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CLINICAL TRIAL REPORT

Long-Term Changes of Urinary Exosomal Peptide Levels After Thyroidectomy in Patients with Thyroid Cancer: A *Prospective Observational Study*

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Background: The recurrence rate of thyroid cancer can be as high as 30%. The purpose of this study was to examine changes of urine exosomal peptide levels after thyroidectomy in patients with thyroid cancer to determine if levels can predict the risk of recurrence.

Methods: Patients >20 years old as newly diagnosed with papillary thyroid cancer who had received a thyroidectomy were recruited. Urine samples were collected at 12 months after enrollment to the study, and 1 year later. Urine exosomes containing different peptides were identified and compared.

Results: A total of 70 patients were enrolled in the study, and were classified by the interval between surgery and enrollment: 42 patients with < 5 years between surgery and enrollment, 14 patients between 5–10 years, and 14 patients longer than 10 years. No recurrence was observed in any patient during the 2 years after enrollment. No significant differences were found in the levels of serum proteins or urine exosomal peptides between groups, or between intervals. Known risk factors for high-risk thyroid cancer had only a mild correlation with serum protein levels and urine exosomal peptides.

Conclusion: Our study revealed the long-term basal fluctuation ranges of serum proteins and urine exosomal peptides in patients with thyroid cancer who underwent thyroidectomy. For high-risk patients after thyroidectomy, concentrations of serum proteins or urine exosomal peptides within the ranges may indicate there is a lower risk of thyroid cancer recurrence during long-term follow-up.

Trial Registration: ClinicalTrials.gov: NCT03488134.

Keywords: thyroid cancer, urine exosome, peptide, thyroglobulin, recurrence

Introduction

Thyroid cancer is not uncommon, and annually there are about 53,000 new cases of thyroid cancer in the United States (US).[1–3] The disease affects both women and men, and is commonly diagnosed in the fourth through sixth decades of life.[1–3] The most common pattern of thyroid cancer is papillary, which accounts for about 80% of patients and it responds well to treatment and is rarely fatal.[1–3] Follicular thyroid cancer accounts for about 15% of cases, and metastasis to bones and lungs is common.[1–3] Medullary and anaplastic thyroid cancers each account for about 2% of cases, and are aggressive diseases.[1–3] Surgery and radioiodine are the most common treatment for thyroid cancer, and radiation therapy and chemotherapy may be used for advanced disease.[1–3]

Despite advances in understanding the pathogenesis of thyroid cancer and novel treatments, the recurrence rate of thyroid cancer after thyroidectomy ranges from 10% to 28%.[4,5] Risk factors for recurrence of thyroid cancer after thyroidectomy include male sex, tumor diameter, lymph node metastasis, and pathological type.[4] While monitoring serum thyroglobulin levels is recommended to identify recurrence after thyroidectomy,[6] however, several studies and meta-analysis have shown that serum thyroglobulin level is not useful for predicting recurrence of thyroid cancer with lobectomy.[7–10] As such, the development of non-invasive and more sensitive biomarkers for early detection of recurrence is crucial for the lifelong follow-up required by all these patients.

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Exosomes are micro-vesicles with a diameter of around 100 nm formed by endocytosis that are produced by most if not all cells types, and are excreted into the extracellular matrix or body fluids including serum, urine, saliva, sweat and ascites.[11–13] Exosomes can carry nucleic acids, proteins, and lipids that are unique to the origin cell.[11–13] Since the contents of exosomes are based on the donor cell, studies are revealing that they hold promise for the diagnosis of diseases and malignancies.[13–16] A number of studies have indicated that exosomes hold promise in the diagnosis and management of thyroid diseases, including malignancy.[17–19]

Our previous study found that urine exosomal thyroglobulin level revealed statistically significant higher in patients with thyroid cancer (stage T3), or with lymph node metastasis.[20] In another pilot study that included 21 patients with thyroid cancer, preoperative urine exosomal tissue inhibitors of metalloproteinases (TIMP) and angiopoietin-1 levels were associated with lymph node metastasis.[21] These results show that preoperative urine exosomes containing certain peptides have the potential to be used as biomarkers for screening high-risk patients before surgery. We hypothesized that urine exosome levels of some proteins might act as predictive biomarkers for monitoring recurrence of thyroid cancer after thyroidectomy. Therefore, this prospective study aimed to determine if urine exosome levels of difference proteins can predict recurrence of thyroid cancer.

Methods

Patients

This prospective study recruited patients diagnosed with thyroid cancer who ever underwent surgical treatment in National Taiwan University Hospital, all these patients had complete medical records. The study inclusion criteria were age >20 years, newly diagnosed as well-differentiated thyroid carcinoma at that time, and had received thyroidectomy. There were no exclusion criteria. Urine samples were collected at 12 months after enrollment to the study, and 1 year later, and urine exosomes containing different peptides were identified and compared.

