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Sahar D. YONIS

Introduction:

The butterfly – shaped thyroid gland which situated infront of the neck, which has been described and named since the Renaissance when the anatomist Wharton links its shape to the ancient shield, is one of the large endocrine glands in the human body (Ellis, 2011). Carcinomas of thyroid gland account for the most common types of endocrine carcinoma, from which the papillary type take the lead in the incidence for about 85% of all thyroid cancers. The diagnosis of the cancer depends primarily on the whole histopathological picture of papillary carcinoma with the classical nuclear criteria. The accurate diagnosis is the milestone in the proper further management of such tumours and predicts the prognosis of the disease. Nevertheless, the diagnosis may be challenging to pathologist when the nuclear criteria is not so clear or the specimen is poor in cells, some benign tumors may have pattern similar to papillae with nuclear features close enough to that of papillary carcinoma (Wu *et al*., 2013).

The normal cells are converted to cancerous cells by the process that involves mutations which include the activation of oncogenes and inactivation of suppressor gene. In normal conditions, the mutated cells are removed by processes that ensure stability of genome and repair of the defect within the cells. Though, some mutations occur that lead to neoplastic transformations. Of these mutations that encountered which occur in RET and BRAF genes (Ghossein, *et al*. 2013).

Rearrangement in RET has been observed in thyroid neoplasms. The fusion of RET tyrosine kinase domain with 5’ leads to activation of the intracellular receptor domain and enhance the mitogen activating protein kinase (MAPK) pathway, followed by proliferation of cells and appearance of malignancy (Santoro*et al.*, 2006). The occurrence of these mutations were noticed as a

consequences of exposure to ionizing radiations, as well as, it was noticed that the incidence is higher in children than adult attributing these finding to the higher rate of thyroid cell proliferation making the susceptibility to DNA damage more frequent. The other gene involved in the mutation is BRAF V600E, which is the B-isoform of RAF kinase. This gene mutation was detected in several tumours, such as gastrointestinal and colorectal adenocarcinomas, pulmonary adenocarcinoma, papillary thyroid carcinoma, etc (Hall and Kudchadkar, 2014).The point mutation of BRAF V600Ewas noticed in about half of cases of papillary thyroid carcinomas, and more frequent in tall cell variant. The BRAF mutation is generally negative in normal thyroid tissue and benign tumours, adding to that follicular and medullary carcinomas(Ghossein, *et al*., 2013). Recent studies suggest the presence of a link between BRAF mutation and the risk of recurrence, metastasis and extrathyroid extension. Hence the detection of BRAF mutation by using BRAF mutation specific antibody in thyroid tissue specimen play an important role in the diagnosis and prognostic determination of the disease(Zhu *et al*., 2019).

Though, it is not precisely confirmed that the cancer is caused by these gene mutations or the mutations were occur in coexistence with the tumours. From the above it is become to need the existence of more diagnostic criteria that confirm and enhance the accuracy of the diagnosis; of these diagnostic methods are the immunohistochemical studies and genetic markers that can differentiate the other thyroid carcinoma from the papillary one.

Aim of the Study:

The present work was aimed to study the immunohistochemical and pathological features, furthermore, some alterations in genes of patients diagnosed as having papillary thyroid carcinoma (PTC) in Babylon province by the following objectives:

- Study the sociodemographic characteristics.
- Gross examination of thyroid gland in patients with papillary carcinoma.
- Routine histopathological study by using haematoxyline and eosin stain.
- Immunohistochemical study by using CD56, CK19 and HMBE-1markers.
- Studying the mutation of RET and BRAF genes by using fluorescence *insitu* hybridization (FISH) technique.

Chapter Two: Review of Literature

2.1. Historical background

The first description of thyroid diseases as they are known today was that of Graves' disease by Caleb Parry in 1786, but the pathogenesis of thyroid disease was not discovered until 1882-1886. Thyroidectomy for hyperthyroidism was first performed in 1880, and antithyroid drugs and radioiodine therapy were developed in the early 1940s. Thomas Curling first described hypothyroidism (myxoedema) in 1850 and the cause and suitable treatment were established after 1883(Ahmed and Ahmed, 2005).

The most common presentation of thyroid cancer is an asymptomatic thyroid mass or a nodule that can be felt in the neck. For any patient with a thyroid lump that has developed recently, record a thorough medical history to identify any risk factors or symptoms. In particular, obtain a history regarding every prior exposure to ionizing radiation and the lifetime duration of the radiation exposure. Some patients with thyroid cancer have persistent cough, difficulty breathing, or difficulty swallowing. Pain is seldom an early warning sign of thyroid cancer (Pynnonen*et al*. 2017).

2.2. Embryology and development of thyroid gland:

The thyroid gland is the earliest endocrine gland in development; it begins in approximately the 24th day of development. The origin of thyroid gland is a budat the midline from endodermal proliferations at the level of the foramen cecum where the future tongue will appear, between the second and third pouches of pharynx, then it descends in front of the trachea, at the first tracheal rings'level (Sadler, 2019). The last pouch to develop is the 5th pharyngeal pouch; it is actually

part of the 4th pouch, from this pouch the ultimobranchial body develops. The later incorporated into the thyroid gland, into which the parafollicular (C cells) originating from the neural crest is, incorporated. These cells responsible for the secretion of calcitonin – the hormone regulating calcium level.(Dudek, 2014).

The diverticulum that appears in the mid-line caudal to median tongue bud extends caudally in front of the hyoid bone as the thyroglossal duct. The end of the duct will divides to form the subsequent thyroid lobes and isthmus of the gland. The gland is relatively large in newborns, and it reaches half the adult size at 2 years old(Lumley *et al.*, 2019).

The thyroid capsule and the septae which lies between the lobules are derived from the mesenchymal neural crest tissue. The failure or disappearance of the parts of the glands which arise from the floor of the pharynx leads to develop ectopic tissue of thyroid, in the tongue, for example (Sadler, 2019).

2.3. Anatomy and histology of thyroid gland:

The thyroid gland situated in front of the neck and it is characterized by its butterfly appearance with usually two lateral lobes, Each lateral lobe is pear-shaped with a narrow upper pole and a broader lower pole, and appears approximately triangular on cross-section with lateral, medial and posterior surfaces (Neel *et al.*, 2016).The lateral (superficial) surface is under cover of sternothyroid and sternohyoid muscles. The medial surface lies against the lateral side of the larynx and upper trachea, with the lower pharynx and upper oesophagus immediately behind the gland. This surface is related to the cricothyroid muscle of the larynx and the inferior constrictor of the pharynx, as well as to the external and recurrent laryngeal nerves (Hansen, 2010). The posterior surface overlaps the medial part of the carotid sheath, i.e. the part containing the common carotid

artery; if enlarged, the lobe may extend across the more laterally placed internal jugular vein. The parathyroid glands usually lie in contact with this surface, between it and the fascial sheath. The relationship of the recurrent laryngeal nerves to the thyroid lobes has importance in thyroid surgery. Which are roughly conical in shape, united in mid-line by the isthmus. The latter lay in front the tracheal rings, the second, third, and forth. The gland enclosed by its connective tissue capsule (Moore *et al.*, 2014).

The architecture of the gland is made by lobules which are separated by the connective tissue septae, that extend from the capsule, each lobule consist of the 20-40 functional unit of the gland, that is the thyroid follicle. The follicle is spherical with 0.02-0.9 cm in diameter (Figure 2-1). These follicles made of a centrally located colloid surrounded by a layer of cuboidal or columnar cells that rest on a basal lamina (Mescher, 2018). The colloid is made of glycoproteins which are iodinated, iodothyroglobulin, T3, and T_4. The activity of the gland is controlled by the circulating thyroid stimulating hormone (TSH), which is released from the hypophysis. Different levels of activities may be seen at the same time (Eroschenko, 2017).

In response to hypothalamic factors, TSH (thyrotropin) is released by thyrotrophs in the anterior pituitary into the circulation. The binding of TSH to its receptor on the thyroid follicular epithelium results in activation of the receptor, allowing it to associate with a G-protein. Activation of the G protein stimulates downstream events that result in an increase in intracellular cAMP levels, which stimulates thyroid growth and thyroid hormone synthesis and release via cAMP-dependent protein kinases (Barrett *et al.*, 2010).

Thyroid follicular epithelial cells convert thyroglobulin into thyroxin (T4) and lesser amounts of triiodothyronine (T3). T4 and T3 are released into the

systemic circulation, where most of these peptides are reversibly bound to circulating plasma proteins, such as thyroxin-binding globulinand transthyretin. The binding proteins act as a buffer that maintains the serum unbound ("free") T3 and T4 concentrations within narrow limits, while ensuring that the hormones are readily available to the tissues. In the periphery, the majority of free T4 is deiodinated to T3; the latter binds to thyroid hormone nuclear receptors in target cells with tenfold greater affinity than does T4 and has proportionately greater activity. Binding of thyroid hormone to its nuclear thyroid hormone receptor (TR) results in the assembly of a multi protein hormone-receptor complex on thyroid hormone response elements (TREs) in target genes, up regulating their transcription (Eroschenko, 2017).

2.4. Physiology of thyroid gland

Thyroid hormones stimulate O_2 consumption by most of the cells in the body, help regulate lipid and carbohydrate metabolism, and thereby influence body mass and mentation. Consequences of thyroid gland dysfunction depend on the life stage at which they occur (Hall and Hall, 2021). The thyroid is not essential for life, but its absence or hypofunction during fetal and neonatal life results in severe mental retardation and dwarfism. In adults, hypothyroidism is accompanied by mental and physical slowing and poor resistance to cold. Conversely, excess thyroid secretion leads to body wasting, nervousness, tachycardia, tremor, and excess heat production. Thyroid function is controlled by the TSH (Barrett *et al.*, 2010).

The thyroid hormones are synthesized and stored in the thyroid gland and incorporated into macromolecules of the protein thyroglobulin, which is stored in large follicles within the thyroid gland. Hormone secretion occurs when the amines aresplit from thyroglobulin and the free hormones are then released into the blood

stream. After entering the blood, most of the thyroid hormones combine with plasma proteins, especially thyroxin-binding globulin, which slowly releases the hormones to the target tissues (Widmaier *et al.*, 2019).

Thyroid hormones circulate in the blood while being mainly bound to plasma proteins. Usually less than 10 percent of thyroid hormones in the plasma exist free in solution. For example, more than 99 percent of the thyroxin in the blood is bound to plasma proteins. However, protein-bound hormones cannot easily diffuse across the capillaries and gain access to their target cells and are therefore biologically inactive until they dissociate from plasma proteins. The relatively large amounts of hormones bound to proteins serve as reservoirs, replenishing the concentration of free hormones when they are bound to target receptors or lost from the circulation. Binding of hormones to plasma proteins greatly slows their clearance from the plasma (Pappa *et al*., 2015).

Thyroid hormones are distinguished by their predominantly intracellular sites of action, since they can diffuse freely through the cell membrane. They bind to a family of largely cytoplasmic proteins known asnuclear receptors (Rosai, 2011). Upon ligand binding, the receptor–ligand complex translocates to the nucleus where it either homodimerizes, or associates with a distinct liganded nuclear receptor to form a heterodimer (Heldin *et al*., 2016). In either case, the dimer binds to DNA to either increase or decrease gene transcription in the target tissue. Individual members of the nuclear receptor family have a considerable degree of homology, and share many functional domains, such as the zinc fingers that permit DNA binding. However, sequence variations allow for ligand specificity as well as binding to specific DNA motifs. In this way, the transcription of distinct genes is regulated by individual hormones. The primary hormone secreted by the thyroid is T4, along with much lesser amounts of T3.T3 has much

greater biological activity than T4 and is also specifically generated at its site of action in peripheral tissues by deiodination of T4. Both hormones are iodine containing amino acids. Small amounts of reverse triiodothyronine (RT3) and other compounds are also found in thyroid venous blood. Whether RT3 is biologically active remains unclear (Barrett *et al.*, 2010).

Thyroid function is regulated primarily by variations in the circulating level of pituitary TSH. TSH secretion is increased by the hypothalamic hormone TRH and inhibited in a negative feedback manner by circulating free T4 and T3. The effect of T4 is enhanced by production of T_3 in the cytoplasm of the pituitary cells. TSH secretion is also inhibited by stress, and in experimental animals it is increased by cold and decreased by warmth (Bianco *et al.* 2014).

