Urinary Neopterin Levels in Patients with Thyroid Cancer

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Abstract Increased urinary neopterin concentrations have been described in many cancers. We aimed to evaluate the urinary neopterin levels in thyroid cancer. Sixty-nine patients with thyroid cancer, 76 patients with benign thyroid pathology and 33 healthy subjects were evaluated. First morning urine samples were collected from the patients and normal subjects for neopterin and creatinine measurement and stored at -80°C until analysed. Neopterin levels were 149.3 (15.2–1,602.2) $\mu\text{mol/mol}$ creatinine in the malignant group, 32 (5.2–275.6) $\mu\text{mol/mol}$ creatinine in the benign group and 9.2 (2.7–78.7) $\mu\text{mol/mol}$ creatinine in normal subjects ($p \leq 0.001$). Urinary neopterin levels were significantly higher in patients with thyroid cancer than patients with benign thyroid pathologies and normal subjects. Also the patients with benign thyroid pathologies had a higher urinary neopterin level than the normal subjects. Malignant group was divided to two groups; patients with/without chronic thyroiditis (confirmed histologically). There were 22 (31.9 %) patients with and 47 (68.1 %) patients without chronic thyroiditis. Urinary levels of neopterin

didn't differ in both groups (168.6 (21.3–716.8) $\mu\text{mol/mol}$ creatinine and 135.3 (15.2–1,602.2) $\mu\text{mol/mol}$ creatinine respectively; $p = 0.381$). Urinary neopterin levels are high in thyroid cancer patients independently from the presence of chronic thyroiditis.

Keywords Neopterin · Thyroid cancer · Inflammation · Hashimoto's thyroiditis

Introduction

Thyroid nodules are becoming more important in clinical setting and thyroid cancer is becoming more prevalent. The annual increase in the incidence of thyroid cancer is 2.4 %. [1]. The clinical importance of thyroid nodules is the need to exclude thyroid cancer which occurs in 5–15 % of the patients. The most commonly seen type is papillary cancer and especially papillary thyroid cancers have a good prognosis. Papillary thyroid cancers grow slowly but some characteristics make them more aggressive with a poor prognosis [2].

Imaging techniques and/or by biopsy are used for diagnosis of cancers. Biomarkers that are being used in the early diagnosis of cancers is favoring [3]. Non-invasive screening methods are preferred by the physician and patient. Using a biomarker which can be used while diagnosing and following the patient can be very helpful.

Neopterin is a pteridine group molecule which is secreted from monocyte and macrophages. It indicates the activation of cellular immunity after IFN-gamma stimulation and shows activation of cellular immunity [4]. Thus neopterin can be used as a marker of immune activation. Studies have shown that neopterin can be found in different body fluids when the immune system is activated [5]. It is

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increased in diseases where the cellular immune system is activated; autoimmune diseases [multiple sclerosis (MS), rheumatoid arthritis (RA)], infections (HIV), and also in some cancer types (ovarian carcinoma, uterine tumors, colon cancer, breast cancer, leukemia and lymphoma) [6–12]. It is used as a prognostic marker [13–16] and can show the stage of the disease [17]. Additionally it can increase in metastatic rather than local diseases [18]. High neopterin levels have been related with advanced tumor stage, increased relapse rates, metastasis and poor prognosis in certain cancer types [13, 19–22]. Although neopterin has been studied and found to be high in autoimmune diseases it has not been studied in autoimmune thyroiditis.

There are limited amount of studies in thyroid disorders. Our aim was to evaluate urinary neopterin in thyroid carcinoma whether it can differentiate between malign and benign cases and if it is effected by chronic thyroiditis.

Materials and Methods

Our study was performed in Ankara Atatürk Education and Research Hospital, Endocrinology and Metabolism Department, thyroid disease outpatient clinic between January 2010 and January 2011. One hundred and forty-five patients were enrolled in the study. Blood samples for thyroid stimulating hormone (TSH), thyroid autoantibodies (Anti-TPO and Anti-Tg) were taken in the morning, after a 12 h fasting period. Thyroid ultrasonography (USG) was performed by the same machine and by one experienced physician. USG guided fine needle aspiration biopsy was performed to every nodule found suspicious. 145 patients who had USG or cytologically suspicious result, cytologically malign nodules or nodules which were symptomatic for the patient were sent to surgery. The control group consisted of 33 healthy subjects. Total thyroidectomy was performed to all of the patients. Sixty-nine of these patients were diagnosed as thyroid cancer and 76 patients were benign histopathologically. Urine samples and blood samples were taken from the control group which was selected from 33 healthy patients.

