cancer TNM classification.

Thyroid cancer prognostic biomarkers

A significant proportion of patients (as high as 20-40%) may experience a recurrence that does impact survival. Follow-up relies on the combination of several methods of monitoring serum thyroglobulin (Tg), radioactive iodine whole body scans (WBS), neck ultrasound, and fine-needle aspiration biopsy. Many thyroid markers have been examined; the most convenient and promising (in terms of extent or assessment in clinical settings) have been the circulating cytokines or molecular markers such as mRNA.

Serum thyroglobulin (Tg) is a highly specific and sensitive tumor marker for detecting persistent or recurrent thyroid cancer and for monitoring clinical status (Whitley & Ain, 2004). The reappearance of circulating thyroglobulin after total thyroid ablation is pathognomonic for the presence of tumor. Human thyroglobulin is a 670 kDa glycoprotein that is synthesized by thyroid follicular cells and it serves as a prohormone for thyroxine (3,5,39,59-tetraiodothyronine; T4) and liothyronine (3,5,39-triiodothyronine; T3) production. A clear increasing trend of serum Tg levels during follow-up is a sensitive hallmark of possible recurrence. Measurement of thyroglobulin mRNA to detect circulating tumor cells may help to overcome some of the limitations of current protein detection methods (Barbosa & Milas, 2008).

Serum TSH in patients with thyroid nodules may aid in diagnosis, in conjunction with clinical, radiological and cytological criteria for thyroid cancer. The systematic review and meta-analysis confirmed that higher serum TSH concentration was associated with higher odds of thyroid cancer (McLeod et al., 2012). Their models predicted that the odds of thyroid cancer being present were 3-fold greater in patients with a serum TSH level of 4 mU/liter compared with a serum TSH of 0 mU/liter. It is also important for cancer surveillance and screening in patients with a history of chronic TSH elevation. However, it is important to recognize that a suppressed TSH alone does not rule out a diagnosis of thyroid cancer.

Leptin is a neuroendocrine hormone that affects glucose metabolism, sexual maturation, reproduction, the pituitary-adrenal axis, the immune system, the thyroid, and growth hormone level. Increased expression of leptin and its receptor in papillary thyroid cancer have been proved by several studies as tumor markers for thyroid cancer. The

oncogenic effects of leptin on papillary thyroid carcinoma cells are related to stimulation of cell proliferation and apoptosis inhibition. In a study of the Iranian population, serum leptin levels in thyroid cancer group were significantly higher than control group(Hedayati, Yaghmaei, Pooyamanesh, Zarif Yeganeh, & Hoghooghi Rad, 2011). Uddin demonstrated that leptin plays an important role in papillary thyroid cancer pathogenesis through the PI3K/AKT pathway via its receptor, Ob-R, and is a potential prognostic marker associated with an aggressive phenotype and poor survival (Uddin et al., 2010). Leptin stimulates expression of some molecules such as CyclinD1, CDK2 and c-Myc that result in cell cycle progression and cell proliferation. Molecular pathways that are important in many types of cancer, such as JAK/STAT3, PI3K/AKT, ERK/MAPK, can be activated by leptin/leptin receptors. Leptin also plays an important role in tumorigenesis via induction of VEGF and VEGF-R2 expression.

The main function of human immune systems is the protection from a diverse range of harmful agents such as tumor cells. In a malignant environment, immune system homeostasis and control of self-tolerance are altered. Tumor–associated antigen–specific T cells are unable to eliminate cancer cells, mainly because the tumor microenvironment is highly immunosuppressive and tumor-associated anti-gen-specific T cells are functionally inhibited. An increased number of lymphocytes T cells, B cells, and NK cells were found near or within tumors. Increased numbers of infiltrating and proliferating $CD4^+$ and $CD8^+$ effectors T lymphocytes are correlated with low stage disease and longer disease-free survival in PTC patients (S. Gupta et al., 2001). There are extensive diversities of phenotype and function T lymphocytes. T helper ($CD4^+$) and cytotoxic ($CD8^+$) lymphocytes are directly involved in cell-mediated tumor destruction,

whereas natural killer (NK) cells are important in tumor rejection (Albertsson et al., 2003).

Function and activity of above effector T cells are tightly controlled by suppressor, $CD4^{+}CD25^{+}FoxP3^{+}$ regulatory T cells (Tregs). Increased Tregs tissue infiltration was positively correlated with advanced disease stage, whereas NK infiltration was negatively correlated (Gogali et al., 2012). T regs inhibit T cell proliferation and maintain tolerance to self-antigen. Tregs showed greater infiltration in thyroid tissue of PTC patients compared with patients with thyroid nodular goiter (TNG), which is the most common coexisting benign thyroid disease in PTC patients. But blood samples showed no difference between these two groups. NK cells in PTC tissue are significantly increased compared with TNG tissue, whereas blood samples showed no difference. French et al. reported that PTC patients with tumor-associated lymphocytes (TAL) exhibited higher disease stage and increased incidence of invasion and lymph node metastasis compared with patients without lymphocytes or with thyroiditis (LT) (French et al., 2010).

Tumor necrosis occurs when tumors outgrow their blood supply and it has been proposed as indicator of tumor aggressiveness (Caruso, 2012). The tumor necrosis factor family of receptors and ligands has been found to mediate immune effector cell interactions with tissue cells (Wallach, 1999). The Fas/FasL pathway, including sFasL in the serum, appears to be involved in the regulation of survival and death of circulating T cells in patients with cancer. Head and neck cancer (HNC) patients with active disease had the highest proportions of circulating Fas_ annexin_V_ T lymphocytes (Hoffmann et al., 2002). Human HNC cells express both Fas and FasL at the mRNA and protein levels.

This indicates that the Fas/FasL pathway is involved in spontaneous apoptosis of circulating T lymphocytes in cancer patients. Fas/FasL interactions might lead to excessive turnover of T cells in the circulation and, consequently, to reduced immune competence in patients with HNC.

Study goal

It is important to identify biomarkers that accurately distinguish disease presence and recurrence in thyroid cancer patients. Early detection of biomarkers with meaningful clinical application would aid in the formulation of an effective and comprehensive treatment plan. This could help set up clear criteria for patients who require special attention to monitor disease prognosis. In this study, we were interested in identifying biomarkers which were significantly associated with progression-free-survival (PFS) and overall survival (OS), and defining their applicability as biomarkers for thyroid cancer occurrence and recurrence. This cross-sectional cohort study recruited 35 patients (20 in remission and 15 with recurrence) and 21 controls. The associations between disease presence or recurrence with biomarkers, and other risk-factors (T, N, M, cancer stage, age, I-131 treatments and radiation treatment) were evaluated through univariate and multivariate analyses.

Chapter II

Methods

Study population and peripheral blood sample analyses data collection

Study design and sample collection referred to the paper of Owonikoko (Owonikoko et al., 2013). This study was conducted under an Institutional Review Board (IRB)-approved protocol. The patients with a diagnosis of papillary or follicular thyroid cancer identified through the tumor registry records and from the thyroid oncology clinic were eligible for the study and they were enrolled between September 2010 and September 2011. Healthy subjects without prior cancer history or actively treated medical conditions were recruited using advertisement fliers. Clinical and demographic data were collected from patients but not from healthy volunteers based on the scope of IRB approval. Peripheral blood samples were collected from each participant only at a single time point and were used for flow cytometry within 4 hours of collection. Simultaneously collected blood samples were centrifuged at 1500xg. The resulting plasma and sera were aliquotted and stored at -80°F until cytokine assay analysis.

Flow cytometry and immune function assays were performed in the Flow Cytometry Core laboratory of the Winship Cancer Institute of Emory University (Atlanta, GA). Complete blood count (CBC) enumeration was performed immediately (generally within 4 hours of sample collection) using Coulter AC*T diff (Beckman Coulter, Miami, Fl). The total WBC count per ml of blood was used to estimate the absolute numbers of the immune cells subsets on fluorescent-activated sorting (FACS) analysis. Flow cytometry was performed using 200-300 µL of erythrocyte-lysed samples. Samples were

stained with antibodies against CD3, CD4, CD8, CD69, programmed death-1 (PD-1), cytotoxic T-lymphocyte antigen 4 (CTLA-4), CD64, programmed death ligand-1 (PD-L1), and PD-L2 in order to enumerate various subsets of B lymphocytes, T lymphocytes and antigen presenting cells (APCs). B lymphocytes were characterized by staining with antibodies against CD3, CD19, CD38, and CD27. Natural Killer (NK) cells were characterized by panel of antibodies against CD3, CD4, CD94, NK group 2A (NKG2A), CD16 and CD56 while regulatory T cells (Tregs) were identified with a panel of antibodies against CD3, CD4, CD25, and forkhead boc P3 (Foxp3) along with isotype control after fixation and permeabilization using commercial anti-human Foxp3 Kit (eBioscience, San Diego, Calif).

Human 51-plex kits purchased from Affymetrix Inc. (Santa Clara, Calif) were used for Luminex assay. The assay was performed in a blinded fashion at the immunology core lab at Stanford University (Stanford, CA). Briefly, samples were mixed with antibody-linked polystyrene beads on 96-well filter plates and incubated at room temperature for 2 hours followed by overnight incubation at 4°C. The plates were vacuum filtered and washed twice prior to 2-hour incubation with biotinylated detection antibody. Samples were then filtered as above, washed twice and re-suspended in streptavidin-phycoerythrin (PE). After incubation for 40 minutes at room temperature, two additional vacuum washes were performed, and the samples re-suspended in reading Buffer. All samples were assayed in a single batch, and each sample was measured in duplicate. Plates were read using a Luminex 200 instrument (Luminex Corp., Austin, Tex) with a lower bound of 100 beads per sample per cytokine.

Statistics analyses

This study enrolled 35 thyroid cancer patients (20 in remission and 15 with recurrence) and 21 health controls. The dataset contained 83 cellular- and serum-based immunologic mediators as potential biomarkers as well as demographic and clinical characteristics covariates such as age, sex, diagnosis, stage, T, N, M, radiation, I-131, anatomical recurrence, and biochemical recurrence. The primary outcomes are overall survival (OS) time and progression free survival (PFS) time. Status indicator for overall survival was the event of death. The status indicator for progression free survival was event of recurrence, which was classified by anatomical examination. Only the enrolled patients with thyroid cancers were followed up for OS and PFS and their demographic and clinical characteristic data were collected. Blood samples from both disease and control groups were collected and all biomarkers were measured. The values of some biomarkers that were outside of measurement range were replaced with the lower or upper limit values.

