بسم الله الرحمن الرحيم

**Sudan University of Science and Technology**

**College of Graduate Studies**

# Molecular Detection of KRAS Gene Mutation Codon12 Exon 1 in Thyroid Tumors Among Sudanese Patients

# الكشف الجزيئي عن الطفرة لجين KRAS كودون 12 إكسون 1 في أورام الغدة الدرقية لدى المرضي السودانين

**A dissertation Submitted in Partial Fulfillment of the Requirement of M.Sc Degree in Medical Laboratory Science (Histopathology and Cytology)**

**By**

**Hajar Eltayeb Abdelhameed Mahmoud**

**B.Sc. Medical Laboratory Science (Histopathology and Cytology)**
**Sudan University of Science and Technology (2007)**

**Supervisor**

**Dr. Mohamed Siddig Abdelaziz**

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# Dedication

To . . .

The candle that burnt to light

My life, my mother.

To . . .

The source of supercilious

My father.

To . . . .

My family

To . . . .

Those who made it

Possible teachers and friends.

# Acknowledgment

First I thank my god who gave me strength to carry this work.

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# Abstract

This is descriptive retrospective analytical case study, was conducted in Sudan university of science and technology at research laborotary and Omdurman teaching hospital during the period from September to December 2017.the study aimed to detect the expression of KRAS gene mutation codon 12 exon 1 in thyroid tumors among Sudanese patient's using allele specific oligonucleotide (ASO) PCR technique.

Forty formalin fixed paraffin embedded blocks previously diagnosed as thyroid tumors were selected for this study, they included thirty with malignant thyroid tumors and ten with benign thyroid tumors. The data was analyzed using SPSS computer to calculate frequency, mean and Chi square test.

The distribution of gender in study population was 12(30%) males and 28(70%) was females.

The age of study population was ranged from 11 to 67 years with mean as 36.7 most of them were less than 50 years 33(82.5%) and the remaining 7 (17.5%) were above 50 years.

The histopathological diagnosis showed that malignant tumors as papillary thyroid cancer (PTC) 16(40%), follicular thyroid cancer 9(22.5%), medullary thyroid cancer 3(7.5%), undifferentiated cancer2 (5.0%), while benign tumors show 8(20.0%) from multi nodular goiter, 1(2.5%) from hashimoto thyroiditis and also 1(2.5%) from follicular adenomas.

The distribution of KRAS gene (normal and mutant) among study population presented as normal KRAS gene 15(37.5%) were negative and 25(62.5%) while mutant KRAS gene 31(77.5%) were negative and 9(22.5%) were positive.

The relation between histopathological diagnosis and KRAS gene mutation (codon 12 exon 1) in benign tumors were 3 positive and 7 were negative while in malignant tumors 6 were positive and 24 were negative and P.value (0.512).

The study concluded that KRAS gene mutation (codon 12 exon 1) does not play role to cause thyroid tumors may another codon and exon caused thyroid tumors.

According to my study showed that female, age less than 50 years were most affected with thyroid tumors and the papillary thyroid tumor is most common malignant tumor in Sudanese patients.

## المستخلص

اجريت هذة الدراسة الوصفية الإسترجاعية التحليلية في جامعة السودان للعلوم والتكنولوجيا في مختبر الابحاث و مستشفي امدرمان التعليمي خلال الفترة من سبتمبر إلي ديسمبر 2017 . هدفت الدراسة للكشف الجزيئي للحمض النووي للطفرة في جين كى آرأى أس كودون 12 إكسون 1 في اورام الغدة الدرقية لدي المرضي السودانين بإستخدام تقنية الحمض النووي محددة الأليل (ASO ) في تفاعل البلمرة (PCR ) . تم إختيار اربعين قالب نسيج محفوظة بالفورمالين ومثبتة في شمع البرافين تم تشخيصها سابقا باورام الغدة الدرقية , شملت ثلاثين عينة من أورام الغدة الدرقية الخبيثة وعشرة عينات من أورام الغدة الدرقية الحميدة . تم تحليل البيانات بإستخدام برنامج الحزم الإحصائية للعلوم الإجتماعية (SPSS ) لحساب التردد والمتوسط وإختبار مربع كاي .وفقا لبيانات المرضي التي تم اخذها من الملفات كان عدد الذكور 12(30%) و إلاناث 28(70%) . وتراوحت اعمارهم بين 11 سنة الي 67 سنة وكتن متوسط العمر للمرضي 36.7 ومعظم المرضي كانت اعمارهم اقل من 50 سنة 33(82.5%) اماالبقية كانو 7(17.5%). وحسب التشخيص النسيجي ل اورام الغدة الخبيثة وجد أن سرطان الغدة الدرقية الحليمي 16(40%) وسرطان الغدة الدرقية الجريبي 9(22.5%) وسرطان الغدة الدرقية النخاعي 3(7.5%) و سرطان الغدة الدرقية غير المتميز 2 (5.0%),و وجدت الأورام الحميدة من تضخم الغدة الدرقية متعددة العقيدات 8(20.0%) و الورم الجريبي الحميد 1(2.5%) و الهاشميتو 1(2.5%).

اعطي تفاعل البلمرة لجين كراس (كودون 12 إكسون 1) في مجتمع الدراسة فكان الجين الطبيعي 15(37.5%) سالبة و25(62.5%) كانت موجبة اما الطفره في الجين كانت 31(77.5%) سالبة و9(22.5%) كانت موجبة .

اعطت تفاعلات البلمرة لطفرة جين كراس (كودون 12 إكسون 1) مع الاورام الحميدة 3 موجبة و7 سالبة واما مع الاورام الخبيثة كانت 6 موجبة و24 سالبة وكانت القيمة الحتمالية (0.512 ) .

خلصت الدراسة إلي عدم وجود علاقة ذات دلالة احصائية بين طفرة جين كراس (كودون 12 إكسون 1) و انواع اورام الغدة الدرقية وذلك قد تكون هنالك طفرة جينية في كودون وإكسون مختلفين سببا في اورام الغدة الدرقية .