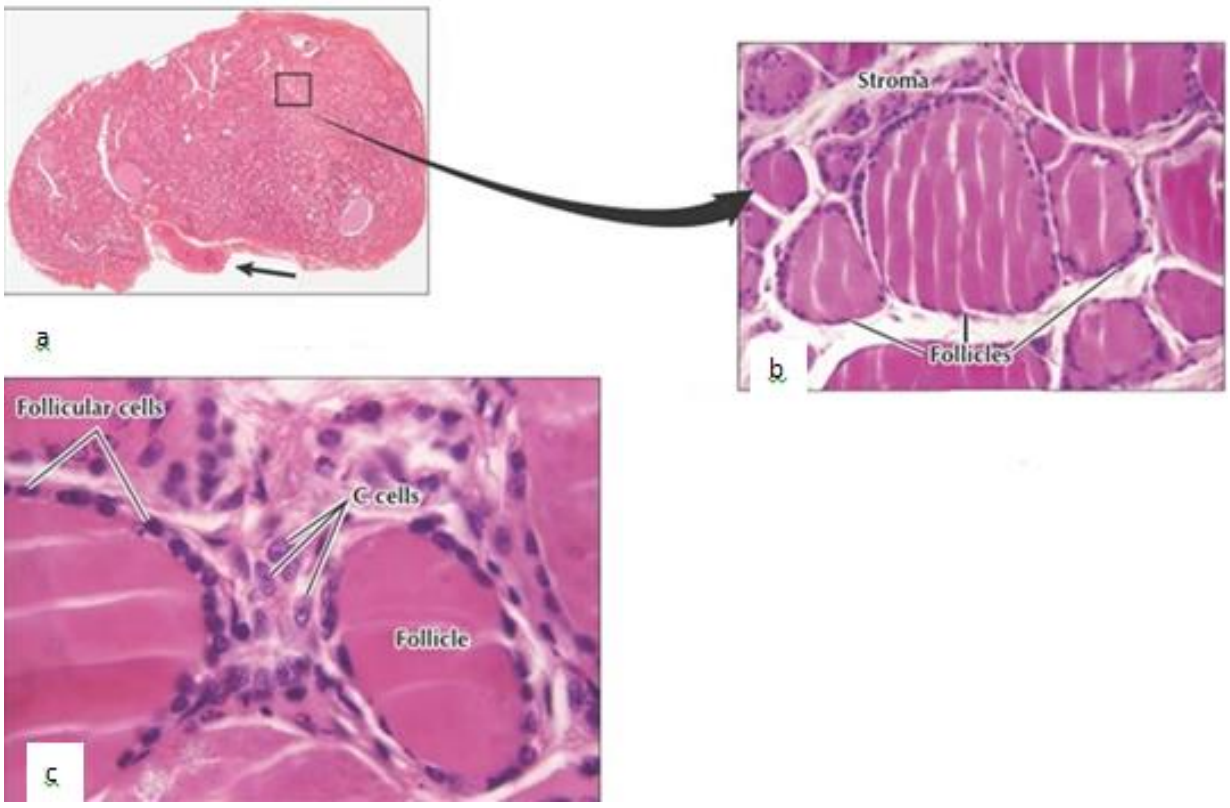


Figure (2-1): a. lobe of the thyroid at low magnification, Blood vessels (arrow) penetrates the very thin outer capsule.40X. H&E. b. closely packed follicles—functioning units of the gland—have varied sizes and shapes. One layer of flattened to cuboidal epithelial cells lines each one. Lumina contain thyroglobulin, which appears homogeneous and eosinophilic, with some crack-like fixation artifacts. Loose connective tissue makes up the delicate stroma that contains a network of capillaries, which are hard to see. 100×. H&E. c. A small clump of parafollicular (C) cells is in the stroma between follicles. Large size and clear, lightly stained cytoplasm identify these cells. Each contains a spherical euchromatic nucleus. Follicular cells around each follicle are low to high cuboidal and have darkly stained nuclei. They are fairly small compared with the parafollicular cells. 400×. H&E. (Eroschenko, 2017)

2.5. Diseases of thyroid gland

Diseases of the thyroid are of great importance because most are amenable to medical or surgical management. They include conditions associated with excessive release of thyroid hormones (hyperthyroidism), those associated with thyroid hormone deficiency (hypothyroidism), and mass lesions of the thyroid(Raphael *et al.*, 2015).

2.5.1. Hyperthyroidism

Thyrotoxicosis is a hypermetabolic state caused by elevated circulating levels of free T_3 and T_4. Because it is caused most commonly by hyperfunction of the thyroid gland, it is often referred to as *hyperthyroidism*. However, in certain conditions the oversupply is related to either excessive release of preformed thyroid hormone (e.g., in thyroiditis) or to an extra thyroidal source, rather than hyperfunction of the gland (Table 2-1). Thus, strictly speaking, hyperthyroidism is only one (albeit the most common) cause of thyrotoxicosis (Leo *et al.* 2016). The terms *primary* and *secondary hyperthyroidism* are sometimes used to designate hyperthyroidism arising from an intrinsic thyroid abnormality and that arising from processes outside of the thyroid, such as a TSH-secreting pituitary tumor. With this disclaimer, we will follow the common practice of using the terms *thyrotoxicosis* and *hyperthyroidism* interchangeably. The three most common causes of thyrotoxicosis are also associated with hyperfunction of the gland and include the following:

- *Diffuse hyperplasia* of the thyroid associated with Graves disease (accounts for 85% of cases)
- Hyperfunctional *multinodular goiter*
- Hyperfunctional *adenoma* of the thyroid. (Kumar *et al.*, 2021)

Table (2-1): Disorders associated with thyrotoxicosis(Kumar *et al.,* 2015)

Associated with Hyperthyroidism
Primary
Diffuse toxic hyperplasia (Graves disease)
Hyperfunctioning ("toxic") multinodular goiter
Hyperfunctioning ("toxic") adenoma
Hyperfunctioning thyroid carcinoma
Iodine-induced hyperthyroidism
Neonatal thyrotoxicosis associated with maternal Graves disease
Secondary
TSH-secreting pituitary adenoma (rare)
Not Associated with Hyperthyroidism
Subacute granulomatous thyroiditis (*painful*)
Subacute lymphocytic thyroiditis (*painless*)
Struma ovarii (ovarian teratoma with ectopic thyroid)
Factitious thyrotoxicosis (exogenous thyroxine intake)

2.5.2. Hypothyroidism

Hypothyroidism is caused by any structural or functional derangement that interferes with the production of adequate levels of thyroid hormone. It can result from a defect anywhere in the hypothalamic-pituitary-thyroid axis(Persani*et al.,* 2019). As in the case of hyperthyroidism, this disorder is divided into *primary* and *secondary* categories, depending on whether the hypothyroidism arises from an

intrinsic abnormality in the thyroid or occurs as a result of pituitary disease; rarely, hypothalamic failure is a cause of *tertiary* hypothyroidism (table 2-2). Primary hypothyroidism accounts for the vast majority of cases of hypothyroidism (Persani, 2012). It can be *thyroprivic* (due to absence or loss of thyroid parenchyma) or *goitrous* (due to enlargement of the thyroid gland under the influence of TSH). The causes of hypothyroidism include the following.

Table (2.2): Causes of Hypothyroidism(Kumar *et al.*, 2015)

Primary
Developmental (thyroid dysgenesis: PAX-8, TTF-2, TSH-receptor mutations)
Thyroid hormone resistance syndrome (TRβ mutations)
Postablative
Surgery, radioiodine therapy, or external radiation
Autoimmune hypothyroidism
Hashimoto thyroiditis
Iodine deficiency
Drugs (lithium, iodides, p-aminosalicylic acid)
Congenital biosynthetic defect (dyshormonogenetic goiter)
Secondary
Pituitary failure
Tertiary
Hypothalamic failure (rare)

2.5.3. Neoplasms of thyroid gland

Cancer is term for diseases in which abnormal cells divide without control and can invade nearby tissues. Cancer cells can also spread to other parts of the body through the blood and lymph systems. Carcinoma is a cancer that begins in the skin or in tissues that line or cover internal organs.

The solitary thyroid nodule is a palpably discrete swelling within an otherwise apparently normal thyroid gland. The estimated incidence of solitary palpable nodules in the adult population of the United States varies between 1% and 10%, although it is significantly higher in endemic goitrous regions. Single nodules are about four times more common in women than in men. The incidence of thyroid nodules increases throughout life (Acquaviva *et al.*, 2018).

From a clinical standpoint, the possibility of neoplastic disease is of major concern in patients who present with thyroid nodules. Fortunately, the overwhelming majority of solitary nodules of the thyroid prove to be localized, non-neoplastic conditions (e.g., nodular hyperplasia, simple cysts, or foci of thyroiditis) or benign neoplasms such as follicular adenomas (Nguyen *et al.*, 2015). In fact, benign neoplasms outnumber thyroid carcinomas by a ratio of nearly 10:1. Carcinomas of the thyroid are thus uncommon, accounting for well under 1% of solitary thyroid nodules and representing about 15,000 new cancer cases each year. Moreover, as will be seen subsequently, most are indolent, permitting a 90% survival at 20 years. Several clinical criteria might provide a clue to the nature of a given thyroid nodule.

- Solitary nodules, in general, are more likely to be neoplastic than are multiple nodules

- Nodules in younger patients are more likely to be neoplastic than are those in older patients
- Nodules in males are more likely to be neoplastic than are those in females
- A history of radiation treatment to the head and neck region is associated with an increased incidence of thyroid malignancy
- Nodules that take up radioactive iodine in imaging studies (hot nodules) are more likely to be benign than malignant (Raphael, *et al.,* 2015).

Such general trends and statistics, however, are of little significance in the evaluation of a given patient, in whom the timely recognition of a malignancy, however uncommon, can be life-saving. Ultimately, it is the morphologic evaluation of a given thyroid nodule, in the form of fine-needle aspiration biopsy and histologic study of surgically resected thyroid parenchyma, that provides the most definitive information about its nature (Rosai, 2011).

2.5.3.1 Adenomas

Adenomas of the thyroid are typically discrete, solitary masses. With rare exception, they are derived from follicular epithelium and so might all be called follicular adenomas. A variety of terms have been proposed for classifying adenomas on the basis of degree of follicle formation and the colloid content of the follicles. Simple colloid adenomas (macrofollicular adenomas), a common form, resemble normal thyroid tissue; others recapitulate stages in the embryogenesis of the normal thyroid (fetal or microfollicular, embryonal or trabecular). There is limited utility in these classifications because mixed patterns are common, and most of these benign tumors are nonfunctional (Yanhua *et al.,* 2020). Clinically, follicular adenomas can be difficult to distinguish from dominant nodules of follicular hyperplasia or from the less common follicular carcinomas. Numerous

studies have made it clear that adenomas are not forerunners of cancer except in rare instances. Although the vast majority of adenomas are nonfunctional, a small proportion produce thyroid hormones and cause clinically apparent thyrotoxicosis. Hormone production in functional adenomas ("toxic adenomas") occurs independent of TSH stimulation and represents another example of thyroid autonomy, analogous to toxic multinodular goiters (Leitha and Staudenherz, 2003).

2.5.3.2. Other benign tumors

Solitary nodules of the thyroid gland may also prove to be cysts. The great preponderance of these lesions represents cystic degeneration of a follicular adenoma; the remainder probably arises in multinodular goiters. They are often filled with a brown, turbid fluid containing blood, hemosiderin pigment, and cell debris. Additional benign rarities include dermoid cysts, lipomas, hemangiomas, and teratomas which seen mainly in infants (Kumar *et al.*, 2021).

2.5.3.3. Carcinomas

Carcinomas (CA) of the thyroid are relatively uncommon in the United States, accounting for about 1.5% of all cancers. Most cases occur in adults, although some forms, particularly papillary carcinomas, may present in childhood (Staniforth *et al.,* 2016). A female predominance has been noted among patients who develop thyroid carcinoma in the early and middle adult years, perhaps related to the expression of estrogen receptors on neoplastic thyroid epithelium. In contrast, cases presenting in childhood and late adult life are distributed equally among males and females. Most thyroid carcinomas are well-differentiated lesions (Pezzolla *et al.,* 2014). The major subtypes of thyroid carcinoma and their relative frequencies include the following:

- Papillary carcinoma (75% to 85% of cases)
- Follicular carcinoma (10% to 20% of cases)
- Medullary carcinoma (5% of cases)
- Anaplastic carcinoma (<5% of cases)

Most thyroid carcinomas are derived from the follicular epithelium, except for medullary carcinomas; which are derived from the parafollicular or C cells. Each variant of thyroid carcinoma has its unique biological and clinical features (Banasik and Copstead, 2019).

2.6. Papillary Carcinoma

Papillary carcinomas are the most common form of thyroid cancer. They occur at any age but most often in the twenties to forties, and account for the majority of thyroid carcinomas associated with previous exposure to ionizing radiation (Abdullah *et al.*, 2019).

2.6.1. Morphology

Papillary carcinomas are solitary or multifocal lesions. Some tumors may be well-circumscribed and even encapsulated; others may infiltrate the adjacent parenchyma with ill-defined margins. The lesions may contain areas of fibrosis and calcification and are often cystic. On the cut surface, they may appear granular and may sometimes contain grossly discernible papillary foci. The definitive diagnosis of papillary carcinoma can be made only after microscopic examination (Kumar *et al.*, 2021). The characteristic hallmarks of papillary neoplasms include the following:

- Papillary carcinomas can contain branching papillae having a fibrovascular stalk covered by a single to multiple layers of cuboidal epithelial cells. In most neoplasms, the epithelium covering the papillae consists of well-differentiated, uniform, orderly, cuboidal cells, but at the other extreme are those with fairly anaplastic epithelium showing considerable variation in cell and nuclear morphology. When present, the papillae of papillary carcinoma differ from those seen in areas of hyperplasia. In contrast to hyperplastic papillary lesions, the neoplastic papillae are more complex and have dense fibrovascular cores (Rossi *et al.,* 2019).
- The nuclei of papillary carcinoma cells contain finely dispersed chromatin, which imparts an optically clear or empty appearance, giving rise to the designation ground glass or Orphan Annie eye nuclei. Change of nuclear size and shape; nuclear enlargement, elongation and overlapping (Lloyd *etal*., 2017). In addition, invaginations of the cytoplasm may in cross-sections give the appearance of intranuclear inclusions ("pseudo-inclusions") or intranuclear grooves. As currently used, the diagnosis of papillary carcinoma is based on these nuclear features even in the absence of papillary architecture.
- Concentrically calcified structures termed psammoma bodies are often present within the lesion, usually within the cores of papillae. These structures are almost never found in follicular and medullary carcinomas, and so, when present, they are a strong indication that the lesion is a papillary carcinoma. It is said that whenever a psammoma body is found within a lymph node or perithyroidal tissues, a hidden papillary carcinoma must be considered.

