Iraqi Journal of Cancer and Medical Genetics

Histopathological and Molecular Analysis of Thyroid Papillary and Medullary Carcinoma in a Samples of Iraqi Patients

Rebah Najah Jabbar

Biotechnology Research Center, Al – Nahrain University

Abstract :

This study included, 1500 patients showing thyroid cancer (500 female and 1000 male), were selected to determine the histopathic disorder and DNA aberration in thyroid tissue compared with 200 healthy people of both genders in four years study (2006 – 2010). Histopathological examination of thyroid disorders showed the presence of papillary carcinoma in patients with goiter and most of benign tumors appeared in female patients and were less in male. Cancer study showed that cancer studied were mostly of papillary carcinoma, whereas medullary carcinoma was less frequent. DNA analysis using hexamere RAPD primers from patients with thyroid carcinoma and thyroid disorders compared with healthy persons showed different patterns analyzed as follow: Patients with hyper and hypo thyroidism showed no DNA aberration when compared with each other and with healthy persons, whereas different patterns of DNA electrophoresis were noticed with patients with cancer cases.

wThis pattern showed that patients with medullary and papillary carcinoma showed bands in location significantly different from those patients with thyroditis and healthy subjects. Further analysis with specific primers showed significant change in bands at location amplified compared with healthy persons suggesting a sever change in DNA sequence in patients with carcinomas which may attributed to BRAF mutation.

Keyword: *thyroid hormones, thyroid cancer, DNA analysis of human, DNA mutation detection by PCR*

Introduction:

A thyroid nodule is any abnormal growth of thyroid cells forming a lump within the thyroid gland. Though most thyroid nodules do not cause any symptoms, occasionally a nodule will cause pain, difficulty swallowing or breathing, hyperthyroid symptoms. About 90-95% of thyroid nodules are benign (non-cancerous) (1).

Thyroid cancer is a common endocrine malignancy. The majorities, derived from follicular epithelial cells, represent a model of malignant transformation from benign adenomas and well differentiated carcinomas to poorly differentiated thyroid carcinoma and the rare but rapidly lethal undifferentiated thyroid carcinoma. This spectrum of progression has been linked with a pattern of cumulative intragenic defects that correlates with tumor differentiation, aggressiveness, and metastatic potential (2). Gene rearrangements involving the RET proto-oncogene or activating point mutations along the Ras / BRAF pathway account for the majority of these carcinomas. These intragenic abnormalities are considered to be early events in thyroid carcinogenesis; cancer behavior and progression are further modified by dysregulation of growth factors and their cognate receptors (3).

Diagnostic tests for thyroid dysfunction included ultrasound tests to determine the presence of enlargement, nodules, and cysts. The nodules can be hot or cold depending on whether they are producing hormones or not. A fine needle aspiration may be necessary, if cancer is suspected. However, the development of molecular RT-PCR-based methods to diagnose viral diseases that identify specific mRNAs, rather than proteins, suggested that, a highly sensitive method for detecting thyroid cancer that would be unencumbered by antibody interference could be developed (4).

Corresponding Address:
Rebah Najah Jabbar
Biotechnology Research Center, Al – Nahrain University
Email: rebahalgafari@gmail.com
Tel.: 07714479000

Material and Methods:

Patients , blood, and tumor collection

Blood and tissue was collected for 4 years (2006 – 2010) from patients at the AL- Yarmok Teaching Hospital

(Baghdad), AL- Kadhymia Teaching hospital (Baghdad), St. Rafael hospital (Baghdad), Marjan /general surgery hospital (Babylon), AL-Zahra hospital (Najaf), AL-Basra general hospital (Basra), AI- Ramadi general hospital (Ramadi), AL- Shifaa general hospital (Mousel), and Karkuk general hospital (Karkuk). A total of 1500 patients were recruited for this study, including 1000 male and 500 female and 200 healthy persons as control. Their ages between (40-70) years.