اظهرت الدراسة ان الاناث و الفئة العمرية الاقل من 50 سنة هي الاكثر اصابة باورام الغدة الدرقية وان ورم الغدة الدرقية الحليمي غير الحميد اكثر انتشارا بين المرضي السودانيين.

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| Abbreviation | Full name |
|---|---|
| Ala | Alanine |
| APUD | Amine precursor up take decarboxylation |
| Arg | Arginine |
| ASCO | American cancer society organization |
| ASO | Allele specific oligonucleotide |
| Asp | Aspartate |
| bp | base pair |
| B.V | Blood vessel |
| CD | Cluster of differentiation |
| C.T | Connective tissue |
| DNA | Deoxyribo nucleic acid |
| EGFR | Epidermal growth factor receptor |
| GDP | Gauanosine diphosphate |
| Gly | Glysin |
| GTP | Guanosine triphosphate |
| HLA | Human leukocyte antigen |
| KRAS | Kirsten rat sarcoma virus oncogen |
| MAPK | Mitogen activated protein kinase |
| MEN | Mutiple endocrine neoplasia |
| MTC | Medullary thyroid carcinoma |
| PCR | Polymerase chain reaction |

| | |
|---|---|
| PI3K/AKT | Phosphatidylinositol 3 kinase & protein kinase B |
| RET | Rational emotive therapy |
| SDS | Sodium dodocyle sulfate |
| T 3 | Triiodothyronine |
| T 4 | Thyroxin |
| Tg | Thyroglobulin |
| TP53 | Tumor protein 53 |
| Val | Valine |

# *Chapter One*

# *Introduction*

# HAPTER ONE

## 1- INTRODUCTION

Thyroid cancer is the most common endocrine malignancies, represented as more than 1% of all human tumors. The incidence during last decades increased globally according to geographic areas, age and sex. In United State incidence of all types of thyroid cancer about 7.7 per 100.000 person with rates of women 11.3 per 100.000 in years and 4.1 per100.000 men in years. the incidence of papillary thyroid cancer5.7per 100.000 person in years(8.8 women and 2.7 men) rates are high among whites and lower among blacks.in USA the incidence follicular thyroid cancer is 0.82 per 100.000(1.06 women and 0.59 men) dosenot affected with ethnicity. The incidence rates of medullary thyroid cancer and anaplastic thyroid cancer 0.11 and 0.21 per 100.000 person in years (Aschebrook, 2011).

The estimation of thyroid cancer in united state for 2017 about 56,870 new cases of thyroid cancer (42,470 women and 14,4 men), about 2,010 death from thyroid cancer(1.090 women and 920 men). Thyroid cancer commonly diagnosed at younger age than adult and 3 from 4 cases are found in women and 2%of thyroid cancer occur in children and teens (ATA, 2016).

In last several years continuously increasing incidence of thyroid cancer in European countries, USA and Canada it fastest growing cancer type and the sixth of most common cancer (Nikiforov, 2010).

Thyroid malignancy in South Africa is major problems of thyroid disease due to high prevalence of iodine deficiency which play important role in follicular thyroid cancer in South Africa (Ferlay, 2000).

The prevalence of thyroid goiter among school children In Darfur state in west of Sudan about 85% which 23.9% was large (stage II or III), the prevalence high in pre pubertal children in both sexes and in adult the female is more frequent 40.9%. In Port Sudan in east of Sudan the prevalence of thyroid goiter was 13.5%among school children. The rate of goiter was increased to 17 %( Izzeldin, 2007).

Thyroid tumors detected through palpation with neck ultrasound then fine neddle aspiration ,thyroid hormone estimation (T3 , T4, TSH and Tg), immunohistochemical markers (CK19, Fibronectin1 and HBME1) and molecular testing for thyroid nodules (BRAF,RAS, RET\PTC and PAX8\PPARɤ mutation) (Pacini,2006).

The RAS gene encoded to a family of three highly homologous proteins (NRAS, HRAS and KRAS). These has 21KD, it's a membrane associated protein play a important role in signals transduction from tyrosine kinase

and G protein coupled receptors to effectors of the MAPK and PI3K-AKT signaling pathways,which mediate cell differentiation proliferation and survival.RAS activity is tightly regulated by GTP mediated hydrolysis of inactivated GTP bound RAS to inactivated GDP bound RAS. point mutation produce oncogenic alleles of RAS that exhibit either increased affinity for GTP (codon 12 and 13 ) or inhibition of autocatalytic GTPase function (codon 61) both mechanism result in constitutive activation of the downstream MAPK and P13\AKT signaling pathway in thyroid cancers (Saavedra, 2000).

The mutation of KRAS gene is essential step in the development of many cancers particularly the mutation in the coding region of the KRAS gene, one of the major oncogenic mutations found in human cancer which result in hyper activation of the protein. The expression of KRAS can be regulated on the gene level and affected by single nucleotide polymorphism (SNP) which located within the promoter or other regulatory regions. The genotype and allele of SNP in the carcinogenesis related genes might provide a simple and valuable method to predict the risk and the prognosis of cancers (Chin, 2008).

RAS mutation suggesting that activation protooncogene in medullary thyroid cancer were detected in thyroid tumor sample and most common detected H-RAS and K-RAS gene mutation codon s 12,13 and 61.(Moura,2011).

RAS mutation may role in thyroid nodules malignancy and the sample that give negative repeat fine needle aspiration or surgical excision test. (Clinkscales, 2017).

Treatment of thyroid cancer depend on type thyroid cancer, size of nodules and cancer spread they treated with surgery , thyroid hormone treatment, radioactive iodine therapy and chemotherapy(Kapiteijin,2012).

## 1.2. Objectives

### 1.2.1. General objective

To detect KRAS gene mutation in thyroid tumors among Sudanese patients.

### 1.2.2. Specific objectives

- To detect KRAS gene mutations (codon 12 exon 1) in thyroid tumors using ASO – PCR technique.

- To correlate detection of KRAS gene mutation with histopathological diagnosis.