Basic descriptive statistics of demographic and clinical characteristics were assessed. Frequency for categorical factors such as sex, diagnosed cancer type, stage, between recurrence and remission group, were compared by either Fisher's exact test or Chi-square test. Two sample Student's t tests were applied to compare continuous factors between recurrence and remission groups. First, the equality of two sample population variances was determined by F-test. If the population variances were equal, pooled variance was used for the Student's t-test. Otherwise, Satterthwaite method variance was used for the Student's t-test.

Univariate analysis of each biomarker between recurrence and remission groups, and also between disease and normal groups, were accessed by either two sample student's t test or Wilcoxon rank-sum test. The distribution of each biomarker was tested to assess whether the data satisfied the assumption of normal distribution (Gaussian condition). If the Kolmogorov-Smirnov goodness-of-fit test p-value was > 0.05, the hypothesis of underlying Gaussian distribution was not rejected and two sample Student's t-test was employed to compare the biomarker means of recurrence and remission groups; otherwise, nonparametric Wilcoxon rank-sum test was used instead.

Time of Overall survival (OS) is calculated as the time from study enrollment to death or last contact. Time of Progression free survival (PFS) is calculated as the time from study enrollment to disease progression date, death date, or last contact whichever comes first. The survivor functions for OS and PFS in thyroid cancer patients (group of disease) were estimated by the method of Kaplan and Meier. The logrank test was used to test the difference in the overall PFS or OS between different groups stratified by the demographic and clinical characteristic factors. A COX proportional hazards model was employed to estimate the un-adjusted effect of all demographic and clinical characteristic factors and biomarkers on PFS or OS. Point estimates of hazard ratio and 95% CI were calculated, and their significance was tested by Wald statistics. From the results of univariate survival analysis, all demographic and clinical characteristic factors and biomarkers with p-value of 0.1 or less were considered possibly significant and were retained for the multivariable analysis. A Cox proportional hazards model was employed in the multivariable survival analysis to estimate the adjusted effect of significant biomarkers after adjustment for all the significant biomarkers on PFS and OS. Spearman

or Pearson correlation coefficients were estimated to measure the relationship among significant biomarkers selected in the multivariate survival model. The same multivariate survival analysis was employed to estimate the adjusted effect of significant biomarkers after adjustment for all the significant biomarkers and significant demographic or clinic characteristic factors on PFS and OS. A backward stepwise analysis with a threshold p-value of 0.1 was carried out to select a best predictive model with statistically significant predicting variables on both outcomes, PFS and OS.

Receiver operating characteristic (ROC) analyses were performed for the significant biomarkers identified from univariate analysis between recurrence and remission groups, and between disease and normal groups. The cut-off values in the ROC curves were estimated to obtain 90% sensitivity and 90% specificity. The optimal cut-off values to achieve the maximum sum of sensitivity and specificity were calculated from the estimations from the logistic regression model that included the specific biomarker. The cut-off values of each biomarker for more than 50% chance of recurrence or disease were also reported.

The significance levels were set at 0.05 for all tests. The SAS statistical package V9.2 (SAS Institute, Inc., Cary, North Carolina) was used for data management and analyses.

Chapter III

Results

Demographic and Clinical characteristics

The classification of recurrence was according to anatomical appearance of cancer. There were 15 recurrence cases and the remaining 20 patients were in the remission status at the end of the study period. If classifying the disease recurrence by biochemical method, there were 13 cases of recurrence and 22 cases of remission. The anatomical method is normally used as the gold standard in medicine practice. McNemar's test between anatomical recurrence and biochemical recurrence had p-value of 0.157, indicating that these two classification methods agreed with each other.

Three out of 35 patients in this study died and the other 91% patients survived. Fifteen out of 35 patients (43%) had recurrence. The survival curves of OS and PFS by Kaplan-Meier methods were shown in Figure 1 and 2. The mean follow up time for for remission group patients was 7.02 ± 1.98 years, and the mean follow up time for recurrence group patients were 12.40 ± 13.24 years. The three patients died in this study, including two male and one female, all had recurrence. They were all at stage III upon initial diagnosis and they all received surgery and I-131 treatment. The two males were in their 50s when cancer was diagnosed. One male who had both T and N at original diagnosis had recurrence after 1.2 years and passed away after 3.7 years. The other one only had T at the original diagnosis, and he had recurrence around 5 years and passed away at about 22 years. The female who died was diagnosed with T stage at 14 years old. She had recurrence after 46 years and passed away 48 years after diagnosis.

Demographic and clinical characteristics data were collected only from thyroid cancer patients and they were summarized in **Table 1**. The information collected included age, sex, cancer type at diagnosis, stage at diagnosis, T, M, N staging, treatment of I-131 and radiation. Among the 35 thyroid cancer patients, 33% of females (8 out of 24) and 64% of males (7 out of 11) had recurrence. The risk of developing recurrence was lower in females than in males, but the difference was not statistically significant. The mean age in the recurrence group was 48.13 ± 17.61 years old, while it was 47.11 ± 11.37 years old in the remission group. The mean age between these two groups was not significantly different.

At the time of diagnosis, there were 1 case of anaplastic, 6 cases of FC or HCT, and 28 cases of PTC. PTC was most prevalent type of cancer among this group of study patients (80%). The one only patient with anaplastic cancer did not experience recurrence. One patient out of 6 (17%) FC/HCT patients had recurrence, and 14 out of 28 (50%) PTC patients had recurrence. There were no significant differences in the risk of recurrence among the three originally diagnosed cancer types. There were 10 patients in stage I, 11 in stage II, 10 in stage III, and 3 in stage IV. The rates of recurrence in stage I, II, III, and IV were 10%, 36%, 80% and 33%, respectively. The highest risk of recurrence was observed in stage III and the risk difference among stages was significant ($p=0.015$).

The TMN staging classification gives a measurement of the extent of the tumor at the primary site and at regional lymph nodes. T1 refers to a small primary tumor, 2 cm or less in largest diameter, whereas T4 is a massive tumor with extension to adjoining tissue. T2 and T3 refer to intermediate cases. Two out of 12 (17%) patients in T1, 3 out of 9

(33%) patients in T2, and 8 out of 10 (80%) patients in T3 had recurrence, while the one only patient in T4 did not have recurrence. In particular, T3 patients had the highest risk of disease recurrence. The overall risk of recurrence in these four T stages was significant different (p=0.017). M stage refers to distant metastasis: 11 out of 31 (35%) patients not in M stage had recurrence, while all patients (100%) in M stage had recurrence. There was a marginally significant difference in M stage status between remission and recurrence groups (p=0.061). N stage refers to clinical evidence of a lymph node metastasis. Seven out of 16 (44%) patients that were not in N stage had recurrence; while 7 out of 11 (64%) patients in N stage had recurrence; while the difference of the risk was not significant (p=0.44). Radiation and I-131 were the treatment methods recorded in this study population. There were 30 patients receiving I-131 treatment and half of them (50%) had recurrence. Five patients did not have I-131 treatment and they were all in remission at the end of study. The Fisher's exact test showed a marginally significant difference (p=0.057) in the risk of recurrence upon I-131 treatment. There were 6 patients receiving radiation treatment, and five of them (83%) had recurrence. Among the 29 patients who did not receive radiation treatment, 10 of them (34%) had recurrence. The Fisher's exact test showed a marginally significant difference (p=0.064) in the risk of recurrence upon radiation treatment.

In summary, the significant demographic and clinical factors for recurrence in thyroid patients were stage at original diagnosis and stage; the marginal significant factors were M stage, I-131 treatment and radiation treatment.

Significant biomarkers between disease and normal groups

To investigate which biomarker differed significantly between disease and normal groups, the means of biomarkers in these two groups were tested by either Student's t test or Wilcoxon rank-sum test depending on the satisfaction of normal distribution (**Table 2**). Among the 83 biomarkers, there were 30 significant biomarkers ($P<0.05$) and 5 marginally significant biomarkers ($P < 0.1$) (IL-12P70, IL-13, TGF-β, TNF-α and plasma cells). The levels of following biomarkers were significantly higher in disease patients: CD40Ligand, GM-CSF, ICAM-1, IL-12P40, IL-7, LEPTIN, MIG, PDGFBB, RANTES, Trail, V-CAM-1, DP-T, PD-1+CD4, PD-1+CD8, PD-1+DP, CD64-PD-L1, Absolute NK-CD94-ml-blood, Absolute-T-regs, T-regs, Gra-B-CD8-T-post-G, Gran-B-NK-post G, IFN-γ-CD4-T post G and PMA, IFN-γ-CD8-T post G and PMA, Gra-B-CD8-T-post G and PMA, TGF-β, and plasma cells. The levels of following biomarkers were significantly lower in the disease group than normal group: IFN-γ, IL-10, IL-15, IL-5, LIF, NGF, IL-12P70, IL-13, and TNF-α.

Significant biomarkers between recurrence and remission groups

Similarly, the means of biomarker levels in remission and recurrence groups were tested by either student's t test or Wilocoxon rank-sum test depending on the satisfaction of normal distribution (**Table 3**). Among the 83 biomarkers, 12 biomarkers were statistically significant ($P < 0.05$) and there were two marginally significant ($p < 0.1$) biomarkers (IL-12P40 and plasma cells). The 12 significant biomarkers were CD40Ligand, sFAS ligand, TGF-β, DN T, PD-1+CD4, PD-1+CD8, PD-1+DP, PD-1+DN, CTLA-4+CD8, CTLA-4+DP, CD64+PD-L1, absolue T-regs. These 14 identified

biomarkers were all significantly higher in remission group. The following five biomarkers were significant in both the comparisons between disease and control groups, and between remission and recurrence groups: CD40Ligand, PD-1+CD4, PD-1+CD8, PD-1+DP, IL-12P40 and plasma cells.