Patient demographic information, serum thyroglobulin examinations, and clinical characteristics were obtained from the medical records. The primary outcome was recurrence of thyroid cancer during the study period. Secondary outcomes were the concentrations of serum proteins and different urine exosomal peptides at 12 months (Time 1) and 24 months (Time 2) after enrollment.

The study (201711082RINA) was approved by the ethics committees of the Institutional Review Board at National Taiwan University Hospital. Informed consent was obtained from all patients with related regulations and guidelines, and we confirm that our study complies with Declaration of Helsinki.

Thyroglobulin and Anti-Thyroglobulin Antibody

Thyroglobulin concentrations were determined through the use of the IMMULITE 2000 Thyroglobulin device, a solid-phase, chemiluminescent immunometric test that boasts an analytical sensitivity of 0.2 ng/mL, produced by Siemens in Erlangen, Germany. The levels of anti-thyroglobulin antibodies were assessed utilizing the ARCHITECT Anti-Tg assay, a 2-step quantitative immunoassay designed to measure thyroglobulin auto-antibodies in human serum, developed by Abbott Laboratory located in Abbott Park, IL, 60,064, USA. This assay has a detection threshold of ≤ 1.0 IU/mL.

Exosome Collection

A 200 mL fresh urine sample was collected to precipitate exosomes. Initially, the urine was spun at 3000×g for 15 minutes at 4°C to eliminate cells and debris, followed by a centrifugation at 10,000×g for 30 minutes at 4°C to clear out microvesicles. The volume of the urine samples was then reduced to 5–10 mL using Amicon® Ultra 15-centrifugal filters with a 100K molecular weight cutoff (Millipore). To separate urine exosomes, the Exo Quick-TC assay (SBI) was employed. The supernatants were decanted into fresh tubes and stored at −80°C with a complete, EDTA-free Protease Inhibitor Cocktail (Roche). The exosomes were subsequently resuspended in lysis buffer containing 7 M urea, 2 M thiourea, and 4% CHAPS, and then pelleted. The exosome protein samples were preserved at −80°C until analysis was performed using a multiple reaction monitoring (MRM) technique.

Tryptic Digestion

To precipitate the urine exosomes, three volumes of cold methanol at −20°C were added, followed by centrifugation at 10,000×g for 10 minutes. The resulting pellet was resuspended in lysis buffer, consisting of 4 M urea and 25 mM ammonium bicarbonate at a pH of 8.5. These samples were then denatured and subsequently reduced using 200 mM dithiothreitol at room temperature for one hour, and thereafter alkylated in darkness with 200 mM iodoacetamide. Excess iodoacetamide was neutralized with 200 mM DTT and allowed to react for 20 minutes at room temperature. The samples underwent digestion with modified sequencing-grade trypsin from Promega, located in Madison, WI, USA, which was carried out for 16 hours at 37°C.

MRM Q1/Q3 Ion Pair Selection Using Direct Infusion

Synthetic peptides were prepared at a concentration of 2 µg/mL in 0.1% formic acid and infused at a rate of 10 µL/min using a syringe pump. The peptide solutions were analyzed using an AB SCIEX QTRAP 5500 mass spectrometer (MS) equipped with a Turbo V source, operated through Analyst software 1.5. The MS conducted analyses in positive ion mode, setting the ion spray voltage at 5500 V and the source temperature at 550°C. The nebulizer pressure was adjusted to 60 psi, with a drying gas flow of 45 psi. Using Analyst software, a comprehensive list of b- and y-series fragment ions for 2+ and 3+ precursor ion-charge states was generated, covering an m/z range of 100 to 1000. For the optimization of MRM Q1/Q3 ion pairs, MRM scans were performed with both Q1 and Q3 at unit resolution (0.7 Da FWHM). The collision energy (CE) was progressively increased from 5 to 55 V in 1 volt steps, with each transition given a dwell time of 150 ms. From these experiments, the four transitions yielding the highest signal strengths were identified for each peptide. Subsequently, the three transitions that provided the most intense signals without interference were selected from the initial four.

