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RESEARCH PAPER

Molecular Detection of Cytomegalo, CDK2 and P27 in Tissues from Patients with Thyroid Carcinoma

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Abstract

Background: Thyroid cancers are related to many environmental and genetic predisposing factors. Cytomegalovirus infection may activate the protein kinase pathways, where in papillary thyroid cancer aberrant activation of these pathways is frequently associated with BRAF mutation. The bulk of thyroid cancers has maintained the expression of p27 which is the inhibitor of Cdk2.**Objective:** The study was designed to examine cellular dysregulation mediated by the concordant protein expressions of CDK2 and P27 tumor suppressor genes with CMV in tissues from thyroid goiter and thyroid cancers. **Patients and Methods:** A seventy thyroid tissues were examined for HCMV-DNA and CDK2 & P27 genes expression. Those samples belonged to (30) patients diagnosed with thyroid cancer and (25) thyroid goiter tissues blocks as well as (15) autopsies from apparently normal thyroid tissues. The detection of HCMV was by in situ hybridization (ISH) whereas CDK2 & P27 genes expression by immunohistochemistry (IHC). **Results:** HCMV DNA-ISH reaction in thyroid cancers was found in 14 / 30 (46.7%), whereas 32% (8 out of 25cases) HCMV- positivity was detected in thyroid goiter group. None of healthy thyroid tissues revealed ISH reaction. The difference of the HCMV in thyroid cancers and control was highly significant. Detection of CDK2 in thyroid cancer was found in 13 out of 30 (43.3%) and benign thyroid goiters group was 28 % (7 out of 25). Detection of p27- expressions in thyroid cancers in 18 out of 30 (60%) was noticed; In thyroid goiter was detected in 36% (9 out of 25) and none of the examined healthy thyroid in the control group revealed such IHC- reactions for both CDK2 &P27 IHC reactions. The difference between the percentages of CDK2- as well as P27 proteins detection in thyroid tissues & control group was statistically significant (<0.05).**Conclusions:** The significant detection of HCMV along with CDK2 & P27genes expression in thyroid cancer patients could support an etiologic role for that virus along with these genes in thyroid carcinogenesis.

Keywords: *Thyroid Cancer, HCMV, ISH, IHC, CDK2&P27.*

Introduction

Thyroid cancers account for up to 2.5% of all cancers [1,2] with an incidence rising faster than all these cancers, the Increase in incidence as well as mortality of thyroid cancers point for additional factors behind this increase [3]. Majority of thyroid cancers develop from follicular epithelial cells and are divided grossly into two groups; well differentiated thyroid cancers, include the usually slowly growing papillary cancers (account for 80%) and follicular cancers (account for 5-15%)[4]. In contrast, anaplastic thyroid cancers (ATC), the poorly differentiated thyroid cancers, comprise only 1-2% of all thyroid malignancies [5]. In addition, approximately 5% of all thyroid cancers are diagnosed as medullary carcinoma, originating from para- follicular C cells [6].

From 2000 to 2009, increased rates among men for 6 cancers were reported (kidney, pancreas, liver, thyroid, skin melanoma, myeloma) while in women, increased rates have found for 7 cancers (thyroid, melanoma, kidney, pancreas, leukemia, liver, uterus). The rates of palpable thyroid nodules was reported to be approximately 5% in females while 1% in males worldwide [7]. However, ultrasound investigations have detected nodules in thyroid among 19%–68% of individuals, and yet higher in females and elderly [8]. Thyroid nodules should not be regarded as cancers of thyroid, occurring in

7%–15% of cases for factors such as age, sex, radiation, familial factor. etc. [9,10]. Differentiated thyroid cancers are the majority of all thyroid cancers [11].

Human cytomegalovirus (HCMV) is responsible for a lifelong persistent infection which ranged from 50% to 90% in adult population, and is related to either socioeconomic status or geographic location. On serological and molecular basis, Human cytomegalovirus is also associated, and assumed to be responsible for the development or etiology of a range of human cancers [12] where the detection of viral DNA, mRNA, and/ or its antigens in the tissues have led to hypothesis of on comodulation, which could catalyzed an oncogenic processes, resulting in a more malignant phenotype. In addition, HCMV infection might lead to buildup tumor cells via the protection of certain tumor cells from apoptosis and modulating angiogenesis [13].