Univariate analysis of progression free survival in disease patients

The event of interest for progression free survival was recurrence of the disease. Univariate PFS analyses for all the demographic and clinical characteristic factors were summarized in **Table 4**. To make Log-rank test feasible, the factors had more than 2 levels of categories were regrouped to final 2 groups only. For type of cancer at diagnosis, group 1 included Anaplastic, FC or HCT; and the group 2 included PTC only. For stage at diagnosis, group 1 included stage I and II; and group 2 included stage III and IV. For T stage, group 1 included T1 and T2; and group 2 included T3 and T4. The significant factors on PFS identified from Log rank test were stage at diagnosis, T, M; and the marginal significant factors were I-131 and radiation treatment. The effect of demographic and clinical factors on PFS function was estimated from Wald test in Cox proportional hazard model. The most significant factor identified by Wald test was T group. The hazard rate of recurrence in T3+T4 group was 4.87 times of T1+T2 group. (HR, 4.874; 95%CI: 1.489 – 15.956; p = 0.009). The marginal significant factor was Stage at diagnosis group. The hazard rate of recurrence in Stage III+IV was 2.979 times of stage I+II (HR, 2.979; 95% CI (0.957 - 9.276); p=0.060). Another marginal significant factor was Radiation treatment. The hazard rate of recurrence in patients received Radiation treatment was 2.954 times higher than the patients did not have

Radiation treatment (HR, 2.954; 95% CI (0.907 - 9.620); p=0.072). The tests for these three factors were consistent with Log Rank test. The Wald test could not provide valid test for the effect of M (comparing the group had M to the group had no M) and I-131 (comparing the group had I-131 treatment to the group did not have I-131 treatment) since the estimates of Hazard rate was un-normally higher.

Both the Log rank test and Wald test for following characteristics were not significant, yet they were informative to understand the disease progression. The hazard rate of recurrence in male was 2.068 times of women (p = 0.175). The hazard rate of recurrence in PTC patients was 3.624 times of patients in other thyroid cancer type (p = 0.215). The hazard rate of recurrence in patients had N was 1.96 times of patients had no N (p = 0.229). Older age was associated with higher hazard rate of recurrence. In the thyroid cancer patients with one year older, the hazard rate of recurrence was increased by 2.7% (p = 0.240).

The association of each biomarker with progression free survival time was evaluated by Cox proportional hazard regression model (**Table 5**). There were 9 significant biomarkers (P < 0.05): G-CSF, IL-12P40, PAI-1, sFAS ligand, TGF-β, PD-1+CD4, PD-1+CD8, and CD64-PD-L1, and IFN-γ + CD8+ T post G. There were 4 marginally significant biomarkers (P<0.1): IFN-α, SCF, PD-1+DP, and Gran-B+ NK - post G. They were un-adjusted estimation since they were estimated from not controlling for other factors in the model. With one unit increase in G-CSF, IL-12P40, PAI-1, sFAS Ligand, TGF-β, IFN-α, and SCF, the hazard rate of recurrence was decreased by 60.5%, 12.7%, 0.4%, 15.8%, 26.7%, 11.2%, and 13.5%, respectively. With one unit increase in PD-1+CD4, PD-1+CD8, CD64-PD-L1, and PD-1+DP, the hazard rate of recurrence was

decreased by 100%. In contrast, increasing levels in IFN-γ + CD8+ T - post G and Gran-B+ NK - post G were associated with dramatically increase in hazard rate of recurrence.

Multivariate analysis of progression free survival in disease patients

The multivariate Cox proportional hazard model was employed to select final model for PFS from a starting model containing all 13 significant biomarkers identified from univariate PFS analysis. The estimates of the significant biomarkers in the final model were adjusted effect on PFS after adjustment for other significant biomarkers. Six biomarkers (PAI-1, sFAS Ligand, TGF-β, IFN-a, PD-1+CD8, and PD-1 + DP) were significantly associated with thyroid cancer recurrence (**Table 6**). sFAS Ligand was the most important factor independently associated with PFS (adjusted HR, 0.011; 95% CI (0 – 0.943); p = 0.047). With one unit increase in sFAS_Ligand, TGF-β, PD-1+CD8, and PD-1+DP, the hazard rate of recurrence was reduced by almost 100%. In contrast, with one unit increase in PAI-1, the hazard rate of recurrence was increased by 5.6%. The hazard rate of recurrence was increased by 99-fold when comparing patients with one unit higher in IFN-a. All six of these biomarkers fit the assumptions for Cox proportional hazard.

The correlations of significant biomarkers identified in multivariate survival (PFS) analysis were assessed by Pearson correlation coefficient (**Table 7**). PAI-1 was highly correlated with sFAS Ligand, TGF-β, and IFN-α. sFAS Ligand was highly correlated with PAI-1 and IFN-α. TGF-β was highly correlated with PAI-1 and PD-1-CD8. PD-1-DP was only correlated with PD-1-CD8.

When including significant demographic and clinical factors in addition to 13 significant biomarkers identified from univariate PFS analysis in the starting Cox PH model, the final selected model included five significant biomarkers (**Table 8).** sFAS ligand, IFN alpha, and PD_1_DP were the three significant biomarkers also identified from the multivariate PFS analysis in **Table 7**. One unit increase in sFAS Ligand level was associated with 67% decrease in hazard rate of recurrence (adjusted HR, 0.330; 95% CI (0.136 – 0.799); p = 0.014). One unit increase in IFN-α level was associated with 4.061 times higher hazard rate of recurrence (adjusted HR, 4.061; 95% CI (1.453 - 11.347); p = 0.008). Higher level of PD_1_DP was associated with higher hazard rate of recurrence (p=0.0098).

Univariate and multivariate analysis of overall survival in disease patients

Univariate analyses of overall survival using Cox proportional hazard model were performed for all demographic and clinical factors in thyroid cancer patients, in which case, the event of interest was death (**Table 9**). No significant factors were identified neither by Log-rank test, nor by Wald test. The hazard rate of death in male was higher than female. The hazard rate of death in PTC cancer type was higher than other type of cancer (Anaplastic, FC or HCT). The hazard rate of death in stage III+IV was much higher than stage I+II. The hazard rate of death in T3+T4 was 2.178 time of hazard rate of death in T1+T2 patients. The hazard rates of death were all much higher in the patients had the following clinical characteristics (M, N, I-131 treatment, Radiation treatment) compared to the patients without those characteristics. The hazard rate of death was increased by 5.6% in the patients group with one year older in age.

The association of each biomarker with overall survival time was evaluated by the Cox proportional hazard regression model (**Table 10**). There were 4 marginal significant biomarkers (P<0.1) identified by Wald-test: IL-15, IL-17F, IL-4, and VEGF. With one unit increase in IL-15, IL-17F, and VEGF, the hazard rate of death was increased by 35%, 107% and 16%. Oppositely, with one unit increase in IL-4, the hazard rate of death was decreased by 83%. There were other 14 biomarkers showed extreme values in the estimates by Cox PH model. They might be also significantly associated with OS.

Multivariate analysis of overall survival of in disease patients was assessed by Cox proportional hazard regression model by starting with the only 4 marginally significant biomarkers in the model. No significant biomarkers were retained for the multivariate model for OS.

Receiver operating characteristic (ROC) analyses of significant biomarkers between recurrence and remission group

The overall performance of a biomarker to predict recurrence or disease was represented by area the curve (AUC). The AUC was compared to chance which has an AUC of 0.5. Statically significant test provides evidence that the biomarker has discrimination ability of classifying disease status. The areas under ROC curve for the 14 significant and 2 marginally significant biomarkers were computed. The p-values computed were quite consistent with the results of univariate analysis (**Table 11**). The cut-off points for 90% sensitivities, 90% specificities, maximum sum of sensitivity and specificity, and more than 50% of recurrence for each biomarker were listed in **Table 12**. For example, the mean level of sFAS_Ligand in Recurrence group was 13.08 ± 2.63, and

it was 18.87 ± 8.73 in remission group. Lower level of sFAS_Ligand was associated with higher risk of recurrence. When using 17.45 as a cutoff value, test sensitivity was 90%, which meant among the people had recurrence, 90% of them had sFAS_Ligand level lower than 17.45. Meanwhile, among the people did not have recurrence, 35% of them had sFAS_Ligand level higher than 17.45. When using 10.88 as a cutoff value, test specificity was 90%, which meant among the people didn't have recurrence, 90% of them had sFAS_Ligand level higher than 10.88. Among the patients who had recurrence, 20% of them had sFAS_Ligand level lower than 10.88. To choose the best cutoff value for predicting disease recurrence status, the one giving the highest sum of sensitivity and specificity will be preferred. In this study population, the best value was 15.00 for sFAS_Ligand. When using 15.00 as a cutoff value, test sensitivity was 80%, which meant among the people had recurrence, 80% of them had sFAS_Ligand level lower than 15.00. Among the people who didn't have recurrence, 70% of them had sFAS_Ligand level higher than 15.00. If a person had sFAS_Ligand level lower than 12.73, the chance of thyroid cancer recurrence was more than 50%.

Receiver operating characteristic (ROC) analyses of significant biomarkers between disease and normal group

The same types of Receiver operating characteristic (ROC) analyses were performed for 35 significant biomarkers that attained statistical significance between disease and normal groups (**Table 13**). Also, the cut-off points for 90% sensitivities, 90% specificities, maximum sum of sensitivity and specificity, and more than 50% of recurrence for each biomarker were listed in **Table 14**. For example, CD40Ligand was a

biomarker which was significant in both event of recurrence and disease. The mean CD40Ligand in disease patients were 63.44 ± 30.88, while it was 43.40 ± 19.75 in normal people. A higher level of CD40Ligand was associated with higher risk of disease. When using 37.21 as a cutoff value, test sensitivity was 90%, which meant among the people who had thyroid cancer, 90% of them had CD40Ligand level higher than 37.21. Meanwhile, among the people who didn't have thyroid cancer, 43% of them had CD40Ligand level lower than 37.21. When using 84.08 as a cutoff value, test specificity was 90%, which meant among those who didn't have thyroid cancer, 90% of them had CD40Ligand level lower than 84.08. Meanwhile, among the people who had thyroid cancer, 11% of them had CD40Ligand level higher than 84.08. The cutoff value giving the highest sum of sensitivity and specificity for CD40Ligand was 50.66. When using 50.66 as a cutoff value, test sensitivity was 69%, which meant among the people who had thyroid cancer, 67% of them had CD40Ligand level higher than 50.66. Among the people who didn't have thyroid cancer, 76% of them had CD40Ligand level lower than 50.66. If a person had CD40Ligan level higher than 43.34, the chance of thyroid cancer was more than 50%.