The study was approved by the Local Ethics Committee to be in concordance with the Helsinki Declaration. All of the patients gave written informed consent.

Patients under 15 years of age, patients who had a thyroid operation before, patients who had pure cystic nodules, history of radiotherapy to the neck, patients with an active infection, patients with a second malignancy, cardiac disease were excluded from the study. Patients who had diseases or treatment that effect neopterin levels were also excluded from the study (hyperlipidemia, antihyperlipidemic drugs, anemia, diabetes mellitus, autoimmune diseases, coronary heart disease).

Ultrasonography and Ultrasonography Guided Fine Needle Aspiration Biopsy

Conventional thyroid US was performed using an Esaote color Doppler machine (Esaote 796FDII, Taipei, Taiwan) and superficial probe (Esaote LA523- 5.5–12.5 MHz, Taipei, Taiwan). Localization, diameters (mm) were recorded. Presence of halo, echogenicity, marginal regularity, micro and macrocalcification, and vascularization pattern were determined. Absence of blood flow and peripheral vascularization were classified as type 1, while intranodular vascularization and marked intranodular vascularization were classified as type 2 vascularization. FNAB was performed ultrasonography guided (GE, Logiq pro 200, Gyeon, Korea).

Sampling

Blood and urine samples were collected for every patient and control group. In the patient group urine samples were collected early in the morning on the day of surgery (8–9 am). In the control group to minimize any interference due to different sampling times, urine samples were collected each morning at the same time (8–9 am). Collected urine samples were kept from direct sunlight and stored at –80 °C until assayed. Urine samples were collected to measure neopterin and creatinine. Blood samples were collected to measure for hsCRP (highly sensitive C reactive protein), complete blood count (CBC), total cholesterol, low density lipoprotein (LDL), high density lipoprotein (HDL), triglycerides (TG), TSH, free T4, free T3, thyroid autoantibodies (Anti-TPO and Anti-Tg). All subjects were euthyroid at the time of operation.

Measurements

At the time for blood collection, the health of the control group subjects was evaluated by the same physician for any acute infection. All subjects were negative for the diseases such as hypertension, diabetes mellitus, any known or detectable cancers, autoimmune diseases, and any drugs that could interfere with the immune system. A commercial neopterin enzyme-linked immunosorbent assay (ELISA) kit (IBL, Germany) was used to analyse urinary neopterin levels. Before analysed the urine samples were dissolved at room temperature. A subset of urine was diluted 10 times with deionized water and centrifuged at 13,000 rpm for 5 min. The supernatant was then diluted 10 times with the assay buffer (phosphate buffer) found in the kit. The urine, which was diluted 100 times, underwent the reactions as mentioned in the manufacturer's instructions and the color density was measured at 450 nm with a photometer. The final concentrations were calculated from the calibration

curve based on the neopterin standard solutions included in the kit (0–111 nmol/l). Samples that showed concentrations above the highest standard were diluted as described by the manufacturer's instructions. In another subset of urine creatinine was analysed by using photometric method on Cobas 6000-c501 analyser, Roche kits. Neopterin in urine was correlated to creatinine and the results were given as neopterin to creatinine ratio in μmol neopterin/mol creatinine.

Statistical Analysis

The results are expressed in mean $\pm$ SD, except for variables with non-normal distribution, which are expressed as medians (minimum–maximum). Shapiro–Wilk test was used for normality. For comparison between 3 groups, one way repeated measures analysis of variance (ANOVA), Mann–Whitney *U* and Kruskal–Wallis test was used. When results were found significant post hoc Turkey test or Conover's non-parametric multiple comparison test were used. Pearson's chi-square test was used for categorical variables. Spearman's correlation test was used for continuous variables. Results which were $p < 0.05$ were considered significant.

Results

Total of 145 patients and 33 cases were enrolled in the present study. Demographical and clinical characteristics of the study group are given in Table 1.

The patient group consisted of 146 patients, 114 females and 32 males. In the malign group there were 61 females and 8 males and in the benign group there were 59 females and 17 males. The control group consisted of 26 females and 7 males. Mean age in the malign, benign and control group was 47.7 ± 12.8 , 50.2 ± 11.3 and 45.1 ± 8.5 years respectively. Patient and control group did not differ for age and sex.

