Pak. J. Biotechnol. Vol 15 (3) 653-658 (2018) ISSN Print: 1812-1837
www.pjbt.org ISSN Online: 2312-7791

ESTIMATION OF PAX8/PPAR FUSION GENE BY FISH TECHNIQUE IN FOLLICULAR THYROID LESIONS

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Article received 29.7.2018, Revised 2.9.2018, Accepted 9. 9. 2018

ABSTRACT

The purpose of the study was to detect PAX8/PPAR G gene rearrangement by fluorescent in situ hybridization (FISH) technique, the present study is the first time in Iraq used this technique to detect fusion gene in follicular thyroid lesions, follicular carcinoma (FC) follicular variant papillary of carcinoma (FVPC), follicular adenoma (FA) and follicular hyperplasia. A total of 120 paraffin block were included in the study, 30 blocks were (FC), 30 blocks were (FVPC). 30 blocks were (FA), 30 blocks were blocks thyroid follicular hyperplasia.20 blocks endocervical epithelium.20 paraffin blocks of colonic epithelium. The clinicopathological parameters were obtained from patients' admission case sheets and pathology reports (age, gender). The description of measures regarding fluorescent instu hybridization of PAX8/ PPAR G fusion gene there was significantly highest in follicular carcinoma, followed by follicular variant of papillary carcinoma, then follicular adenoma and lastly by follicular hyperplasia The area under the curve for all three markers was >0.8.The p-value was highly significant for all three markers ($P<0.001$). Cutoff values that predict malignant thyroid follicles lesion were as following: score of >1 for Galectin-3 marker, score of >2 for HBME-1 marker and number of positive cells per 50 of >13 (>26%) for PAX8 /PPAR G fusion gene marker. The sensitivities for the three markers were 100.00 %, 90.00 % and 66.67 %, respectively, whereas the specificities were 88.30 %, 98.33 % and 100.00 % respectively.

Key words: Thyroid, PAX8 gene, FISH Technique.

INTRODUCTION

Constitutive activation of the MAP kinase/ERK pathway leads to tumor genesis by up regulating cell division and proliferation (Roberts, and Der, (2007)). Thyroid tumor harbors in about 5% of thyroid nodules (Popoveniuc, and Jonklaas (2012). This improvement can be utilized as a diagnostic marker for the differentiation between follicular thyroid carcinoma and adenoma, the PAX8/PPAR G rearrangement was recognized by fluorescence in situ hybridization (FISH) (Algeciras-Schimnich, et al., (2010)). Papillary thyroid carcinoma (PTC) is known to harbor BRAF most commonly, followed by RAS and RET/PTC, whereas follicular thyroid carcinoma (FTC) is characterized by the presence of either RAS or PAX8/PPARG (Kondo, et al., (2006)). The fusion gene PAX8/PPARG denoted as PAX8/PPAR G fusion protein (PPFP). In vitro and in vivo evidence indicate that PPFP can act as an oncoprotein, PAX8/PPAR G rearangement is created by a translocation between chromosomal regions 2q13 and 3p25 (Ross, et al., (2009)).

This translocation results in a fusion transcript wherein most of the coding sequence of PAX8 (2q13) is fused in frame with the entire coding exons of PPARG 1.

MATERIALS AND METHODS

The study was done in the period between (2015-2017) in the department of Biology/ College of Education for Pure Science /Ibn-Haitham /Baghdad University and in the department of Pathology College of medicine/ Al-Nahrain University. Paraffin blocks of thyroid tissue samples used in this study were collected from laboratories of Baghdad Teaching Hospital, Al-Khadhmiya Teaching Hospital, Al-Yarmouk Teaching Hospital, Al-Kindi Teaching Hospital, Al-Karama Teaching Hospital, Ghazi Al-Hariri Hospital for surgical specialist in Baghdad, Al-Hussein Hospital (Kerbala Health Office) in Karbala, Al-Sadder Medical City in Al-Najaf, Al-Sadder Teaching Hospital(Al-Ashraf/ pathology unit) in Basra, Rizgary Teaching Hospital in Erbil, Kalar Educational Hospital in Al-Sulaymaniyah and private laboratories, for the years (2006-2016).