Thyroid Histopathological Examination

After surgical removal of the follicular epithelial cells, it was subjected to histopathological examination. The tissue was fixed in small containers containing 20 ml of formalin 10% and dehydrated in progressively more concentrated alcohol embedded in paraffin and cut into section 4-5 µm thickness, stained with haematoxyline and eosin (H&E) for the microscopically examination under 40x and 100x magnification (5).

Thyroid Hormone concentration measurement

Hormones T3, T4 and TSH were measured using Mini-VIDAS Bio – Merieux/ France kit Sa. 69280 marcy I, Etoile – France, as instructed by the manufacturer (6).

RNA extraction

Total RNA was isolated from 1 ml venous blood obtained from standard venipuncture and transferred immediately into sterile tubes containing 3 ml RNXTM reagent [RNXTM total RNA isolation system kit from CinnaGen Iran (RN7713C)] according to the manufacturer's recommendations (7). RNA isolated from 50-100 mg thyroid tissue obtained from patients undergoing thyroidectomy was used.

RT-PCR

Total RNA (10µg) was reverse transcribed to complementary DNA (cDNA) in a final volume of 40µL using 250ng random hexamer primers (Sigma, U.S.A.), 10 U ribonuclease inhibitor (Sigma, U.S.A.), 200 U for each M-MLV Revers transcriptase and AMV Revers transcriptase (Sigma, U.S.A.), 50mmol/L Tris–HCl (pH 8.3), 75 mmol/L KCl, 3mmol/L MgCl2, 500µmol/L of each deoxy-NT P, and 10 mmol/L dithiothretol.

PCR was performed using 5µL first strand cDNA in a 25µL reaction volume containing 10mmol/L Tris–HCl (pH 8.3), 50 mmol/L KCl, 1.5mmol/L MgCl2, 200µmol/L of each deoxy-NT P, 1U Taq polymerase (Sigma, U.S.A.), and 40 pmol of specific primer (Cyclin A: sense primer : 5́ – GTCACCACATACTATGGACATG – 3́, antisense primer: 5́ -AAGTTTTCCTCTCAGCACTGAC-3́).

The cycling condition used to protocol included of 45oC for 45 min., 94oC for 2 min., followed by 40 cycle of 94oC for 30 second., 60oC for 1 min., 68oC for 2 min., and 68oC for 7 min. after the amplification, 5µL of each PCR reaction were electrophoresis through 2% agarose gel and visualized with ethidium bromide.

Statistical analysis

Statistical analysis was done using Minitab 15 statistical analysis software. ANOVA test was used to compare different groups among each other and with control. All values were expressed as Mean ± Standard Deviation of the mean (M ± SD). P value < 0.05 was regarded as statistically significant.

Results:

The 1500 patients with thyroid carcinomas were subjected for investigation. All of them suffered from hyperthyroidism and evolved to cancer with neglecting treatment in some cases, while others especially elderly patients developed cancer as a result of change in their body physiology and deterioration of immune and DNA repair system.

Categorizing cancer type with age and gender is listed in tables (1) and (2).

Table (1). Identification of cancer type and it's manifestation among different age groups in male patients.

Age / years	Patients with Papillary carcinoma	Patients with Medullary carcinoma
31 – 40	Non	Non
41 – 50	200	Non
51 – 60	200	100
61 – 70	300	200

Table (2). Identification of cancer type and it's manifestation among different age groups in female patients.

Age / years	Patients with Papillary carcinoma	Patients with Medullary carcinoma
20 – 30	Non	Non
31 – 40	Non	Non
41 – 50	100	Non
51 – 60	200	Non
61 – 70	100	100

Tables (1) and (2) show that cancer initiation was more in male than female and all of those patients were identified as hyperthyroidism patients as shown in table (3).

Table (3). Thyroid hormone concentration in patients with thyroid cancer.