Malign group had 59 multinodular goiter, 2 nodular goiter and 8 patients with huge nodules (nodule size >3 cm). Benign group had 58 patients with multinodular goiter, 13 patients with nodular goiter and 5 patients with huge nodule (nodule size >3 cm). The indication for surgery were; cytology with/suspicion of papillary thyroid cancer, 3 nondiagnostic cytology results, pressure symptoms and a suspicious USG result. Histopathology results in the malign group was papillary thyroid cancer in 21 (30.4 %), papillary microcarcinoma in 18 (26 %) and papillary carcinoma together with microcarcinoma in 11 (15.9 %), multicentric microcarcinoma in 13 (18.8 %), follicular carcinoma in 4 (5.7 %), hurtle cell carcinoma in 1 (1.44 %) and atypia of undetermined significance in 1 (1.44 %) of the patients.

The parameters (total cholesterol, LDL, Hb, hs CRP) that could have an effect on neopterin levels were determined for the patients and control subjects and were found to be similar in the 3 groups. Total cholesterol levels in the malign, benign and control group were 188.4 ± 33.3 , 194.7 ± 42.5 and 180.5 ± 24.3 mg/dl respectively ($p = 0.216$). Medians levels of LDL cholesterol in the malign, benign and control group were 110 (45.8–164) mg/dl, 114.5 (43.2–193.2) mg/dl, 109 (54.5–145) mg/dl, respectively ($p = 0.252$). Hs-CRP levels in the malign, benign and control group were 0.33 (0.0–1.15), 0.10 (0.0–0.98), 0.10 (0.0–0.96), respectively ($p = 0.778$).

Neopterin levels between groups are given in Table 2 and Fig. 1. The median urinary neopterin levels in the malign, benign and the control group were 149.3 μmol /mol creatinine (15.2–1,602.2), 32 μmol /mol creatinine (5.2–275.6), 9.2 μmol /mol creatinine (2.7–78.7), respectively ($p < 0.001$). Neopterin levels were higher in the malign group than in the benign and control groups ($p < 0.001$). Additionally the neopterin levels were significantly higher in the benign group than in the control group ($p < 0.001$).

The patients with papillary carcinoma were divided into four groups. The neopterin levels of the patients with papillary carcinoma together with papillary microcarcinoma, papillary

Table 1 Demographical and clinical characteristics of patients and controls

Variables	Malign ($n = 69$)	Benign ($n = 76$)	Control ($n = 33$)	p value
Age	47.7 ± 12.8	50.2 ± 11.3	45.1 ± 8.5	0.096
Gender F/M	61/8	59/17	26/7	0.209
Total Cholesterol (mg/dl)	188.4 ± 33.3	193.3 ± 40.3	180.5 ± 24.3	0.216
LDL (mg/dl)	110 (45.8–164)	114.5 (43.2–193.2)	109 (54.5–145)	0.252
HDL (mg/dl)	53 (32.1–219)	47.9 (25.1–166)	49 (28.9–92)	0.108
Triglyceride (mg/dl)	104 (36–289)	120 (36–416)	95 (41–381)	0.108
hs-CRP (mg/dl)	0.33 (0.0–1.15)	0.10 (0.0–0.98)	0.10 (0.0–0.96)	0.778
Hemoglobin (g/dl)	13.9 ± 1.6	14.1 ± 1.2	13.8 ± 1.2	0.361
Hematocrit	40.3 ± 4.8	40.7 ± 3.9	39.9 ± 3.7	0.661
Chronic thyroiditis	22 (31.9 %)	24 (31.6 %)	–	0.969

F female, *M* male, *LDL* low density lipoprotein, *HDL* high density lipoprotein, *hs-CRP* highly sensitive C reactive protein

Table 2 Preoperative urinary neopterin levels in patient and control group

Groups	Preoperative urinary neopterin ($\mu\text{mol/mol}$ creatinine)
Malign ($n = 69$)	149.3 (15.2–1,602.2) ^{a,b}
Benign ($n = 76$)	32.0 (5.2–275.6) ^{a,c}
Control ($n = 33$)	9.2 (2.7–78.7) ^{b,c}
<i>p</i> value	<0.001

^a The difference between malign and benign group was found statistically significant ($p < 0.001$)

^b The difference between malign and control group was found statistically significant ($p < 0.001$)

^c The difference between benign and control group was found statistically significant ($p < 0.001$)

Fig. 1 Preoperative urinary neopterin levels between malignant, benign and control group $p < 0.001$

carcinoma, multicentric papillary microcarcinoma, papillary microcarcinoma was 252.5 (23.6–602.3) $\mu\text{mol/mol}$ creatinine, 158.5 (37.6–532.7) $\mu\text{mol/mol}$ creatinine, 168.6 (43.1–1,602.2) $\mu\text{mol/mol}$ creatinine, 100.3 (15.2–568.4) $\mu\text{mol/mol}$ creatinine respectively ($p = 0.109$) (Table 3).