- Most of papillary thyroid carcinoma are infiltrative while some are encapsulated or well demarcated (usually follicular variant)
- Foci of lymphatic invasion by tumor are often present, but involvement of blood vessels is relatively uncommon, particularly in smaller lesions. Metastases to adjacent cervical lymph nodes are estimated to occur in up to half the cases (Rossi *et al*., 2019).

There are variant forms of papillary carcinoma that are important to recognize because they can resemble other lesions and have unique clinical features. The encapsulated variant constitutes about 10% of all papillary neoplasms. It is usually confined to the thyroid gland, is well encapsulated, and rarely presents with vascular or lymph node dissemination, and so it can easily be confused with a benign adenoma. In most cases, this variant has an excellent prognosis (Kumar *et al*., 2021).

The follicular variant has the characteristic nuclei of papillary carcinoma but has an almost totally follicular architecture. Grossly, the tumor may be encapsulated, and focally, psammoma bodies may be seen. These follicular variants still behave biologically as usual papillary carcinomas as long as they meet the nuclear criteria for diagnosis of papillary cancers. The true follicular carcinoma, in contrast, lacks these nuclear features, frequently demonstrates capsular and vascular invasion, and has a less favorable prognosis (Haugen *et al.*, 2016). A differential diagnosis of thyroid lesions with a follicular architecture is summarized in table (2.3).

Table (2-3): Thyroid Lesions with a Follicular Architecture (Kumar *et al.*, 2021).

Non-Neoplastic
Hyperplastic nodule in goiter
Neoplastic
Follicular adenoma *
Follicular carcinoma *
Follicular variant of papillary carcinoma †

* Differentiating follicular carcinoma from follicular adenoma requires histologic evidence of capsular or blood vessel invasion, or documented metastasis: †: The diagnosis of papillary carcinoma is rendered on the presence of characteristic nuclear features, irrespective of the presence or absence of papillae.

A tall cell variant is marked by tall columnar cells with intensely eosinophilic cytoplasm lining the papillary structures. Typically, the cells are at least twice as tall as they are wide (hence the eponym "tall cell" variant) (Jung, 2020). These tumors tend to occur in older individuals and are usually large with prominent vascular invasion, extra-thyroidal extension, and cervical and distant metastases. It has been recently demonstrated that more than half the tall cell variants harbor a *RET/PTC* translocation that confers greater mitogenic potential than the *RET/PTC* observed in usual papillary thyroid cancers. The presence of this genetic abnormality might result in more aggressive behavior (Trovisco *et al.*, 2007)

An unusual diffuse sclerosing variant of papillary carcinoma occurs in younger individuals, including children. These tumors do not present with a mass, but rather with a bilateral goiter. There is a characteristic "gritty" sensation to the cut surface of the lesion due to the presence of abundant psammoma bodies. The tumor demonstrates a prominent papillary growth pattern, intermixed with solid

areas containing nests of squamous cells (squamous morules). The neoplastic cells exhibit classic nuclear features of a papillary neoplasm. As the name suggests, there is extensive, diffuse fibrosis throughout the thyroid gland, often associated with a prominent lymphocytic infiltrate, simulating Hashimoto thyroiditis. The neoplastic cells have a peculiar propensity to invade intrathyroidal lymphatic channels; hence, nodal metastases are present in almost all cases (Chow *et al.*, 2003)

Hyalinizing trabecular tumors, a group that includes both adenomas and carcinomas, have recently been reconsidered as a variant of papillary carcinomas, based on the presence of *ret/PTC* gene rearrangements in 30% to 60% of these tumors. They are characterized by an "organoid" growth pattern, with nests and trabeculae of elongated tumor cells within a fibrovascular stroma; at first glance, the tumor may resemble an extra-adrenal paraganglioma. Both intracellular and extracellular hyalinization are prominent and confer a pink hue on the tumor on low-power microscopic examination. The nuclear features resemble those seen in classic papillary carcinomas, and psammoma bodies may be present. Hyalinizing trabecular adenomas are well encapsulated, while carcinomas demonstrate capsular and/or vascular invasion (Kumar *et al.*, 2021)

2.7. Epidemiology

The incidence of thyroid cancer has increased dramatically in recent decades due in large part to identification subclinical disease. The rate of increase may be plateauing, perhaps due to efforts to discourage behaviors that lead to their detection, such as aggressively biopsying small nodules and performing extensive surgeries. For people who are identified with low risk papillary thyroid cancer, the new management strategy of active surveillance holds promise as a potential path

to avoid the harms of aggressive treatment while remaining cost effective (Vigneri *et al.*, 2015).

Increased incidence of thyroid cancer with attribution to over diagnosis has also been described outside of the United States. Using observed versus expected data from the Cancer Incidence in five continents, International Agency for Research on Cancer (IARC) estimates, the incidence of thyroid cancer in South Korean women aged 50–59 in early years of twenty-first century was 120 cases per 100,000, as compared to 22.2 in the United States in 2014 (Sanabria*et al.*, 2018).

Similar large increases in incidence were seen in other developed nations where patients have high access to healthcare, the main driver of increased detection. The study estimated the rate of over diagnosis, concluding that among women in the United States between, 228,000 cases of thyroid cancer, representing between 70–80% of cases, were asymptomatic lesions that would have gone undetected during a patient's lifetime if ultrasound and other imaging studies were not available. Similarly, 90% of cases in South Korea, 70–80% of cases in Italy, France, and Australia, and 50% of cases in Japan, the Nordic countries, and England and Scotland likely represented over diagnosis among women during that time period (Vigneri *et al.*, 2015).

Although several reports examining the distributstion of thyroid carcinoma (TC) in the Middle East, in general, and in the Arab Gulf states, in particular, have been documented. Epidemiological studies have reported a progressive increase in the overall incidence over the past 20 years (Salim *et al.*, 2009). Thyroid cancers may produce a wide range of clinical presentations, from highly differentiated carcinoma with a good prognosis to undifferentiated anaplastic cancers that occur mainly in older people and have a poor prognosis. Different factors contribute to

this variability, such as histological pattern, tumor stage, age at diagnosis (worse in those /40 years) and gender, worse in men (Ho *et al.*, 2020).

In Iraq, the thyroid cancer occupy the second common cancer in female after the breast cancers, whereas, in male it not appear in the top ten common cancers. These studies according to Iraq Cancer Registry ICR (2019). The incidence of thyroid cancers in female appears to be 7.34 per 100000 of population which account approximately 6.96% of registered cancer patients. The age group varies widely with as young as childhood till the geriatric age group, with the peak of incidence in the fourth decade.

Babil province registered 76 cases of thyroid cancers out of 1802 (about 4.76 %) in Iraq in 2019, which are about 3.59 per 100000. Of those 63 were female the percentage was 3.94 incidence was 6.01, while in male it was 13 cases (0.81 % with incidence 1.21) (ICR, 2019).

2.8. Markers used in detection of papillary thyroid carcinoma:

2.8.1. Cluster of differentiation CD56.

CD56 or also called neural cell adhesion molecule (NCAM) is a glycoprotein homophilic binding, one of immunoglobulin superfamily. The antibody targeting an isoform of NCAM and it is normally expressed in natural killer cells, activated T cells, lymphocytes, brain tissue in follicle cells of normal thyroid glandand endocrine glands, it is believed that this protein regulate motility of cells, neuron binding and outgrowth. There are three main isomers found of CD56 (NCAM-120, NCAM-140, and NCAM-180), they are differing in their intracellular domain length and all are generated by alternative splicing from one single gene (Ditlevsen *et al.*, 2008).

In some metastatic malignancies and tumors, loss of expression of CD56 is reported by some studies (Jeong*et al.*, 2016). Adding to that, CD56 is expressed in benign thyroid nodule, that's why it can be counted as a good diagnostic marker for PTC. There is a little data about the expression of CD56 in different variants of PTC other than classic PTC or about the utility of CD56 as a diagnostic marker in rare PTC variants (Pyo *et al.*, 2018).

The expression of CD 56 affect the migration ability of tumour cells, that's to say, the loss of CD 56 is correlated with potentiality for metastasis and related to poor prognostic features of some malignancies(El Demellawy *et al*, 2008).

CD56 is often considered a marker of neural lineage commitment due to its discovery site. However, CD56 expression is also found in, among others, the hematopoietic system. Here, the expression of CD56 is most stringently associated with, but certainly not limited to, natural killer (NK) cells. Also, in the bone marrow, at the site where hematopoiesis occurs, CD56 fulfills a pivotal role. Mesenchymal stromal cells provide niches for hematopoietic stem cells by, inter alia, the expression of adhesion molecules comprising CD56, maintaining long-term hematopoiesis. On the other hand, aberrant CD56 expression is seen in a range of hematological malignancies, e.g. multiple myeloma and leukemia as well as solid tumors e.g., lung cancer, ovarian cancer, and neuroblastoma (Teicher, 2014).

The CD56 has been used as a diagnostic aid for the immunohistochemical diagnosis of small cell lung carcinoma (SCLC). It showed a strongly diffuse cytoplasmic staining in all SCLC. One of the most frequent difficulties in thyroid pathology is differentiating follicular variant of PTC from follicular adenoma (Kontogianni *et al.*, 2005).

2.8.2. Cytokeratin 19 (CK19):

Cytokeratins (CKs) are one that represent the largest intermediate filament protein subgroup and constitute a multi-gene family with more than 20 different types of polypeptides that are divided into acidic type I (CK9-CK20) and basic type II (CK1-CK8) keratins. In normal epithelium the cells express one type I and one type II keratin, at least. CKs are forming the cytoskeleton of the epithelial cells, and the main function of them is to maintain the integrity of epithelial cell. Nevertheless, some other functions that include their roles in stress responses, cell signaling, and apoptosis (Keyvani *et al.*, 2016). CKs undergo a series of complex regulation including modifications occur post-translational and interactions with self and with various classes of associated proteins. During the process of apoptosis, proteolysis which is caspase – mediated that targeting the epithelial cell. There are three keratins mainly expressed in epithelium, whether simple or stratified, and in various types of carcinomas like breast cancers, these keratins are; CK19, CK8 and CK18. The CK 19 is cleaved by caspase 3, and in cancer patient it can detect the released soluble fragments. In contrast, the release of intact, non-degraded CK molecules has not yet been demonstrated (Panabières *et al.*, 2009).

CK19 is a low-molecular-weight cytokeratin found in a wide range of normal and neoplastic tissues. In the thyroid, previous studies have shown a strong and diffuse expression in PTC, as well as heterogeneous expression in follicular adenomas (FA) and follicular carcinoma (FCA). CK19 is represented in a glandular and simple epithelium of gastrointestinal tract, pancreas, liver and biliary tract. It presents in both normal and pathologic tissues of these organs. In the thyroid gland, the normal follicle does not show a detectable amount of CK19, while studies reported the presence of CK19 expression in papillary thyroid

carcinomas. That's to say, this marker can be used as a diagnostic marker for PTC (Liu *et al*., 2015).

2.8.3. Human bone marrow endothelium-1(HBME-1)

Human bone marrow endothelium marker-1, also named Hector Battifora mesothelial-1 (HBME-1).is a common molecular marker of tumors, it has been suggested for its potential use in diagnosis and prognosing differentiated of thyroid carcinoma. It is a monoclonal antibody directed against an unknown antigen on mesothelial cells that has been proven useful in the diagnosis of malignant follicular-derived lesions of the thyroid (FDLT), particularly in PTC (Barroeta *et al.,* 2006). HBME-1 was strongly expressed in the malignant cases (FCA: 54%; PTC: 91%; FVPTC: 75%; anaplastic carcinoma: 80%; poorly differentiated carcinoma: 100%); however, up to 25% of the normal thyroid and 17% of the cases of lymphocytic thyroiditis showed positive staining. Currently, there are few studies that report the expression levels of HBME-1 protein in different types of differentiated thyroid carcinoma tissues, and their correlation with ultrasonic manifestation of thyroid (Qiao *et al.*, 2017).

HBME-1 is an antigen constituent of the microvilli on the surface of mesothelial cells in humans, and hyaluronic acid (HA) is the main ingredient of HBME-1(El-Mahdy *et al.*, 2011).

In the papillary carcinoma group and the follicular carcinoma group, the level of expression of HBME-1 in affected tissues and the immunohistochemical finding of HBME-1 expression were all higher than those in the normal thyroid tissue. In the papillary carcinoma group, the HBME-1 expression in affected tissues was higher than that in the follicular carcinoma group. (Qiao *et al*., 2017).

Anti-HBME-1 has been most extensively investigated as an immunochemical (IHC) marker in thyroid cancer. It has a sensitivity of 78.3% and specificity of 85.4% in 10 studies using preoperative fine needle aspirate (FNA) specimens. Similar sensitivity of 77% and specificity of 83% have been reported in meta-analysis of 21 IHC studies on formalin-fixed paraffin embedded tissue sections (Rodrigues *et al.*, 2012). However, their results and applications are still controversial since these molecules have not proved to have specificity and – more critically, to avoid an eventual diagnostic thyroidectomy – enough sensitivity in the differentiation of follicular lesions because of persistent variable rates of, respectively, falsepositive and false-negative results (Alexander *et al.*, 2012).

2.9. Molecular markers for diagnosis of PTC

Most papillary carcinomas have gain-of-function mutations in the serine/ threonine kinase BRAF or involving the genes encoding the RET or neurotrophic receptor tyrosine kinase 1(NTRK1) receptor tyrosine kinases, which lies in the MAPK pathway (Al rasheed and Xu, *2019*).Activation of the MAPK pathway has emerged as the defining molecular genetic feature of papillary carcinoma. This pathway regulates important cellular functions (e.g., proliferation, differentiation, and survival) and is frequently altered in many different types of tumor. In the thyroid gland, MAPK pathway activation is the consequence of three distinct events: gene rearrangements (RET/PTC or the less common TRK rearrangements), BRAF, or RAS activating mutations (Goldblum *et al.*, 2018).

