

RESEARCH ARTICLE

Gene Expression of CYP1A1 and its Possible Clinical Application in Thyroid Cancer Cases

JA Gallegos-Vargas^{1*}, J Sanchez-Roldan^{1*}, MD Ronquillo-Sanchez², L Carmona-Aparicio³, E Floriano-Sanchez⁴, N Cárdenas-Rodríguez³

Abstract

Background: Thyroid cancer is the most common endocrine malignancy, and exact causes remain unknown. The role of CYP450 1A1 (CYP1A1) in cancer initiation and progression has been investigated. The aim of this work was to analyze, for the first time, CYP1A1 gene expression and its relationship with several clinicopathological factors in Mexican patients diagnosed with thyroid cancer. **Materials and Methods:** Real-time PCR analysis was conducted on 32 sets of thyroid tumors and benign pathologies. Expression levels were tested for correlations with clinical and pathological data. All statistical analysis were performed using GraphPad Prism version 3.0 software. **Results:** We found that female gender was associated with thyroid cancer risk ($P<0.05$). A positive relationship was identified between CYP1A1 mRNA levels and the presence of chronic disease, alcohol use, tumor size, metastasis and an advanced clinical stage ($P<0.05$). **Conclusions:** The results suggest that CYP1A1 gene expression could be used as a marker for thyroid cancer.

Keywords: Thyroid cancer - cytochrome P450 1A1 - molecular marker

Asian Pac J Cancer Prev, 17 (7), 3477-3482

Introduction

The worldwide incidence of thyroid cancer has been increasing over the last four decades (Jung et al., 2014). The most commonly diagnosed thyroid cancers include papillary (80%) and follicular (11%) cancers (Pusztaszeri et al., 2014). In Mexico, thyroid cancer exhibited an incidence of 2.6 per 100,000 in 2012 (2:1, female:male) and a mortality of 0.8 per 100,000 (GLOBOCAN, 2012). Until 2007, thyroid cancer was the third leading cause of head and neck malignancies in Mexico (Tirado and Granados, 2007).

Thyroid carcinoma is generally an indolent cancer characterized by slow progression (Kim et al., 2014). The exact cause of thyroid cancer is still unknown, but some environmental, dietary and genetic factors have been associated with thyroid cancer (Rossing et al., 2000).

The cytochrome P450 (CYP) enzymes are key enzymes in the metabolism of diverse pro- carcinogenic and carcinogenic antineoplastic drugs and are involved in tumor development and the tumor response to therapy (Dees and Watkins, 2005). CYP1A1 (aryl hydroxylase or aryl hydrocarbon hydroxylase) contributes to the toxicity of carcinogens. This enzyme acts on a wide range of substrates, including xenobiotics, steroids, fatty acids,

vitamins, and prostaglandins. The relationship between the presence of polymorphic variants of CYP1A1 and the risk of or protection against lung cancer, head and neck cancer, liver cancer, leukemia, brain cancer, prostate cancer, breast cancer, colorectal cancer, cervical cancer and bladder cancer has been demonstrated in several studies (Beresford, 1993; He et al., 2014; Wahid et al., 2013; Khlifi et al., 2013; Lin et al., 2014). Moreover, an association between the presence of CYP1A1 polymorphisms and the risk of developing thyroid carcinomas has been observed in various populations (Bufalo et al., 2006; Siraj et al., 2008; Reis et al., 2010; Barbieri et al., 2013).

The aim of this work was to analyze CYP1A1 gene expression and its relationship with several clinicopathological factors, for the first time, in Mexican patients diagnosed with thyroid cancer.