The malign group was divided into groups according to the histopathology characteristics. 60 patients had vascular invasion while 9 patients did not. Neopterin levels were higher in the vascular invasion group but the result was not statistically significant [151.3 (82.8–430) $\mu\text{mol/mol}$ creatinine vs. 147.8 (15.2–1,602.2) $\mu\text{mol/mol}$ creatinine, respectively; $p = 0.682$].

Table 3 Preoperative urinary neopterin measurements regarding for postoperative histopathology results

	Preoperative urinary neopterin ($\mu\text{mol/mol}$ creatinine)
Papillary carcinoma	158.5 (37.6–532.7)
Papillary microcarcinoma	100.3 (15.2–568.4)
Papillary ca + papillary microcarcinoma	252.5 (23.6–602.3)
Papillary microcarcinoma multicentric	168.6 (43.1–1,602.2)
<i>p</i> value	0.109

Fourteen of the patients had capsule invasion 55 did not. Neopterin levels were higher in the capsule invasion group but the result was not statistically significant [177.2 (21.3–602.3) $\mu\text{mol/mol}$ creatinine vs. 146.3 (15.2–1,602.2) $\mu\text{mol/mol}$ creatinine, respectively; $p = 0.541$].

Six patients had lymphatic invasion, 63 patients did not. Neopterin levels were higher in the lymphatic invasion group but the result was not statistically significant [250.8 (102.5–317.5) $\mu\text{mol/mol}$ creatinine vs. 146.1 (15.2–1,602.2) $\mu\text{mol/mol}$ creatinine, respectively; $p = 0.274$] (Table 4).

The patients were divided to seven groups according to the tumor size. Tumor size in twelve patients was <0.5 cm, 0.6–0.9 cm in 12 patients, >1 cm in 19 patients. Six patients had more than one tumor with sizes <0.5 cm and 0.6–0.9 cm. Three patients had more than one tumor with sizes <0.5 and >1 cm and five patients had more than one tumor with tumor sizes <0.5, 0.6–0.9 and >1 cm. We found a positive relation between the tumor size and neopterin levels ($r = 0.272$, $p < 0.001$) although the size of the groups were too small (Fig. 2).

In order to determine the effect of chronic thyroiditis on neopterin levels we divided 145 benign and malign patients into two groups. One group ($n = 46$) consisted of patients who had and the other group ($n = 99$) who didn't have chronic thyroiditis confirmed histologically. Neopterin levels did not differ in patients with or without chronic thyroiditis [101 (5.24–716.8) $\mu\text{mol/mol}$ creatinine vs. 80.85 (5.62–1,602.25) $\mu\text{mol/mol}$ creatinine, respectively; $p = 0.55$].

In the malign group, 22 patients had 47 patients did not have chronic thyroiditis. Neopterin levels didn't differ between two groups [168.6 (21.3–716.8) $\mu\text{mol/mol}$ creatinine vs. 135.3 (15.2–1,602.2) $\mu\text{mol/mol}$ creatinine, respectively; $p = 0.381$].

Discussion

A tumor marker must be specific to a tumor and should be easily evaluated. Neopterin is a marker of activated immune response and has been found to be increased in

Table 4 Preoperative urinary neopterin measurements regarding for characteristics within malign group

	Preoperative urinary neopterin ($\mu\text{mol/mol}$ creatinine)
Carcinoma	
Papiller microcarcinoma	121.6 (15.2–716.8)
Papiller carcinoma	204.0 (21.3–1,602.2)
<i>p</i> value	0.160
Vascular invasion	
With	151.3 (82.8–430.0)
With out	147.8 (15.2–1,602.2)
<i>p</i> value	0.682
Capsule invasion	
With	177.2 (21.3–602.3)
With out	146.3 (15.2–1,602.2)
<i>p</i> value	0.541
Lymphatic invasion	
With	250.8 (102.5–317.5)
With out	146.1 (15.2–1,602.2)
<i>p</i> value	0.274
Chronic thyroiditis	
With	168.6 (21.3–716.8)
With out	135.3 (15.2–1,602.2)
<i>p</i> value	0.381

Fig. 2 Preoperative urinary neopterin levels in the malignant group according to tumor size $r = 0.272$, $p = 0.024$

certain malignancies. Many studies have determined its predictive value [13–16]. There is limited number of studies about neopterin in thyroid diseases [17, 23, 24].