2.9.1. The role of the RET proto-oncogene in PTC

The RET proto-oncogene was first identified in 1985(Takahashi *et al.*, 1985). The RET proto-oncogene is located on the long arm of chromosome 10

(10q11.2). The proteins that RET encodes is a cellular tyrosine kinase transmembrane receptor that is divided into the following three domains: an N-terminal extracellular domain containing four cadherin-like regions; a cysteine-rich region with a transmembrane domain; and a cytoplasmic domain with tyrosine kinase activity (Takahashi *et al., 2020*).

The RET/PTC rearrangements produce genes that encode fusion proteins with constitutive tyrosine kinase activity. Similarly, paracentric inversions or translocations of NTRK1 on chromosome 1q21 are present in 5% to 10% of papillary thyroid cancers. These genetic events also produce constitutively active NTRK1 fusion proteins. Activation of RET stimulates multiple downstream pathways that promote cell growth, proliferation, survival and differentiation (Arighia *et al.*, 2005).

These pathways include the MAPK, the phosphoinositide 3-kinase (PI3K) and protein kinase B, signal transducer and activator of transcription, proto-oncogene tyrosine-protein kinase Src1 and focal adhesion kinase pathways. Three versions of the RET messenger ribose mono nucleic acid (mRNA) transcript exist due to alternative splicing of the 3′ end; RET9, RET43 and RET51 contain 9, 43 and 51 amino acids behind the RET Gly1063 residue, respectively (Pasini *et al.*, 1995 and Myers *et al.*, 1995). RET9 and RET51 have distinct sub-cellular localizations, trafficking properties and downstream signal capacities, thus determining different functional effects and individual roles for each transcript in cellular development (Richardson *et al.*, 2012).

The correct anchoring of the RET receptor to the plasma membrane is dependent on N-glycosylation of the extracellular domain in the Golgi apparatus, and post-translational modification produces the functional 170 kilo Dalton (kDa) protein (Takahashi *et al.*, 1993). The activation of the RET receptor is induced by

binding with ligands that belong to the glial cell-line derived neurotrophic factor (GDNF) family of ligands (GFLs) (Baloh *et al.,* 2000). These ligands include GDNF, neurturin, artemin and persephin (Arighi *et al.*, 2005).

The RET gene is important in the normal development of the central and peripheral nervous systems as well as the excretory system (for example, in the development of the Wolffian duct and ureteric bud epithelium). The few studies that have investigated the expression of the RET protein in human tissues have demonstrated that this protein is only present in a few organs during adulthood, mainly those derived from the neural crest (Santoro *et al.,* 2004 and Takaya, *et al.*, 1996).

Medullary thyroid carcinomas typically contain activating point mutations and few deletions or insertions, whereas only chromosomal rearrangements are found in PTC. Of note, this dual mechanism of oncogene activation is seen with other genes associated with thyroid carcinoma. For example, the BRAF gene, another key oncogene in PTC development, can be activated by both point mutations (Kimura *et al.*, 2003) and chromosomal rearrangements (Ciampi *et al*., 2005), which suggests that distinct mutational mechanisms could be due to different aetiological factors responsible for thyroid carcinogenesis. Activating RET alterations, and chromosomal rearrangements and point mutations, were discovered in 1987 and 1993, respectively (Mulligan *et al*, 1993 and Fusco *et al.*, 2005).

In 1987, Fusco and co-authors analyzed DNA extracted from five PTCs and two of their respective lymph node metastases and observed a chromosomal rearrangement generated by the fusion of the RET tyrosine kinase domain with the 5′ terminal region of CCD6 (formerly known as H4/D10S170) (Grieco *et al.*,

1990). This genetic alteration was named RET/PTC1. Shortly after this report, another RET rearrangement (RET/PTC3), generated by the fusion of the tyrosine kinase domain of RET to the nuclear receptor co-activator 4 gene (NCOA4), was described in a human PTC (Santoro *et al.*, 1994).

The fusion of the RET tyrosine kinase domain with the 5′ portion of ubiquitously expressed partner genes determines the constitutive activation of the intracellular domain of the receptor, followed by uncontrolled activation of the MAPK and PI3K pathways that, similarly to the result of activating RET point mutations, leads to the proliferation of the follicular cells and the development of malignancy, as demonstrated by in vitro and in vivo studies (Santoro *et al.*, 2006). The most frequent RET/PTC rearrangements identified thus far in PTC are the RET/ PTC1 and RET/PTC3 rearrangements (Santoro *et al.*, 1994). In the majority of patients, these rearrangements are characterized by the presence of the same RET portion and totally different partner genes, whereas in a few cases, a different breakpoint in an already known RET/PTC rearrangement has been described, which result in longer or shorter rearrangements than the most common rearrangements (Elisei *et al.*, 2001). RET/PTC1 and RET/PTC3 are intrachromosomal rearrangements of the long arm of chromosome 10 and can be induced in vitro by irradiating normal thyroid cells (Caudill *et al.* 2005 and Ameziane-El-Hassani *et al.*, 2010). Strong evidence for the role of chromosomal architecture in the formation of rearrangements has been demonstrated. The spatial proximity of the loci involved in RET/PTC rearrangements predisposes their mis-joining as a consequence of double-stranded breaks produced by ionizing radiation (Evdokimova *et al*., 2012). Moreover, it has been demonstrated that the higher the number of cells with the spatial proximity of the loci involved in different chromosomal rearrangements (RET, BRAF and tyrosine kinase receptor (TRK),

the higher the prevalence of the corresponding rearrangements (Gandhi *et al.,* 2012).

One hypothesis to explain the finding that the incidence of PTC is high in children is related to the high proliferation rate of thyroid follicular cells in children, which make them more susceptible to DNA damage than adult cells (Elisei *et al.*, 2001, and Jarzabandand Handkiewicz-Junak, 2007). Other studies have also shown that the occurrence of these rearrangements could be due to the presence of fragile sites in specific regions where mutagens (radiation or chemicals) are more likely to induce rearrangements. In particular, fragile sites have been identified in the regions of RET and CCDC6 that are involved in RET/PTC1, and chemical induction of fragility at these sites is able to induce rearrangement (Gandhi *et al.*2010).

The prevalence of RET/PTC rearrangements was up to 87% in the first series of cases reported after the Chernobyl accident. However, in the latest series, characterized by a long latency period from the nuclear accident to the diagnosis, the reported prevalence of RET/ PTC rearrangements declined (Rabes *et al.*, 2000). This evidence suggests that the most recent cases might have a different pathogenesis to the earlier cases. A similarly significant decrease in the prevalence of RET/PTC rearrangements was also observed in spontaneous PTC, which is currently ~20% (compared with >30% in the 1990s) (Romei *et al.*, 2012).

RET/PTC rearrangements have also been reported in benign thyroid diseases (Guerra *et al.*, 2011and Domingues *et al.*, 2005). A high prevalence of the RET/PTC rearrangements has been found in patients with Hashimoto thyroiditis (Rhoden *et al.*, 2006), mainly using a highly sensitive method such as nested reverse transcription polymerase chain reactions (PCR). Of note, the presence of Hashimoto thyroiditis was only associated with a PTC in a few patients (Sheils *et*

al., 2000 and Wirtschafter *et al.*, 1997). RET/PTC rearrangements have also been reported in nodules classified as benign at histology with a variable prevalence (13.3–21.0%) (Guerra *et al.*, 2011and Ishizaka *et al.*, 1991), particularly in patients who had previously been exposed to radiation (Bounacer *et al.*, 1997). Although rare, RET/PTC rearrangements have also been found in poorly differentiated thyroid carcinoma and ATC, mainly in carcinoma associated with a differentiated component (Mochizuki *et al.*, 2010; Liu *et al.*, 2019). This finding is not unexpected, as the progression of PTC with RET/PTC rearrangements towards dedifferentiated and advanced forms is a rare event (Soares *et al.*1998; Mayr *et al.*, 1998).

RET/PTC in other human malignancies. An unexpected fusion between RET and MYH13 has been observed in one patient with MTC (Grubbs *et al.*, 2015). RET/PTC rearrangements have also been detected in 3 of 15 (20%) cases of primary peritoneal carcinoma; however, these genetic alterations were not thought to be directly implicated in tumour growth but might instead be ‘passenger’ mutations that reflect RET instability in secondary tumour subclones (Flavin *et al.*, 2009). A few cases of chromosomal translocations causing the fusion of the tyrosine kinase domain of RET to FGFROP1 or BCR genes have been described in haematopoietic malignancies (Bossi *et al.*, 2014; Ballerini *et al.*, 2012).

2.9.2. The role of BRAF gene mutation in papillary thyroid carcinoma:

BRAF is the B-type Raf kinase, is a protooncogene that encodes a serine/ threonine kinase, it is located on chromosome7, and is a potent activator of the mitogen-activated protein kinase/extracellular-signal-regulated kinase (MEK-ERK) pathway. This pathway is hyperactivated in approximately 30% of human cancers (Hoshino *et al.*, 1999). The mutation of BRAF is more specific for

epithelial derivatives tissue, particularly papillary thyroid carcinoma PTC and poorly differentiated thyroid carcinoma. There are three related RAF genes in mammals: first RAF paralogue (ARAF), BRAF and human homologue of v-RAF (CRAF) (RAF-1). The BRAF gene, located at 7q34, contains 18 exons and encodes a serine–threonine kinase in the RAS/RAF/ MAPK signaling pathway (Raman *et al.*, 2007). All the main RAF proteins share three highly conserved regions: CR1, CR2 and CR3; however, BRAF has a number of structural differences from the other RAF proteins (ARAF and CRAF). CR1 contains the RAS binding domain and the cysteine-rich domain. CR2 is rich in serine and threonine and contains regulatory phosphorylation sites. CR3 contains the P-loop and the important kinase domain with the activation segment (Morrison and Cutler, 1997).

Activating BRAF mutations lead to unchecked signaling. Most BRAF mutations occur in the CR3 domain, in the P-loop and the activating segment of the kinase domain. The most common activating mutation which is a thymine to adenine transversion involves exon 15 at the nucleotide position 1799. and is the most common hotspot mutation in the BRAF gene. This results in the substitution of valine by glutamate. This mutation often referred to as BRAF V600E encodes a protein that has 10 times more kinase activity than the wild type protein.(Davies *et al.*, 2002). It has been proven to be an oncogene in human cancer (Garnett and Marais, 2004).

Although many different BRAF mutations have been detected, BRAFV600E accounts for the majority of activating mutations (Wan *et al.*, 2004). BRAF kinase domain mutations, the majority of which are V600E, occur in approximately 8% of human cancers. Mutant BRAF acts as an oncogene. V600E promotes tumour cell viability, proliferation and growth (Gorden *et al.*, 2003).

In general, the rates of BRAF mutations encountered in thyroid papillary carcinoma vary from 29% to 69% (Namba *et al.*, 2003). Point mutations of exon 15 involving BRAFV600E is the most common alteration in sporadic papillary carcinoma (Xing, 2005).

The gene mutation was the focus of investigations, as it may be an indicator for the prognosis and therapy in the treatment of PTC, in addition to its value as a diagnostic factor, this gene is BRAF mutation. The mutation of this gene is the area of intensive investigation (Kakarmath *et al.*, 2016).

Many investigations have identified molecular markers that may carry diagnostic, prognostic, and therapeutic value in the management of PTC. This complementary information can supplement characterization of the clinical and pathologic features of the disease and may help provide a tailored approach with the goal of mitigating the risk of recurrence. BRAF mutation has been the subject of intensive investigation, as many investigators have tried to assess if the mutated gene is associated with a worse prognosis for PTC. Since the first reports describing the BRAF mutation in melanoma, glioma, colorectal, ovarian, lung, and liver cancers and sarcoma cells, numerous studies have been published correlating mutated BRAF with thyroid malignancy, and in particular PTC. This mutation is specific for papillary and poorly differentiated and anaplastic thyroid carcinomas of epithelial derivation. It is not seen infollicular, Hürthlecell, and medullary carcinomas (Tufano *et al.*, 2012).

The BRAF V600E mutation has been widely reported to be associated with PTC prognosis, showing an adverse influence on tumor aggressiveness. However, some studies did not find a significant association with prognosis. The aggressiveness of the PTC and an increased risk of recurrence/persistence has been observed with clinicopathologic factors such as age 15 years and 45 years, male

sex, history of familial thyroid cancer, and tumor factors such as diameter 9.2 cm, multifocal, bilateral, extrathyroidal extension, subtypes like tall and columnar cell types, nuclear atypia and tumor necrosis, vascular invasion, lymph node metastasis, distant metastasis, and low iodine uptake.75 BRAF V600E mutation has been related to almost all of these factors, which are used in predicting a disease recurrence/persistence event (Wang *et al.*, 2010).

Interestingly, in an integrated genomic characterization of PTC performed by the Cancer Genome Atlas Research Network that was published in 2014, a low prevalence of RET/PTC rearrangements (33 of 484, 6.8%) was found(Cancer Genome Atlas Research Network., 2014). In parallel to the reduced prevalence of RET/PTC rearrangements, an increased prevalence of the BRAF Val600Glu alteration has been also reported (Romei *et al.*, 2012 and Smyth *et al.*, 2005); this mutation is now the most frequent genetic alteration in PTC. With the exception of about 10% of advanced PTCs or poorly differentiated thyroid cancers (Fenton *et al.*, 2000), in which several molecular alterations can be found in the same sample, RET/ PTC rearrangements and the BRAF Val600Glu alteration are usually mutually exclusive, as are other less frequent genetic alterations such as (Rat sarcoma) RAS point mutations (Nakata *et al.*, 1999; JarzabandHandkiewicz-Junak, 2007).