Many findings indicated that thyroid gland is a reservoir for latency of CMV, where in a small numbers of cases, human CMV was the only one found in thyroid tumors [14] and in another study was found in 3 out of 8 thyroid autopsies [15]. In 1970s–1980s, Cyclin-dependent kinases were first identified to be involved in cell cycle regulation [16] and are associated with regulatory subunits, the cyclins family (periodically expressed and degraded), to form functional heterodimeric complexes [17].

Action of cdk 2 is inhibited by Cip/Kip family members, including p21Cip1, p27 Kip1 and p57 Kip2. The absence of free Cip/Kip proteins is necessary for cyclin E-cdk2 activation and progression through G1 phase. Cyclin Ecdk2, in addition, phosphorylates p27Kip1, targeting it for degradation [18, 19].

The P27 is a CDK-inhibitor and belongs to the Cip/Kip proteins playing an important role finking extracellular regulatory growth signals to progress or exit from cellular cycle [20].In S – phase, P27 Kip is phosphorylated by cyclin E-CDK2 then recognized to be a target for ubiquitination by SCFKip2(S-phase kinase – associated protein2 [21].The P27 expression was found to decrease during tumor development and progression [22].The p27kip1 levels was reported to be reduced or absent in 50% of cancers [23,24]. A Sequestration of P27 protein was recognized as a mechanism of cancers to overcome p27-inhibition. These findings have been noticed among colonic [25], esophageal, [26], thyroid, [27], ovarian, (28], and breast cancers [29-31]. The p27kip1 is a marker of shorter disease-free and/or survival in patients with thyroid cancers.[24 ,31]. This study is aiming to analyze the rate of concordance of CDK2&P27-gene translational expression as well as HCMV in thyroid tissues from a group of patients with malignant thyroid tumors.

Materials and Methods

The study has 70 selected FFPE thyroid tissues blocks; which included (30) malignant thyroid tumor divided into grade I,II & III, ; [25] thyroid goiter tissues as and (15) autopsies from healthy thyroid autopsies used as control groups .Cases were choice for testing depended on 2 featuers: (i) found the sufficient component for testing and (ii) re-diagnostic by two consultant pathology that investigate the cases contained at least 30% malignant tumor cells (cancer cases).

In one hand, the detection of HCMV by ISH kit (Zyto Vision GmbH. Fischkai, Bremerhaven. Germany) was performed on 4µm paraffin embedded tissue sections using digoxigenin-labeled oligo-nucleotides probe which targets Human cytomegalovirus DNA. One section was mounted on ordinary glass slide and stained with hematoxyline and eosin, while another slide was mounted on charged slide to be used for ISH for detection of HCMV.

Immunohistochemistry/ Detection system (Abcam . England) was used to demonstrate the CDK2 and P27 tumor suppressor genes. Chi –square test was used to detect the significance between variables of our study. All the statistical analyses was done by SPSS program (Version–21)&P value was considered significant when $p < 0.05$.

Result

Distribution of patients with thyroid cancers according to their Age

Female patients with thyroid cancers have mean age of (53.4+ 6.8 years) ,while the mean age of those who have thyroid nodular goiter was (51.6 + 5.9 years). Lastly, the mean age of those who have apparently healthy thyroid tissues was (65.7+ 6.1years) (Table 1).

Table 1: Studied groups according to the mean age

Study Groups	N	Mean (years)	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Malignant thyroid nodular goiter	30 25	53.4 51.6	6.8 5.9	1.3 1.6	47.9 48.9	58.2 60.2	41.00 43.00	72.00 70.00
Control	15	65.7	6.1	1.9	57.4	62.7	47.00	72.00
Total	70	55.6	9.3	1.5	52.7	58.4	38.00	76.00

	Statistical Analysis						
	Independent Samples Test:					95% CI for Difference	
	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Age	-2.967	42	.002	-9.00000	3.97665	-14.76444	-3.54332

Histopathological Grading of Papillary Thyroid Carcinomas

Table (2) shows the results of grading of papillary thyroid cancers. The rate of well differentiated grade was 56.7% (17 / 30 cases) and moderate and poor grades ranked 26.7% and 16.6% (5 / 30 cases), respectively. Statistically, analysis revealed significant differences when well grade compared to poorly and moderate grade differentiated thyroid carcinomas.