# *Chapter Two*

# *Literature Review*

# Chapter Two

# 2. Literature Review

## 2.1. Anatomy, physiology and histology of thyroid:

Thyroid is located in lower part of the neck at the level of fifth cervical vertebra to the first thoracic vertebra.It is composed of two conical pyramidal lobes joined by narrow bridge of glandular tissue called the isthmus, the weight varies approximately 20-30 grams.The thyroid gland in female enlarged slightly during menstruation and pregnancy. Normal thyroid gland has a butterfly shaped. Thyroid gland is major endocrine gland, it synthesis hormones which secreted in to bloodstream and then act as messengers to effect on body metabolisim, growth and development (Marshall, 2004).

The thyroid gland is composed of roughly spherical follicles, lined by low cubical to columnar epithelium and filled with thyroglobulin rich colloid. In response to TSH released by thyrotrophs in the anterior pituitary, the follicular epithelial cells of the thyroid pinocytize colloid and ultimately convert thyroglobulin into thyroxin (T4) and lesser amount of triiodothyronine (T3). T4 and T3 are released in to systemic circulation. (Robbin, 2002).

Thyroid is composed of acinar spaces with uniform size lined by cuboidal epithelium.the acini in normal gland filled with clear colloid material which take pink to red color by H&E stain. (Dey, 2013).

Thyroid gland has 2 capsules the outer is fascial sheath and the inner is firm thin fibro elastic capsule, trabeculae is very thin formed of C.T cells and fibers.the parenchyma of thyroid gland is formed of thyroid follicles which are the structural units of thyroid gland lined with simple cuboidal cells and surrounded with very thin basement membrane.it has uniform shape in young and irregular shape in adult. Each thyroid follicle compose of principal follicular cells and parafollicular(Clear or C) cells. Thyroid tissue is composed of 20 -30 million microscopic spheres called thyroid follicle which lined by simple epithelium and the central cavity contain gelatinous substance (is compose of glycoprotein with high molecular mass 660KDa called thyroglobulin). (Marshall, 2004).

## 2.2. Pathological Condition of thyroid gland:

### 2.2.1. Benign Condition of thyroid gland:

#### 2.2.1.1. Multi nodular goiter:

Goiter is simple enlargement and most common thyroid gland disease. The incidence of goiter reflects to impaired synthesis of thyroid hormone and associated with hyperthyroidism, it caused by iodine deficiency, induction of thyroid cell hyperplasia and mutation in TSH receptor. is more common in female than in male, which increased incidence in puberty due to increased physiologic demand of thyroxin also may result from hereditary enzymatic defects that interfere with thyroid hormone synthesis. Follicles are lined by crowded columnar cells.Microscopically the follicular epithelium may be hyperplasic in early stages of disease which lined by crowded columnar cells with abundant Colloid. It may be uninodular goiter or multinodular or diffuse goiter, the changes in older lesions includes areas of fibrosis, hemorrhage, calcification, and cystic change.(Frilling,2004).

### 2.2.2. Inflammation of thyroid gland:

#### 2.2.2.1. Chronic lymphocytic (Hashimoto thyroiditis):

Hashimoto thyroditis is the most common its autoimmune inflammatory disorder it caused by defect in T cells which lead to hypothyroidism. of TSH,further contributing to hypothyroidism . Hashimoto disease is most prevalent between 45 and 65 years of age, and it's more common in females than males. Hashimoto disease is increased risk for the development of B cell non Hodgkin lymphoma with in thyroid. (Dey,2013 ).

#### 2.2.2.2. Sub acute granulomatous:

Sub acute granulomatous thyroiditis, also known as De quervain thyroiditis is less common than Hashimoto disease.it's most common between the ages of 30 and 50 years, like other of thyroiditis occurs more in women than in men. It trigger by viral infection. (Dey,2013 ).

#### 2.2.2.3. Sub acute lymphocytic thyroiditis:

Sub acute lymphocytic thyroiditis is known as silent or painless thyroiditis .The disease is most likely autoimmune in etiology, .It most affects middle age women. Caused by autoimmune response which damage thyroid gland lead to hyperthyroidism then hypothyroidism. (Dey, 2013).

**2.2.2.4. Other form of thyroiditis:**

**2.2.2.4.1. Riedel thyroiditis:**

A rare disorder of unknown etiology, is characterized by extensive fibrosis involving the thyroid and contiguous neck structures .The presence of hard and fixed thyroid mass clinically simulates a thyroid neoplasm .It may be associated with idiopathic fibrosis in other sites in the body such as the retro peritoneum. (Robbin, 2002).

**2.2.2.4.2. Palpation thyroiditis:**

Caused by vigorous clinical palpation of the thyroid gland, results in multifocal follicular disruption associated with chronic inflammatory cells and occasional giant cell formation. (Robbin, 2002).

**2.2.3. Malignant thyroid tumors:**

**2.2.3.1. Adenomas:**

Adenomas of thyroid are benign neoplasms derived from follicular epithelium. Usually are solitary. Clinically and morphologically may difficult to distinguish from hyperplasic nodules from the less common follicular carcinomas. Most adenomas are appear as gray-white to red-brown,spherical lesion that compresses the adjacent non neoplastic thyroid. Microscopically the cells are arranged in uniform follicles that contain colloid. The neoplastic cells has uniform, with well defined cell border. Occasionally the cells are brightly eosinophilic granular cytoplasm (oxyphil or Hurtle cell change). A bout10% of adenoma nodule prove to be malignant. (Robbin, 2002).

**2.2.3.2. Carcinomas:**

Most thyroid carcinomas are derived from the follicular epithelium except medullary carcinomas derived from parafollicular or C cells.Both genetic and environmental factors are play role in the pathogenesis of thyroid cancers. (Robbin, 2002).

The environmental factors like exposure to ionizing radiation is one of the most important factors to the development of thyroid cancer.The incidence of carcinoma of the thyroid is higher among atomic bomb survivors in Japan

and in those exposed to ionizing radiation after the Chernobyl nuclear plant disaster. The majority of cancers arising are papillary cancers and most have RET gene rearrangements. Also long standing of multinodular goiter has been suggested as developing to cancer in some cases, areas with iodine deficiency related endemic goiter have a higher prevalence of follicular carcinomas also race (white and asian people more develop of thyroid cancer) and breast cancer at young age have high risk of thyroid cancer. (Robbin ,2002).