Chapter IV

Discussion

Survival analysis is suited for the growing field of clinical trials in medical research because medical intervention follow-up studies could start without all experiments units enrolled at start of observation time and could end before all experimental units had experienced an event. Censoring enabled researchers to analyze incomplete data due to delayed entry or withdrawal from the study and allow each experimental unit to contribute all the information possible to the model for the amount of time the research was able to observe the unit. In some situations, right censoring arises from different reason such as some individual are still surviving at the time when the study is terminated, or some individual may be withdraw from the study because of a worsening or improving prognosis, or move away from study of area or loss of contact. A right-censoring mechanism is said to be independent if the failure rates that apply to individuals on trial at each time are the same as those would have applied had there been and censoring.

Two popular methods in survival analysis are Kaplan-Meier (KM) method and Cox proportional hazard regression modeling (Stel, Dekker, Tripepi, Zoccali, & Jager, 2011a, 2011b). The log-rank test in KM method can provide p value for comparison of survival of two groups; however, it cannot provide an estimate of the effect and related confidence interval (CI). Instead, Cox regression model analyses can provide unadjusted and adjusted effect estimates with their related 95% CIs. Cox's semi-parametric modeling allows no assumptions to be made about the parametric distribution of the survival time. The proportional hazards assumption refers to the fact that the hazard

functions are multiplicatively related, their ratio is assumed constant over survival time. The use of the Partial Likelihood function makes the Cox model has the flexibility to introduce time-dependent explanatory variable and handle censoring of survival time.

Immunological status such T cells, Tregs, and cytokines are important parameters to understand cancer prognosis. $CD4^+$ and $CD8^+$ T cells are two main subsets for T lymphocytes. The appearance of $CD4^+$ and $CD8^+$ cells is always correlated with lower stage disease and longer disease survival (S. Gupta et al., 2001). But Yu et al. reported that there were no differences in numbers of $CD4^+$ and $CD8^+$ T cells in tissue and peripheral blood of patients with PTC patients plus multi nodular non-toxic goiter (MNG) (H. Yu et al., 2012). Study by French et al. showed that PTC patients with relatively high levels of CD4+ T cells presented with larger tumors and CD8+ cell frequency showed a modest inverse correlation with tumor size (French et al., 2010). In this study, functional activity of B-cell, T-cell as well as NK cell subpopulations was assessed by granzyme-B and interferon gamma (IFN-γ) production with or without PMA/Ionomycin stimulation in vitro. $CD4^+$ T cells (IFN-γ-CD4-T post G and PMA) and $CD8^+$ T cells (Gra-B-CD8-T-post-G, IFN-g-CD8-T post G and PMA, Gra-B-CD8-T-post G and PMA) in the peripheral blood sample in thyroid cancer patients were higher than in controls. Higher levels of $CD8^+$ T cells (IFN--γ + CD8+ T - post G) were associated with poor disease prognosis (higher hazard rate of recurrence). Similar to previous reports of increased number of NK cells were found to be near or within tumors (S. Gupta et al., 2001), this study showed that NK cells (Gran-B-NK-post G) in the peripheral blood sample in thyroid cancer patients were higher than in controls. NK cells (Gran-B+ NK - post G) were associated with poor disease progress (higher hazard rate of recurrence).

Tregs are major negative regulators of tumor immunity and capable of suppressing the proliferation of autologous tumor Ag (TA) specific and Ag-nonspecific $CD4^+$ and $CD8^+$ T cell response. $FoxP3^+$ Tregs can be divided into two subsets, $ICOS^+$ Tregs and $ICOS^-$ Tregs. $ICOS^+$ Tregs secrete much larger amount of interleukin 10 (IL-10) and IL-10 is a negative regulator in tumor escape. $ICOS^+$ Tregs uses IL-10 to suppress plasmacytoid dendritic cell (pDC) function, and pDC could effectively generate Tregs from Naïve $CD4^+$ T cells. $ICOS^-$ Tregs have a high capacity for transforming growth factor (TGF)-β expression, which is also a suppressor for T cell function. ICOS+ Tregs are more immune suppressive than $ICOS^-$ Tregs (T. Ito et al., 2008; T. Ito et al., 2007). Yu et al. suggested that Tregs and pDCs together contribute to the tumor escape in patients with PTC plus MNG (multi nodular non-toxic goiter) (H. Yu et al., 2012). Their study found that in thyroid tissue and peripheral blood, the number of $Foxp3^+$ Tregs and IL-10 level were significantly higher in PTC plus MNG compared to patients with MNG alone. The levels of TGF-β between these two groups were not significantly different. In our study, Tregs cells (Absolute-Tregs, %T-regs) in the peripheral blood sample in thyroid cancer patients were higher than in controls. TGF-β was higher in thyroid cancer patients than in controls. TGF-β was higher in remission patients than in recurrence patients and TGF-β was associated with lower hazard rate of recurrence. IL-10 levels in thyroid cancer patients were significantly lower than normal. Another immune-suppressive cytokines is VEGF, and it acts indirectly to inhibit dendritic cell maturation leading inefficient activation of naïve T cells and Tregs induction. French et al. study showed that PTC tumors expressed high levels of VEGF and TGF-β (French et al., 2010). In our study, VEGF level in thyroid cancer patients was higher than in

normal, but the difference was not significant. VEGF was found to be associated with poor overall survival. It has been suggested that pDCs could limit tumor growth by producing type I interferons (IFNs). Type I IFNs produced by pDCs in breast cancer was moderately reduced and pDC numbers were associated with poor prognosis (Gehrie, Van der Touw, Bromberg, & Ochando, 2011). From the multivariate survival analyses of our study, IFN-α was one of the significant biomarkers and it was associated with higher rate of thyroid cancer recurrence (adjusted HR, 4.061; 95% CI (1.453 - 11.347); p = 0.008).

Another significant cytokine appeared to be associated with higher rate of recurrence was PAI-1 from multivariate survival analysis of progression free survival time. Plasminogen activator inhibitor 1 (PAI-1) and PAI-2 are components in the urokinase plasminogen activator (uPA) system (uPAS). Each uPAS member acts as a multi-tasking factor involved in all steps of tumour progression, from the local growth and spreading of malignant cells to migration and invasion of distant sites, as well as in tumour neoangiogenesis. Some studies documented that an increased expression of uPA, uPAR and PAI-1 in different human malignancies, and they are correlated with a poor prognosis(Baldini et al., 2012). Their study demonstrated that the increased gene expression of uPA and uPAR in PTC tissue is associated with tumour invasiveness, advanced stages and shorter disease-free interval.

In previous studies, the Fas/FasLigand pathway, including sFAS Ligand in the serum, appears to be involved in the regulation of survival and death of circulating T cells in patients with head and neck cancer (HNC) (Hoffmann, 2002). Fas/FasLigand interactions might lead to excessive turnover of T cells in the circulation and,

consequently, to reduced immune competence in patients with HNC. In our study, sFAS Ligand was the most important factor associated with progression free survival (adjusted HR, 0.330; 95% CI (0.136 – 0.799); p = 0.014). Higher level of sFAS Ligand was associated with lower rate of recurrence of thyroid cancer. The findings of this study suggested that sFAS Ligand could be a potential biomarker of thyroid cancer recurrence.

The findings from this study set the stage for important future diagnostic and therapeutic applications and provide novel testable hypotheses of thyroid cancer biology. The strengths of this study were its ability to interrogate a large panel of serum-based cytokines from small volume of blood using the Luminex assay platform, and the use of blood samples processed within a few hours of collection. This provided real-time information about blood composition in the patients and control subjects. Nonetheless, there were some important limitations of the study. The first limitation was a modest sample size. Only 35 thyroid cancer patients were included in this study. Also, there was no demographic information for normal control subject was missing. Consequently, a direct comparison between the disease and normal groups on demographic characteristics was not assessed, but it might be very useful to better understand thyroid cancer induction and progression. Another limitation was that only a single time point assessment was performed in this study. A longitudinal follow-up of patients' blood profiling and serum cytokine profiling would provide clinically significant information on their association with progression free survival and overall survival. Finally, the lack of a significant association of any biomarkers and covariates in multivariate overall survival in this study may be related to the low percentage of deaths in the study, and also may be due to its relatively small sample size.

Despite these limitations, the results in this study provide a useful benchmark on which to build a larger prospective validation studies to definitively establish the predictive and prognostic importance of the identified biomarkers.

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Appendix

Table 1. Clinical characteristics of patients with remission (n=20) and recurrence (n=15) status.

Characteristics	Level	Total N	Remission n (%)	Recurrence n (%)	p^{*+}
Sex	Male	11	4 (37%)	7 (64%)	0.144
	Female	24	16 (67%)	8 (33%)	
Cancer Type	Anaplastic	1	1 (100%)	0 (0%)	0.222
	FC or HCT	6	5 (83%)	1 (17%)	
	PTC	28	14 (50%)	14 (50%)	
Stage	I	10	9 (90%)	1 (10%)	0.0153
	II	11	7(64%)	4(36%)	
	III	10	2 (20%)	8 (80%)	
	IV	3	2 (67%)	1 (33%)	
T	1	12	10 (83%)	2 (17%)	0.0172
	2	9	6 (67%)	3 (33%)	
	3	10	2 (20%)	8 (80%)	
	4	1	1 (100%)	0 (0%)	
M	No	31	20 (65%)	11 (35%)	0.0608
	Yes	3	0 (0%)	3 (100%)	
N	No	16	9 (56%)	7 (44%)	0.440
	Yes	11	4 (36%)	7 (64%)	
I-131	No	5	5 (100%)	0 (0%)	0.057
	Yes	30	15 (50%)	15 (50%)	
Radiation	No	29	19 (66%)	10 (34%)	0.064
	Yes	6	1 (17%)	5 (83%)	
Age (years)	N		20	15	0.837^{+}
	Mean		47.11	48.13	
	Std Dev		11.37	17.61	
Overall survival	N		20	15	0.882*
Time (years)	Mean		7.02	12.40	
	Std Dev		1.98	13.24	
Progression free	N		20	15	0.0015^{+}

Time (years)	Mean		7.02	5.34	
	Std Dev		1.98	11.60	

Note: *p**-value was obtained from student's t test. p^{+} -value was obtained from Wilcoxon rank-sum test. Other *p*-values were obtained from chi-square test.

Table 2. Statistics summary of biomarkers between disease patients and healthy controls.