2.10. FISH Technique used in the study:

Fluorescence in situ hybridization (FISH), like several other molecular techniques, is based on the ability of complementary single-stranded nucleic acid molecules (DNA or RNA) to hybridize to each other. The most important feature of FISH is its ability to localize specific DNA sequences in intact nuclei and chromosome spreads. Since its inception, in the early 1970s, and its first clinical applications, in the early 1990s, investigative and clinical purposes, from gene

mapping studies to the identification of chromosome abnormalities in genetic disorders and cancer (Oliveira and French, 2005).

The FISH procedure essentially requires 2 types of DNA, target and probe. Target DNA is a heterogeneous population of DNA molecules immobilized on a solid surface (e.g., glass slides) as either chromosome spreads (e.g., metaphase spreads) or interphase cells (e.g., tissue sections or cytologic preparations). Probe DNA, by contrast, is a population of labeled DNA molecules specific to certain chromosomes, chromosome regions or genes. Probe DNA is made visible under the microscope by a procedure called nucleic acid labeling (Wilkinson, 1998).

Fluorescence in situ hybridization (FISH) is a technique that allows DNA sequences to be detected on metaphase chromosomes or in interphase nuclei from fixed cytogenetic samples. The technique uses DNA probes that hybridize to entire chromosomes or single unique sequences, and serves as a powerful adjunct to classic cytogenetics. This valuable technique can now be applied as an essential diagnostic tool in prenatal, haematological and pathological chromosomal analysis. Target DNA, after fixation and denaturation, is available for annealing to a similarly denatured, fluorescently labeled DNA probe, which has a complementary sequence. Following hybridization, unbound and non-specifically bound DNA probe is removed and the DNA is counterstained for visualization. Fluorescence microscopy then allows the visualization of the hybridized probe on the target material (Green, 2021).

FISH is widely used for several diagnostic applications: identification of numerical and structural abnormalities, characterization of marker chromosomes, monitoring the effects of therapy, detection of minimal residual disease, tracking the origin of cells after bone marrow transplantation, identification of regions of

deletion or amplification, detection of chromosome abnormalities in non-dividing or terminally differentiated cells, determination of lineage involvement of clonal cells, etc. Moreover it has many applications in research: identification of non-random chromosome rearrangements, identification of translocation molecular breakpoint, identification of commonly deleted regions, gene mapping, characterization of somatic cells hybrids, identification of amplified genes, study the mechanism of rearrangements. FISH is also used to compare the genomes of two biological species to deduce evolutionary relationships (Oliveira and French, 2005).

Generally, three different types of FISH probes are used by researchers, each of which has a different application:

- **Locus specific probes** bind to a particular region of a chromosome. This type of probe is useful when researchers have isolated a small portion of a gene and want to determine on which chromosome the gene is located.
- **Alphoid or centromeric repeat probes** are generated from repetitive sequences found in the middle of each chromosome. Researchers use these probes to determine whether an individual has the correct number of chromosomes. These probes can also be used in combination with "locus specific probes" to determine whether an individual is missing genetic material from a particular chromosome.
- **Whole chromosome probes** are actually collections of smaller probes, each of which binds to a different sequence along the length of a given chromosome. Using multiple probes labeled with a mixture of different fluorescent dyes, scientists are able to label each chromosome in its own unique color. The resulting full-color map of the chromosome is known as a spectral karyotype. Whole chromosome probes are particularly useful for

examining chromosomal abnormalities, for example, when a piece of one chromosome is attached to the end of another chromosome (Bishop, 2010).

3. Materials and methods:

3.1. Materials

3.1.1. Equipment used in the study

All equipment used in the study are listed in table (3-1).

Table 3.1: Equipment used in the study, their manufacturers and company.

Equipment	Manufacturing company/ Country
Automated tissue processor	Leica. USA
Centrifuge	Hettich/Germany
Electric Paraffin dispenser	Model No. 222 Lipshaw, Detroit USA
Fluorescence microscope	Optika, Italy
Fluorescence microscope	Optica /Italy
Forceps	Germany
Freezer	Concord /Lebanon
Glass coplin jars	Germany
Hot plate	FischerScientific Germany
Immunohistochemistry staining device	Tintodetector. Bio SB. USA
Incubator	LabTech. Korea
Micro-centrifuge tubes	Germany
Micropipette tips (10 µl,100 µl,1000µl)	Bio-Basic, Canada
Micropipette(Automatic)(10µl, 50µl, 100µl and1000µl)	Gilson France
Micropipettes of different size (1µ - 200 µ)	Germany
Microscope equipped with camera for photography	(Olympus BIX-61).
Microtome	(Reichert-Jung, Germany).
Oven	(Fisher Scientific Model 615G). USA
Plastic rack	AFCO /Jordan
Positively charged slide	Pathnsitu. UK
Power Supply	CleverScientific, UK
Rubber solution glue	UK
Sensitive Balance	Sartoriusm, Germany
Sensitive electronic balance	KERN/ German
Sensitive humid incubator	LabTech. Korea
Slides and cover slips	Germany

Thermal cycler	CleverScientific UK
Vortex Tube Shaker	FischerScientific Germany
Water bath	LabTech. Korea
Water Bath	FischerScientific Germany
Water Distillatory	4Labtech, China

3.1.2. Chemicals used in the study

The chemicals that used in the study are listed in table (3-2).

Table 3.2: Chemicals used in the study and their manufactures

Chemicals	Manufacturing company/ Country
Absolute ethyl alcohol	BDH, AnalaR
Alum	Sanfeng , China
BRAF breakapart probe	CytoCell, Oxford
CD_{56} kit	PathnSitu, UK
Ck_{19} kit	PathnSitu, UK
DAPI antifad	Thermo Fisher Scientific, USA
Deaminobenzidine	Sigma, USA
Deionized water	Iraq
Dextrin plasticizer xylene (DPX)	Sigma, USA
Distled water	Iraq
Eosin stain	Fluka, Switzerland
Fluorescence probe	Cyto Cell. UK
Formaline	BDH – chem. . Ltd pool , England
Glacial acetic acid (May and Baker)	(May & Baker Ltd. England
HBME1 kit	PathnSitu, UK
Hematoxylin crystal	(Fluka, Switzerland
Horse redish peroxidase	Sigma, USA
Hydrochloric acid	Fluka, Switzerland
Mercuric oxide	(BDH chemicals Ltd., England
Paraffin	solidification point about 56-58°C. E.Merck. Germany

Peroxidase solution	Sigma, USA
Phosphate buffered saline (PBS)	Cytocell, UK
Potassium Aluminum sulfate	AnalaR BDH chemicals Ltd. England
RET breakapart probe	CytoCell, Oxford
Saline sodium citrate	Fluka, Switzerland
Tissue pre-treated solution (reagent 1)	Cytocell, UK
Tri-buffered saline	Sigma, USA
Tween 20	Sigma, USA
Xylene	(BDH, AnalaR)

3.1.3. Chemical solutions:

3.1.3.1. Formalin fixative:

Fixative was prepared according to Bancroft &Stevens (1982) by adding100ml formalin (37-40% stock solution) to900ml water.

3.1.3.2. Mayer's Albumin:

It was prepared according to Presnell and Schreibman (1997) by mixing 50 ml of egg albumin with50 ml of Glycerol and adding 1 gram thimol to prevent fungal contamination.

3.1.3.3. Haematoxylin stain (Harris):

The stain was prepared according to Presnell and Schreibman (1997) as following: The haematoxylin(5 gram)was dissolved in absolute ethyl alcohol 50 ml), and was then added to the potassium alum (100g)which had been previously dissolved in warm distilled water(950ml) in a flask. The mixture was rapidly brought to the boiler and the mercuric oxide (2.5g) was then added. The stain was rapidly cooled by plunging the flask into cold water. When the

solution is cold, the glacial acetic acid (40ml)was added. The stain then stored, and was filtered before use (Presnell and Schreibman,1997).

3.1.3.4. Eosin:

Eosin –yellowish 1% weight /volume (Fluka) was used with the alum haematoxylin as a counter stain, to demonstrate the general histological architecture, and to distinguish between the cytoplasm of different cell types and between different connective tissue fibers and matrices (red –pink). The staining solution was prepared dissolving 1o grams of eosin yellowish in 990 ml of distilled water according to Presnell and Schreibman (1997).

3.2. Study design

The study design was summarized in figure (3-1).

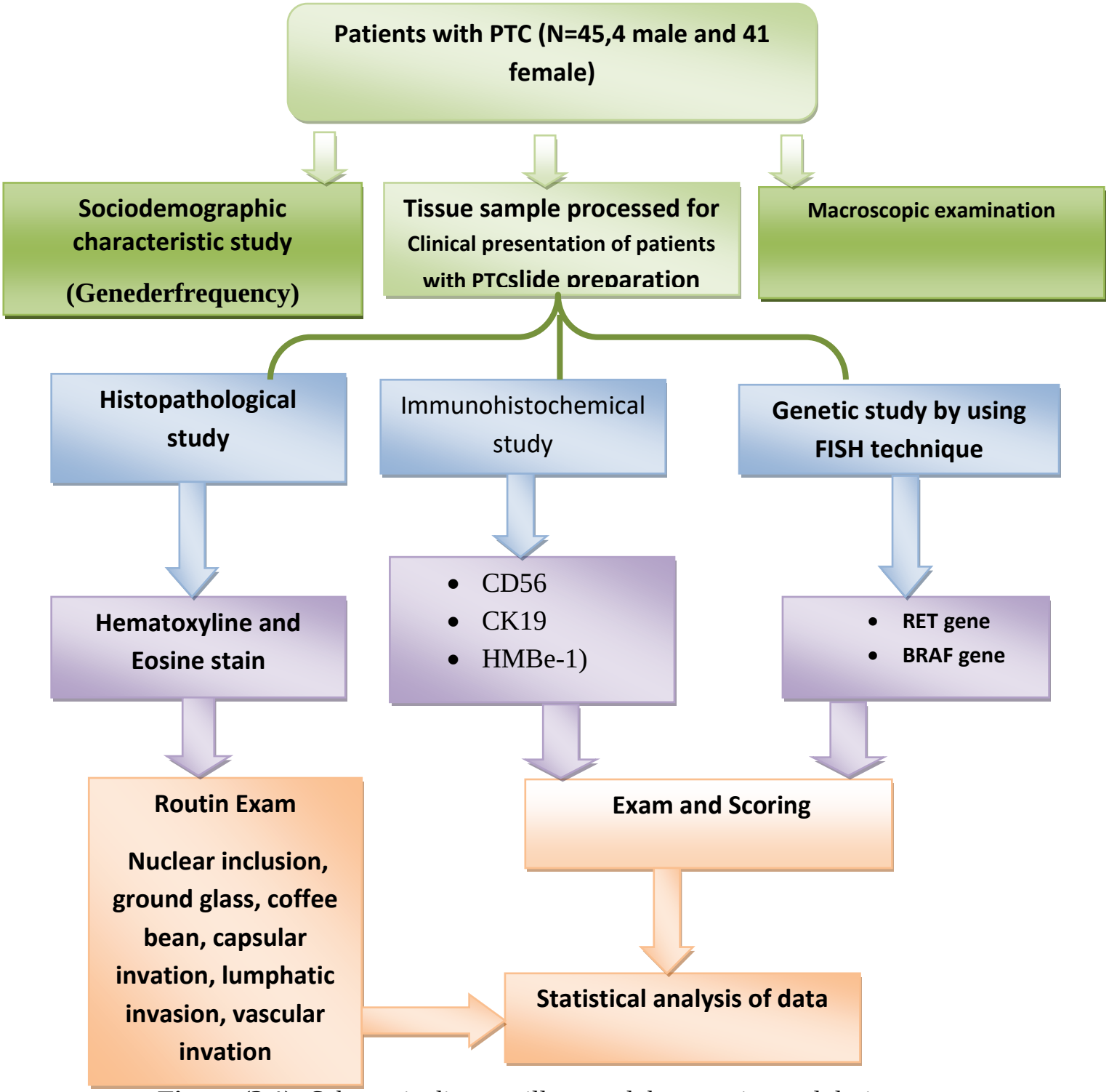


Figure (3.1): Schematic diagram illustrated the experimental design.

3.3. Methods:

3.3.1. Collection of tissue specimens

This cross sectional study was done on45 patients with papillary thyroid carcinoma who have been diagnosed by at least two histopathology specialist following thyroid biopsy. The age was ranged from 26 to 50 years. The study was carried out at Al-Hilla Teaching Hospital and in a number of private laboratories in Babil Province, mid-Euphrates region of Iraq during the period extending from June 2020 and June 2021.

The pathological reports of those patients were reviewed for histopathological diagnosis, histological and cellular criteria of malignancy, invasion, lymphatic and vascular involvement as well as capsular involvement. The sociodemographic features, clinical presentation and other investigations were also retrieved from these reports.

The study was based on taking a thyroid tissue specimen in addition to retrieving paraffin blocks from the central laboratory of the teaching hospital and from private laboratories and performing conventional hematoxylin and eosin stain, immunohistochemical stain and genetic study.

3.3.2. Inclusion and exclusion criteria:

3.3.2.1. Inclusion criteria:

- Any patients with papillary carcinoma of the thyroid gland based on histopathological reports.