Materials and Methods

Patients and samples

All thyroid tissue samples were obtained from patients diagnosed with surgical pathologies of the thyroid (partial or total thyroidectomy) in the Service of Otolaryngology and Head and Neck Surgery of Hospital Central Militar, Secretaría de la Defensa Nacional (SEDENA) in Mexico

¹Service of Otolaryngology and Head and Neck Surgery, Hospital Central Militar, Secretaría de la Defensa Nacional, ²Section of Graduate Studies and Research, Escuela Superior de Medicina, Instituto Politécnico Nacional, ³Laboratory of Neurosciences (Neurochemistry), Instituto Nacional de Pediatría, ⁴Laboratory of Molecular Biology, Escuela Militar de Graduados de Sanidad, Secretaría de la Defensa Nacional, México, D.F, México *For correspondence: noemicr2001@yahoo.com.mx and floriano_esa@yahoo.com

City. The tissues were classified as showing a malignant (cancer group) or benign (control group) histological type by the Department of Pathology of the same hospital, in accordance with standard diagnostic procedures, and frozen at -80 °C. Considering the inclusion, exclusion and elimination criteria in the study, 32 thyroid cancer samples and 32 control group samples were obtained. Clinico-pathological data were obtained from the patient medical records. The study was conducted according to the requirements of the Bioethics Committee (registration number 64) after the patients were informed of the study and signed the consent form.

Total RNA isolation

Samples of approximately 100 mg of cancer and benign thyroid tissue were collected and individually homogenized in the TRIzol reagent (TRI Reagent® Solution, RNA/DNA/Protein Isolation Reagent, Ambion, Waltham, MA, USA). Total RNA was extracted according to the manufacturer's protocol. The concentration of total RNA in each sample was determined with a NanoDrop spectrophotometer (Delaware, USA) according to the 260 nm/280 nm ratio. The quality of isolated RNA was determined based on the integrity of the bands of the 28S, 18S and 5S subunits after electrophoresis in 1% agarose gels stained with ethidium bromide and visualized with a UV transilluminator (290 EDAS Kodak, New Haven, CT). The total amount of RNA obtained (1 µg) was supplemented with 0.5 µL of RNase inhibitor (Boehringer Mannheim GmbH Germany). The total RNA extracted was stored at -80°C.

Real-time PCR

The following specific oligonucleotides were designed and optimized to a temperature of 59 °C for the CYP1A1 gene and the reference genes ribosomal subunit 18S, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), glucose 6-phosphate dehydrogenase (G6PDH) and β-actin (BACT). Sequences were obtained from GenBank™ (Table 1). The endogenous gene (GAPDH) was validated using BestKeeper software as described by Pfaff et al. (Pfaff et al., 2002). RT-PCR conditions were optimized with a thermocycler (gradient Px2 Thermal Cycler Hybaid, Franklin, MA). The reaction mixture was composed of 6.25 µL of 2X SYBR® Green Reaction Mix with ROX, 0.25 µL of SuperScript™ III RT/Platinum® Taq Mix (Invitrogen, Carlsbad, CA), 0.4 µL of MgSO₄, 0.4 µL of the forward primer, 0.4 µL of the reverse primer, 4 µL of total RNA and 0.8 µL of nuclease-free water. Reverse transcription was performed at 52 °C for 10 min. PCR was performed at 94 °C for 5 min, followed by 40 cycles at 95 °C for 20 s, 57 °C for 20 s and 72 °C for 20 s, then a melting temperature of 60-98 °C and a final step of 40 °C for 2 min. The amplification parameters, such as temperature, primer concentrations, dNTPs and volume, were transferred to the amplification protocol in real time with the detection system Rotor-Gene 6.0 (Corbett Life Science, Sydney City, Australia). The amplification products of RT-PCR were analyzed via 2% agarose gel electrophoresis.

Quantitative determination of mRNA CYP1A1

The CP values for endogenous candidates (GAPDH, G6PDH, BACT and 18S) and the CYP1A1 gene were exported to Rotor-Gene 6.0 (Corbett Life Science, Sydney, Australia) software to calculate the obtained efficiencies with the statistical model REST© (Pfaff et al., 2002; Floriano et al., 2009), and the data were plotted to construct a linear regression comparing the logarithmic concentration (total RNA) against the CP values (CP is defined as the number of cycles in which the fluorescence intensity increases above the basal fluorescence of the sample). To obtain the correlations of the endogenous candidate genes and determine the most stable genes, BestKeeper software was used, with the CP values being exported from Rotor-Gene 6.0 software to Excel to reveal the characteristic melting temperature (T_m) of each amplified product. Determination of the most stable endogenous gene was performed using the BestKeeper statistical model, analyzing the CP values through Pearson correlation (Tinzl et al., 2004).