Biomarker	Disease (n=35)		Normal (n=21)		
	Mean	Std Dev	Mean	Std Dev	p^{+}
CD40Ligand	63.44	30.88	43.40	19.75	0.005
ENA78	87.72	88.52	49.31	35.18	0.156
EOTAXIN	9.14	3.52	8.01	2.67	0.231
FGF beta	3.90	1.37	4.42	19.95	0.347
G-CSF	1.48	0.75	1.33	0.59	0.442†
GM-CSF	26.59	12.16	15.35	6.68	0.0001
GRO ALPHA	12.44	4.49	11.03	2.99	0.216
HGF	22.67	10.12	19.78	8.94	0.216
ICAM-1	1233564	3468054	7141.8	13745.1	0.008
IFN-alpha	5.67	4.98	7.28	5.16	0.144
IFN-beta	6.63	6.23	5.04	2.20	0.465
IFN-gamma	3.75	1.06	6.00	4.90	0.003
IL-10	0.86	0.41	1.32	0.79	0.021
IL-12-P70	8.12	2.26	9.67	3.82	0.076
IL-12-P40	19.25	7.79	13.90	6.07	0.010
IL-13	3.65	1.66	4.44	1.57	0.084
IL-15	5.57	3.69	13.14	18.38	0.013
IL-17	1.18	0.45	1.38	0.57	0.125
IL-17F	1.45	1.46	1.04	0.46	0.238
IL-1 alpha	10.49	2.72	10.33	2.54	0.926
IL-1 beta	2.96	0.78	3.47	1.81	0.512
IL-1RA	617	245.8	580.5	93.03	0.933
IL-2	12.80	2.96	13.44	6.03	0.618
IL-4	4.92	0.92	5.60	2.02	0.356
IL-5	11.22	7.30	14.06	6.03	0.017
IL-6	1.43	2.26	1.21	1.98	0.577
IL-7	42.82	12.07	31.79	8.19	0.001
IL-8	26.03	45.27	40.66	40.56	0.269
IP-10	14.68	5.07	14.40	5.82	0.849†
LEPTIN	1457.4	1187.8	447.6	459.4	< 0.001
LIF	1.74	0.45	2.57	1.91	0.011
M-CSF	7.41	3.36	6.04	2.43	0.198
MCP-1	15.21	7.03	12.31	3.42	0.110
MCP-3	10.31	2.94	9.50	1.60	0.193†
MIG	52.37	31.81	35.41	14.88	0.039
MIP-1 beta	90.36	43.52	87.52	51.69	0.534
MIP-1 alpha	17.04	11.06	14.17	3.78	0.269

NGF	6.38	1.34	10.39	10.54	0.035
PAI-1	726	138.6	654.5	115.5	0.052†
PDGFBB	237.7	158.2	147.4	96.78	0.044
RANTES	617.8	399.9	416.7	114.1	0.012
Resistin	199.7	106.3	180.9	120.2	0.440
SCF	17.84	3.91	17.12	7.69	0.173
sFAS ligand	16.39	7.34	14.28	4.06	0.480
TGF alpha	1.41	2.23	1.06	1.19	0.532
TGF beta	5.85	2.27	4.82	2.09	0.095†
TNF alpha	4.26	1.82	5.50	2.21	0.060
TNF beta	0.41	1.03	0.15	0.19	0.230
Trail	41.63	16.55	34.09	5.59	0.011
V-CAM-1	2446.8	1003	1747.5	728.5	0.008
VEGF	7.86	6.16	6.63	3.15	0.717
WBC	6.32	1.51	5.92	1.64	0.373†
CD3+ T	1.74	0.77	1.64	0.54	0.417
CD4+ T	1.06	0.59	0.95	0.27	0.368†
CD8+ T	0.55	0.25	0.57	0.36	0.620
DP T	0.039	0.041	0.013	0.007	0.048
DN T	0.076	0.071	0.089	0.054	0.151
PD-1+CD4	0.303	0.438	0.089	0.173	0.0002
PD-1+CD8	0.099	0.063	0.064	0.047	0.033
PD-1+DP	0.011	0.009	0.005	0.003	0.027
PD-1+DN	0.020	0.018	0.012	0.008	0.095
CTLA-4+CD4	0.019	0.025	0.013	0.018	0.450
CTLA-4+CD8	0.016	0.026	0.012	0.021	0.874
CTLA-4+DP	0.008	0.033	0.001	0.001	0.544
CTLA-4+DN	0.009	0.028	0.001	0.001	0.531
B CELLS	0.408	0.371	0.429	0.214	0.312
Plasma cells	0.027	0.021	0.017	0.019	0.082
CD64+PD-L1+	0.120	0.078	0.044	0.050	0.0009
CD3-CD64+ cells / ml blood(x10^6)	0.32	0.17	0.32	0.15	0.76
Absolute NK+/ml blood	0.23	0.15	0.16	0.07	0.276
Absolute NK+ CD94+/ml blood	0.12	0.10	0.08	0.05	0.034†
Assolute T-regs	0.028	0.0200	0.005	0.004	< 0.0001
% T-regs	55.47	18.39	23.23	13.62	< 0.0001
IFN-g+ CD4+ T post G	0.00194	0.00204	0.00180	0.00210	0.397
Gra-B+ CD4+ T post G	0.12	0.11	0.08	0.100	0.161
IFN-g+ CD8+ T	0.00076	0.00063	0.00091	0.00105	0.925

post G					
Gra-B+ CD8+ T post G	0.46	0.38	0.33	0.54	0.08
IFN-g+ NK - post G	0.00430	0.00590	0.00131	0.00219	0.133
Gran-B+ NK post G	0.47	0.38	0.15	0.13	0.004
IFN-g+ CD4+ T post G and PMA	0.25	0.15	0.15	0.12	0.022
Gra-B+ CD4+ T post G and PMA	0.11	0.08	0.09	0.09	0.354
IFN-g+ CD8+ T post G and PMA	0.42	0.31	0.22	0.37	0.006
Gra-B+ CD8+ T post G and PMA	0.38	0.31	0.22	0.38	0.016

Note: Except $p^{\dagger}$ -values were from of student's t test, other *p*-values were from Wilcoxon rank sum test.

Table 3. Statistic summary of biomarkers between remission and recurrence patients.

Biomarker	Remission (n=20)		Recurrence (n=15)		p^{+}
	Mean	Std Dev	Mean	Std Dev	
CD40Ligand	71.43	35.68	52.78	19.35	0.042
ENA78	89.51	73.78	85.32	107.9	0.401
EOTAXIN	9.39	3.15	8.80	4.04	0.420
FGF beta	3.97	1.50	3.81	1.21	0.553
G-CSF	1.60	0.71	1.33	0.80	$0.301^{\dagger}$
GM-CSF	26.51	10.69	26.69	12.89	0.753
GRO ALPHA	12.22	4.38	12.73	4.77	0.680
HGF	21.44	8.49	24.32	12.07	0.753
ICAM-1	910558	2882844	1664239	4193173	0.779
IFN-alpha	6.54	5.60	4.52	3.89	0.285
IFN-beta	6.90	6.42	6.27	6.17	0.753
IFN-gamma	3.81	1.24	3.67	0.78	0.843
IL-10	0.89	0.41	0.81	0.43	0.448
IL-12-P70	8.42	2.52	7.72	1.87	0.520
IL-12-P40	21.65	8.94	16.04	4.42	0.073
IL-13	3.62	1.90	3.69	1.34	0.420
IL-15	5.15	3.20	6.12	4.31	0.499
IL-17	1.17	0.50	1.20	0.40	0.552
IL-17F	1.53	1.58	1.34	1.31	0.509
IL-1 alpha	10.41	2.77	10.59	2.74	0.921
IL-1 beta	2.92	0.81	3.02	0.77	0.843
IL-1RA	570.10	139.40	679.50	336.2	0.245
IL-2	13.18	3.03	12.30	2.88	0.563

IL-4	4.95	0.85	4.88	1.03	0.908
IL-5	12.43	9.28	9.60	2.75	0.449
IL-6	1.63	2.77	1.16	1.37	0.970
IL-7	44.92	12.96	40.01	10.53	0.301
IL-8	11.04	13.40	46.02	63.11	0.357
IP-10	13.76	4.54	15.91	5.62	0.286
LEPTIN	1629.5	1331.9	1295.8	931.9	0.427
LIF	1.77	0.46	1.71	0.44	0.597
M-CSF	7.88	3.91	6.77	2.43	0.332
MCP-1	14.42	4.13	16.27	9.74	0.987
MCP-3	9.77	2.43	11.01	3.48	0.222†
MIG	52.62	35.47	52.04	27.39	0.934
MIP-1 beta	93.13	42.95	86.65	45.50	0.272
MIP-1 alpha	15.57	6.09	19.01	15.48	0.779
NGF	6.33	1.56	6.44	1.03	0.448
PAI-1	762.5	148.9	677.3	110.1	0.071†
PDGFBB	250.9	163.8	220	154.3	0.680
RANTES	704.3	501.4	502.5	148.9	0.401
Resistin	201.6	108.9	197.1	106.5	0.882
SCF	18.30	4.24	17.23	3.48	0.401
sFAS ligand	18.87	8.73	13.08	2.63	0.017
TGF alpha	1.76	2.72	0.93	1.27	0.585
TGF beta	6.61	2.02	4.84	2.25	0.02†
TNF alpha	4.44	1.89	4.02	1.75	0.509
TNF beta	0.67	1.32	0.06	0.09	0.175
Trail	44.84	20.55	37.35	7.58	0.139
V-CAM-1	2505	1116.5	2369.2	860.4	0.520
VEGF	8.07	6.47	7.58	5.93	0.947
WBC	6.27	1.69	6.37	1.31	0.858†
CD3+ T	1.89	0.64	1.54	0.90	0.777
CD4+ T	1.16	0.52	0.93	0.67	0.29†
CD8+ T	0.589	0.221	0.500	0.284	0.322
DP T	0.044	0.038	0.032	0.044	0.226
DN T	0.091	0.076	0.055	0.060	0.037
PD-1+CD4	0.469	0.530	0.089	0.044	0.001
PD-1+CD8	0.127	0.068	0.062	0.029	0.008
PD-1+DP	0.014	0.009	0.006	0.006	0.017
PD-1+DN	0.026	0.020	0.013	0.012	0.011
CTLA-4+CD4	0.024	0.029	0.013	0.016	0.340
CTLA-4+CD8	0.023	0.031	0.008	0.014	0.015
CTLA-4+DP	0.013	0.044	0.001	0.001	0.031
CTLA-4+DN	0.011	0.034	0.007	0.017	0.464
B CELLS	0.478	0.431	0.319	0.264	0.420
Plasma cells	0.034	0.020	0.020	0.021	0.053
CD64+PD-L1+	0.160	0.069	0.066	0.054	0.002