3.3.2.2. Exclusion criteria

- Patients with benign thyroid lesions such as colloid goiter or toxic adenoma.
- Patients with other malignancy of thyroid gland such as follicular carcinoma, medullary carcinoma and anaplastic carcinoma.

3.4. Macroscopic examination:

The largest diameter of thyroid glands of patient with PTC were measured for size calculation.

3.5. Microscopic examination:

3.5.1. Histological preparation

Preparation of tissue sections and staining according to method using by **(**Spencer and Bancroft, 2013**)**

After specimen fixation in formalin (10%) for 12 hrs., and then transferred to 70% ethanol.

3.5.1.1. Dehydration and clearing

Dehydration was followed by ascending ethyl alcohols; 70% over night, two changes of 80%, 90% and100%, 2hrs for each concentration. Clearing by using two changes of alcohol/xylene for 30-45 minutes.

3.5.1.2. Infiltration

Infiltration was done by using a mix of xylene and paraffin wax (melting point 56-58C^{o}), the samples were put in electric oven at temperature 60C^{o} (2hrs for two changes). Then transferred to molten wax for two stages (1 hour for each stage).

3.5.1.3Embedding:

An electric wax dispenser was used for embedding the sections in a labeled baths of a molten paraffin wax melting point 58 Cº, two changes were performed 2 hours for each using an embedding oven. Then the samples were transferred to be blocked in paraffin wax using a labeled stainless-steel Leuckhard embedding boxes (L- shaped)in transverse and longitudinal orientations of the specimens. and just when the wax surface (in the mould) solidified it transferred into ice cold water, and that rapid cooling gives the wax better properties and reduces the wax crystals size. When the solidification of the blocks completed, the blocks were separated from the molds and were kept at the lab temperature.

3.5.1.4. Trimming and Sectioning

Wax molds containing the samples were trimmed using a sharp scalpel, the samples were sectioned by using the rotary microtome at 5 µm thickness. Sections were then floated on a water bath at 50 ºC; the sections were mounted on a glass slide coated with thin layer of Mayer's albumin.

3.5.1.5. Staining

Paraffin sections of PTC blocks were used for hematoxylin and eosin stain to diagnosis the histological features, immunohistochemical assessment by using appositive charge slide and genetic study.

3.5.1.5.1 Hematoxylin and eosin staining procedure

The hematoxylin and eosin staining procedure had been used for showing the general components of the tissues; this staining procedure had been done as following:

- Dewaxing of the slides by xylene for 30 minutes.
- Rehydration of the sections with descending concentrations of ethanol (100, 90, 80, 70 %) for 2 minutes to each step.
- Immersing the slides in Harris hematoxylin for 3-5 minutes.
- Rinsing the slides in running distilled water for 5minutes for optimum bluing.
- Immersing in 1% Eosin for 1-3 minutes.
- Rising in running tap water for 30 seconds.
- Dehydration through ascending concentrations of ethanol (70,80,90,100 %) for 2 minutes each.
- Clearing for 2-3 minutes in xylene.

3.5.1.6. Mounting

The prepared slides were mounted with Dextrin plasticizer xylene (D.P.X), covered by cover slip, then left to dry. The slides then became ready for microscopic examination and photography.

3.5.1.7. Microscopic examination

In routine stain the slides were examined by using compound light microscope to observe the histological changes in the slides of the groups.

3. 5.1.7.1 Scoring:

The scoring system to assess the staining intensity was used following previous study of Erdogan-Durmus *et al.* (2016); as follow; if the staining cells

in the examined field were less than 5% it regarded as negative. 5-30% regarded as weak, 31-69% as moderate and more than 70% regarded as strong staining.

3.5.1.8. Photography

The tissues sectioned were photographed by using the camera attached with a microscope.

Six Paraffin sections of thyroid papillary carcinoma paraffin blocks were made. One section was made from each paraffin block and stained by the conventional hematoxylin and eosin (H & E) stain to be reviewed by two pathologists who confirmed the diagnosis and other related histological features. Three sections were used for immunohistochemical assessment for which a positive charge slide was used and the other two were used for genetic study. Sections were prepared using Reichert-Jung, Germany microtome with a thin section (4-6 µm).

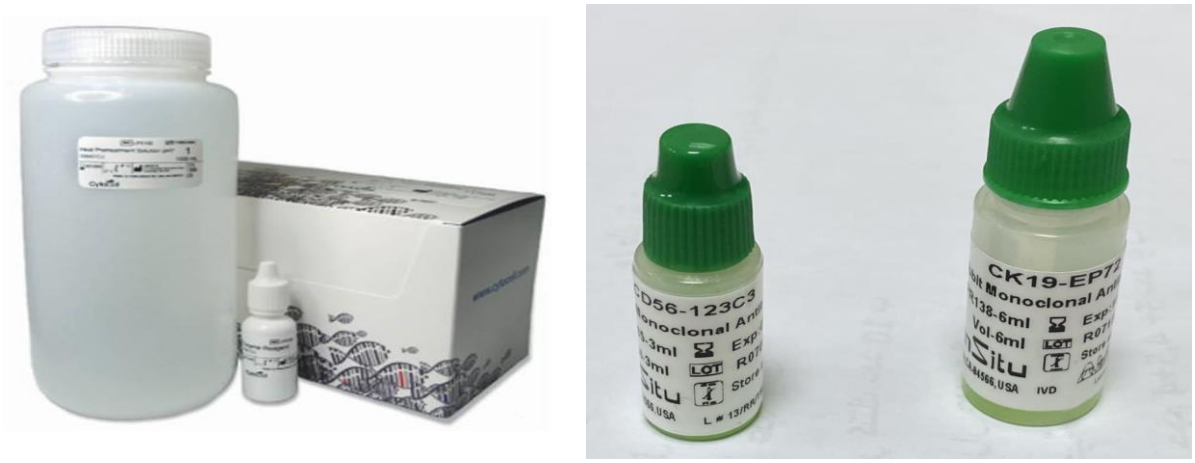
3.6. Immunohistochemical study

Three further sections were made for immunohistochemical staining by CK19, CD56 and HBME-1. The staining was done according to following step: The sections made with 4 µm and were put on positive charged slides.

1. Deparaffinization step was done by being kept in oven 60 °C overnight
2. Dewaxing done by using xylene bath, three times 5 minutes for each.
3. Rehydration step was done using descending ethanol concentrations baths 96%, 90%, 70 % and 50 %, 5 minutes for each then followed by distil water bath for 5 minutes.

4. The antigen retrieval step was carried out at water bath when temperature is 65 the slides were placed in and when temperature reached 99 °C the slides were left for 20 minutes. Then slides were removed from retrieval solution and let to cool.
5. Tri-buffered saline wash for 3 times.
6. Peroxidase solution for 5 minutes.
7. Tri-buffered saline wash for 3 times.
8. Incubation with primary anti-body (CK19 and CD56) at room temperature for 40 minutes.
9. Tri-buffered saline wash for 3 times.
10. Link (secondary anti-body) Incubated for 15 minutes at room temperature.
11. Tri-buffered saline wash three times
12. Horse reddish peroxidase was added and incubated for 15 minutes.
13. Horse reddish peroxidase wash for 3 times
14. diaminobenzidine (DAB) Chromogene substrate and incubation 5 minutes at room temperature.
15. Tri-buffered saline wash for 3 times.
16. Hematoxylin was then used for nuclei counterstaining for 1 minute.
17. Distilled water wash.

18. Dehydration step was then performed with ascending ethanol concentrations baths followed by clearing step and mounting using Canada balsam and covered by cover slip.



A B

Figure (3-2): Immunohistochemistry staining kit. A. HBME1 PathnSitu. B CD 56 and CK19 PathnSitu.

3.7. Genetic Study:

Fluorescence *in situ* hybridization (FISH) of thyroid tissue with PTC:

This technique was used to detect DNA sequence by using fluorescent probes. The manufacturer's guide was used to detect RET proto-oncogene chromosomal abnormalities, FISH CytoCell RET Breakapart (10q11.21) from Oxford Gene Technology-USA. The procedure was as follow: (figure 3-4 a &b)

- Paraffin embeddedtissue from patients diagnosed with papillary carcinoma of thyroid glandwere sectioned (4µm-thickness).

- Mounted on positively charged slides.
- Dewaxed with two passes in xylene 10 minutes each time.
- Hydrated with descending concentration of alcohol (96%, 85% and then 70%) 10 minutes each time.
- Fixed in formalin for 10 minutes.
- Five handeredml of TissuePre-treatment Solution (Reagent 1) was heated to boil or at least to 96 – 98 °C.
- The slides were then incubated for 15 minutes in heated (98C) Pre-treatment solution.
- Washed in phosphate – buffered saline (PBS)or D.W at room temperature for 2 x 3 minutesthen covered with 100µl of enzyme reagent 2 for 10 minutes at room temperature.
- Washed with phosphate – buffered saline (PBS) before dehydrated in ascending concentration of alcohol.
- The probe was thawed to room temperature and centrifuged shortly before it has been used.
- Ten µl of the fluorescent probe was applied on the sections at 37 °C for five minutes.
- Covered with a cover slip and sealed with glue carefully. The glue let to dry completely.
- The slides were then heated to 75°C for five minutes for tissue denaturation,
- The slides were kept in dark, humid incubator at 37 °C overnight.
- Thecover slip was then removed and washed with the washing solution 0.4xSSC (pH 7.0) at 72ºCfor two minutes without agitation.
- The slide drained and immersed in 2xSSC, 0.05% Tween-20 at room temperature (pH 7.0) for 30 seconds without agitation.

- The slides were drained and 10µl - 15µl of DAPI antifadewere applied.
- The slides were covered with a cover slip, bubbles, if present, were removed, and slides kept in dark for 10 minutes to allow the colour to develop.
- All slides were examined by usingwith the aid of the specific fluorescence filters.
- Slide examination and detection by using fluoresce microscope showed that localisation of both green/red fluorescent colours was described as normal chromosomal contents, whereas the dissociated signal of red and green considered rearrangedRET.100 intact nucleus were examined per slide, and samples showed split signal in more than 15% of the entire count were considered positive for RET.

3.8. Statistical analysis

Data were analyzed using statistical package for social sciences (SPSS, IBM, Chicago, USA, version 23). Qualitative data were expressed as number and percentage; whereas, quantitative data were expressed as mean, standard deviation and range. Independent sample t-test was used to compare mean between two groups, whereas, chi-square test was used compare proportions between two groups.Pearson correlations was used to determine correlation between quantitative or ordinal variables. The level of significance was chosen at $P \leq 0.05$.

5. Discussion

5.1. Sociodemographic characteristics

Thyroid cancer is one of the important health issues worldwide and papillary thyroid cancer is the most common form of this malignant neoplasm. The incidence of papillary carcinoma is increasing globally (Ito *et al.*, 2013; Jung *et al.*, 2013; Joshi *et al.*, 2014). According to the Surveillance, Epidemiology and End Results (SEER) program, new cases of thyroid cancer in people age < 20 represent 1.8% of all thyroid malignancies diagnosed in the United States (Howlader *et al.*, 2010). Unfortunately, the incidence appears to be increasing.

Among 15-19years old adolescents, thyroid cancer is the eighth most frequently diagnosed cancer and the second most common cancer among girls (Hogan *et al.*, 2009). Adolescents have a 10-fold greater incidence than younger children, and there is a female to male preponderance (5:1) during adolescence that is not seen in young children. The most common presentation for differentiated thyroid cancer (DTC) in children is that of a thyroid nodule. However, papillary thyroid cancer (PTC) also frequently presents as cervical adenopathy with or without a palpable thyroid lesion, or as an incidental finding after imaging or surgery for an unrelated condition. Occasionally, the diagnosis is made only after the discovery of distant metastases (DeLellis *et al.*, 2004)

The current study included a total of 45 patients, 4 males and 41 female, with a male to female ratio of 1: 10.25. The high prevalence of papillary carcinoma in women in comparison of men has been documented by previous authors (Lim *et al.*, 2017; Rao*et al.*, 2017) and worldwide, it is estimated that the thyroid cancer is approximately 2.9 times more common in women than in men (Rahbari *et al.*, 2010).

PTC can occur at any age and has rarely been diagnosed as a congenital tumour. It is usually detected in the third to fifth decades of the patients' life, with the mean age at 40 years. The incidence of PTC increases with age, and women are more frequently affected than men (Abdullah *et al.*, 2019). In the current study, the mean age of male patients was more than that of female patients, 42.00 ±2.58 years versus 36.76 ±7.79 years, respectively; however, the difference did not reach statistical significance (p = 0.163). The mean age of all patients with papillary carcinoma enrolled in this study was 37.22 ±7.61 years and it ranged from 26 to 50 years.In the current study, the mean age of patients enrolled in the current study was 37.22 ±7.61 years and males appear to be older than females. Indeed, this observation is in line with previous reports that papillary thyroid cancer is diagnosed at a median age of women that is younger than that for female patients (Rahbari *et al.*, 2010).

Some authors have hypothesized that differences in reproductive hormones may be linked to the difference in the incidence rate of papillary carcinoma between men and women but evidences from a variety of reports are inconclusive (Negri *et al.*, 1999; Ortega *et al.*, 2004; Kilfoy *et al.*, 2009). Thyroid cancer overall appears to behave more aggressively in elderly patients. PTC, which is diagnosed mostly in the third and the fifth decades and is often indolent and slow growing, also behaves aggressively in older individuals. Most studies indicate that older individuals, especially over the age of 45–50 years, seem to fare less well overall. The tall columnar variant (TCV) of PTC, which is the most common among the aggressive variants and the most aggressive of all variants, tends to occur in elderly patients (Das, 2005). Nevertheless, the incidence of papillary thyroid carcinoma was reported in pediatric age group in foreign countries, which was attributed to the high proliferation rate of the thyroid cells that make them susceptible to DNA

changes especially RET rearrangement (Jarzaband Handkiewicz-Junak, 2007). In current study we did not met such age group in Babylon province patients.