Statistical analysis

The χ^2 or Fisher exact test was used to estimate the association between individual clinical-pathological factors and the risk of thyroid cancer. Data from the absolute quantification of all samples were normalized against GAPDH and analyzed with Student's t test. In addition, the relationships between CYP1A1 gene expression and gender, place of residence, weight, familiar history of cancer, chronic diseases, tobacco and alcohol use, histology, tumor size, metastasis and clinical stage were evaluated using Student's t test or one-way ANOVA. The Pearson test was employed to assess correlations between age, T3 and T4 levels and cytochrome expression. All statistical analyses were performed using GraphPad Prism version 3.0 software. The results were considered significantly different at P values lower than 0.05.

Results

Characteristics of study subjects

The clinical and histological data from all patients (32 subjects diagnosed with thyroid cancer and 32 subjects diagnosed with benign non-cancerous thyroid disease) are summarized in Table 2. Non-significant differences were observed between age, place of residence, body mass index, family history of cancer, the presence of chronic diseases, the use of tobacco, alcohol and drugs and exposure to radiation. Only female gender was significantly associated with an increased risk for thyroid cancer, of nearly eight-fold.

TNM and clinical stage in the thyroid cancer group

Histopathological analysis in the control group revealed the presence of multinodular goiters in 34.4% (11) and papillary carcinoma in 75% (24) of the subjects in the cancer group. Approximately half of the patients with thyroid cancer (46.9%) were diagnosed with a tumor that invaded the limits of the thyroid capsule and with metastases in the cervical and lymph nodes; in addition, two patients with distant metastases were

observed. Regarding the clinical staging results, the largest proportion of patients (62.5%) exhibited a clinical stage of level IV, indicating that they were diagnosed in an advanced stage with an asymptomatic pathology (Figure 1).

Gene expression of CYP1A1 in thyroid cancer and its relationship with clinicopathological factors

The CYP1A1 gene was expressed in both thyroid cancer (9.55 ± 3.18) and control tissue samples (10.29 ± 3.86). No significant differences were observed in the two groups ($P > 0.05$). The observed variations in the gene expression of CYP1A1 were not related to gender, age, T3 and T4 levels, place of residence, weight, family history of cancer, the use of tobacco or histology. However, positive relationships ($P < 0.05$) were found between CYP1A1

mRNA levels and the presence of chronic diseases, the use of alcohol, tumor size (> 2 cm), the presence of metastasis (gangliar or in other peripheral tissues) and an advanced

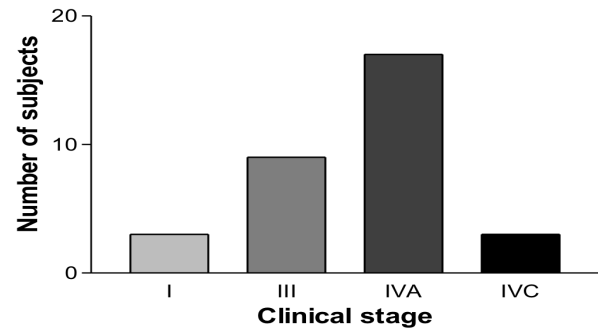


Figure 1. Clinical Stages of Thyroid Cancer

Table 1. Sequence of Primers for Endogenous and CYP1A1 Genes

Symbol	Nucleotide sequence: Primer forward, Primer reverse	Amplicón size (pb)	Primers efficiency
BACT	CTGGCACCCAGCACAAATG GGGCCGGACTCGTCATAC	143	2.7
18S	GTAACCCGTTGAACCCCAT CCATCCAATCGGTAGTAGC	151	2.6
GAPDH	GAGCCAAAAGGGTCATCATC CCTTCCACGATACCAAAGTT	175	2.04
G6PDH	CCTGGAGGAGCTGAGAATG CGGGCTCTCTCGGTACTTG	159	2.66
CYP1A1	TTCTTCGCTACCTACCCAAC CTGCTTCTCCTGACAGTGC	161	2

Table 2. Distribution of Clinical-pathological, Etiological and Sociodemographic Variables and Putative Risk Factors for Thyroid Cancer