CD3-CD64+ cells / ml blood(x10^6)	0.314	0.148	0.327	0.204	0.74
Absolute NK+/ml blood	0.231	0.135	0.223	0.165	0.98
Absolute NK+ CD94+/ml blood	0.119	0.081	0.125	0.119	0.94
Assolute T-regs	0.034	0.016	0.022	0.023	0.03
% T-regs	55	18.40	56.14	19.46	0.95
IFN-g+ CD4+ T post G	0.00189	0.00228	0.00198	0.00191	0.974
Gra-B+ CD4+ T post G	0.09	0.06	0.14	0.13	0.770
IFN-g+ CD8+ T post G	0.00049	0.00031	0.00098	0.00074	0.136
Gra-B+ CD8+ T post G	0.40	0.33	0.51	0.43	0.496
IFN-g+ NK - post G	0.00298	0.00496	0.00540	0.00658	0.236
Gran-B+ NK post G	0.31	0.20	0.61	0.45	0.153
IFN-g+ CD4+ T post G and PMA	0.28	0.12	0.23	0.18	0.350
Gra-B+ CD4+ T post G and PMA	0.09	0.06	0.12	0.09	0.626
IFN-g+ CD8+ T post G and PMA	0.42	0.23	0.42	0.37	0.720
Gra-B+ CD8+ T post G and PMA	0.36	0.25	0.39	0.36	0.974

Note: Except $p^{\dagger}$ -values were from of student's t test, other *p*- values were from Wilcoxon rank sum test.

Table 4. Univariate progression free survival analysis of demographic and clinical characteristics in disease patients.

Characteristics	Level	N	Hazard Ratio (HR)	95% CI of HR	*p-value Wald test*	*p-value Log-Rank test*
Sex	Male	11	2.068	(0.723-5.913)	0.175	0.166
	female	24	1.000			
Cancer Type	Anaplastic, FC or HCT	7	1.000			
	PTC	28	3.624	(0.473 - 27.779)	0.215	0.185
Stage	I and II	21	1.000			
	III and IV	13	2.979	(0.957 - 9.276)	0.060	0.0487
T	1 and 2	21	1.000			
	3 and 4	11	4.874	(1.489 -- 15.956)	0.0089	0.0042
M	No	31	1.000			
	Yes	3	4.05E8	(0.000 - --)	0.994	<.0001
N	No	16	1.000			
	Yes	11	1.960	(0.654 - 5.873)	0.229	0.221
I-131	No	5	1.000			
	Yes	30	11971495	(0.000 - --)	0.994	0.093
Radiation	No	29	1.000			
	Yes	6	2.954	(0.907 - 9.620)	0.072	0.060
Age		35	1.027	(0.982 - 1.074)	0.240	

Table 5. Univariate progression free survival analysis of biomarkers in disease patients.

Biomarker	Hazard Ratio (HR)	95% CI of HR	*p-value Wald test*
CD40Ligand	0.983	(0.959 - 1.007)	0.158
ENA78	1.001	(0.995 - 1.008)	0.749
EOTAXIN	0.931	(0.778 - 1.114)	0.434
FGF beta	0.816	(0.524 - 1.270)	0.367
G-CSF	0.395	(0.158 - 0.987)	0.047

GM-CSF	0.990	(0.941 - 1.041)	0.695
GRO ALPHA	1.007	(0.890 - 1.140)	0.911
HGF	1.025	(0.971 - 1.082)	0.778
ICAM-1	1.000	(1.000 - 1.000)	0.498
IFN-alpha	0.888	(0.772 - 1.021)	0.095
IFN-beta	0.986	(0.897 - 1.084)	0.774
IFN-gamma	0.851	(0.494 - 1.466)	0.560
IL-10	0.602	(0.169 - 2.145)	0.434
IL-12-P70	0.889	(0.682 - 1.160)	0.388
IL-12-P40	0.873	(0.785 - 0.997)	0.012
IL-13	0.952	(0.691 - 1.310)	0.761
IL-15	1.023	(0.889 - 1.178)	0.746
IL-17	0.822	(0.242 - 2.793)	0.753
IL-17F	0.925	(0.610 - 1.402)	0.713
IL-1 alpha	0.957	(0.782 - 1.171)	0.672
IL-1 beta	1.096	(0.535 - 2.247)	0.802
IL-1RA	0.999	(0.997 - 1.002)	0.576
IL-2	0.901	(0.745 - 1.089)	0.280
IL-4	0.871	(0.469 - 1.618)	0.662
IL-5	0.936	(0.815 - 1.074)	0.347
IL-6	0.939	(0.733 - 1.204)	0.621
IL-7	0.968	(0.923 - 1.015)	0.175
IL-8	1.005	(0.996 - 1.104)	0.248
IP-10	1.031	(0.936 - 1.137)	0.536
LEPTIN	1.000	(0.999 - 1.000)	0.440
LIF	0.631	(0.167 - 2.382)	0.496
M-CSF	0.887	(0.749 - 1.051)	0.166
MCP-1	1.014	(0.933 - 1.103)	0.745
MCP-3	1.055	(0.877 - 1.270)	0.569
MIG	0.997	(0.979 - 1.016)	0.756
MIP-1 beta	1.000	(0.987 - 1.013)	0.974
MIP-1 alpha	1.019	(0.981 - 1.057)	0.329
NGF	0.950	(0.643 - 1.403)	0.796
PAI-1	0.996	(0.992 - 1.000)	0.037
PDGFBB	0.999	(0.995 - 1.002)	0.438
RANTES	0.998	(0.996 - 1.001)	0.184
Resistin	0.999	(0.994 - 1.04)	0.690
SCF	0.865	(0.734 - 1.020)	0.085
sFAS ligand	0.842	(0.717 - 0.990)	0.037
TGF alpha	0.834	(0.599 - 1.161)	0.282
TGF beta	0.733	(0.548 - 0.987)	0.036
TNF alpha	0.0863	(0.620 - 1.200)	0.381
TNF beta	0.014	(0.000 - 2.102)	0.157
Trail	0.959	(0.913 - 1.008)	0.101
V-CAM-1	1.000	(0.999 - 1.000)	0.257

VEGF	0.981	(0.888 - 1.083)	0.700
WBC	0.941	(0.655 - 1.350)	0.740
CD3+ T	0.634	(0.281 - 1.434)	0.274
CD4+ T	0.476	(0.152 - 1.492)	0.203
CD8+ T	0.782	(0.081 - 7.507)	0.831
DP T	0.464	(0.000 – 5375832)	0.926
DN T	0.003	(0.000 - 386.83)	0.334
PD-1+CD4	0.000	(0.000 - 0.007)	0.0033
PD-1+CD8	0.000	(0.000 - 0.011)	0.0122
PD-1+DP	0.000	(0.000 – 1701149)	0.097
PD-1+DN	0.000	(0.000 - 16026.84)	0.121
CTLA-4+CD4	0.000	(0.000 – 1336682)	0.376
CTLA-4+CD8	0.000	(0.000 - 4237441)	0.303
CTLA-4+DP	0.000	(0.000 - $2.53^{E}182$)	0.538
CTLA-4+DN	0.001	(0.000 – 88795560)	0.593
B CELLS	0.470	(0.072 - 3.080)	0.430
Plasma cells	0.000	(0.000 - 13231.58)	0.177
CD64+PD-L1+	0.000	(0.000 - 0.001)	0.0011
CD3-CD64+ cells / ml blood(x10^6)	0.742	(0.026 - 21.252)	0.862
Absolute NK+/ml blood	0.620	(0.010 - 40.16)	0.822
Absolute NK+ CD94+/ml blood	1.557	(0.006 - 414.55)	0.877
Assolute T-regs	0.000	(0.000 - 684517)	0.164
% T-regs	0.999	(0.962 - 1.037)	0.967
IFN-g+ CD4+ T post G	$3.652^{E}15$	(0 - $5.75^{E}131$)	0.793
Gra-B+ CD4+ T post G	61.61	(0.279 - 13606.37)	0.135
IFN-g+ CD8+ T post G	--	(116090 - --)	0.047
Gra-B+ CD8+ T post G	1.690	(0.414 - 6.905)	0.465
IFN-g+ NK - post G	1.646E19	(0 - 5.842E56)	0.316
Gran-B+ NK post G	3.007	(0.789 - 11.463)	0.107
IFN-g+ CD4+ T post G and PMA	0.328	(0.003 - 35.518)	0.641
Gra-B+ CD4+ T post G and PMA	60.58	(0.040 - 91283.35)	0.272
IFN-g+ CD8+ T post G and PMA	1.108	(0.158 - 7.778)	0.918
Gra-B+ CD8+ T post G and PMA	1.252	(0.190 - 8.250)	0.815

Table 6. Multivariate Cox proportional hazard model of progression-free survival time (PFS) in disease patients (n = 35) with biomarkers.

Variable	Hazard Ratio (HR)	95% CI of HR	*p-value Wald Test*
sFAS Ligand	0.011	(0.000 - 0.943)	0.047
IFN alpha	99.585	(1.001 - 911.75)	0.050
PD_1+CD8	0.000	(0.000 - 7.759)	0.052
PD_1+DP	--	(0.000 - --)	0.053
TGF beta	0.010	(0.000 - 1.052)	0.053
PAI-1	1.056	(0.998 - 1.117)	0.061

Table 7. Pearson correlation analysis of six significant biomarkers in the selected Cox PH model for PFS.

	Parameters	sFAS Ligand	TGF beta	IFN alpha	PD_1+CD8	PD_1+DP
PAI-1	Coefficient	0.643	0.415	0.457	0.101	0.078
	Probability	<0.0001	0.013	0.0058	0.581	0.671
sFAS Ligand	Coefficient	--	0.177	0.807	0.237	0.0558
	Probability		0.309	<0.0001	0.192	0.762
TGF beta	Coefficient	--	--	0.0658	0.407	0.217
	Probability			0.707	0.021	0.232
IFN alpha	Coefficient	--	--	--	0.188	0.060
	Probability				0.303	0.744
PD_1+CD8	Coefficient	--	--	--	--	0.536
	Probability					0.0016

Table 8. Multivariate Cox proportional hazard model of progression-free survival time (PFS) in disease patients (n = 35) with biomarkers and clinical characteristics.