The clinicopathological parameters were obtained from patients' admission case sheets and pathology reports, including age, gender, type, size of lesion, cancer invasiveness (capsular or vascular). The total number of the thyroid samples used in this study was 120 paraffin blocks, these include 30 thyroid follicular carcinoma, 30 follicular variant of papillary carcinoma, 30 follicular adenoma and 30 follicular hyperplasia. PAX8/PPARG is intended for fluorescent in situ hybridization

(FISH) for the analysis of chromosomal aberrations on fixed cytogenetic specimen. The t (2,3) PAX8/ PPARG is designed as a dual-fusion probe. An orange labeled probe is flanking the breakpoint at 2q13 (PAX8) and a green labeled probe covers the breakpoint region at 3p25 (PPARG). The probe is intended for tissue-FISH applications and analytical and performance characteristics are not established.

FISH procedure including, Deparaffinization, Pretreatment, Denaturation and Hybridization, Post-Hybridization Washing and Counterstain. Fluorescence microscope (Carl Zeiss,Germany) was used to visualling the FISH probe signals.

Data were collected, summarized, analyzed and presented using three statistical software programs: the statistical package for social science (SPSS version 22), Categorical variables were pre-sented as number and percentage whereas numeric variables were presented either as mean and standard deviation (SD) or median and interquartile range (IQR), according to the results of Kolmogrov Smirnov test of normality distribution for numeric variables. The association between categorical variables was assessed using Chi-square test and correction was done as needed. Comparison of mean values between two groups was carried out using either independent samples-t test or Mann Whitney U test, while comparison of mean values among more than two groups was carried out using either one-way analysis of variance (ANOVA) test or Kruskal Wallis tests. Correlation was evaluated using Spearman correlation test. Cutoff value for PAX8- FISH of follicular hyperplasia was calculated using invers beta function in Microsoft Office Excels. Receiver operator characteristic (ROC) analysis was done to calculate cutoff values. P-value was considered significant when it was equal to or less than 0.05.

RESULTS

Figure (1-10) showed the cells expressing PAX8/PPARG fusion gene in study groups using fluorescent *in situ* hybridization (FISH). The description of measures regarding fluorescent in situ hybridization (FISH) of PAX8/ PPARG fusion gene is summarized in table (1) and figure (11). Mean number of cells that showed signals of fusion gene per 50 cells was significantly highest in follicular carcinoma, followed by follicular variant of papillary carcinoma, then follicular adenoma and lastly by follicular hyperplasia; the values were 18.12±10.29, 15.67±6.89, 9.22±1.86 and 5.00±1.00 respectively.

The cut-off value for positive samples was calculated using beta inverse function in Microsoft Office Excel as following: Minimum cut-off = BETAINV (confidence interval, highest number of positive cells in samples with follicular hyperplasia + 1, total number of counted cells). The highest number of cells expression PAX8/PPARG fusion gene in follicular hyperplasia was 6 out of 50 counted cells. When confidence interval was set at 95% the cut-off value was calculated to be 20.1% (10 cells per 50 counted cells). According to this cut-off value the rate of positivity for PAX8/PPARG fusion gene was calculated as > 20% (>10 cells out of 50) and presented in table (2). The total number of sample was forty samples, the positive percentage in follicular hyperplasia 0 consider them as negative, while positive cases were follicular adenoma 11.1%, follicular carcinoma 41.2% and follicular variant of papillary carcinoma 44.4%.

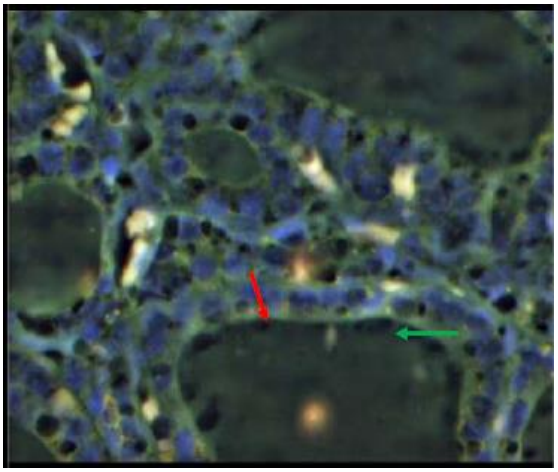

Figure 1: Tissue section of thyroid follicular adenoma by FISH analysis showing two separate green (2G) signals, two separate orange (2O) signals repeating the two normal PAX8-PPAR G loci.