5.2. Clinical presentation of patients with papillary thyroid carcinoma.

Papillary carcinoma is presented mainly as thyroid nodule which is asymptomatic; it may be associated with mass in cervical nodes. The presence or absence of these cervical nodes involvement is not strictly related to bad or good prognosis (Kholmatov *et al.*, 2017). In the current study, from clinical perspective, presentation in the form of solitary nodule was far more frequent than diffuse enlargement, 41 (91.1 %) versus 4 (8.9 %), respectively and there was no significant difference in the mode of clinical presentation, whether solitary or diffuse enlargement, between male and female patients ($p > 0.05$).

Patients with Hashimoto's thyroiditis have higher risk to develop thyroid cancers, i.e., long term Hashimoto's thyroiditis cause persistent elevated TSH serum level, this play as a risk factor for the development of carcinoma (Zhang *et al.*, 2012). Solitary nodule was the main clinical patter of presentation in the present study. Indeed, thyroid cancer is more likely to be associated with solitary nodules and our observation is consistent with previous observations that solitary nodule is the most frequent mode of clinical presentation in association with papillary thyroid carcinoma (Popoveniuc *et al.*, 2012; Jena *et al.* 2015; Shahbaz *et al.*, 2018).

Thyroid carcinomas less than 1 cm are almost exclusively papillary and are termed papillary thyroid microcarcinoma (PTMC) according to the World Health Organization (Roti *et al.*, 2005). The clinical importance of PTMC is debatable. Some authors have observed that PTMCs have a benign behavior and do not progress over follow-up period (Hwang *et al.*, 1992). Incontrast, other authors

have reported a case study of 3rd decade female with PTMC that has local lymph node and distant metastases at the time of diagnosis and during follow-up evaluation (Braga *et al.*, 2002).

5.3. Histopathological evaluation of biopsy from patients with papillary carcinoma

Histologic diagnosis of malignancy is usually very simple, but in some tumors it is challenging. Papillary and follicular carcinomas are the two most common entities, usually referred to as differentiated thyroid cancer. Papillary carcinomas are solitary or multifocal lesions. Some tumors may be well-circumscribed and even encapsulated; others may infiltrate the adjacent parenchyma with ill-defined margins. The lesions may contain areas of fibrosis and calcification and are often cystic (Kumar *et al.*, 2021). The mean size of tumor which was found in the current study was 5.69 ±1.10 cm and there was no significant difference in mean size of nodules between male and female patients (p = 0.139).Thyroid carcinomas less than 1 cm are almost exclusively papillary and are termed papillary thyroid microcarcinoma (PTMC) according to the World Health Organization (Roti *et al.*, 2005). The clinical importance of PTMC is debatable. Some authors have observed that PTMCs have a benign behavior and do not progress over follow-up period (Hwang *et al.*, 1992). In contrast, other authors have reported a case study of 3rd decade female with PTMC that has local lymph node and distant metastases at the time of diagnosis and during follow-up evaluation (Braga *et al.*, 2002). Previous studies about the size of PTC may range from 2-100 mm with average of 12.8 mm. this study give wide range of tumour size, in patients enrolled in our study the tumour size range from 4 to 8 cm. this indicate that the patient is not seeking the medical advice till the tumour become large enough to be seen and observed by naked eye, furthermore, the absence of

routine investigations and periodic examination of patient permits the development of tumour and delay interventions.

It should be kept in mind that the size of the nodule may be large also in benign tumours, in some studies on patient underwent thyroidectomy they found that the tumour size of more than 1 cm has a protective role in their study. They interpret their finding to the fact that small tumour size especially benign one is not transferred to surgery but treated conservatively. While in patient diagnosed as has PTC, the tumour size of more than 1 cm is at risk of metastasis (Zhang *et al.*, 2012).

The hallmark of histopathological diagnosis of papillary carcinoma of the thyroid gland is based on demonstration of nuclear criteria of malignancy including nuclear inclusions, ground glass appearance and coffee bean nuclei on microscopical examination and these were seen in 37 (82.2 %), 37 (82.2 %) and 37 (82.2 %) of cases enrolled in the current study and there was no significant difference in the rate of detection of these nuclear features between male and female patients. Capsular invasion was detected in 31 (68.9 %) and was more frequently seen in women, other studies indicate that the presence of capsular invasion is related to less favorable prognosis (Haugen *et al.*, 2019). lymphatic invasion was seen in 10 (22.2 %) and was more frequently recognized in women and vascular invasion was seen in 22 (48.9 %) and was more commonly encountered in male patients.

Microscopically, papillary carcinomas contain papillary areas with a focal distribution or with a diffuse pattern. The papillae consist of a stromal vascular axis lined by characteristic cells. Other aspects may be associated with the papillae: follicles filled with colloid or a trabecular or lobular aspect, squamous metaplasia,

and psammoma bodies are other distinguishing features present in 40–50% of tumors (Pacini *et al.*, 2018).Ground glass (optically clear) nuclei, which often have a large size and an overlapping quality. The nucleolus is usually inconspicuous and pushed against the nuclear membrane, which appears thickened. This change is present in sections obtained from paraffin-embedded material regardless of the fixative used, but is less apparent or altogether absent in frozen sections or cytology material (Rosia, 2011).

In the present study, nuclear criteria of malignancy were seen in most of patients. This observation is in line with previous authors (Lloyd *et al.*, 2011; Bavle, 2013; Policarpio-Nicolas and Sirohi, 2013; Lee *et al.*, 2016). In this study, capsular invasion was predominating when compared to lymphatic or vascular invasion, but vascular invasion was seen in all enrolled men. These features are very important in identifying the malignant nature of thyroid neoplasm; however, their role in follicular carcinoma is far more important than in papillary carcinoma (Xu and Ghossein, 2018).To note that, It is also not clear from the literature whether the other cytological features peculiar to the tall cell variant of papillary thyroid carcinoma, such as oncocytic cytoplasm and higher frequency of intranuclear cytoplasmic inclusion, have any relationship with the age of the patients.

Since prognosis of PTC cases is related to histological variants and also to the age of the patients, an attempt was made to find out how the morphological parameters, especially those characterizing the most aggressive variant of PTC, correlated with the age of the patients (Das, 2005).

5.4. Immunohistochemical evaluation of biopsy from patients with papillary thyroid carcinoma

The differentiation between papillary, follicular and other form of thyroid cancer relies mainly on histological feature; especially the nuclear characteristics of the malignancies; however, significant overlap in these features exists and it sometime very difficult, based on sole morphological features, to identify specific histological pattern (Arora *et al.*, 2008; Park *et al.*, 2010). Thus, the role of immunohistochemistry has been proposed to aid in the diagnosis of papillary carcinoma of thyroid gland and both CD56 and CK19 gain wide acceptance among pathologists worldwide (Huang *et al.*, 2018). Furthermore, HBME-1 can be used for such purpose.

5.4.1. Immunohistochemical expression of CD56 in patients with papillary carcinoma of thyroid gland.

CD56 is a neural cell adhesion molecule (NCAM) that plays a role in the cohesiveness of cells in nervous systems and neuro-endocrine cells (Chen *et al.*, 2020). The lack of expression of CD56 is a useful immunohistochemical marker in differentiating between PTC and other lesions including cases mimic PTC like Hashimoto thyroiditis (El Demellawy *et al.*, 2008).

Positive CD56 expression was detected in the current study in 48.9 % of cases and distributed as following: weak staining pattern was seen in 13 (28.9 %), 4 (100.0 %) of males and 9 (22.0 %) of females, being more significantly encountered in men ($p = 0.012$); moderate staining pattern was never seen and strong staining pattern was seen in a total of 9 (20.0 %) and was significantly restricted to women ($p< 0.001$).

CD56 is present on follicular epithelial cells of the normal thyroid. One of the most frequent difficulties in thyroid pathology is differentiating follicular variant of PTC from follicular adenoma. The immune profile of follicular derived lesions and neoplasms show some overlap and no single marker or even panel is 100% sensitive and 100% specific for PTC. Hence, the use of immunohistochemistry in the diagnosis of the follicular variant of PTC has to be used with extreme caution (El Demellawy *et al.*, 2008).

CD 56 is expressed strongly in other types of neoplasms like small cell lung cancers. Therefore it is one of the immunohistochemical markers with important diagnostic value in different types of tumours, whether its expression is present or lacked (Kontogianni *et al.*, 2005). Other studies showed there are differences in the expression of CD56 in association with the tumour stage. This observed in pancreatic neoplasms when the CD56 is strongly expressed in lower stages and lacked in advance stages (Chen *et al.*, 2020). As a result, using CD56 marker alone as an immunohistochemical factor to ensure the diagnosis is suggestive but not exclusive, since a debate appear in the result and previous studies.

5.4.2. Immunohistochemical expression of CK19 in patients with papillary carcinoma of thyroid gland

Immunostaining with CK19 showed strong and diffuse cytoplasmic and membrane positivity in the majority of cases of PTC (Barroeta *et al.*, 2006). In the current study, positive CK19 expression was reported in a total of 42 (93.3 %) of cases and it was distributed as following: weak staining pattern was restricted to women accounting for 3 (7.3 %), moderate staining pattern was also restricted to women accounting for 3 (7.3 %) and strong staining pattern was more frequently associated with male patients in comparison with female patients, 4 (100.0 %)

versus 29 (70.7 %), respectively, but the difference was statistically insignificant (p= 1.000).

Previous studies revealed that the CK19 immuno reactivity was stronger in the cells with nuclear features of PTC, while staining in cells lacking nuclear changes of PTC varied from completely negative to moderately positive (Sahoo *et al.*, 2001). Studies have shown that normal thyroid strongly expresses the simple epithelial cytokeratins, CK7 and CK18, and, to a lesser extent, CK8 and CK19, but not stratified epithelium-type cytokeratins such as CK5/6 and CK13.9 The latter also have been found lacking in follicular carcinoma. Instead, PTCs expressed CK5/6 and CK13 in 66% (27/41) and 34% (14/41) of cases, respectively (Fonseca *et al.*, 1997).

In the current study, CD56 immunostaining was seen in about 50% of cases and CK19 immunostaining was seen in 93.3 %5 and strong CD56 staining was more frequently seen in women. Strong CD56 staining was limited to 20 % of cases, while strong CK19 staining was seen in 73.3 % of cases. These findings suggest a more reliable role for CK19 as an aid in the diagnosis of papillary thyroid cancer than CD56.

Previous studies mentioned the preference of PTC to occur in female more than male, but to our knowledge the studies did not explore the expression of CK19 in female and male separately, though a comparison with previous studies in this point is not applicable. According to Abou Hashem and Talaat (2017), CK19 was detected in 87.8 % of patients with papillary thyroid cancer and CD56 was seen in 19.2 % of patients with papillary thyroid cancer, thus our results accord with them concerning CK19 but disagree with them with respect to CD56. According to Huang *et al.*(2018), CK_{19} expression was detected in 116 out of 120

(96.7 %), confirming our results and that CD56 was seen in only 12 out of 120 (10%) which is less than the approximately 50 % rate in our study.

Combining the results of the current study with previous observation one can conclude that combining the immunohistochemical findings of CD56 and CK19 with conventional histopathological evaluation of thyroid lesions greatly increase the accuracy of diagnosing papillary carcinoma.

5.4.3. Immunohistochemical expression of HBME-1 in patients with papillary thyroid carcinoma

The results of current study reported positive HBME-1 expression in a total of 33 (73.3 %) of cases and it was distributed as following: weak staining pattern was seen in 1 (25.0 %) of men and 3 (7.3 %) of women with no significant difference (p = 0.250), moderate staining pattern was seen in 1 (25.0 %) of men and 17 (41.5 %) of women with no significant difference (p = 1.000) and strong staining pattern was more frequently associated with male patients in comparison with female patients, 2 (50.0 %) versus 9 (22.0 %), respectively, but the difference was statistically insignificant (p = 0.212).

HBME-1 is an antigen constituent of the microvilli on the surface of mesothelial cells in humans, and hyaluronic acid (HA) is the main ingredient of HBME-1 (El-Mahdy *et al.*, 2011). HBME-1 a common molecular marker of tumors has been suggested for its potential use in diagnosis and prognosing differentiated thyroid carcinoma (Cantisani *et al.*, 2016). Currently, there are few studies that report the expression levels of HBME-1 protein in different types of differentiated thyroid carcinoma tissues (Qiao *et al.*, 2017).

Currently, there are few studies that report the expression levels of HBME-1 protein in different types of differentiated thyroid carcinoma tissues, and their

correlation with ultrasonic manifestation of thyroid. Clinical practice of Bychkov*et al.*(2016) and Chen *et al.* (2016) has proven that IHC can be used for a differential diagnosis of papillary thyroid carcinoma. In recent years, more and more attention has been paid to some tumor markers with high sensitivity, such as CK19 and HBME-1. These markers have been applied in the clinical diagnosis of many malignant tumors, including thyroid carcinoma (Zhu *et al.*, 2010; Erdogan-Durmus *et al.*, 2016; Liu *et al.*, 2016).