LC-MRM/MS Analysis of Urinary Exosomes Digests

The Agilent 1260 Infinity HPLC system was employed to inject 10 µL of urine digest samples into a reverse-phase analytical column (100 mm × 2.1 mm i.d., 2.7 µm, Agilent Poroshell 120 EC-C18) maintained at room temperature. The separation of samples was accomplished with a 300 µL/min flow rate, using a gradient that increased from 3% to 90% of mobile phase B across a 30-minute run time. Mobile phase A consisted of 0.1% v/v formic acid, while mobile phase B was a mixture of ACN and 0.1% formic acid. The gradient protocol included several linear stages (time in min, % of mobile phase B): 0.1 min at 10%, increasing through various percentages at specified times, reaching 90% at 23.5 min, and returning to 3% by 27 min, maintained until 30 min. Sample analysis for LC-MRM/MS was performed using an AB SCIEX QTRAP 5500 equipped with a Turbo V ionization source and managed by Analyst software. The setup parameters were a 5500 V ion spray voltage, nebulizer and drying gas pressures of 60 and 45 psi, respectively, a source temperature of 550°C, and both Q1 and Q3 were set to unit resolution (0.7 Da FWHM). Initial MRM acquisition methods included four ion pairs per peptide to identify high-signal, interference-free transitions and to develop the LC method. The finalized analytical method featured one verified quantifier ion pair per peptide, showcasing a rapid, high-throughput 30-minute technique checked for common urine interferences. However, for thorough urine sample analysis, methods utilized three verified ion-pair transitions per target peptide to capture any subtle, sample-specific signals. These MRM acquisition methods were designed with tuned collision energy voltages specific to each fragment ion and included retention time constraints to ensure precision.

MRM Data Analysis

MRM dataset analysis was carried out using AB SCIEX Analyst software (version 1.5), employing the Integrator algorithm for peak integration with its default settings. Every integrated peak underwent manual verification to confirm accurate peak identification and precise integration. The calibration curves for quantification were analyzed using linear regression, applying a standard weighting of 1/x^2 (where x represents concentration) to effectively span a broad dynamic range. The concentration for each peptide target was determined by correlating the measured response to the

linear regression equation derived from the standard curve. These concentrations were expressed in micromoles per liter of urine, equivalent to ng/mL when considering the total weight of the processed protein content.

Reagents and Chemicals

All chemicals utilized were of ACS grade or superior quality. Every solvent employed, water included, met the standards for LC/MS grade.

Statistical Analysis

Patient data were summarized using counts (n) and percentages (%) for categorical variables, and medians alongside interquartile ranges (IQR: Q1-Q3) for continuous variables. To evaluate differences between groups, categorical data were analyzed with the chi-square or Fisher's exact tests, while the Kruskal–Wallis test was applied to continuous data. Partial correlation coefficients were employed to examine the relationships between patient characteristics and protein concentrations. The differences among groups were tested using the chi-square test or Fisher's exact test for categorical data, and the Kruskal–Wallis test was used for continuous data, followed by Dunn test for post-hoc pairwise comparison if necessary. The statistical computations were conducted using IBM SPSS software, version 22.0 (IBM Corp., Armonk, NY). For graphical representations, the "ggplot2" package from R was utilized. All the statistical evaluations were bidirectional, adopting a significance threshold of 0.05.

Results

A total of 70 patients diagnosed with thyroid cancer who underwent surgical treatment were included in the study, and their baseline characteristics are summarized in Table 1. Patients were classified by the interval between surgery and enrollment, including 42 with < 5 years between surgery and enrollment, 14 patients between 5 and 10 years, and 14 patients longer than 10 years. The mean age was 48.8 ± 11.4 years, and 84.3% were female. Four of the patients (5.7%)

Table 1 Patient Baseline Characteristics

	All (N = 70)	**Interval Between Operation and Enrollment**			
		< 5 years (n = 42)	**5–10 years (n = 14)**	**> 10 years (n = 14)**	***p-value***
Age, years					0.711
< 55	50 (71.4)	30 (71.4)	9 (64.3)	11 (78.6)	
≥ 55	20 (28.6)	12 (28.6)	5 (35.7)	3 (21.4)	
Sex					0.597
Female	59 (84.3)	35 (83.3)	11 (78.6)	13 (92.9)	
Male	11 (15.7)	7 (16.7)	3 (21.4)	1 (7.1)	
Diagnosis					0.816
PTC	66 (94.3)	39 (92.9)	13 (92.9)	14 (100.0)	
FTC	4 (5.7)	3 (7.1)	1 (7.1)	0 (0.0)	
Tumor size					0.874
T1-T2	48 (69.6)	28 (66.7)	10 (71.4)	10 (76.9)	
T3	21 (30.4)	14 (33.3)	4 (28.6)	3 (23.1)	
Cancer stage					0.988
Stage I	49 (70.0)	28 (66.7)	11 (78.6)	10 (71.4)	
Stage II	14 (20.0)	9 (21.4)	2 (14.3)	3 (21.4)	
Stage IVA/IVB	7 (10.0)	5 (11.9)	1 (7.1)	1 (7.1)	
Microcarcinoma	8 (11.4)	3 (7.1)	2 (14.3)	3 (21.4)	0.341
I-131 treatment cumulative dose					0.076
≤ 100 mCi	50 (71.4)	34 (81.0)	8 (57.1)	8 (57.1)	
>100 mCi	20 (28.6)	8 (19.0)	6 (42.9)	6 (42.9)	