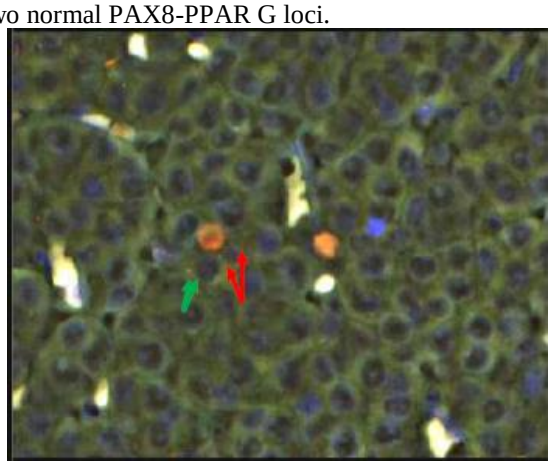

Figure 2: Tissue section of thyroid follicular hyperplasia by FISH analysis showing two separate green (2G) signals, two separate orange (2O) signals repearing the two normal PAX8-PPAR G loci.

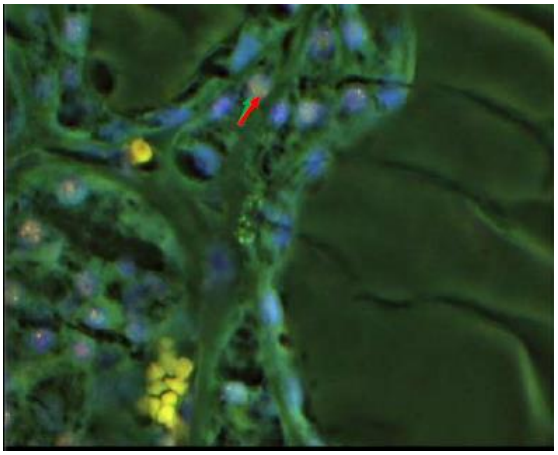

Figure 3: Tissue section of thyroid follicular carcinoma by FISH analysis showing aberrant cell: one green (1G), one orange (1O) two green –orange (2GO) fusion signal, including a fusion of PAX8 and PPARG.

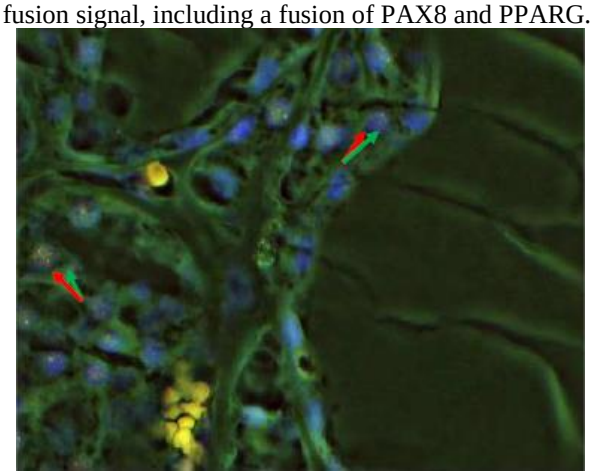

Figure 4: Tissue section of thyroid follicular carcinoma by FISH analysis showing aberrant cell: one green (1G), one orange (1O) two green –orange (2GO) fusion signal, including a fusion of PAX8 and PPARG.

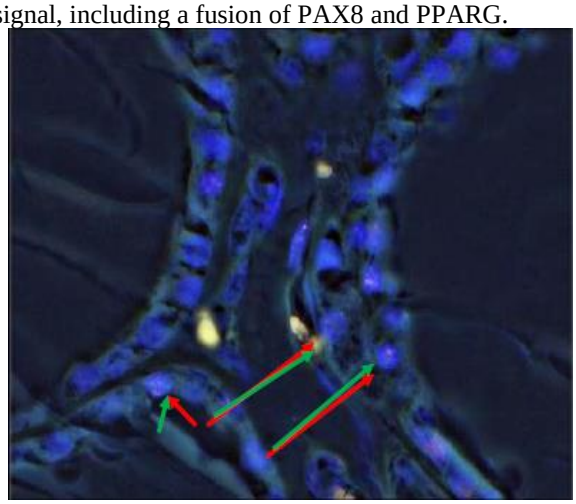

Figure 5: Tissue section of thyroid follicular variant of papillary carcinoma by FISH analysis showing aberrant cell: one green (1G), one orange (1O) two green – orange (2GO) fusion signal, including a fusion of PAX8 and PPARG.