T 3 (normal value 1.2 – 2.8 nmol/l)			
Gender	Age (years)	Hormone concentration	No. of cases
Male	40 – 70	5 – 7	1000
Female	40 – 70	4 – 10	500
T 4 (normal value 60 – 160 nmol/l)			
Gender	Age (years)	Hormone concentration	No. of cases
Male	40 – 70	400 – 700	1000
Female	40 – 70	300 – 600	500
TSH (normal value 0.25 – 0.5 nmol/l)			
Gender	Age (year)	Hormone concentration	No. of cases
Male	40 – 70	0.01 – 0.25	1000
Female	40 – 70	0.1 – 0.3	500

Table (3) shows the unusual elevation in T3 and T4 in cancer patients serum while TSH reduced in blood stream. Analysis of significance of thyroid hormone between the two genders is given in table (4).

Table (4). Statistical analysis of thyroid hormone levels in comparison with normal using two ways ANOVA.

Age / years	T3 (nmol±SD)		T4 (nmol±SD)		TSH (nmol±SD)	
	Male	**Female**	**Male**	**Female**	**Male**	**Female**
41-50	A, a 5.5±0.7071	AB, a 4 ± 1	A, a 500±141.42	A, a 500±100	A, a 0.13±0.16971	A, a 0.2±0.1
51-60	A, a 6.3333±1.1547	A, a 8.5±2.121	A, a 566.67±152.75	A, a 450±212.13	A, a 0.12±0.12124	A, a 0.2±0.14142
61-70	A, a 6±1	A, a 7.5±3.536	A, a 580±130.38	A, a 500±141.42	A, a 0.126±0.09762	A, a 0.25±0.07071
Control	B, a 2.12±0.4638	B, a 1.830±0.49	B, a 114±30.62	B, a 106±34.06	B, a 0.38±0.07888	A, a 0.36±0.08756

A and B = Comparison of hormone concentration of patients of the same gender but differ in age. a and b= Comparison of hormone concentration of patients of the same age category but differ in gender. Different letter means the difference is considered significant when (P<0.05).

Table (4) shows there is a significant difference in thyroid hormones concentration when compared with normal in both genders. It is at an elevated level in all cases; whereas TSH in female did not show significant difference when compared with normal.

Some patients with thyroid goiter in this study who suffered a fibrosis in thyroid gland reported to histopathology laboratories for biopsy, where as thyroid cancer patients were subjected for thyrodectomy. Samples from those patients were used for histopathological examination as shown in figure (1) that shows papillary adenoma in patients with hyperthyroidism and fibrosis, and figure (2) for patients with papillary carcinoma.

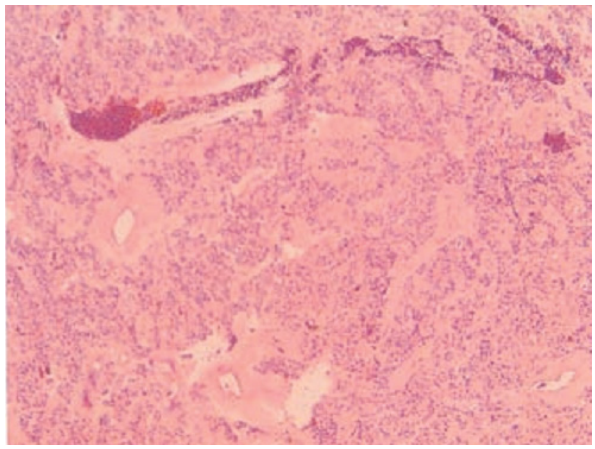

Figure (1). Papillary adenoma section in patients with hyperthyroidism suffering from fibrosis in thyroid gland. Tissue was stained with H&E and examined under 400X magnification power.