Variable	Hazard Ratio (HR)	95% CI of HR	*p-value Wald Test*
sFAS Ligand	0.330	(0.136– 0.799)	0.014
SCF	0.438	(0.211 – 0.907)	0.003
IL-12_P40	0.737	(0.517 – 1.051)	0.009
IFN alpha	4.061	(1.453 – 11.347)	0.038
PD_1_DP	1.626E92	(0.00 – 2.77E20)	0.010

Table 9. Univariate overall survival analysis of demographic and clinical characteristics in disease patients.

Characteristics	Level	N	Hazard Ratio (HR)	95% CI of HR	*p-value Wald test*	*p-value Log-Rank test*
Sex	Male	11	1.14E8	(0.000 - --)	0.998	0.103
	female	24	1.000			
Cancer Type	Anaplastic, FC or HCT	7	1.000			
	PTC	28	13669382	(0.000 - --)	0.998	0.581
Stage	I and II	21	1.000			
	III and IV	13	81336416	(0.000 - --)	0.998	0.177
T	1 and 2	21	1.000			
	3 and 4	11	2.178	(0.412 - 11.513)	0.360	0.515
M	No	31	1.000			
	Yes	3	--	--	--	--
N	No	16	1.000			
	Yes	11	60563509	(0.000 - --)	0.998	0.186
I-131	No	5	1.000			
	Yes	30	4381748	(0.000 - --)	0.997	0.655
Radiation	No	29	1.000			
	Yes	6	2.236	(0.111 - 44.877)	0.599	0.593
Age		35	1.056	(0.904 - 1.233)	0.494	0.475

Table 10. Univariate overall survival analysis of biomarkers in disease patients.

Biomarker	Hazard Ratio (HR)	95% CI of HR	*p-value* *Wald test*
CD40Ligand	0.978	(0.909 - 1.051)	0.542
ENA78	0.963	(0.888 - 1.045)	0.368
EOTAXIN	0.528	(0.230 - 1.213)	0.132
FGF beta	0.928	(0.367 - 2.346)	0.874
G-CSF	0.413	(0.061 - 2.800)	0.365
GM-CSF	0.896	(0.71 - 1.131)	0.357
GRO ALPHA	0.871	(0.534 - 1.420)	0.580
HGF	0.921	(0.754 - 1.126)	0.424
ICAM-1	1.000	(1.000 - 1.000)	0.455
IFN-alpha	0.888	(0.614 - 1.283)	0.526
IFN-beta	1.320	(0.789 - 2.208)	0.291
IFN-gamma	1.086	(0.308 - 3.825)	0.898
IL-10	3.406	(0.071 - 163.995)	0.535
IL-12-P70	0.924	(0.472 - 1.809)	0.818
IL-12-P40	0.750	(0.491 - 1.147)	0.185
IL-13	1.366	(0.662 - 2.801)	0.395
IL-15	1.350	(0.964 - 1.891)	0.081
IL-17	0.911	(0.048 - 17.21)	0.951
IL-17F	2.072	(0.859 - 5.002)	0.105
IL-1 alpha	0.805	(0.386 - 1.681)	0.564
IL-1 beta	1.325	(0.130 - 13.474)	0.812
IL-1RA	0.998	(0.992 - 1.005)	0.617
IL-2	0.920	(0.592 - 1.431)	0.712
IL-4	0.167	(0.022 - 1.272)	0.084
IL-5	0.933	(0.779 - 1.266)	0.957
IL-6	0.986	(0.454 - 2.143)	0.971
IL-7	0.981	(0.869 - 1.107)	0.753
IL-8	0.997	(0.972 - 1.023)	0.819
IP-10	0.999	(0.688 - 1.451)	0.996
LEPTIN	0.999	(0.995 - 1.005)	0.422
LIF	0.565	(0.029 - 10.995)	0.706
M-CSF	0.941	(0.579 - 1.530)	0.807
MCP-1	0.742	(0.478 - 1.150)	0.182
MCP-3	0.907	(0.563 - 1.463)	0.690
MIG	0.980	(0.920 - 1.043)	0.523
MIP-1 beta	0.817	(0.620 - 1.077)	0.152
MIP-1 alpha	1.008	(0.876 - 1.161)	0.907
NGF	1.188	(0.451 - 3.127)	0.728
PAI-1	0.998	(0.987 - 1.009)	0.708
PDGFBB	0.998	(0.988 - 1.008)	0.653
RANTES	0.998	(0.989 - 1.007)	0.628
Resistin	0.914	(0.782 - 1.068)	0.260

SCF	0.608	(0.318 - 1.164)	0.133
sFAS ligand	0.932	(0.635 - 1.368)	0.720
TGF alpha	1.272	(0.726 - 2.229)	0.401
TGF beta	0.640	(0.250 - 1.642)	0.353
TNF alpha	0.657	(0.225 - 1.924)	0.444
TNF beta	0.336	(0 - 547.71)	0.773
Trail	0.985	(0.853 - 1.137)	0.834
V-CAM-1	0.999	(0.998 - 1.001)	0.524
VEGF	1.163	(0.969 - 1.397)	0.105
WBC	0.091	(0.001 - 7.655)	0.290
CD3+ T	0.426	(0.025 - 7.369)	0.558
CD4+ T	0.341	(0.012 - 9.441)	0.526
CD8+ T	1.757	(0.001 - 3743.57)	0.885
DP T	418.24	(0.000 - 8.112^{E}22)	0.800
DN T	0.000	(0.000 - --)	0.791
PD-1+CD4	0.000	(0.000 - 3.7224^{E}8)	--
PD-1+CD8	0.000	(0.000 - 3.324^{E}13)	0.261
PD-1+DP	0.000	(0.000 - 1.58^{E}164)	0.442
PD-1+DN	0.000	(0.000 - 7.377^{E}39)	0.954
CTLA-4+CD4	0.000	(0.000 - 1.32^{E}170)	--
CTLA-4+CD8	0.000	(0.000 - --)	--
CTLA-4+DP	0.000	(0.000 - --)	--
CTLA-4+DN	0.000	(0.000 - --)	0.676
B CELLS	0.000	(0.000 - 62812.44)	--
Plasma cells	0.000	(0.000 – 7.32E69)	0.360
CD64+PD-L1+	0.000	(0.000 - 2.403E9)	0.592
CD3-CD64+ cells / ml blood(x10^6)	404.14	(0.025 - 647688.3)	0.224
Absolute NK+/ml blood	0.000	(0.000 - 1988.83)	0.191
Absolute NK+ CD94+/ml blood	0.000	(0.000 - 1.3995E8)	0.394
Assolute T-regs	0.000	(0.000 - --)	--
% T-regs	0	(-- - --)	--
IFN-g+ CD4+ T post G	815095	(0.000 - --)	--
Gra-B+ CD4+ T post G	6.8749E99	(0.000 - --)	--
IFN-g+ CD8+ T post G	--	(-- - --)	--
Gra-B+ CD8+ T post G	4.33E64	(0.000 - --)	--
IFN-g+ NK - post G	--	(0.000 - --)	--

Gran-B+ NK post G	1.55E40	(0.000 - --)	--
IFN-g+ CD4+ T post G and PMA	0.104	(0.000 – 1718659)	0.790
Gra-B+ CD4+ T post G and PMA	7022	(0.000 - 2.214E12)	0.375
IFN-g+ CD8+ T post G and PMA	4.636E55	(0.000 - --)	0.999
Gra-B+ CD8+ T post G and PMA	1.166E29	(0.000 - --)	--

Table 11. ROC analysis of significant biomarkers between remission and recurrence patients.

Biomarker	AUC	Std Error	95% CI of AUC	*p-value chi-square test*
CD40 ligand	0.7133	0.0901	(0.5367 - 0.8900)	0.018
sFAS ligand	0.7533	0.0848	(0.5871 - 0.9196)	0.0028
TGF beta	0.7550	0.0899	(0.5789 - 0.9311)	0.0045
DN_T	0.7302	0.0994	(0.5353 - 0.9250)	0.021
PD_1_CD4	0.9087	0.0519	(0.8070 - 1.0000)	<0.0001
PD_1_CD8	0.7976	0.0812	(0.6385 - 0.9567)	0.0002
PD_1_DP	0.7659	0.0855	(0.5983 - 0.9334)	0.0019
PD_1_DN	0.7857	0.0875	(0.6141 - 0.9573)	0.0011
CTLA_4_CD8	0.7698	0.0935	(0.5866 - 0.9531)	0.0039
CTLA_4_DP	0.7381	0.0950	(0.5518 - 0.9244)	0.012
CD64_PD_L1	0.8718	0.0634	(0.7475 - 0.9961)	<0.0001
Assolute Tregs	0.7821	0.1016	(0.5830 - 0.9811)	0.0055
Plasma cells	0.7367	0.1030	(0.5348 - 0.9386)	0.022
IL_12_P40	0.6867	0.0925	(0.5053 - 0.8680)	0.044
PAI-1	0.6767	0.0936	(0.4933 - 0.8601)	0.059
IFN alpha	0.6100	0.0986	(0.4168 - 0.8032)	0.265

Table 12. Summary of cutoff points for different sensitivities and specificities of significant biomarkers between remission and recurrence patients.