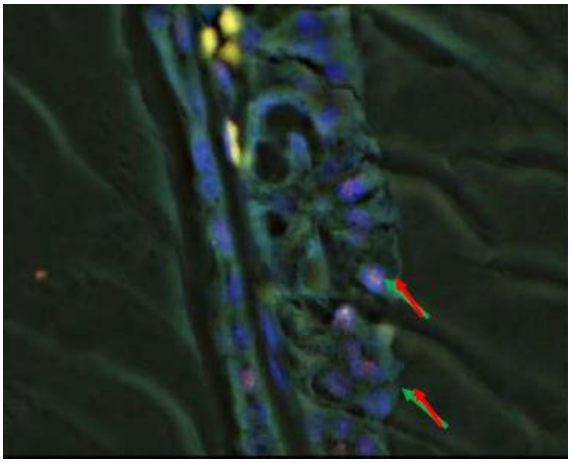

Figure 6: Tissue section of thyroid follicular carcinoma by FISH analysis showing aberrant cell: one green (1G), one orange (1O) two green –orange (2GO) fusion signal, including a fusion of PAX8 and PPARG.

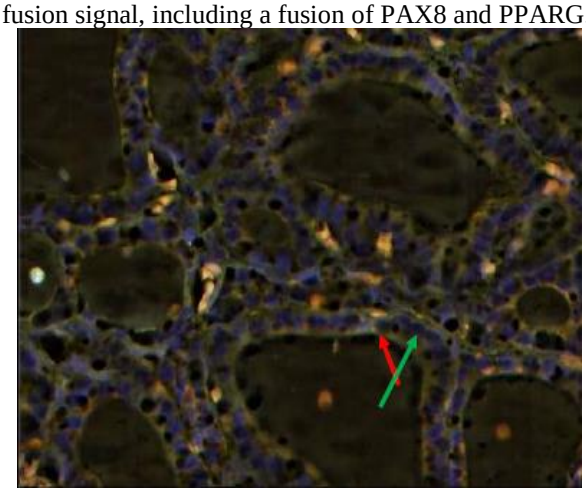

Figure 7: Tissue section of thyroid follicular hyperplasia by FISH analysis showing two separate green (2G) signals, two separate orange (2O)signals reappearing the two normal PAX8-PPAR G loci.

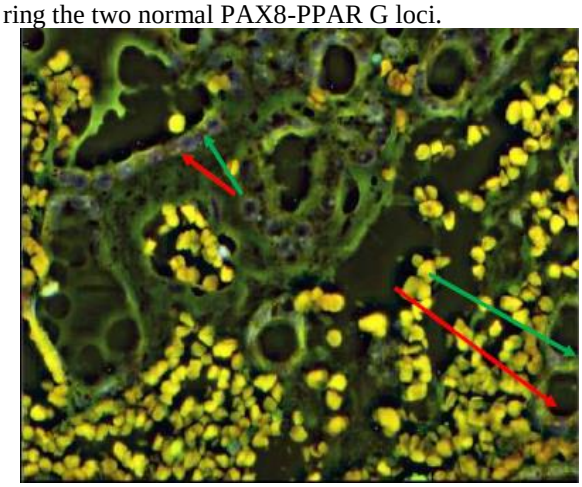

Figure 8: Tissue section of thyroid follicular adenoma by FISH analysis showing two separate green (2G) signals, two separate orange (2O) signals reappearing the two normal PAX8-PPAR G loci.

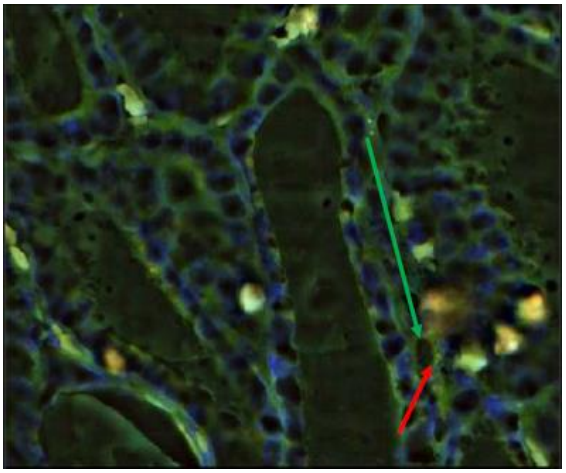

Figure 9: Tissue section of thyroid follicular adenoma by FISH analysis showing two separate green (2G) signals, two separate orange (2O) signals reappearing the two normal PAX8-PPAR G loci.