**2.2.3.2.1. Papillary thyroid carcinoma:**

Papillary carcinomas are the most common form of well differentiated thyroid cancer represent as 80% of all cases. It occur at any age and the majority of papillary thyroid carcinomas associated with previous exposure to ionizing radiation. The lesions contain areas of fibrosis and calcification and are often cystic, appear as granular and sometimes contain grossly discernible papillary foci. diagnosis of papillary carcinoma is based on nuclear features(the nuclei of papillary carcinoma cells contain very finely condensed chromatin which appearance ground glass nuclei,the cytoplasm may contain intranuclear inclusions (pasmmoma bodies). Papillary thyroid cancer is slow growing but can spread via lymphatic within thyroid to lymph nodes. (Lam, 2005).

**2.2.3.2.2. Follicular thyroid carcinoma:**

Follicular carcinoma is the second form of thyroid cancer repsented as15% of all cases, usually present at older age. The incidence of follicular carcinoma is increased in areas of dietary iodine deficiency. There is no evidence that follicular carcinomas arise from preexisting adenomas. Microscopically most follicular carcinomas are composed of fairly uniform cells forming small follicles,extensive invasion of adjacent thyroid parenchyma makes the diagnosis of carcinoma obvious in some cases.The lesion may require extensive histologic sampling before they can be distinguished from follicular adenomas. Follicular carcinomas present as solitary thyroid nodules. Can metastasize through the bloodstream to the lung, bone and liver.(Robbin ,2002).

**2.2.3.2.3. Medullary thyroid carcinoma:**

Medullary carcinoma of the thyroid is derived from the parafollicular cells or C cells of thyroid .represent as 5% - 8% of all cases.Microscopically medullary carcinomas are composed of polygonal to spindle shape cells which may form nests, trabeculae and even follicles with cellular amyloid deposits derived from altered calcitonin molecules present in the adjacent stroma.can spread by lymphatic to regional lymph node in the neck. It may be familial arises as part of multiple endocrine neoplasia syndrome type 2A or 2B which important prognostic factors. (Kloos, 2009).

**2.2.3.2.4. Anaplastic thyroid carcinoma:**

Anaplastic carcinoma of the thyroid the most aggressive thyroid tumor, affect more women than men.it arises from follicular cell. Occur predominantly in elderly patients (55-65 years) particularly in areas of endemic goiter. Morphologically anaplastic carcinomas found as bulky masses that grow rapidly from thyroid capsule into adjacent neck structures .Microscopically the neoplasm compose of high anaplastic cells with pleomorphism, gaint , spindle ,squamous and hurthle cell types are seen.the prevelance very low about 2% of all thyroid tumors.(Neff,2008).

**2.3. Diagnosis of thyroid tumor:**

**2.3.1. Blood examination:**

There are several types of blood tests that may be done in diagnosis and monitor the patient during and after treatment,this includes: thyroid hormone levels (T3, T4, thyroglobulin and TSH) and thyroid tumor marker (carcinoemberyonic antigen, calcitonin, rational emotive therapy). (Willamson, 1994).

**2.3.2.Ultrasound scan:**

Real time ultrasound scanning with high resolution transducer probe to show size and other information about nodules. ( Willamson, 1994).

### 2.3.3. Thyroid biopsy:

This done by 1 of 2 ways as fine needle aspiration or surgical biopsy. (Willamson, 1994).

### 2.4. Signs and symptos of thyroid tumor:

The most common symptoms and signs of thyroid tumors as lump in the front of the neck near the Adam's apple , hoarseness or problem speaking with normal voice, swollen gland in the neck , difficulty breathing , Pain in the throat or neck. (Willamson, 1994).

### 2.5. Treatment of thyroid tumor:

Thyroid tumor treated by one or combination treatment according to type and stage of thyroid tumor. (Willamson, 1994).

### 2.5.1. Surgery:

It's the main treatment for most people with thyroid tumor it depend on the size of tumor,rapidly effective and low incidence of recurrence but have complication like haemorrhage,liquefying haematoma, wound infection,air embolism and unsatisfactory scar. (Willamson, 1994).

### 2.5.2. Hormone treatment:

Patient who treated with surgery usually require thyroid hormone therapy. In addition to replacing the hormone that is needed, the most Thyroid hormone used is levothyroxine. (Willamson, 1994).

### 2.5.3. Radioactive iodine therapy:

The radiation therapy (I-131 or RAI) used to destroy thyroid cells not removed by surgery.it simply treatment and avoid risk of operation and long term of antithyroid drug therapy but the efficiency slow. (Willamson, 1994).\

### 2.6. RAS gene:

RAS gene is family of small G proteins with intrinsic GTPase activity that has important roles in different cellular signal transduction pathways. RAS traffic from cytosol to plasma membrane, where it resides and communicates external signals to the nucleus. The family of RAS genes is consisting of three functional genes H-ras, N-ras and K-ras which encodes to proteins highly molecular weight 21,000 KDa. The three genes in human are most common proto-oncogenes in human cancers , mutation of these genes lead to permanent activate RAS which found in 20% - 25% and sometimes up to 90% of human cancers .(Good ,1999).

RAS activates several pathways such as the mitogen activated protein kinase (MAPK) cascade, which transmits signals downstream and result in the transcription of genes involved in cell growth and division. Another RAS activated signaling pathway is PI3K/AKT/mTOR pathway which is intracellular signaling important in regulating to cell cycle, stimulates proteins synthesis and cellular growth and inhibits apoptosis. RAS is a G protein or guanosine nucleotide binding protein, it function as binary signaling switch "on" and "off" state. It bound to nucleotide guanosine diphosphate (GDP) in "off" state, while in "on" state it bound to guanosine triphosphate (GTP) which has extra phosphate group when compare with GDP. (Lodish, 2000).

#### 2.6.1. KRAS gene:

KRAS gene is proto oncogene located in short arm of chromosome 12(12p12.1). The gene is member of RAS family of small guanosine nucleotide binding proteins. The first identification as cellular transforming gene in the Kirsten rat sarcoma virus. Activating mutation of KRAS found in several human cancers like pancreas, lung, thyroid and colorectal. The frequency of KRAS mutation found in exon1 (codon 12 and 13) and exon 2(codon61), most mutation of KRAS in codon 12 and 13 associated with lack response to EGFR. (Whitehall, 2009).