Characteristics	Thyroid cancer		P value	OR (95% CI)
	N	Control N		
Age (mean±SD)	(54.81±14.77)	(53.66±13.62)		
	(32-82 years)	(23-73 years)		
< 45	5	11	0.08	0.4 (0.1-1.1)
≥ 45	27	21		
Gender				
Male	11	2	0.005*	7.9 (1.6-39.2)
Female	21	30		
Place of residence				
North	1	0	0.6	–
Central	25	26		
South	6	6		
Obese or overweight				
Yes	13	11	0.8	1.3 (0.5-3.6)
No	19	21		
Familiar history of cancer				
Yes	7	5	0.8	1.5 (0.4-5.3)
No	25	27		
Comorbidities				
Yes	20	16	0.5	1.7 (0.6-4.5)
No	12	16		
Tobacco				
Yes	4	7	0.5	0.5 (0.1-2)
No	28	25		
Alcohol				
Yes	5	1	0.2	5.7 (0.6-52.6)
No	27	31		
Drugs				
Yes	2	0	0.5	5.3 (0.2-115.6)
No	30	32		
Exposure to radiation				
Yes	1	0	1	3.1 (0.1-78.9)
No	31	32		

Table 3. CYP1A1 Gene Expression and Clinicopathological Parameters in Thyroid Cancer Patients

	CYP1A1 mRNA levels	P value
Gender		
Male (n=11)	10.67±3.4	0.13
Female (n=21)	8.90±2.94	
Age	Corr. Coef. 0.30	0.11
T3 levels	Corr. Coef. 0.0009	0.99
T4 levels	Corr. Coef. -0.078	0.68
Place of residence		
Central (n=25)	9.15±3.19	0.28
Other (n=7)	10.66±3.07	
Weight		
Normal (n=13)	9.06±3.42	
Overweight (n=10)	10.45±3.13	0.59
Obese (n=9)	9.46±3.05	
Familiar history of cancer		
Yes (n=7)	10.37±2.75	0.48
No (n=25)	9.39±3.28	
Comorbidities		
Yes (n=20)	10.77±2.88	0.032*
No (n=12)	8.32±3.07	
Tobacco		
Yes (n=4)	11.03±3.25	0.23
No (n=28)	9.01±3.04	
Alcohol		
Yes (n=5)	13.47±1.72	0.0022*
No (n=27)	8.95±2.92	
Histology		
Medullary carcinoma (n=3)	9.87±3.4	0.82
Papillary carcinoma (n=24)	9.66±2.81	
Others (n=5)	8.66±5.54	
Tumor size		
T(1-2 cm) (n=3)	4.13±1.75	0.0022*
T(3-4 cm) (n=29)	9.94±2.93	
Metastasis		
Yes (n=24)	10.37±2.86	0.001*
No (n=8)	6.29±2.24	
Clinical stage		
I-II (n=3)	4.19±1.24	0.0011*
III-IV (n=29)	10.14±2.73	

clinical stage (Table 3).

Discussion

Papillary thyroid cancer accounts for approximately two-thirds of thyroid cases in both males and females. Our results are consistent with this statistic (Thamnirat et al., 2015). Papillary thyroid cancer is the most indolent form of thyroid cancer (Brito et al., 2013) which may account for the advanced clinical stages detected among our patients.

Various factors are related to cancer development. These factors include environmental, genetic and lifestyle factors, which cause approximately 50% of all cancer deaths (Kye et al., 2015). Additionally, there is evidence that the following risk factors are associated with papillary thyroid cancer and papillary thyroid microcarcinoma: age, gender, multifocal location, extra-thyroid extension, lymph node metastasis, tumor size, distance of metastasis and I-131 therapy (Cooper et al., 2009; Guo et al., 2014).