Biomarker	Sensitivity= 90%		Specificity= 90%		Maximum Sum of Sens. and Spec.			≥50% Recurrence
	Spec.	Cutoff	Sens.	Cutoff	Sens.	Spec.	Cutoff	Cutoff
CD40 ligand	50%	68.61	7%	33.14	93%	50%	68.61	≤ 50.57
sFAS ligand	35%	17.45	20%	10.88	80%	70%	15.00	≤ 12.73
TGF beta	15%	9.26	33%	3.99	67%	85%	5.05	≤ 4.75
DN_T	17%	0.118	50%	0.032	64%	89%	0.040	≤ 0.043

PD_1_CD4	72%	0.143	71%	0.112	100%	72%	0.163	≤ 0.128
PD_1_CD8	67%	0.098	29%	0.045	100%	67%	0.11	≤ 0.078
PD_1_DP	39%	0.016	36%	0.0028	71%	72%	0.0056	≤ 0.0072
PD_1_DN	33%	0.029	64%	0.00079	64%	94%	0.010	≤ 0.013
CTLA_4_CD8	28%	0.027	64%	0.0012	64%	94%	0.0012	≤ 0.0039
CTLA_4_DP	33%	0.0019	50%	0.00027	50%	100%	0.00025	≤ 0.00064
CD64_PD_L1	61%	0.15	62%	0.072	77%	83%	0.091	≤ 0.092
Assolute Tregs	54%	0.036	33%	0.012	83%	77%	0.021	≤ 0.024
Plasma cells	23%	0.059	46%	0.091	77%	69%	0.028	≤ 0.026
IL_12_P40	40%	21.27	13%	10.99	80%	60%	19.82	≤ 15.89
PAI-1	40%	799.31	20%	576.87	67%	70%	682.84	≤ 657.51
IFN alpha	25%	8.931	20%	0.610	53%	70%	2.711	≤ 2.340

Table 13. ROC analysis of significant biomarkers between disease patients and healthy controls.

Biomarker	AUC	Std Error	95% CI of AUC	*p-value chi-square test*
CD40 ligand	0.7333	0.0723	(0.5916 - 0.8751)	0.0013
GM-CSF	0.8293	0.0597	(0.7123 - 0.9462)	< 0.0001
ICAM-1	0.8016	0.0627	(0.6787 - 0.9245)	< 0.0001
IFN-gammar	0.7517	0.0676	(0.6192 - 0.8842)	0.0002
IL-10	0.6912	0.0802	(0.5340 - 0.8483)	0.017
IL-12-P40	0.7156	0.0724	(0.5738 - 0.8575)	0.0029
IL-15	0.7075	0.0726	(0.5652 - 0.8498)	0.0043
IL-5	0.6986	0.0750	(0.5517 - 0.8456)	0.0081
IL-7	0.7735	0.0624	(0.6512 - 0.8958)	< 0.0001
LEPTIN	0.8571	0.0557	(0.7479 - 0.9664)	< 0.0001
LIF	0.7136	0.0705	(0.5755 - 0.8517)	0.0024
MIG	0.6707	0.0727	(0.5282 - 0.8133)	0.019
NGF	0.6741	0.0768	(0.5237 - 0.8246)	0.023
PDGFBB	0.6667	0.0739	(0.5218 - 0.8115)	0.024
RANTES	0.7088	0.0692	(0.5732 - 0.8445)	0.0025
Trail	0.7116	0.0699	(0.5746 - 0.8485)	0.0025
V-CAM-1	0.7224	0.0714	(0.5825 - 0.8624)	0.0018
DP T	0.6688	0.0752	(0.5213 - 0.8162)	0.025
PD-1+CD4	0.8391	0.0634	(0.7148 - 0.9633)	< 0.0001
PD-1+CD8	0.6828	0.0761	(0.5337 - 0.8319)	0.016
PD-1+DP	0.6898	0.0730	(0.5468 - 0.8329)	0.0093

CD64+PD-L1	0.7968	0.0674	(0.6648 - 0.9288)	< 0.0001
Abssolute Tregs	0.9410	0.0342	(0.8738 - 1.0000)	< 0.0001
% Tregs	0.9329	0.0345	(0.8652 - 1.0000)	< 0.0001
Gran-B+NK post G	0.7828	0.0726	(0.6405 - 0.9252)	< 0.0001
IFN-g+ CD4+ T post G and PMA	0.7222	0.0840	(0.5576 - 0.8869)	0.0082
IFN-g+ CD8+ T post G and PMA	0.7727	0.0804	(0.6151 - 0.9304)	0.0007
Gra-B+ CD8+ T post G and PMA	0.7348	0.0865	(0.5653 - 0.9044)	0.0066

Table 14. Summary of cutoff points for different sensitivities and specificities of significant biomarkers between disease patients and healthy controls.

Biomarker	Sensitivity= 90%		Specificity= 90%		Maximum Sum of Sens. and Spec.			≥50% Disease
	Spec.	Cutoff	Sens.	Cutoff	Sens.	Spec.	Cutoff	Cutoff
CD40 ligand	43%	37.21	11%	84.08	69%	76%	50.66	≥ 41.34
GM-CSF	48%	13.50	46%	26.74	83%	81%	16.91	≥ 16.47
ICAM-1	28%	2598.10	26%	61006.53	69%	94%	8179.73	≥ 1234.12
IFN-gammar	33%	5.05	51%	3.39	57%	90%	3.58	≤ 5.05
IL-10	38%	1.41	9%	0.25	71%	71%	0.99	≤ 1.29
IL-12-P40	38%	10.99	29%	21.19	71%	71%	15.27	≥ 12.31
IL-15	29%	9.743	14%	2.751	71%	67%	5.592	≤ 11.12
IL-5	19%	16.12	14%	6.902	83%	62%	11.993	≤ 16.58
IL-7	38%	28.85	43%	46.94	66%	81%	37.50	≥ 31.97
LEPTIN	43%	363.19	38%	1371.95	81%	90%	584.41	≥ 698.74
LIF	24%	2.47	29%	1.39	69%	71%	1.77	≤ 2.22
MIG	24%	24.68	29%	66.097	46%	86%	43.061	≥ 27.843
NGF	43%	7.76	34%	5.56	91%	43%	7.761	≤ 8.50
PDGFBB	24%	67.62	23%	329.576	54%	81%	204.901	≥ 96.83
RANTES	29%	357.52	34%	613.06	54%	81%	520.6	≥ 386.72
Trail	29%	31.403	31%	43.387	46%	90%	39.916	≥ 31.12
V-CAM-1	52%	1635.44	26%	3018.88	91%	52%	1635.54	≥ 1566.84
DP T	0%	0.0037	47%	0.024	47%	95%	0.0266	≥ 0.0117
PD-1+CD4	75%	0.060	25%	0.207	91%	75%	0.06	≥ 0.044
PD-1+CD8	25%	0.0393	44%	0.093	59%	80%	0.075	≥0.0448

PD-1+DP	10%	0.0017	34%	0.0123	53%	85%	0.0066	≥ 0.004
CD64+PD-L1	30%	0.0205	52%	0.1098	87%	75%	0.0349	≥ 0.064
Abssolute Tregs	90%	0.0103	80%	0.0122	92%	90%	0.0103	≥ 0.0102
% Tregs	81%	35.71	68%	47.11	91%	81%	35.707	≥ 37.907
Gran-B+NK post G	33%	0.085	50%	0.408	50%	100%	0.4198	≥ 0.229
IFN-g+ CD4+ T post G and PMA	44%	0.1051	14%	0.4417	64%	78%	0.2051	≥ 0.182
IFN-g+ CD8+ T post G and PMA	44%	0.1059	0%	1.6471	73%	83%	0.2128	≥ 0.213
Gra-B+ CD8+ T post G and PMA	11%	0.0382	59%	0.3290	59%	94%	0.329	≥ 0.165

Figure 1. Kaplan-Meier curve of overall survival (OS).

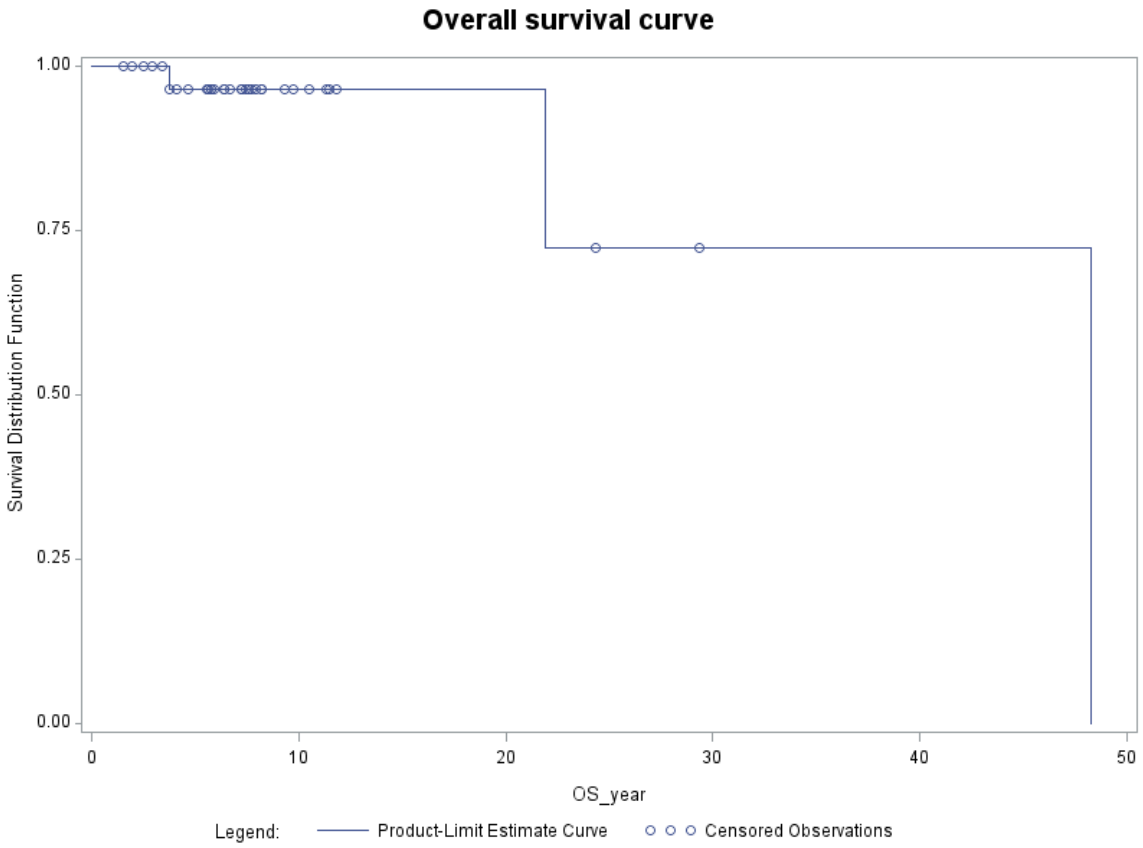

Figure 2. Kaplan –Meier curve of progression free survival (PFS).