Table 2: Tumor grading of thyroid cancers group

Thyroid Cancer Grades (Differentiated)	N	%
Well *	**17**	**56.7**
Moderately	**8**	**26.7**
Poorly	**5**	**16.6**
Total	**30**	**100.0**
Thyroid Cancer Grades(Differentiated)	**N**	**%**
Well *	17	56.7
Moderately	8	26.7
Poorly	5	16.6
Total	30	100.0

***Significant differences when well grade compared to poorly and moderate grade**

HCMV -Associated Thyroid Tumors

The signals of HCMV In situ hybridization reactions were detected as blue discoloration that was detected by their specific probes (Figure 1). Table (3) shows the positive results of HCMV-ISH reactions, where 46.7% (14 of total 30) thyroid cancers showed positive signals while 32% (8 out of 25cases) HCMV positivity was detected in thyroid nodular goiter group. None of healthy thyroid tissues revealed positive for HCMV-ISH reaction. The statistical analysis shows significant differences among patient and control groups (p =0 .117).

Table 3: Frequency of HCMV in situ hybridization reactions among the study groups

	Study Groups		HCMV-ISH		Total
			Positive	Negative	
Type	Thyroid cancer	Count	14	16	30
		% within Type	46.7%	53.3%	100.0%
	thyroid nodular goiter	Count % within Type	8 32%	17 68%	25 100%
		Control count	0	15	15
		% within Type	00.0%	100%	100.0%
Total		Count	22	48	70
		% within Type	31.4%	68.6%	100.0%

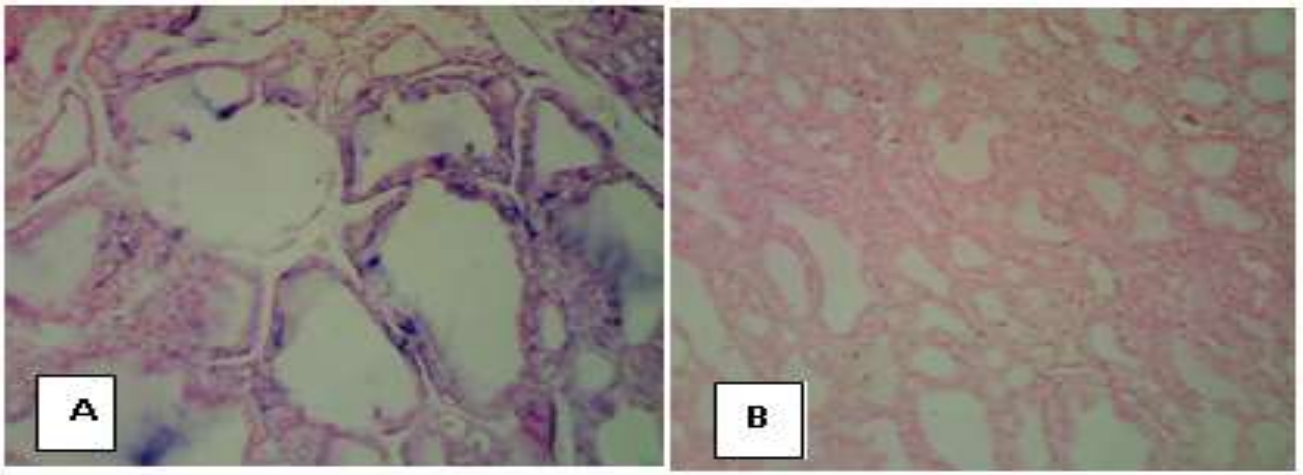


Figure 1: Infiltrative Thyroid Cancers Tissues Processed For (ISH) for HCMV Detection: B- Negative HCMV – reactions (20X) A-Positive HCMV -Ireaction (40X).

Correlation of HCMV Infection with Age of Patients

There was no statistical significant difference between the frequencies of HCMV- ISH reactions according to the age of the study groups (Table 4).