The KRAS gene approximately 38 kb and encode to a 188 amino acid with molecular weight 21.6KDa. (Malumbres, 2003).

KRAS play important role in EGFR signaling network, it activate the signal pathway through the downstream effectors of the mitogen protein kinase(MAPK) pathway, which affect on cellular proliferation, adhesion, angiogenesis, migration and survival. The effect by blocking EGFR with monoclonal antibodies (cetuximab or panitumumab) inhibits all downstream effects of the receptor. When KRAS gene mutated the EGFR becomes ineffective. (Monzon, 2009).

Mutation found in oncogenic form of RAS p21protien impair GTPase activity and make KRAS protein unresponsive to GAP protein, mutated on p21 lead to exchange GDP for GTP.( Scheffzek, 1997)

The KRAS mutation can subdivided into transversions and transition both lead to exchange of amino acid glycine to another amino acid .A transition is replacement of one purine base to other or one pyrimidine base to other and transversion is replacement from purine to pyrimidine or vice versa.(Riely , 2008).

The allele mutation of KRAS most amino acid changed in condon 12 exon 1is from Gly to Asp, Ala ,Ar , Ser ,Val and Cys .( Clayton ,2000)

Activating mutation of KRAS gene impair the ability of KRAS protein to switch between active and inactive states lead to cell transformation and increased resistance to chemotherapy and biological therapy targeting in epidermal growth factor receptors.( Scheffzek,1997).

Detection of KRAS mutation in tissue diagnosed with cancer done by different methods such as DNA sequencing and real time PCR (polymerase chain reaction). DNA sequencing direct (Sanger) sequencing and pyrosequencing can used for KRAS mutation detection, but this time consuming and sensitivity low in Sanger method (gold standard technique) (20% of mutant allele is required for detection) while pyrosequencing is faster than Sanger with less amount of mutant allele required for mutation detection. Real time PCR the presence of KRAS mutation can detected either

by allele specific oligonucleotide (ASO) real time PCR amplification or by post PCR fluorescent melting curve analysis, both methods are closed PCR system, with it can reduce the risk of contamination. ASO available as commercial kits and can estimate to 1% mutant allele but it have disadvantage as reagent is high cost and it need more tissue amount for analysis compared with other methods. (Monzon, 2009).

RAS mutation frequency targeting the Korean population shows a 45.7% from follicular thyroid carcinomas and 35.7% in follicular thyroid adenomas. K-RAS codon 12-13 was most common RAS mutation in follicular thyroid carcinomas when compared with other RAS gene mutation (Jeong , 2015).

In other study the K- RAS 12-13 mutation was associated with a significantly lower carcinoma outcome 41.7% when compared with other RAS genes. The most common K-RAS 12 -13 mutation found in medullary thyroid carcinoma (Radkay , 2014).

# *Chapter Three*

# *Materials and Methods*

# CHAPTER THREE

# Materials and Methods

## 3.1. Study design:

This is a descriptive retrospective analytical case study, aimed to detect the frequency of KRAS gene mutation in thyroid tumors using molecular technique.

## 3.2. Study area:

The study was carried out at Omdurman Teaching Hospital and research laboratory sudan university of science and technology.

## 3.3. Study duration:

The study was conducted in period from September to December 2017.

## 3.4. Study samples:

The samples in this study were formalin fixed paraffin embedded tissues previously diagnosed as thyroid tumors.

## 3.5. Sample size:

Forty blocks previously diagnosed as thyroid tumors, they include thirty blocks were malignant and ten were benign tumors. the patients data was collected from patients files in hospital.

## 3.6. Ethical consideration:

Permission to carry out this study was taken from college of graduate studies in Sudan University of science and technology and from Omdurman teaching hospital (histopathology department).

## 3.7. Data collection:

Check lists were used to record all patients' data (age, gender and diagnosis) and also used check list to record the PCR results.

## 3.8. Sample processing:

The samples obtained from formalin fixed paraffin embedded tissue, the sections were cut using rotary microtome, the thickness for each sample was 10µm (3times) collected in screw capped Eppendrof tube (1.5ml). To avoid cross contamination each block was cut with new gloves and new disposable microtome knife.

## 3.9. Method of detection of KRAS gene mutation:

PCR technique (ASO method) is used to detect KRAS gene mutation in thyroid tumors in both malignant and benign tissues.

## 3.10. DNA extraction from FFPE tissue:

About 10µm(3times) was cut from each blocks using rotary microtome ,remove to excess paraffin to avoid contamination and collected in to labeled Eppendrof tube (1.5ml).

### 3.10.1. Removal of paraffin:

Added 1ml xylene and centrifuged at 14,000rpm for 10 minutes, aspirated supernatant and repeated addition of xylene then aspirated supernatant. Added 1ml 100% ethanol and centrifugated at 14,000rpm for 10 minutes aspirated supernatant and repeated addition of 100% ethanol then aspirated supernatant. Added 1ml of 80% ethanol and centrifugated at 14,000rpm for 10 minutes, aspirated supernatant and repeated addition of 80% ethanol then aspirated supernatant. Added 50% ethanol and centrifugated at 14,000rpm for 10 minutes, aspirated supernatant and repeated addition of 50% ethanol then aspirated supernatant. Added 1ml of H2o and incubated at 4°C overnight.

### 3.10.2. Digestion of protein:

Centrifuged sample at 14,000rpm for 10 minutes then aspirated supernatant, added 700ul of Nucleic Acid Lysis Buffer (NALB) + 50ul of proteinase K and incubated for 24 hours at 56°C. Added additional 50ul of proteinase K and incubated for additional 24 hours at 56°C.