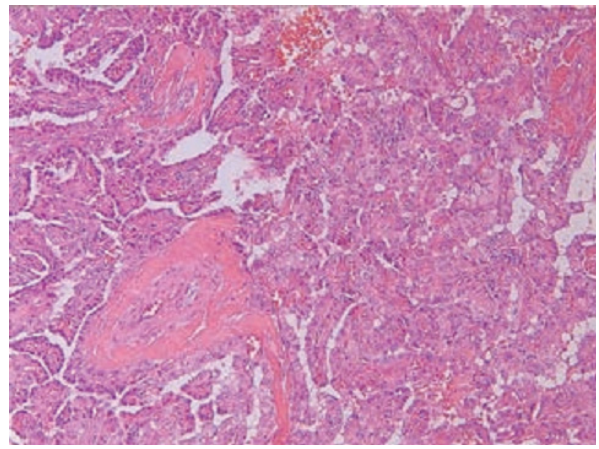

Figure (2). Papillary carcinoma section in patients with thyroid cancer. Tissue was stained with H&E and examined under 400X magnification power.

Molecular analysis of thyroid mRNA from blood

Blood stream contains many components; some of them are proteins, minerals, lipids, and cells that the body is composed of. Some literatures referred that thyroid cells are circulating blood and can be isolated by magnetic cell sorting technique (8).

Exploiting this fact, blood samples from patients of thyroid dysfunction was used to search for markers that can be useful to determine the genetic change in their thyroid and the possibility to diagnose patients that may develop thyroid cancer at early stage. This required isolation of mRNA from blood and generating DNA by using it as a template for RT – PCR. This was made by using a kit developed for this purpose, and. Result of using random hexamere primers is shown in figure (3).

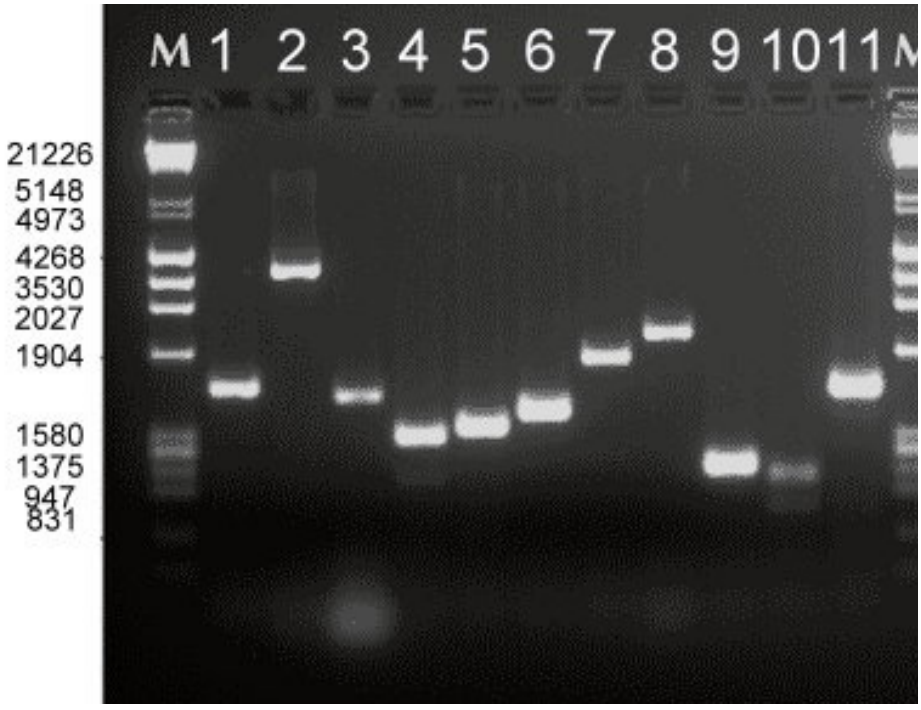

Figure (3). RT – PCR amplification of mRNA isolated from patients with thyroid disorders using hexamere primers. M is a marker of Lambda DNA digested with EcorI and HindIII, lane 1, 3, and 11 is the result of healthy persons, lane 2 the result of patient of medullary carcinoma, lane 4, 5, 6 is the result of patients with hyperthyroidism, lane 7 and 8 is the result of patients with hypothyroidism, lane 9 and 10 is the result of patients with papillary carcinoma. Gel electrophoresis was performed at 10 v/cm for 2 hrs in 1 % agarose.