Notes: The differences among groups were tested using the chi-square test or Fisher's exact test for categorical data.
Abbreviations: FTC, follicular thyroid cancer; PTC, papillary thyroid cancer.

were diagnosed with follicular thyroid cancer (FTC), while the remaining patients had papillary thyroid cancer (PTC). The distribution of cancer stages was as follows: 49 patients (70.0%), stage I, 14 patients (20.0%) stage II, and 7 patients (10.0%) stage IV (1 stage IVA and 6 stage IVB). Additionally, 8 patients (11.4%) had microcarcinoma, and 20 (28.6%) had received I-131 treatment with a cumulative dose exceeding 100 mCi. There were no statistically significant differences in baseline characteristics between the 3 groups categorized as time from surgery to enrollment.

Observations in Protein Level

No recurrence was observed in the patients during the 2-year follow-up after enrollment. The levels of different serum proteins between groups are shown in Figure 1, and the levels of urine exosomal peptides between groups at 12 and 24 months after enrollment are shown in Figures 2 and 3. No significant differences were observed between groups, or between intervals.

Significant differences were observed among the 3 groups in the distribution of high-sensitivity thyroid stimulating hormone (hsTSH) at Time 1 (p=0.021), Keratin-19 at Time 2 (p=0.036), and TIMP peptide14 at Time 2 (p=0.026) (Supplementary Table 1). A summary of the percentage of abnormal blood test results based on the defined normal range ranges is shown in Supplementary Table 2. There was a significant increase in the proportion of abnormal free T4 levels observed at Time 1 as the time from surgery to enrollment increased (<5 years: 20.0%, 5–10 years: 35.7%, >10 years: 61.5%; p=0.005).