### 3.10.3. Precipitation and isolation of DNA:

Centrifuged sample at 14,000rpm for 10 minutes then aspirated supernatant, added 250ul of 6M NaCl(saturated) at room temperature for 10 minutes then Pelleted sample at 14,000rpm for 10 minutes, aspirated supernatant , carefully transfer supernatant to clean Eppendrof tube , added 1ml of ice cold (-20°C) of 100% ethanol, well mix carefully and incubated at -20°C for 20 minutes, then Pelleted sample at 14,000rpm for 10 minutes, carefully discarded the supernatant , washed the pellet with 1.5 ml of 70% ethanol then Pelleted sample at 14,000rpm for 10 minutes , carefully discharged the supernatant and allow the pellet to air dry for 15 minutes. Finally added 80ul of TE buffer.

## 3.11. KRAS gene mutation detection by PCR:

In this study examined to genomic DNA from benign and malignant formalin fixed paraffin embedded tissue from thyroid tissue which amplified by polymerase chain reaction using allele specific oligonucleotide technique to screened for KRAS gene mutation in codon 12 exon 1 in both types of tissues, the PCR product size 95bp, using 3 specific primers sequences which synthesized by macrogen as:

**Mutant (M) F: 5´ ACTTGTGGTAGTTGGAGCTG 3´**

**Normal (N) F: 5´ACTTGTGGTAGTTGGAGCTGG 3´**

**Common(C) R: 5´ CTATTGTTGGATCATATTCGTCC 3´**

To ensured a positive mutation result and avoided false positive result was used common or wildtype of KRAS sequence as control.

Each sample was detected two times used the tube of master mix (0.2ml) which contain magnesium chloride, iTaq polymerase and dNTP mixture .added 0.5 µl from mutant(F) primer, common (R) 0.5µl, 2µl from genomic DNA isolated and 25µl of DW.for the same sample instead used of mutant(F) primer we used normal(F) primer . The product size of PCR about 95 bp in length. The PCR cycles in thermo cycler 35 cycles, each cycle was begin by a denaturation step at 94°C for one minutes, the second step an annealing at 55°c for one minutes and the third step extension at 72°C for 30second.

In electrophoresis detection firstly prepared 1x TBE buffer, 2% agarose gel then added 1.5 µl from ethidium bromide. Then added 5µl of PCR product in to well of gel electrophoresis and wait for 20 minutes, then readed with ultra violet system to detect presence of KRAS gene mutation band.

## 3.12. Statistical analysis:

The data was analyzed using SPSS (version 11.5) computer program to calculate the frequencies, mean and chi-square values.

# *Chapter four*

# *Results*

# Chapter Four

## 4. Results

A total of 40 paraffin blocks previously diagnosed as thyroid tumors were collected in this study. A cording to histopathological diagnosis thirty patients were classified as malignant tumors and ten patients are benign tumors. All tissues samples in this study were screened for KRAS gene mutation (codon 12 exon 1) using Allele Specific Oligonucleotide (ASO) - PCR technique.

Figure (4.1) show the frequency of gender among study population revealed that as males 12(30%) and females 28(70%).

The distribution of study population according to the age which divided into two group , group 1 less than 50 years 33(82.5%) and group 2 more than 50 years 7(17.5%) show in table(4.1).

In table (4.2) The distribution of study sample according to the histopathological diagnosis founded as papillary thyroid carcinomas (PTC) 16 (40%), follicular carcinomas(FTC) 9 (22.5%) ,Medullary Thyroid Carcinomas(MTC) 3 (7.5%), undifferentiated thyroid carcinomas 2 (5.0%) ,while in benign tumors showed 8(20.0%) from multi nodular goiter (MNG) , 1(2.5%) of hashimoto thyroiditis and 1(2.5%) of follicular adenomas.

According to distribution of KRAS gene (normal & mutant) among study population, the normal KRAS gene show 15(37.5%) was negative and 25(62.5%) positive. while mutant KRAS gene show 31(77.5%) negative and 9(22.5%) was positive showed in table(4.3).

The relation between KRAS gene mutations and histopathological diagnosis founded in benign 3 positive and 7 negative while in malignant tumors 6 positive and 24 negative showed in table (4.4). P.value (0.512)

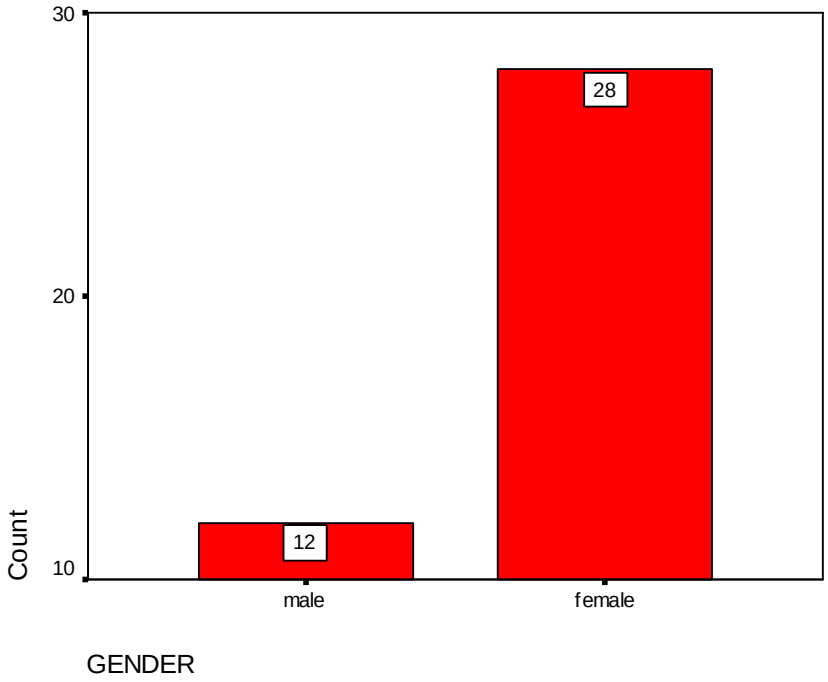



Figure (4.1) Frequency of gender among study population.