For further confirmation of the results, another RT – PCR process was performed using Cycline A primers designed specifically for thyroid gene analysis. The result is shown in figure (4).

Discussion:

This study showed that papillary adenoma had over rated medullary carcinoma. This may be attributed to the genetic alterations, some of which are seen only in this cancer (13). The classical oncogenic genetic alterations commonly seen in thyroid cancer include Ras mutations (14), RET / PTC rearrangements (15), and PAX8 peroxisome proliferators activated receptor g (PPARg) fusion oncogene (16). Various activating Ras mutations, widely seen in other cancers as well, occur mainly in FTC and the follicular variant of PTC (17). However, our results are pointing to a sever mutation at BRAF gene. In most patients with previously treated well differentiated thyroid carcinoma, this highly sensitive technique can detect small amounts of thyroid tissue even during TSH-suppressive thyroid hormone therapy. The assay of using circulating mRNA developed derives its sensitivity from the combined use of RT-PCR, a technique that allows specific amplification of small numbers of mRNA molecules.

RT-PCR has been used to detect small foci of malignant tissue as well as circulating cancer cells in patients with metastatic prostate cancer (18), malignant melanoma, breast cancer, and thyroid cancer (19).

The assay for circulating thyroglobulin mRNA used in this study appears to have performance characteristics different from those of other assays recently reported. Ditkoff et al., 1996 detected circulating thyroglobulin mRNA in all 7 patients with metastatic thyroid cancer whom they studied, but in less than 10% of 78 patients without metastases (20). Moreover, they did not detect thyroglobulin mRNA in blood from normal subjects. Tallini et al., 1998 analyzed thyroglobulin mRNA in blood samples obtained from patients with thyroid cancer before surgery (21). Although these patients had intact thyroid glands, circulating thyroglobulin mRNA was detected in only 4 of 7 patients with thyroid cancer and in 4 of 17 patients with benign thyroid nodules before thyroidectomy.

Several lines of evidence argue that the thyroglobulin mRNA detected in peripheral blood arises from circulating thyroid cells rather than from lymphocytes, in which thyroglobulin gene expression might represent ectopic transcription. First, negative and positive control lane did not show amplified bands after electrophoresis. Second, thyroglobulin mRNA can be detected in peripheral blood RNA after just one round of amplification. By contrast, detection of the extremely low levels of ectopically expressed mRNAs typically requires two consecutive rounds of nested amplification (22).

Figure (3) can be interpreted as follows: rapid hexamere primers that were designed for analysis of thyroid genes had succeeded in pairing with target sites giving different patterns of DNA bands. In healthy persons lane 1, 3, and 11 showed a DNA band of the same size without significant difference that are useful as a reference point for comparison of other bands. Lane 2 which is a DNA amplification of patients with medullary carcinoma showed a DNA fragment of size 3.91 kbp whereas lane 9, and 10 showed DNA fragments of patients with papillary carcinoma of size 1.016 and 0.939 kbp. Comparing these fragments with each other and with normal showed a significant difference relating their size and distance traveled. The reason for this may be explained as the presence of mutation within genes controlling thyroid function of different types in both disorders. The mutation in medullary carcinoma patients may be of amplification and rearrangement type resulted in presence of multiple DNA fragments of the same sequence that increased the specificity of primers used for amplification to associate with the target site. The result was a DNA fragment of large size whereas in case of papillary carcinoma, a point mutation or deletion in gene(s) controlling thyroid function reduced the specificity of primers to associate with target size resulted in a faint and less size fragment. In addition, no significant difference was found among patients with hyperthyroidism and hypothyroidism when compared with normal suggesting the change only occurred at physiological level.