In the present study, only female gender was found to be associated with an increased risk for thyroid cancer, but an age greater than 45 years also presented a non-significant association. Factors such as ionizing radiation (Ron et al., 1995), dietary and environmental factors (Bosetti et al., 2002) common somatic mutations in the human oncogene B-RAF (V600E), rearrangements in transformation/papillary thyroid carcinomas (RET/PTC) and neurotrophin receptor-tyrosine kinase (NTRK) (Shibru et al., 2008) do not appear to play a role in the gender disparity in thyroid cancer. However, several studies in high-risk populations have found an association between menstrual and pregnancy history, sex hormones and thyroid cancer risk (Brindel et al., 2008; Pham et al., 2009). With respect to age, many publications have shown that age is a predictor of outcome in thyroid cancer: an age of ≤ 45 years is linked to better outcomes than an age >45 years (Thamnirat et al., 2015). It has been demonstrated that the prevalence of thyroid cancer varies from 19 to 67% and increases with age, affecting approximately 50% of the population older than 40 years of age (Cooper et al., 2009; Abel et al., 2015).

CYP1A1 and CYP1A2 play roles in the metabolic activation of carcinogenic polycyclic aromatic hydrocarbons (PAH) and heterocyclic aromatic amines/amides (HAA), the demethylation of aminoazo dyes, and the dealkylation of phenacetin, caffeine and other therapeutic agents to electrophilic reactive intermediates that form DNA and protein adducts, leading to tumor formation and toxicity (Kim and Guengerich, 2005). The induction of CYP1A by PAHs is mediated through AhR. Binding of PAH to AhR elicits sequential signaling events leading to the activation of AhR and transcription of CYP1A genes through the dioxin response element located in the enhancers of these genes (Ma, 2001).

We observed no change in the mRNA levels of CYP1A1 in thyroid cancers in comparison with the control group. CYP1A1 is one of the most important detoxification enzymes, showing a wide distribution throughout the body. We hypothesize that dietary and ambient factors can also influence the expression of this monooxygenase detected in cancer and normal tissues (Ito et al., 2007).

In this study, we observed a significant increase of CYP1A1 gene expression in patients with thyroid cancer in the presence of comorbidities and alcohol use, possibly because chronic disease and alcohol induce inflammation or because cells are exposed to toxic metabolites that lead to oxidative stress and major cell damage (Torres et al., 2015). It has been demonstrated that oxidative stress and inflammation increase CYP1A1 expression (Lingappan et al., 2014; Hussain et al., 2014). Moreover, in 1976, Williams proposed the hypothesis that alcohol intake may increase the level of TSH, which regulates the growth and function of the thyroid gland (Williams, 1976). Several animal and cell culture studies have demonstrated that alcohol exposure increases benzo- α -pyrene adduct formation (Autrup et al., 1992; Izzotti et al., 1998; Barnes et al., 2000). Changes in thyroid function and PAH metabolism induced by alcohol consumption might be another reason for the increase in CYP1A1 mRNA expression associated with cancer.

Finally, CYP1A1 mRNA levels increased significantly with an increase in tumor size, the presence of metastasis and an advanced clinical stage. CYP1A1 has been demonstrated to be associated with tumor development in non-small cell lung cancer, and a positive relationship between CYP1A1 expression and adenocarcinoma progression has been demonstrated (Oyama et al., 2007). We suggest that CYP could serve as a prognostic marker indicating the grade of tumor severity in thyroid cancer patients.

In conclusion, these data showed, for the first time, that female gender is a risk factor for thyroid cancer in a Mexican population. Moreover, a significant increase of CYP1A1 gene expression was demonstrated to be associated with tumor size, the presence of metastasis and advanced clinical stage for the first time in this study. These results suggest that CYP1A1 gene expression could be used as a marker to evaluate the severity of tumors or to identify an aggressive phenotype and that this marker could complement histopathological screening, diagnosis and therapeutic strategies for the possible personalized treatment of thyroid cancer patients.

Acknowledgements

This work was partially supported by A022 program obesity project, SEDENA, Mexico, D.F.