Table 4: Frequency of HCMV in situ hybridization reactions according to the age of the study groups

	HCMV	N	Mean (Year)	Std. Deviation	Std. Error Mean
Age malignant	**Positive**	**14**	**49.7666**	**7.78699**	**2.96656**
	Negative	**16**	**51.8765**	**8.88799**	**1.56711**
Age thyroid nodular goiter	**Positive**	**8**	**50.5675**	**7.64436**	**2.33689**
	Negative	**17**	**52.5438**	**8.22456**	**1.21678**
	HCMV	**N**	**Mean (Year)**	**Std. Deviation**	**Std. Error Mean**
Age malignant	Positive	14	49.7666	7.78699	2.96656
	Negative	16	51.8765	8.88799	1.56711
Age thyroid nodular goiter	Positive	8	50.5675	7.64436	2.33689
	Negative	17	52.5438	8.22456	1.21678

Correlation of HCMV Infection with Grading of Thyroid Carcinoma

On matching each score (from 1 to 3) with each grade (from well to poor) it was found that fourteen cases out of 30 have showed positive HCMV-ISH reaction. It was found that the percentage of HCMV-ISH test reactions in thyroid cancer tissues with poorly grades constituted (60%) followed by moderate grade (50.0%) and well grade (35.3%)(Table 5).. The statistical analysis according to the grading distribution HCMV – ISH reactions in breast carcinoma reveals no significant differences.

Frequency Distribution of Immune Histo Chemistry Results of CDK2 Protein expression Among Study Groups

The signals of immunohistochemical reactions for CDK-2antigen with their specific primary antibodies were observed as brown discoloration at the specific antigenic sites of these reactions (Figure 2).

The positive- signal of CDK-2-immunohistochemical reactions were found in 43.3% % (13 out of total 30) thyroid cancers ,while in thyroid nodular goiter group was detected in 28 % (7 out of 25). Lastly, no tissue in the control group has showed such IHC signals. The l Chi-Square analysis shows significant difference between the patients and control groups (<0.05) Table 6.

Table 5: Correlation of HCMV infection with grading of thyroid carcinoma

HCMV infection		Thyroid Cancer Grading						P
		Well differentiated (n=17)		Moderately differentiated (n=8)		Poorly differentiated (n=5)		
		N	%	N	%	N	%	
Negative		11/17	64.7	4/8	50.0	2/5	40.0	0.62 [N.S]
Positive		6/17	35.3	4/8	50.0	3/5	60.0	
Scoring	I	3/6	50.0	2 /4	50.0	1/3	33.3	
	II	2/6	33.3	1/4	25.0	2/3	66.7	
	III	1/6	16.7	1/4	25.0	0.0	0.00	
Mean rank		72.1		61.7		66.5		

Correlation of CDK2 Protein Expression with Grading of Thyroid Carcinoma

Table (7) illustrated the correlation of infection with CDK2-IHC reactions with oral thyroid cancer grading. It was found that the percentage of P27-IHC test reactions in thyroid cancer tissues with moderate grades constituted (62.5%) followed by poorly grade (40.0%) and well grade (35.3%).

The statistical analysis according to the grading distribution HCMV – ISH reactions in breast carcinoma shows non-significant differences (Table 5).

Statistically, no significant correlations between LMP1 infection and the oral squamous cell cancer grade p- value = 0.310**.**

Evaluation of Signal Positive P27-IHC Reactions

The p27 gene expression by IHC was detected in tissue blocks obtained from patients with thyroid tumors. The signals of p27 - IHC were detected as brown discoloration at the specific antigenic sites of these reactions (Figure 3).

Table 8 illustrated the positive results of P27 -IHC detection, where 60% (18 out of 30 cases) malignant thyroid tumors found that positive signals.