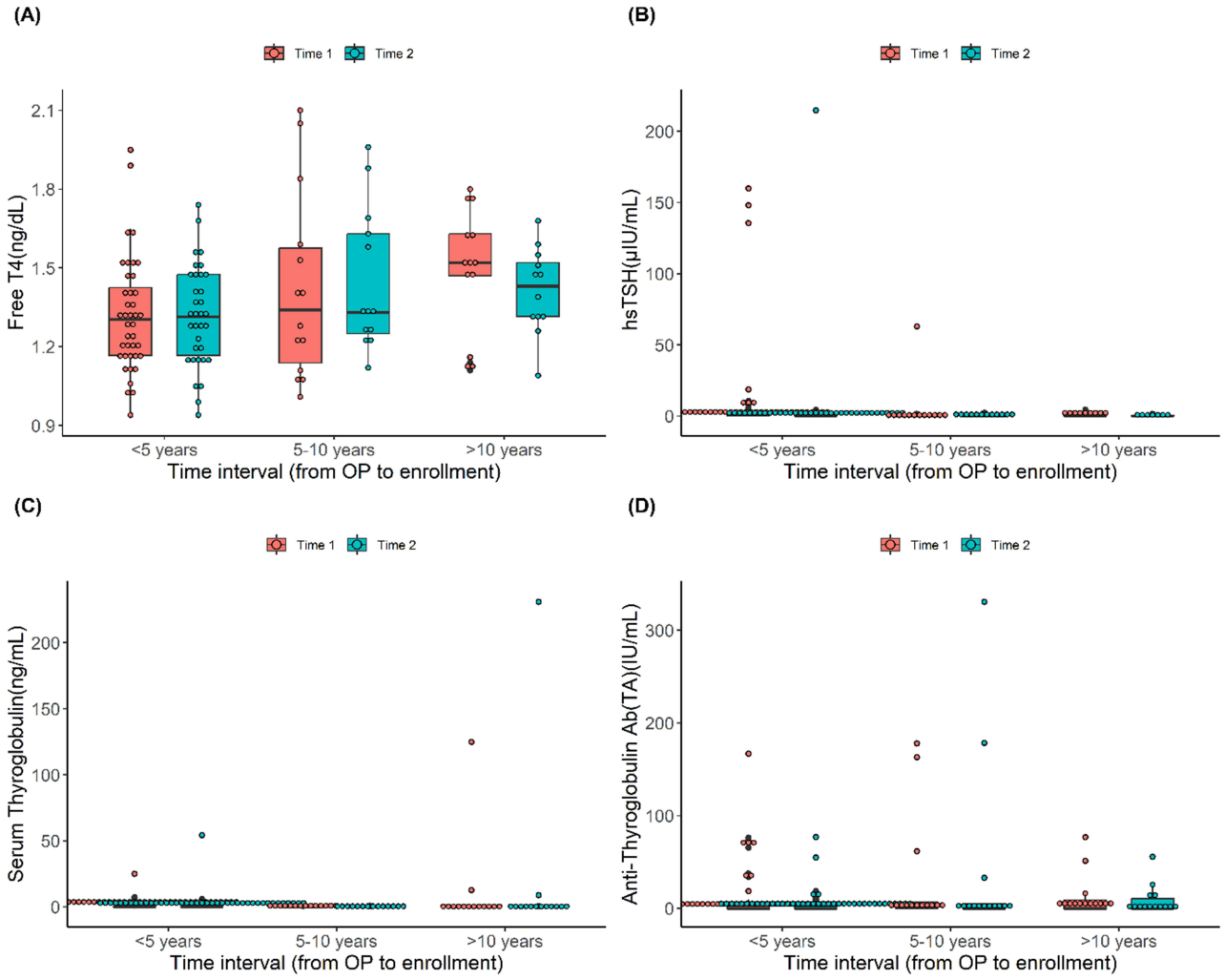

Figure 1 Serum protein levels between groups. (**A**) Free T4. (**B**) hsTSH. (**C**) Serum Thyroglobulin. (**D**) Anti-Thyroglobulin Ab.