Table (4.1) Frequency of age group among study population.

| **Age group** | **Frequency** | **Percent** |
|---|---|---|
| ≥ 50 years | 33 | 82.5 |
| < 50 years | 7 | 17.5 |
| **Total** | 40 | 100 |

Table (4.2) Distribution of histopathological diagnosis among study sample

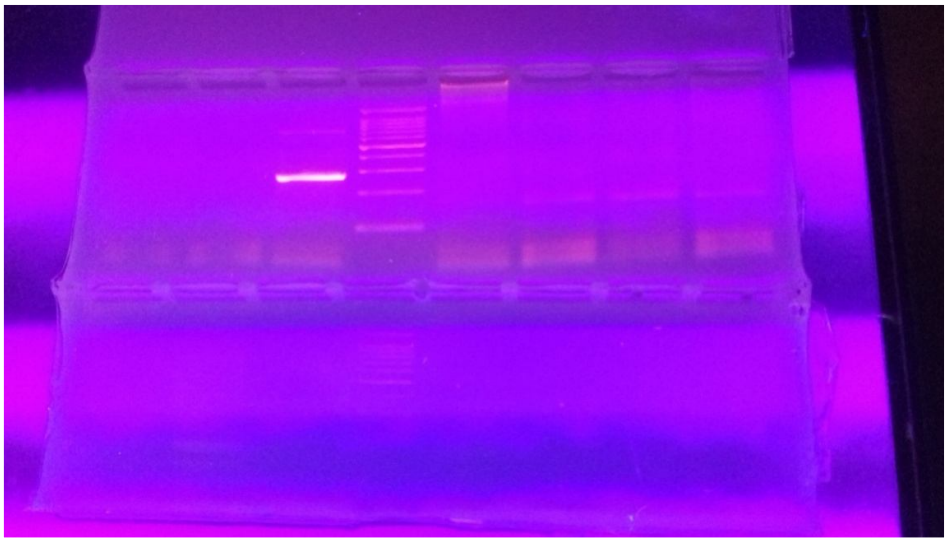
| **Diagnosis** | **Frequency** | **Percent** |
|---|---|---|
| Multi nodular goiter | 8 | 20 |
| Hashimoto thyroiditis | 1 | 2.5 |
| Follicular adenoma | 1 | 2.5 |
| Papillary thyroid carcinoma | 16 | 40 |
| Follicular thyroid carcinoma | 9 | 22.5 |
| Medullary thyroid carcinoma | 3 | 7.5 |
| Undifferentiated thyroid carcinoma | 2 | 5 |
| **Total** | 40 | 100 |

Table (4.3) Distribution of KRAS gene codon 12 exon 1 among study sample

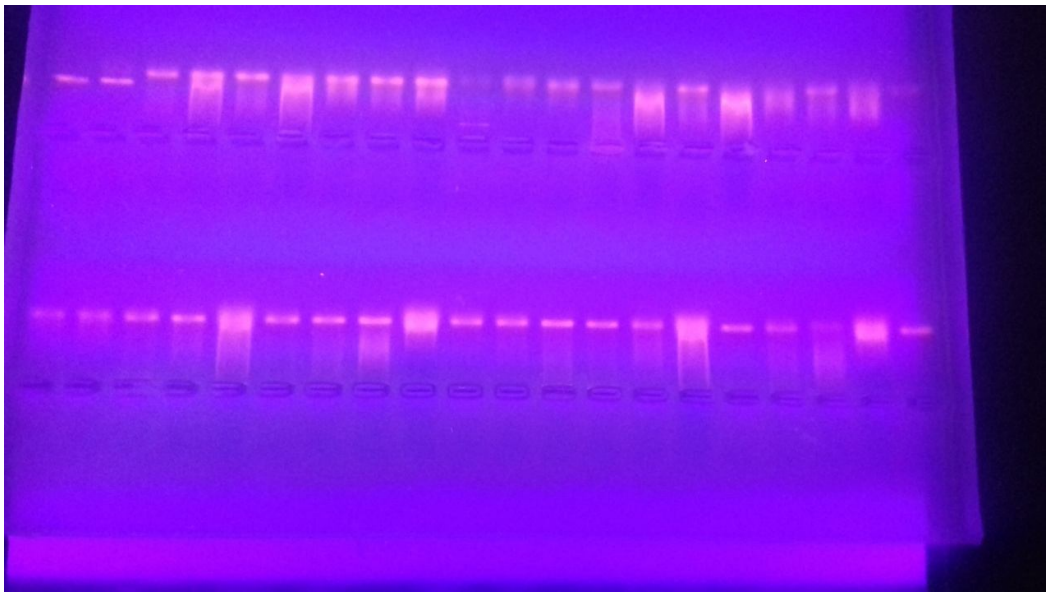
| KRAS gene | | Frequency | Percent |
|---|---|---|---|
| **Normal gene** | Positive | 25 | 62.5 |
| | Negative | 15 | 37.5 |
| **Mutant gene** | Positive | 9 | 22.5 |
| | Negative | 31 | 77.5 |

Table (4.4) Relation between KRAS gene codon 12 exon 1 mutation and histopathological diagnosis.