Yeşil*et al.* (2015) and Chao *et al.* (2016) have reported that the high expression of HBME-1 can be detected in papillary thyroid carcinoma tissues.Previous studies confirm the usefulness of HBME-1 and suggest that the combination of HBME-1 and CK19 attains a high sensitivity and specificity for the diagnosis of papillary thyroid carcinoma (Nasr *et al.*, 2006). Cheung *et al.* (2001) reported HBME-1 positivity in 38/54 (70%) classic papillary thyroid carcinomas and 38/84 (45%) follicular variant of papillary thyroid carcinomas. Similarly, Prasad *et al.*(2005) demonstrated HBME-1 expression in 57/67 (85%) papillary thyroid carcinomas.

A high level of HBME-1 immuno-expression was also documented in the fine needle aspiration (FNA) samples by previous studies (Ozolins *et al.*, 2012). No HBME-1 immunoexpression was observed in benign lesions. A study by Saleh *et al.* (2009) showed that the sensitivity and specificity of immunoexpression for HBME-1 to distinguish benign from malignant lesions was one of the highest among all the markers.

Our findings of high sensitivity and specificity of HBME-1 in the diagnosis of papillary thyroid carcinoma confirm previous studies that have found this antibody to be diagnostically useful, since the dependence on histopathological features alone is not exclusive for determining neither the further management

northe prognosis of the disease, due to the large similarity between other types of thyroid carcinomas.

5.5. Genetic study

Fluorescence *in situ* hybridization (FISH) is a laboratory technique for detecting and locating a specific DNA sequence on a chromosome. The technique relies on exposing chromosomes to a small DNA sequence called a probe that has a fluorescent molecule attached to it. The probe sequence binds to its corresponding sequence on the chromosome. In this approach, a fluorescent dye is attached to a purified piece of DNA, and then that DNA is incubated with the full set of chromosomes from the originating genome, which have been attached to a glass microscope slide. The fluorescently labeled DNA finds its matching segment on one of the chromosomes, where it sticks. By looking at the chromosomes under a microscope, a researcher can find the region where the DNA is bound because of the fluorescent dye attached to it. This information thus reveals the location of that piece of DNA in the starting genome (Green, 2021).

FISH has greatly expanded the capabilities of cytogenetics and pathology laboratories through its high sensitivity, specificity and rapid turnover with a high efficiency of hybridization and detection. Material for FISH can be processed in 4–24 hrs, and the analysis of 1000–2000 cells accomplished in 15–45 min, enabling the information on the cytogenetic pattern of tumour cells to be achieved within a sufficient time frame for use in treatment strategies (Gozzetti and Le Beau, 2000)

The power of FISH ability to identify specific genetic aberrations has propelled FISH-based techniques to the forefront of screening procedures for prenatal, paediatric and adult cases in a wide variety of cell types, including paraffin-embedded tissue, making FISH analysis data a useful tool in the decision

of therapy to combat cancer. This is supported by a recently conducted survey by Wordsworth *et al.* (2008) and Bishop (2010) who reported that the most common techniques used for the testing of somatic mutations in laboratories were IHC (Bishop, 2010).

5.5.1. RET mutation in patients with papillary carcinoma of thyroid gland

In the present study, the mutation of RET gene was either in the form of deletion or in the form of rearrangement. Out of 45 patients, 11 had RET gene deletion accounting for 24.4 %. All males (100.0 %) had the RET gene deletion, whereas, 7 (17.1 %) of female patients had the RET gene deletion and the difference was significant ($p = 0.002$). In addition, in this study, out of 45 patients, 11 had RET gene rearrangement accounting for 24.4 %. None of males (0.0 %) had the RET gene rearrangement, whereas, 11 (26.8 %) of female patients had the RET gene rearrangement and the difference was not significant ($p = 0.558$).

FISH technique was adopted to detect the RET gene mutation. In the current study, out of 45 patients, 15 showed RET gene mutation accounting for 36.6 %. All male patients (100.0 %) had RET gene mutation, whereas, only 36.6 % of females had the RET gene mutation and the difference was statistically significant (0.026). other studies showed that the Positive RET/PTC was present in 75.00% of male patients with PTC, which was significantly greater compared with female patients with PTC (Zhou *et al.* 2018). This result is close to our observations that the expression of RET is greater in male than female.

Thyroid epithelium is very prone to chromosomal rearrangement, these include RET and neurotrophic receptor tyrosine kinase1 (NTRK1) oncogenes in PTC. This predisposition to gene rearrangements is a peculiarity of thyroid epithelium, at variance with other epithelia, and the understanding of the molecular

basis underlying such predisposition. Oncogenic activation of RET results in the constitutive activation of the kinase which, in turn, causes auto phosphorylation, recruitment of intracellular substrates, and activation of diverse signaling pathways (Melillo *et al.*, 2004)

Papillary thyroid carcinomas are now known to harbor different types of RET rearrangements, which are called RET/PTC. These rearrangements consist of the fusion of the RET tyrosine kinase (TK) domain to the 5-terminal region of heterologous genes, which results in RET/PTC chimeric oncogenes. At least 15 such chimeric mRNAs involving 10 different genes have been reported, of which RET/PTC1 and RET/PTC3 are by far the most common. In 1999, Miki *et al.* showed that expression of RET/PTC was significantly increased in patients with PTC who had local invasion. Giannini *et al.*(2000) showed that H4-RET expression was significantly associated with lymph node metastasis in PTC. The findings of these previously published studies support the relationship between RET/PTC and metastasis in thyroid cancer (Zhou *et al.*, 2018).

The mean frequency of RET mutation in sporadic papillary carcinoma is 20%-30% in adults, rising up to 45%-50% in pediatric and young patients, and being highest (50%-80%) in patients with a history of accidental or therapeutic radiation exposure (Rabes *et al.*, 2000; Sadetzki *et al.*, 2004). Accordingly, it appears quite reasonable to believe that the pathomorphologically identical papillary carcinomas must have followed different routes of carcinogenesis characterized by different clinical manifestation. In the last decades, there was a significant increase in the number of PTC, so one of the possibilities was the influence of radiation like that due to Chernobyl nuclear power plant disaster (LiVolsi*et al.*, 2011). Knowing that RET mutation has been proven to be a good marker for its influence (Punda *et al.*, 2018).

Nuclear factor- kB (NF-kB) has a role in cell apoptosis, in promoting inflammation, and in the immune response. Ludwig *et al.* (2001) showed that high levels of expression of NF-kB were related to RET activation.

Rearrangement of the tyrosine kinase receptor gene (RET gene named RET/PTC) is the most common structural genetic alteration which, however, shows great geographical variability ranging from 0 to 80% in different studies (Punda *et al.*, 2018).

Papillary thyroid carcinoma (PTC) is the most frequent thyroid cancer and consists in a well-differentiated carcinoma, originating from thyroid follicular cells and associated to exposure to ionizing radiation (Sherman, 2003; Williams, 2008). Consistently, typical molecular features of PTCs are chromosomal aberrations generated as a consequence of ionizing radiation-induced double-strand breaks and unfaithful repair. In particular, PTCs display chromosomal rearrangements of chr.10q, causing the rupture of the RET gene and its fusion to heterologous genes due to unfaithful repair (Carlomagno, 2012).

Ionizing radiation is a well-known risk factor for PTC (Vuong *et al.*, 2017; Schneider, 1995 and Schneider, 1990). Furthermore, the risk of developing thyroid cancer is proportional to the absorbed dose (Schneider, 1995; Cardis *et al.*,2005 ;Cardis *et al.*, 2006).

In agreement with evidence that the development of RET/PTC rearrangements is mainly caused by exposure to ionizing radiation (Caudill *et al.*2005; Gandhi *et al.*, 2010), RET/PTC rearrangements have a higher prevalence in radiation-induced PTC than in sporadic PTC (Elisei *et al.*, 2001; Nikiforov, 2002). These rearrangements are particularly prevalent in PTC that developed as a consequence of the radioactive fallout of the Chernobyl nuclear accident (~50%),

even if they have also been found to occur in sporadic tumours. The PTC induced by the radiation resulting from the Chernobyl accident remains an example of tumorgenesis driven by chromosomal rearrangement that can activate several effectors of the mitogen activating protein kinase (MAPK) pathway (Ricarte-Filho. *et al.*, 2013).

The prevalence of RET/PTC rearrangements was up to 87% in the first series of cases reported after the Chernobyl accident. However, in the latest series, characterized by a long latency period from the nuclear accident to the diagnosis, the reported prevalence of RET/ PTC rearrangements declined (Rabes *et al.*, 2000). This evidence suggests that the most recent cases might have a different pathogenesis to the earlier cases.

In addition to a higher prevalence of RET/PTC rearrangements in radiation-induced thyroid tumours, these genetic alterations are also more frequent in children than in adults for both irradiated and not irradiated PTC. The partner genes encode heterogeneous proteins all containing protein-protein interaction domains such as coiled-coil motifs (Nikiforov *et al.*, 2011). RET/PTC1 and RET/PTC3 represent over 90% of all RET/PTC rearrangements identified so far. In both cases, the chromosomal aberration consists in a paracentric inversion of the long arm of chromosome 10 where, together with RET the corresponding fusion partner of RET/PTC1, CCDC6 (H4) and of RET/PTC3, NCOA4 (RFG, ELE1, ARA70) map.RET/PTC3 is mainly associated with radiation-induced carcinomas and is frequently found in more aggressive PTC variants such as the solid-follicular or the tall cell histotypes. The other 11 RET/PTC isoforms are very rare and have been found only in few cases of radiation-induced PTCs (Carlomagno, 2012).

Previous study results on the relationship between PTC-related risk factors like effect of radiation exposure, female gender, and young age on RET/PTC rearrangement were controversial, Young age was also associated with higher prevalence of RET/PTC3, and this association was more significant in the subpopulation exposed to radiation. These studies also demonstrated an association between female gender and higher prevalence of the RET/PTC (Su *et al.*, 2016). In our study, RET mutation was seen in all males and this may explain the poor prognosis of thyroid cancer in males in comparison with females; however, this issue is still controversial. As several factors (such as hypoxia, caffeine and ethanol) are able to induce chromosome fragility, these factors could explain the presence of RET/PTC rearrangements in PTCs that are not linked to radiation exposure. Moreover, as the development of RET/PTC rearrangements (mainly RET/PTC1) can also be induced by high levels of H_2O_2 through increased concentrations of free radicals (Ameziane-El-Hassani *et al.*, 2010), theoretically all substances able to produce free radicals can induce RET/PTC rearrangements.

Much debate surrounds the question of whether the finding of RET/ PTC rearrangements in benign lesions, and Hashimoto thyroiditis, is related to the high sensitivity of the methodology used, which might be able to detect subclonal mutations (Nikiforov, 2006; Zhu *et al.*,2006).

5.5.2. BRAF mutation in patients with papillary carcinoma of thyroid gland

In the current study, out of 45 patients, 14 had BRAF gene rearrangement accounting for 31.1 %. All males (100.0 %) had the BRAF gene mutation, whereas, 10 (24.4 %) of female patients had the BRAF gene mutation and the difference was significant ($p = 0.007$).

Mutations of the BRAF gene were initially found to be present in around 66% of melanomas and also approximately 12% of colon cancers. Two unique somatic mutations of the BRAF gene have been identified in papillary thyroid carcinoma. BRAF is a gene that has a critical role in the MAPK signaling pathway. The most common mutation in the BRAF gene is V600E, which occurs as a result of a base substitution in exon 15 of thymine for adenine that converts valine to glutamic acid at amino acid residue 600 (BRAF V600E) (Kimura *et al.*, 2003).

As the most common BRAF mutation, V600E promotes the continuous activation of BRAF kinase. Previously published studies have shown that up to 45% of patients with PTC have a BRAF mutation, most of them being mutations in BRAF V600E.Several factors are associated with an increased prevalence of BRAF V600E mutation. High iodine intake, sometimes implicated in PTC pathogenesis, is one such factor. Male patients have also been reported to have an increased prevalence of BRAF V600E mutation (Jung *et al.*, 2010).

The analysis of several hundred PTC samples by the Cancer Genome Atlas (TCGA) has demonstrated that the most common PTC lesions are those targeting BRAF (about 60% of the cases), mainly the V600E point mutation (Cancer Genome Atlas Research Network, 2014), furthermore, Previous studies on papillary thyroid carcinomas revealed that the positive BRAF was present in 72.97% of female patients with PTC, which was significantly greater compared with male patients with PTC (Zhou *et al.*, 2018).

The BRAF V600E mutation has been observed in 18% to 87% of thyroid cancers (Xing, 2005; Trovisco *et al.*, 2006). It is most commonly present in PTC,

and some forms of poorly differentiated thyroid cancer and anaplastic thyroid cancers that coexist with, or arise from, PTC (Begum *et al.*, 2004).

Both in vitro studies and transgenic models of BRAF suggest that the BRAF V600E mutation promotes thyroid cancer progression and is associated with invasive thyroid cancer phenotype (Mesa *et al.*, 2006 ; Knauf *et al.*, 2005). On the basis of these findings, several investigators have evaluated whether the presence of a BRAF V600E mutation in thyroid cancer is associated with an aggressive tumor phenotype. Some studies suggest an association between the presence of BRAF V600E mutation and poor prognostic factors, such as older age, male gender, extrathyroidal tumor invasion, lymph node and distant metastases, higher tumor stage, and even higher rates of recurrent disease. However, several investigators have not found the presence of the BRAF V600E mutation to be associated with aggressive thyroid cancer phenotype (Kebebew *et al.*, 2007)

Certification

I certify that the preparation of this thesis was made by***(Sahar Dakhil Yonis Saffah)*** under my supervision at the Department of Biology , College of Science , Universityof Babylon , as a partial fulfilment of the requirement for the degree of Ph.D in Biology/ Zoology.

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