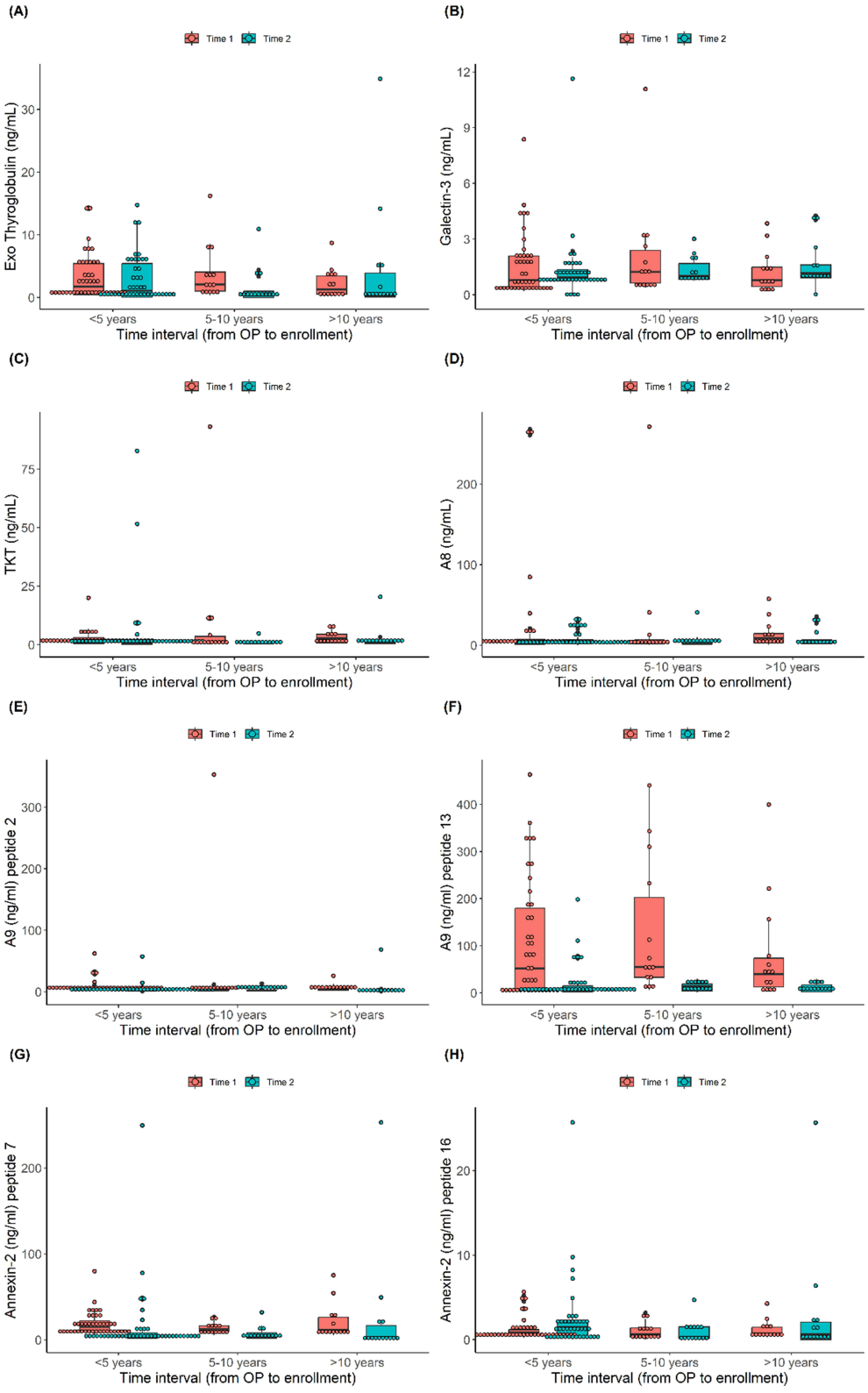

Figure 2 Concentrations of urine exosomal peptides between groups, including (**A**) Thyroglobulin. (**B**) Galectin-3. (**C**) TKT. (**D**) A8. (**E**) A9 peptide 2. (**F**) A9 peptide 13. (**G**) Annexin-2 peptide 7. (**H**) Annexin-2 peptide 16.

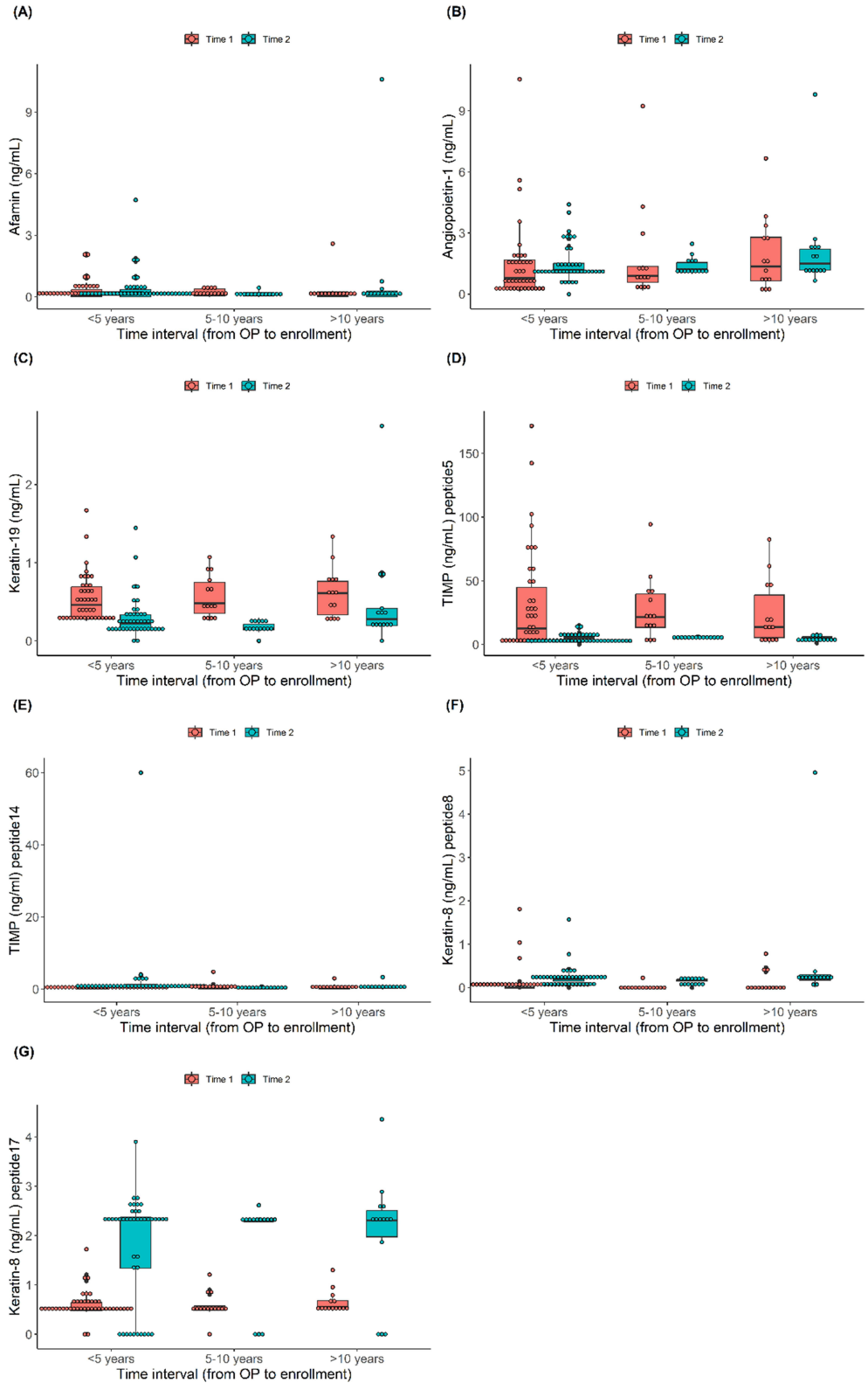

Figure 3 Concentrations of urine exosomal peptides between groups, including (**A**) Afamin. (**B**) Angiopoietin-1. (**C**) Keratin-19. (**D**) TIMP peptide 5. (**E**) TIMP peptide 14. (**F**) Keratin-8 peptide 8. (**G**) Keratin-8 peptide 17.