Figure (4) can be interpreted as follows: the Cycline A primer set was specific for thyroid gene analysis. The comparison among patients with hyperthyroidism and hypothyroidism with normal (healthy) did not yield a significant difference, suggesting no DNA alteration occurred at the molecular level. However, no DNA band was observed in patients with thyroid carcinomas (lanes 1, 2, 3, and 8) from RT – PCR amplification. This can be attributed to the presence of a mutation within gene(s) controlling thyroid function and normal development and response. This mutation had altered the gene(s) in a way that the primer used for this purpose (Cycline A) was not sensitive to the target site, thus no amplification occurred as a result of the process.

References:

1. Morreale de Escobar, G.; Obregon, M. J.; and Escobar del Rey, F. (2004). Role of thyroid hormone during early brain development. European Journal of Endocrinology, 151: U25–U37.
2. Ringgel, M.D . (2004). Molecular Detection of Thyroid Cancer: Differentiating “Signal” and “Noise” in Clinical Assays. The Journal of Clinical Endocrinology and Metabolism, 89(1):29–32.
3. Nikiforov, Y. E. (2011). Molecular analysis of thyroid tumors. Modern Pathology 24: 534 – 543.
4. Yen P. M.; Ando, S.; Feng, X.; Liu, Y.; Maruvada, P.; and Xia, X. (2006). Thyroid hormone action atthecellular, genomic and target gene levels. Molecular and Cellular Endocrinology, 246: 121–127.
5. Bancroft, J. D. and Stevens, A. S. (1992). Theory and Practice of Histological Techniques. 2nd (ed.) Churchill Living Stone, Edinburgh, London.
6. Biersact ,H. J.; and Hotze, A. (1991). The clinician and the thyroid. Eur. J. Nucl. Med., 18: 761-778.
7. Chomezynski, P.; and Sacchi, N. (1987). Anal Biochem. 162: 156-159.
8. Takano T.; Matsuzuka, F.; Sumizaki, H.; Kuma, K.; and Amino, N. (1997). Rapid detection of specific messenger RNAs in thyroid carcinomas by reverse transcription-PCR with degenerate primers: specific expression of oncofetal fibronectin messenger RNA in papillary carcinoma. Cancer Res., 57: 3792–3797.
9. Da Silva, J. A. (1995). Sex Hormones, glucocorticoids, and autoimmunity: facts and hypotheses. Ann. Rheum. Dis., 54:6–16.
10. Reed Larsen, P.; Davies, T. F.; and Hay, I. D. (1998). The thyroid gland. In: Wilson, J. D.; Foster, D. W.; Kronenberg, H. M.; and Reed Larsen, P. eds. Williams textbook of endocrinology. Philadelphia: Saunders; 389–516.