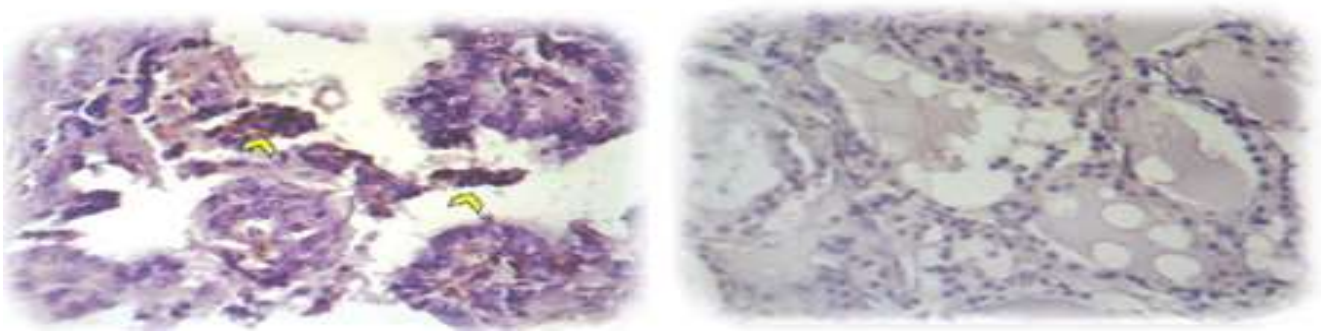
The thyroid nodular goiter group revealed 36% positive signals which represented 9 out of 25 cases in this group. Lastly, none in the healthy control group revealed positive signals. Statistically, highly significant differences p-value >0.001were found on comparing the results of these study groups.

Correlation of P27 Protein Expression with Grading of Thyroid Carcinoma

On matching each score (from 1 to 3) with each grade (from well to poor) it was found that eighteen cases out of 30 have showed positive p27-IHC reaction. Well grade showed (70.4%) positivity, poor grade (60.0%) and moderate grade (37.5%). The statistical analysis did not show significant differences (Table 9)

Table 6: The CDK2 - gene expression in patients with thyroid tumors

Groups		CDK2- IHC Reaction		Total
		Positive	Negative	
Malignant	Count	13	17	30
	% within Type	43.3%	56.7%	100.0%
thyroid nodular goiter	Count	7	18	25
	% within Type	28%	72%	100.0%
Control	Count	0	15	15
	% within Type	0%	100.0%	100.0%
Total	Count	20	50	70
	% within Type	35.7%	64.3%	100.0%



A B

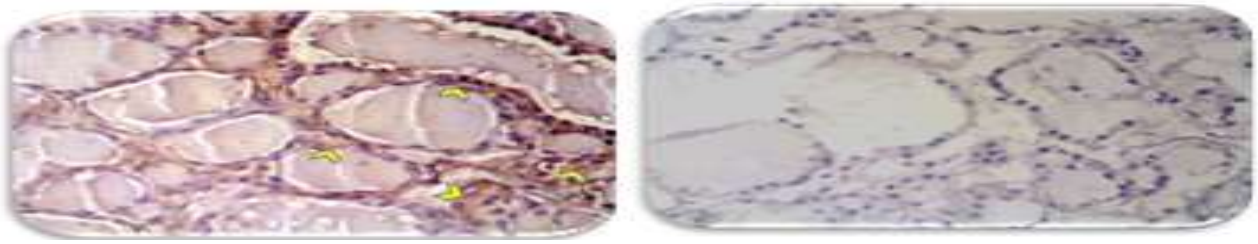
Figure 2: Immunohisto chemical results for CDK2 expression detection in Thyroid tumor; DAB chromogen stained (brown) and counter stained by Mayer's hematoxyline (blue); A. Thyroid cancer with negative CDK2IHC reaction (40X).B. Thyroid Carcinoma with positive CDK2- IHC reaction (40X)

Table 7: CDK2-IHC according to the grades of malignant thyroid tumors

		CDK2-IHC		P value
		-	+	
Grade	Poorly differentiated (n=5)	3	2	0.275
		60%	40%	
	Moderately differentiated (n=8)	3	5	
		37.5%	62.5%	
	Well differentiated (n=17)	11	6	
		64.7%	35.3%	
Total		17	13	
		56.7%	43.3%	

Table 8: The P27 - gene expression in patients with thyroid tumors

		P27-IHC Reaction				
		negative		positive		
		N	%	N	%	
Groups	Malignant thyroid cancers (n=19)	12	40	18	60	0.001
	Thyroid nodular goiter (n=16)	17	64	8	36	
	Control(n=15)	15	100	0	0.00	
Total		44	62.9	26	37.1	