Associations Between Patient Characteristics and Protein Level

Analysis for the association between known risk factors for recurrence and urine exosomal peptide levels was performed using partial correlation coefficients adjusted for time (Table 2). Age was negative correlated with urine exosomes of thyroglobulin (partial $r = -0.212$, $p=0.031$) and galectin-3 (partial $r = -0.194$, $p=0.049$). Male sex (partial $r = 0.295$, $p=0.002$) and follicular thyroid cancer (partial $r = 0.412$, $p<0.001$) were correlated with serum thyroglobulin level. Cancer stage was correlated with serum thyroglobulin (partial $r = 0.316$, $p=0.001$) and hsTSH (partial $r = 0.237$, $p=0.015$) levels, and stage was negatively correlated with the urine exosome of A9 peptide 13 (partial $r = 0.258$, $p=0.008$). Patients with microcarcinoma exhibited higher levels of anti-thyroglobulin Ab (partial $r = 0.217$, $p=0.027$) and the urine exosome of angiopoietin-1 (partial $r = 0.215$, $p=0.028$).

The cumulative dose of I-131 treatment was correlated with free T4 level (partial $r = 0.213$, $p=0.030$). The associations between each risk factor and levels of serum protein and urine exosomal peptides at each timepoint are shown in. Supplementary Tables 3–9 List of urinary exosomes containing different peptides is in Supplementary Table 10.

Discussion

This is the first study to investigate whether levels of urine exosomal peptides can be used as a biomarker to predict the recurrence of thyroid cancer after thyroidectomy. Because no recurrence was observed in the study, our results showed the long-term basal fluctuations of urine exosomal peptides after thyroidectomy. For patients after thyroidectomy, concentrations of serum protein and/or urine exosomal peptides within this range may provide additional information indicating a lower risk of recurrence during long-term follow-up. Known risk factors for high-risk thyroid cancer had only a mild correlation with serum protein levels and urine exosomal peptides.

Urinary exosomes have been shown to be promising biomarkers for a vast number of diseases including lung diseases, diabetic kidney disease, gastrointestinal cancers, prostate cancer, bladder cancer, and hepatocellular carcinoma.[13] Urinary exosomes also hold promise in the diagnosis of thyroid diseases, including malignancies.[17–19] Exosomes secreted by thyroid cancer cells have been shown to be important for tumor progression, angiogenesis, and metastasis.[17] In the current study, no significant differences were observed in levels of urine exosome in patients without recurrence, regardless of the intervals from thyroidectomy to trail enrollment; thus, because no recurrences were detected in the study the detected exosomes were not released from tumor, but from other cell sources.[11]

Monitoring serum thyroglobulin level after surgical treatment is recommended for detecting recurrence of thyroid cancer.[6] However, whether this method is effective for predicting recurrence is controversial because a number of studies have shown no significant difference in serum thyroglobulin levels between patients with and without recurrence.[7–9] In a prior study, we analyzed urinary exosomal proteins, including thyroglobulin and galectin-3, preoperatively and postoperatively in 16 patients with papillary thyroid carcinoma and follicular thyroid carcinoma being treated with surgery and radioactive iodine.[20] Urine samples were collected before surgery, immediately after surgery, and at 3 and 6 months after surgery. Trends in urinary thyroglobulin concentration were detected in all patients, and importantly, serum thyroglobulin was not detected in 5 patients after operation and radioactive iodine treatment, while urinary exosome thyroglobulin showed an increasing trend, suggesting probable recurrence of thyroid cancer. These findings suggested that urinary exosomal thyroglobulin may be useful for predicting the risk of thyroid cancer recurrence.