Urinary neopterin can determine the extent of unspecific cancer related inflammation [25]. Our aim was to determine if neopterin could be a marker in differentiating malign and benign nodules.

The present data demonstrates that neopterin levels are elevated in thyroid malignancies. Our data is consistent with the data of Sahin et al. [17]. Urinary neopterin levels were increased in 24 papillary thyroid cancer patients. No difference was found in patients with benign thyroid pathologies and control cases. In our study neopterin levels were increased in thyroid cancers patients more than in the benign group and controls.

Epidemiologic studies have shown that, tumors may develop under resistant inflammation (chronic infection, autoimmune diseases) [26–28]. Neopterin levels are increased in autoimmune diseases (RA, SLE) and it predicts disease activity [6, 7].

Ugolini et al. [29] demonstrated that 20–50 % of papillary thyroid cancer patients have chronic thyroiditis and suggested that this was due thyrocytes that expressed RET/PTC3 which caused increased proinflammatory cytokines [30]. Accordingly, it can be hypothesized that chronic thyroiditis may cause increased urinary neopterin levels. In one study neopterin was not different in chronic thyroiditis and multinodular goiter. Our study also showed that the coexistence of chronic thyroiditis does not affect urinary neopterin levels in thyroid carcinoma patients.

On the other hand Kondera-Anasz et al. [31] found that serum neopterin levels were increased in patients with increased levels of Anti Tg and Anti TPO although not statistically significant. In our study because chronic thyroiditis was proven histopathologically, we did not need to study the correlation between urinary neopterin and Anti Tg and Anti TPO.

Kondera-Anasz et al. [31] studied serum neopterin levels in 71 patients with different thyroid pathologies (11 thyroid cancer, 33 nontoxic nodular goiter, 22 thyroid adenoma). Serum neopterin levels were normal in the malign, benign and control groups but were significantly higher in the malign and benign groups than in controls.

Wagner et al. [23] demonstrated that serum neopterin levels were higher in Graves's disease patients than in autoimmune thyroiditis, nontoxic and normal subjects. Urinary neopterin levels are increased in autoimmune disorders. Interestingly, in our study, urinary neopterin levels did not differ in patients with chronic thyroiditis when compared with patients without chronic thyroiditis. Although our findings were consistent with the former results studies have to be done to determine an explanation.

Although Wagner et al. [23] suggested that neopterin levels did not change with thyroid functions all our subjects were euthyroid and did not use any medications that could affect neopterin levels. Hyperthyroidism affects the immune system

by increasing the levels of IL-2 [32]. Additionally the use of thyroxin in hypothyroidism increases the levels of soluble IL-2R alpha and the use of methimazole in hyperthyroidism decreases the levels of IL-2R alpha [33]. Botella-Carretero et al. [34] demonstrated that thyroxin suppression treatment used in differentiated thyroid cancer, effects cellular immune reaction by increasing the levels of interleukin-18, soluble interleukin-2 (IL-2) and natural killer cells. After being stimulated by IL-2, lymphoid cells secrete neopterin [35].

Neopterin has been related with tumor stage, distant metastases, advanced disease, long term prognosis in colon cancer, breast and gastric cancer patients [12–14, 18]. In one study, serum neopterin levels were found to be increased in patients with lymph node metastases [18]. Neopterin levels were related with tumor size in one study [16] and in another no relation was found [10]. We found a positive relation between the tumor size and neopterin levels. Additionally, neopterin levels were higher in patients who had papillary carcinoma together with papillary microcarcinoma than the patients who had uni or multicentric papillary microcarcinoma. Oncogenes (*RET/PTC*, *BRAF*, *RAS*) that cause neoplastic transformation in papillary carcinoma, activate proinflammatory genes and stimulate an inflammatory reaction around the tumor [36, 37]. The inflammatory cells found around the tumor cells play a role in the progression of the tumor [38]. May be the extent and number of the cancer found in the thyroid gland may affect the neopterin levels.

In malignancies other than thyroid, neopterin levels correlated with vascular and lymphatic invasion [14–16]. In our study, neopterin levels were higher in patients with vascular, lymphatic and capsular invasion but the results didn't reach statistical significance. This might be due to our small sample size. Although we found that urinary neopterin levels are higher in thyroid cancer the present data cannot differentiate benign and malign pathologies. A cut of point may be demonstrated in a study with a larger study population.

Conclusions

As a conclusion our study shows that activation of cell-mediated immunity, defined as increased formation of neopterin, is increased in patients with thyroid cancer compared with patients with benign thyroid disorders.

Conflict of interest Authors declare no conflict of interest.

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