References

- Abel GA, Shelton J, Johnson S, Elliss-Brookes L, Lyratzopoulos G (2015). Cancer-specific variation in emergency presentation by sex, age and deprivation across 27 common and rarer cancers. *Br J Cancer*, **112**, 129-36.
- Astrup JL, Hansen C, Astrup H (1992). Detection of tobacco smoke carcinogen-DNA adducts in cultured rat buccal mucosa cells following exposure to ethanol and total cigarette smoke condensate or chewing tobacco. *Chem Biol Interact*, **85**, 141-50.
- Barbieri RB, Bufalo NE, Cunha LL, et al (2013). Genes of detoxification are important modulators of hereditary medullary thyroid carcinoma risk. *Clin Endocrinol (Oxf)*, **79**, 288-93.
- Barnes S, Singletary K, Frey R (2000). Ethanol and acetaldehyde enhance benzo[a]pyrene-DNA adduct formation in human mammary epithelial cells. *Carcinogenesis (Lond)*, **21**, 2123-8.
- Beresford AP (1993). CYP1A1: Friend or foe? *Drug Metab Rev*, **25**, 503-15.
- Bosetti C, Negri E, Kolonel L, et al (2002). A pooled analysis of case-control studies of thyroid cancer. VII. Cruciferous and other vegetables (International). *Cancer Causes Control*, **13**, 765-75.
- Brindel P, Doyon F, Rachedi F, et al (2008). Menstrual and reproductive factors in the risk of differentiated thyroid carcinoma in native women in French Polynesia: a population-based case-control study. *Am J Epidemiol*, **167**, 219-29.
- Brito JP, Morris JC, Montori VM (2013). Thyroid cancer: zealot imaging has increased detection and treatment of low risk tumours. *BMJ*, **347**, 4706.
- Bufalo NE, Leite JL, Guilhen ACT, et al (2006). Smoking and susceptibility to thyroid cancer: an inverse association with CYP1A1 allelic variants. *Endocr Relat Cancer*, **13**, 1185-93.
- Cooper DS, Doherty GM, Haugen BR, et al (2009). American Thyroid Association (ATA) Guidelines TaskForce on Thyroid Nodules and Differentiated Thyroid Cancer. *Thyroid*, **19**, 1167-214.
- Dees EC, Watkins PB (2005). Role of cytochrome P450 phenotyping in cancer treatment. *J Clin Oncol*, **23**, 1053-5.
- Floriano-Sanchez E, Cardenas-Rodríguez N, Castro-Marín M, Alvarez-Grave P, Lara-Padilla E (2009). DD3(PCA3) gene expression in cancer and prostatic hyperplasia. *Clin Invest Med*, **32**, E258-64.
- GLOBOCAN 2012. <http://globocan.iarc.fr/Default.aspx>. Accessed December 4, 2015.
- Guo K, Wang Z (2014). Risk factors influencing the recurrence of papillary thyroid carcinoma: a systematic review and meta-analysis. *Int J Clin Exp Pathol*, **7**, 5393-5403.
- He XF, Wei W, Liu ZZ, et al (2014). Association between the CYP1A1 T3801C polymorphism and risk of cancer: evidence from 268 case-control studies. *Gene*, **534**, 324-44.
- Hussain T, Al-Attas OS, Al-Daghri NM, et al (2014). Induction of CYP1A1, CYP1A2, CYP1B1, increased oxidative stress and inflammation in the lung and liver tissues of rats exposed to incense smoke. *Mol Cell Biochem*, **391**, 127-36.
- Ito S, Chen C, Satoh J, Yim S, Gonzalez FJ (2007). Dietary phytochemicals regulate whole-body CYP1A1 expression through an arylhydrocarbon receptor nuclear translocator-dependent system in gut. *J Clin Invest*, **117**, 1940-50.
- Izzotti A, Balansky R, Blagoeva P, et al (1998). DNA alterations in rat organs after chronic exposure to cigarette smoke and/or ethanol ingestion. *FASEB J*, **12**, 753-8.
- Jung CK, Little MP, Lubin JH, et al (2014). The increase in thyroid cancer incidence during the last four decades is accompanied by a high frequency of braf mutations and a sharp increase in RAS mutations. *J Clin Endocrinol Metab*, **99**, 276-85.
- Khlifi R, Messaoud O, Rebai A, Hamza-Chaffai A (2013). Polymorphisms in the human cytochrome P450 and arylamine N-acetyltransferase: susceptibility to head and neck cancers. *BioMed Res Int*, **2013**, 1-20.
- Kim D, Guengerich FP (2005). Cytochrome P450 activation of arylamines and heterocyclic amines. *Annu Rev Pharmacol Toxicol*, **45**, 27-49.
- Kim TY, Kim WG, Kim WB, Shong YK (2014). Current status and future perspectives in differentiated thyroid cancer. *Endocrinol Metab*, **29**, 217-25.
- Kye SY, Kwon JH, Kim Y, et al (2015). Cancer risk factors in Korean news media: a content analysis. *Asian Pac J Cancer Prev*, **16**, 731-6.
- Lin J, He B, Cao L, et al (2014). CYP1A1 Ile462Val polymorphism and the risk of non-small cell lung cancer in a Chinese population. *Tumori*, **100**, 547-52.
- Lingappan K, Jiang W, Wang L, et al (2014). Mice deficient in the gene for cytochrome P450 (CYP)1A1 are more susceptible than wild-type to hyperoxic lung injury: evidence for protective role of CYP1A1 against oxidative stress. *Toxicol Sci*, **141**, 68-77.
- Ma Q (2001). Induction of CYP1A1. The AhR/DRE paradigm: transcription, receptor regulation, and expanding biological roles. *Curr Drug Metab*, **2**, 149-64.
- Oyama T, Sugio K, Uramoto H, et al (2007). Cytochrome P450 expression (CYP) in non-small cell lung cancer. *Front Biosci*, **12**, 2299-308.
- Pfaffl MW, Horgan GW, Dempfle L (2002). Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res*, **30**, 36.
- Pham TM, Fujino Y, Mikami H, et al (2009). Japan Collaborative Cohort Study Group: Reproductive and menstrual factors