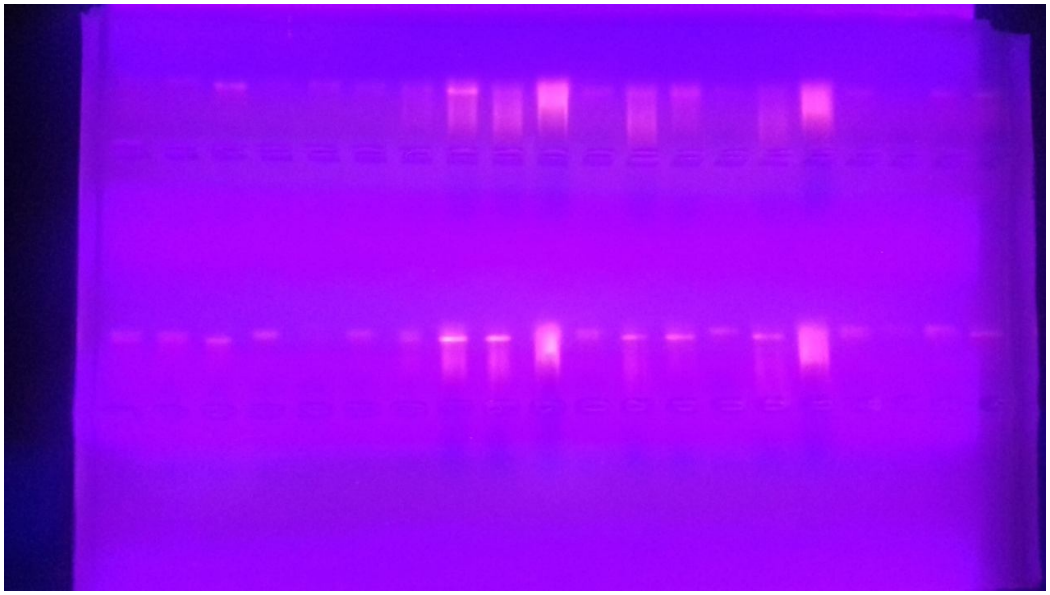
| **Diagnosis** | **KRAS gene mutation** | | **Total** | **P. value** |
|---|---|---|---|---|
| | **Positive** | **Negative** | | 0.512 |
| **Benign** | 3 | 7 | 10 | |
| **Malignant** | 6 | 24 | 30 | |
| **Total** | 9 | 31 | 40 | |



Microphotograph (4.1) negative result of KRAS gene codon 12 exon 1 mutation. 1. Ladder (100Pb) and 2,3,4,5 negative mutant KRAS gene.



Microphotograph (4.2) positive results of normal KRAS gene mutation codon 12 exon1.



Microphotograph (4.3) positive results of mutant KRAS gene mutation codon 12 exon 1.

# *Chapter Five*

# *Discussion , conclusion and recommendation*

# Chapter Five

# 5. Discussion, conclusion and recommendation

## 5.1. Discussion:

A total sample of 40 formalin fixed paraffin embedded blocks were previously diagnosed as thyroid tumor which include 30 samples diagnosed as thyroid cancer and 10 samples with benign thyroid tumors.

Most of patients affected with thyroid tumors under 50 years old according to my study. Teunvall (1986) represented the incidence of thyroid cancer is higher in patients under 40 years of age. Kauffmann (2018) paitents age $\geq$ 60 years have worse after diagnosis with papillary thyroid cancer cross all stages of disease. Zirilli(2018) founded patients younger than 30 years have risk to increase developing of differentiated thyroid cancer in last 3$^{rd}$ decade . is more sever in children younger than 18 years .

According to distribuation of gender in study samples represented females more affected than males. Mazzaferri(1994) thyroid cancer affects women more commonly than men.

According to histopathological diagnosis presented as papillary thyroid cancer most common then follicular thyroid cancer then medullary thyroid cancer and then undifferentiated thyroid cancer while in benign tumor multi nodular goiter most common then follicular adenoma and then hashimoto thyroiditis all among samples study. Hanumanthappa(2012) founded the prevelance of thyroid malignancy was papillary thyroid cancer followed by follicular thyroid cancer .

According to frequency of KRAS gene mutation codon12 exon 1 and histopathological diagonosis represented as in benign tumor most of them in multi nodular goiter and in malignant tumor most of them in papillary thyroid cancer. P.value (0.512) the KRAS gene mutation codon 12 exon 1 doesn't play role to cause thyroid tumor. Schulten(2013) RAS mutation commonly associated with follicular thyroid lesions and new discovered of KRAS codon 59 . Radkay(2014) done study for RAS gene mutation in thyroid gland result in activation of signaling patway and associated with follicular growth and probability of carcinoma outcome ,the KRAS gene 12\13 mutation was associated with low carcinoma outcome when compared with other RAS genes, medullary thyroid carcinoma is most presented. Jeong(2015) investigate frequency RAS mutation for follicular thyroid adenoma and

follicular thyroid carcinoma in korean population show KRAS codon 12 -13 as 6 (31.6) and in codon 61 as 3(15.8). KRAS gene codon 12 -13 was most common RAS mutation in follicular thyroid carcinoma. Clinkcales(2017) RAS mutation may role in malignancy of thyroid nodules.

The present study showed female under 50 years were most affected with thyroid tumors and papillary thyroid carcinoma were most common in Sudanese patients.

Most of KRAS mutation codon 12 exon1 presented in samples diagnosed as papillary thyroid cancer and multi nodular goiter.

## 5.2. Conclusion:

According to this study we concluded that:

- The patients age under 50 years were most affected with thyroid tumors.
- The female were most common affected with thyroid tumors.
- The papillary thyroid carcinoma were most common in Sudanese patients..
- The most of KRAS gene mutation codon 12 exon1 presented in samples diagnosed as papillary thyroid cancer and multi nodular goiter.

### 5.3. Recommendation

On basis to this study we recommended that:

- Further study with large sample size should be done.
- Detection for all RAS gene mutation (KRAS, NRAS and HRAS) in codon 12, 13 and 61 in exon 1 and 2 in thyroid tumors.
- Sequencing methods should be used to detect KRAS gene mutation.

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# *Appendices*

# Appendices

**Appendix I:**

Material and instruments used polymerase chain reaction (PCR):

● **Instruments:**

Micro centrifuge.

Eppendrof tubes.

Pipettes.

Vortex mixer.

Permanent marker.

Disposaple gloves.

Rotary microyome .

Disposable knife.

Incubater(56°C) .

Refrigerator(-20 °C and 4°C).

● **Materail:**

Xylene .

Ethyl alcohol (100%, 80%, 50%, 70%).

DNA lysis buffer:

Nacl: 3.24 g.

EDTA: 0.075 g.

SDS: 0.7 g.

Tris base: 0.121 g.

D.W: 100 ml.

PH: 8.0.

Commercial protienase K.

6M Nacl (saturated):

Nacl: 17.5 g.

D.W: 500ml.

10 x (TBE) buffer:

Tris base: 2.695 g

Boric acid: 1.376 g.

EDTA: 0.186 g.

D.W:250ml.

PH: 8.3.

1 x buffer (running buffer):

10 x (TBE) buffer: 10 ml.

D.W: 90 ml.

**Appendix II:**

**Kit's leaflets**