A B

Figure 3: Immunohistochemistry for P27 detection in thyroid Cancers: A. Negative P27IHC Results (40X).B. Thyroid Carcinoma with positive Results (40X)

Table 9: Correlation of P27-IHC according to the grades of malignant thyroid tumors

		P27-IHC		P value
		-	+	
Grade	Poorly differentiated (n=5)	2	3	0.364
		40%	60%	
	Moderately differentiated (n=8)	5	3	
		62.5%	37.5%	
	Well differentiated (n=17)	5	12	
		29.4%	70.6%	
Total		12	18	

Correlations among Signals of Studied Markers (Wide Spectrum HCMV, P27, & CDK2) in Patients with Thyroid Tumors

There is a strong positive relationship (with highly significant correlation) between the signals of HCMV and CDK2 markers in

thyroid tumor. Also Table (10) shows a positive relationship (with a significant correlation) between the signals of P27 marker and HCMV in patients with thyroid cancers as well as CDK2. However, there are no significant correlations among the signals of HCMV, P27& CDK2 and other markers in patients with thyroid cancers.

Table 10: Spearman's rho statistical testing to evaluate the signals of studied molecular markers in relation with HCMV infections in thyroid tumor

Signals (Thyroid tumors)						
Spearman's rho		Age groups /Year	Grade	HCMV	P27	CDK2
HCMV	r.	.055	.115			
	P-value	.736	.480			
CDK2	r.	.178	-.311	.505**	.406**	
	P-value	.272	.051	.001	.009*	
P27	r.	.214	.100	.369*	.206	.228
	P-value	.019	.539	.185	.203	.157

Correlation is significant (P<0.05). **Correlation is highly significant (P<0.01).

Discussion

Worldwide, the malignant neck and head tumors have ranked the 6th most common type of malignant tumors, and more than seventy percent of them happened in third world counteries. In Iraq thyroid cancer ranks the eighth among commonest ten cancers in female [32].

The malignant neck and head tumors that develop in the oral as well as sino- nasal cavities, gland of thyroid, glands of salivary, throat, laryngeal and lips were found to be predominately of squamous cell carcinomatous type. However, the majority of molecular events in their genesis are unknown [33]. It was found that the relationship between inflammatory chronic and risk for developing some malignant tumor is well documented [34].

Herein, and in compatible with that, malignant thyroid tumor is also influenced by modulates inflammation [35].Hashimoto's thyroiditis, is the most common autoimmune diseases of thyroid, has been more associated with malignant thyroid tumor [36].

Recent evidence has implicated viruses in the tumor progression, yet little is known regarding the roles of these viruses in thyroid malignancies [37].

In the study of Thomas, D et al.,[38], who detection DNA -herpes viruses in specimens from four nodular goiter disease and eighteen autoimmune thyroid infection found that the % of at least one type of DNA-herpes viruses (HSV 1, HSV2, HHV6 & HHV7) is highly significant in thyroid infection than in goiter disease (Seventy percent vs. twenty five ercent). Moreover some researchers, that found some viral components such as HCV, B19, and Coxsackie virus, in the tissues of thyroid [39].

In the current research, the rate of detection of positive results of HCMV DNA-ISH reaction in tissues with thyroid cancer was observed in 14 out of 30 (46.7%), while 32% (8 out of 25cases) HCMV- positivity was detected in nodular goiter group. None of healthy thyroid tissues revealed ISH reaction.

We thought, the present study is the 1st in Iraq to investigate the HCMV in a group of thyroid tissues from an Iraq patients with malignant & benign thyroid lesions. In addition, and on reviewing the available scientific research works in this entity, we found an extreme shortage of the articles in this respect. However, the present results are in disagreement with a work done by Tung-Sun Huang et al.,[40] who have not able to find HCMV-DNA in the examined thyroid lesions.

Microorganisms that cause severe infectious or inflammation have become increasingly detection as a possible for promoters of malignant tumor. Although inflammation was implicated in the development of thyroid cancer, no previous study has investigated the human cytomegalovirus in thyroid tumors, and herein, their suggested that human cytomegalovirus infectious is properly to be associated with malignant papillary thyroid tumor. These viral infectious may be an alteration expression of IE protein which act to accessory production of viruses and immune evasion [41].