- and thyroid cancer among Japanese women: the Japan Collaborative Cohort Study. *J Womens Health (Larchmt)*, **18**, 331-5.
- Pusztaszeri MP, Bongiovanni M, Faquin WC (2014). Update on the cytologic and molecular features of medullary thyroid carcinoma. *Adv Anat Pathol*, **21**, 31-40.
- Reis AAS, Silva DM, Curado MP, da Cruz AD (2010). Involvement of CYP1A1, GST, 72TP53 polymorphisms in the pathogenesis of thyroid nodules. *Genet Mol Res*, **9**, 2222-9.
- Ron E, Lubin JH, Shore RE, et al (1995). Thyroid cancer after exposure to external radiation: a pooled analysis of seven studies. *Radiat Res*, **141**, 259-77.
- Rossing MA, Cushing KL, Voigt LF, Wicklund KG, Daling JR (2000). Risk of papillary thyroid cancer in women in relation to smoking and alcohol consumption. *Epidemiol*, **11**, 49-54.
- Shibru D, Chung K, Kebebew E (2008). Recent developments in the clinical application of thyroid cancer biomarkers. *Curr Opin Oncol*, **20**, 13-8.
- Siraj AK, Ibrahim M, Al-Rasheed M, et al (2008). Polymorphisms of selected xenobiotic genes contribute to the development of papillary thyroid cancer susceptibility in middle eastern population. *BMC Med Genet*, **9**, 61.
- Thamnirat K, Utamakul C, Chamroonrat W, et al (2015). Factors affecting disease-free status of differentiated thyroid carcinoma patients. *Asian Pac J Cancer Prev*, **16**, 737-40.
- Tinzl M, Marberger M, Horvath S, Chypre C (2004). DD3PCA3 RNA analysis in urine--a new perspective for detecting prostate cancer. *Eur Urol*, **46**, 182-6.
- Tirado L, Granados M (2007). Epidemiología y etiología del cancer de la cabeza y el cuello. *Cancerología*, **2**, 9-17.
- Torres A, Cachafeiro V, Millan J, et al (2015). Red wine intake but not other alcoholic beverages increases total antioxidant capacity and improves pro-inflammatory profile after an oral fat diet in healthy volunteers. *Rev Clin Esp*, **215**, 486-94.
- Wahid M, Mahjabeen I, Baig RM, Kayani MA (2013). Expression of CYP1A1 and GSTP1 in human brain tumor tissues in Pakistan. *Asian Pac J Cancer Prev*, **14**, 7187-91.
- Williams R (1976). Breast and thyroid cancer and malignant melanoma promoted by alcohol-induced pituitary secretion of prolactin, T.S.H. and M.S.H. *Lancet*, **307**, 996-9.