In a more recent study, we enrolled 21 patients from 2017 to 2018 with newly diagnosed papillary and follicular thyroid cancer.[21] Preoperative urine samples were collected, and the associations of urinary exosomal protein concentrations with lymph node metastasis and MACIS score (distant Metastasis, patient Age, Completeness of resection, local Invasion, and tumor Size) were analyzed. The concentration of urine exosomal TIMP was significantly higher in patients with lymph node metastasis ($p = 0.01$). Multiple logistic regression analysis showed associations of urine exosomal TIMP (adjusted odds ratio (aOR) = 3.09, 95% confidence interval (CI): 0.99–9.6, $p = 0.052$) and angiopoietin-1 (aOR = 2.24, 95% CI: 0.97–5.15, $p = 0.058$) with lymph node metastasis. No association was noted between MACIS score and various urine exosomal protein candidates. Taken together, the results of this and the aforementioned studies and the current study suggest that urine exosomal protein concentrations may be useful for identifying patients with thyroid

Table 2 Correlation Coefficients Between Known Risk Factors for Thyroid Cancer and Serum Protein/Urine Exosomal Peptide Levels

	Age, Years (≥ 55 vs < 55)	Sex (M vs F)	Tumor Histology (FTC vs PTC)	Tumor Size (T3 vs T1, T2)	Cancer Stage (I/II/IVa vs IVb)	Microcarcinoma (Yes vs No)	I-131 Treatment (> 100mCi vs ≤ 100mCi)
Serum protein							
Free T4 (ng/dL)	−0.016	−0.071	−0.084	−0.034	0.086	−0.091	**0.213**
hsTSH (μIU/mL)	−0.014	0.042	−0.048	−0.119	**0.237**	−0.071	0.136
Serum Thyroglobulin (ng/mL)	−0.113	**0.295**	**0.412**	0.171	**0.316**	0.020	0.184
Anti-Thyroglobulin Ab (TA) (IU/mL)	−0.045	0.151	−0.059	0.021	0.077	**0.217**	0.061
Urine exosomal peptide							
Exo Thyroglobulin (ng/mL)	**−0.212**	−0.068	−0.030	0.053	−0.093	−0.153	−0.072
Galectin-3 (ng/mL)	**−0.194**	−0.177	−0.022	0.104	−0.154	−0.112	−0.046
TKT (ng/mL)	−0.011	−0.120	−0.036	0.112	−0.001	−0.091	−0.080
A8 (ng/mL)	−0.115	−0.130	−0.073	0.157	−0.129	−0.066	−0.004
A9 peptide 2 (ng/mL)	−0.073	−0.063	−0.043	−0.013	−0.061	−0.049	−0.027
A9 peptide 13 (ng/mL)	−0.162	−0.141	−0.007	0.078	**−0.258**	−0.059	−0.043
Annexin-2 peptide 7 (ng/mL)	−0.182	−0.148	−0.022	0.153	−0.147	−0.070	−0.139
Annexin-2 peptide 16 (ng/mL)	−0.126	−0.037	−0.084	0.120	−0.100	−0.014	−0.179
Afamin (ng/mL)	−0.003	0.161	−0.063	0.010	0.105	−0.002	−0.138
Angiopoietin-1 (ng/mL)	−0.096	−0.079	0.022	−0.102	−0.149	**0.215**	−0.010
Keratin-19 (ng/mL)	−0.133	−0.059	−0.080	−0.068	0.002	−0.018	−0.086
TIMP peptide 5(ng/mL)	−0.123	−0.154	−0.090	−0.048	−0.194	−0.054	−0.120
TIMP peptide14 (ng/mL)	−0.073	−0.038	−0.017	0.166	−0.047	−0.036	−0.072
Keratin-8 peptide8 (ng/mL)	0.008	0.077	−0.116	0.073	0.171	0.032	−0.004
Keratin-8 peptide17 (ng/mL)	0.027	0.151	−0.015	−0.034	0.083	−0.075	−0.059

Notes: Partial correlation coefficients were adjusted for Time 1 and Time 2. The differences among groups were tested using the chi-square test or Fisher's exact test for categorical data. Significant values ($p<0.05$) are shown in bold.

cancer who have a high-risk of advanced disease and\or recurrence after treatment. Urinary exosome peptides panel should be considered as the future aspect for earlier detection of cancer recurrence,[22] including thyroid cancer.

Limitations

The primary limitation of this study is that the small number of patients may limit the generalization of the results to other populations or locations. Therefore, we will interpret the results of our study with humility.

Conclusions

Our prospective study revealed the long-term basal fluctuation ranges of serum proteins and urine exosomal peptides in patients with thyroid cancer who underwent thyroidectomy. For high-risk patients after thyroidectomy, concentrations of serum proteins or urine exosomal peptides within the ranges may indicate there is a lower risk of thyroid cancer recurrence during long-term follow-up.

Statement of Data Sharing

We intend to share individual deidentified participants data. We could share all raw data with other researchers. We could also provide our prior publications in other scientific journals. If researchers interested in this topic, please contact with the corresponding author (Chih-Yuan Wang) via email. We will share requested data via Google drive immediately after upon requests.

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Disclosure

All authors have declared that no competing interests exist for this work.

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