Activation of BRAF is included in the Ag expression of IE-human cytomegalovirus [42].

In the current study, the percentage of p27-IHC in cases with thyroid cancer is 60% (18 / 30), where as in nodular goiter cases group was detected in 36% (9 / 25).

Maria Letizia et al., [43] study has provided a complex happened for pathway of PI3K/ AKT & inhibitor cycline kinase p27kip1 have played important way in carcinogenesis of thyroid, especially in malignant thyroid tumor cells progression or transformation [44-47].

The novel finding of a study Maria et al.,[43] who explained the additional signals of PI3K/AKT pathway may be p27kip1 missing cytoplasm in malignant thyroid tumor cell ;where, localized and phosphorylated of p27kip1 in these cells is regulation in a AKT -and PI3K -dependent pattern. AKT activation is significant correlation with p27kip1 missing in cytoplasm, whereas p27kip1 was excluded nuclear in cancer with AKT inactivation [43].

Role of p 27kip1 in missing nuclear localized to-cytoplasm in advance stage of tumor of thyroid is that it explain the important of p27kip1 into nuclear of cell decrease the amount of p27kip1 in nuclei that preventing p27kip1-promote suppression the activation of cyclin E-CDK2 [48].

A wide studies from the researches has given more supports the notion that p27kip1play important role in promote and initiation cell cancer [49]. It was noticed, in tumors of thyroid the inhibitor cyclin-dependent kinase p27kip1 only missing from localization in nuclear and cytoplasm. By similarity within the correlated cyclin-dependent kinase inhibitor (p21cip1) [50] p27kip1 in cytoplasm play important role in apoptosis suppression , this help malignant cells tumor to down-regulation many functions of cellular with one to another (hit), and p27kip1 in cytoplasm was found to be correlated with migration activation in expression of AKT- of malignant thyroid cells [51].

The result of CDK2-IHC in cases with thyroid cancer was detected in 43.3% (13 / 30), followed by 28 % (7 out of 25) in benign thyroid goiter group .

In tumors of thyroid, induced of CDK2 was found to be inversed association with the contained of nucleus Cdk2-binding p27. Tumor of thyroid derived cell lines and may be excluded malignant tumor by surgery, lower activation of Cdk2 has detection high levels of p27 binding to nucleus Cdk2 than did those cases with higher Cdk2 activation, these lead to suggest that cyclin-dependent kinase inhibitor p27 available is highly importance from its absolutely amount in regulate of p27 functions [48].

The regulation of the cell cycle involves CKIs that potentially act as tumor suppressor genes, of which two classes of genes whose products can inhibit CDK activities have recently been identified. The first class consists of p21, p27 and p57 [52, 53], which can inhibit a variety of CDK subtypes. This class of genes is mainly involved in development of tumor [54, 55], since mutations in this class of genes have not been identified in tumors.

Also table (10) shows a positive relationship (within a significantly association) between signals of cyclin-dependent kinase inhibitor P27 and cyclin-dependent kinase -2 in patients with thyroid cancers.

P27Kip1 is known as cyclin-dependent kinase inhibitor of cyclin E-cdk2 [56]. However, an observed that act as oligonucleotides antisense to p27Kip1 are not able to re-storage complete proliferation, led to the induced the cyclin-dependent kinase inhibitor p27Kip1 may not be mediator as unique to inhibition in malignant thyroid tumor cells. Other regulation of cell cycle, such as cyclin D1 or A, have been indicated in the establish of contacted inhibitor [57], In vivo, cyclin-dependent kinase inhibitor p27Kip1 its play important role in arrest of growth, in some tissues or cases [58].

The investigation changes in cyclin-dependent kinase inhibitor p27Kip1 protein in malignant thyroid tumor cells are correlation with changes of concomitant in both levels of Kip1 mRNA and in the degradation rate of p27Kip1 which has been showed in endothelial cells, where these findings have suggested to be achieved by a

inhibition of p27Kip1 protein degradation and Kip1 mRNA, and induced gene transcription of Kip1 [59].

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