11. Vitti, P.; Rago, T.; and Chiovato, L. (1997). Clinical features of patients with Graves' disease undergoing remission after antithyroid drug treatment. Thyroid, 7:369–375.
12. Nordyke, R.A., Gilbert F.I.; Harada A.S.M. (1988). Graves' disease Influence of age on clinical findings. Arch. Intern. Med., 148:626–631.
13. Aizawa, T.; Ishihara, M.; Hashizume, K.; Takasu, N.; and Yamada, T. (1989). Age-related changes of thyroid function and immunologic abnormalities in patients with hyperthyroidism due to Graves' disease. J. Am. Geriatr. Soc., 37:944–948.
14. Fagin, J.A. (2002). Minireview: branded from the start-distinct oncogenic initiating events may determine tumor fate in the thyroid. Molecular Endocrinology, 16: 903–911.
15. Nikiforov, Y. E. (2002). RET / PTC rearrangement in thyroid tumors. Endocrine Pathology, 13: 3–16.
16. Chrisoula, D. (2004). Histopathology of Thyroid Tumors. An Overview. Hormones, 3(2): 100 – 110.
17. Vasko, V.; Ferrand, M.; Di Cristofaro, J.; Carayon, P.; Henry, J. F.; and de Micco, C. (2003). Specific pattern of RAS oncogene mutations in follicular thyro id tumors. J. Clin. End. Metab., 88: 2745–2752.
18. Ghossein, R.A.; Scher H.I.; Gerald W.L. (1995). Detection of circulating tumor cells in patients with localized and metastatic prostatic carcinoma: clinical implications. J Clin Oncol. ,13:1195–1200.
19. Kruger, W.; Krzizanowski, C.; and Holweg, M. (1996). Reverse transcriptase/ polymerase chain reaction detection of cytokeratin-19 mRNA in bone marrow and blood of breast cancer patients. J. Cancer Res. Clin. Oncol., 122:679–686.
20. Ditkoff, B. A.; Marvin, M. R.; Yemul, S.; Shi ,Y. J.; Chabot, J.; Feind, C.; and Gerfo, P. L. (1996). Detection of circulating thyroid cells in peripheral blood. Surgery, 120:959 –965.
21. Tallini, G.; Ghossein, R.; and Emanuel, J. (1998). Detection of thyroglobulin, thyroid peroxidase, and RET/PTC1 mRNA transcripts in the peripheral blood of patients with thyroid disease. J. Clin. Oncol. ,16:1158 –1166.
22. Matthew Ringel, D.; Paul W. Ladenson, and Michael, A. Levine, 1998. Molecular Diagnosis of Residual and Recurrent Thyroid Cancer by Amplification of Thyroglobulin Messenger Ribonucleic Acid in Peripheral Blood. J. Clin.End. Metab., 83, No. 12: 4435 – 4442.

تحليل نسيجي ومرضي وجزيئي للسرطان الحلمي والنخاعيني للغدة الدرقية في عينات مرضى عراقيين

رباح نجاح جبار

مركز بحوث التقنيات الاحيائية / جامعة النهرين

الخلاصة:

شملت هذه الدراسة 1500 مريض اظهروا سرطان الغدة الدرقية (500 انثى و 1000 ذكر) تم انتخابهم لدراسة التغير النسيجي والوراثي في المادة الوراثية في نسيج الغدة الدرقية مقارنة مع 200 شخص من كلا الجنسين من الذين لا يعانون هذا المرض خلال اربع سنوات (2006 – 2010) . الدراسة النسيجية أظهرت في المرضى وجود غدد حلمية في المرضى الذين أظهروا تضخم الغدة وأن الاورام الحميدة وجدت في الاناث بنسبة أعلى منها في الذكور. دراسة السرطان أظهرت ان معظم أنواع السرطان المشخصة كانت من النوع الغدي الحلمي بينما كان السرطان النخاعي أقل وجودا. أظهر تحليل الدنا بإستخدام بادئات سداسية القواعد النتروجينية وبطريقة التضاعف العشوائي المتسلسل لعينات مرضى الغدة الدرقية مقارنة بالاشخاص الاصحاء وجود تغاير واضح في نواتج التضاعف تم تفسيره كما يلي:- ان المرضى المصابين بإرتفاع أو إنخفاض إفراز الغدة الدرقية لم يظهروا تغيرا في الدنا مقارنة بالاشخاص الاصحاء بينما كان هناك فرقاً واضحاً في نواتج الترحيل الكهربائي للعينات المأخوذة من مرضى سرطان الغدة الدرقية. أظهر تحليل عينات مرضى سرطان الغدة الدرقية بإستخدام بادئات متخصصة وطريقة التضاعف العشوائي المتسلسل أن هناك تغيراً شديداً في تسلسل الدنا بهؤلاء المرضى يمكن ان يعزى الى وجود طفرة في جين BRAF .