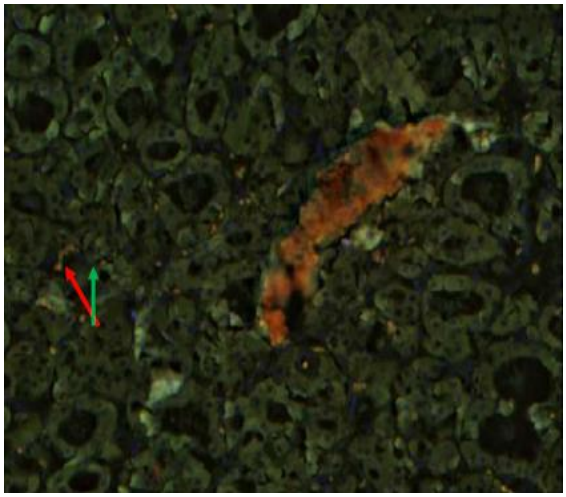

Figure 10: Tissue section of thyroid follicular hyperplasia by FISH analysis showing two separate green (2G) signals, two separate orange (2O) signals reappearing the two normal PAX8-PPAR G loci.

Table 1: Mean number of cells showing PAX8/PPARG fusion gene signals using FISH.

Parameter	Cases in Study Groups				P-value
	Follicular Carcinoma	FVPC	Follicular Adenoma	Follicular Hyperplasia	
Mean ±SD	18.12±10.29	15.67±6.89	9.22±1.86	5.00±1.00	<0.001
Median (IQR)	10.00 (18.50)	10.00 (12.5)	9.00 (1.50)	5.00 (2.00)	
Range	10.00-35.00	10.00-26.00	6.00-13.00	4.00-6.00	

*Kruskal Wallis test; HS: highly significant; IQR: interquartile range. FVPC; Follicular variant of papillary carcinoma.

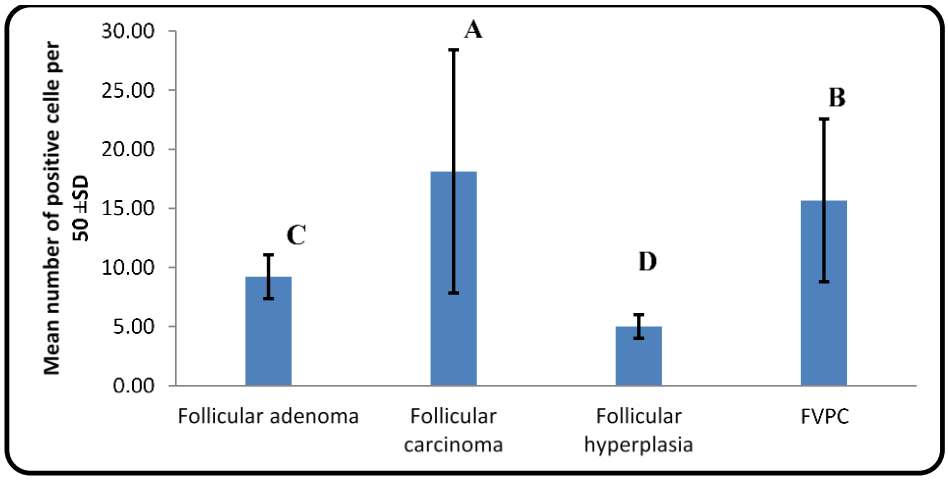

Figure 11: Mean number of cells expressing PAX8/ PPARG fusion gene in the study groups using FISH. FVPC; Follicular variant of papillary carcinoma.

Table 2: Number and percentage of cases with positive PAX8/PPARG fusion gene using FISH.

Cases in Study Group	Total Cases	Positive PAX8/PPARG fusion gene N%
Follicular carcinoma	17	7 (41.2)
Follicular variant of papillary carcinoma	9	4 (44.4)
Follicular adenoma	9	1 (11.1)
Follicular hyperplasia	5	0 (0.0)
Total	40	12 (30.0)

DISCUSSION

This study showed, following fluorescent in situ hybridization (FISH) of PAX8/PPARG fusion gene, mean number of cells per 50 cells was significantly highest in follicular carcinoma, followed by follicular variant of papillary carcinoma, then follicular adenoma and lastly by follicular hyperplasia. In this study two methods were used to

identify a cut-off value: the first one was to identify the level of expression at which a positive value is labeled since some cases of normal thyroid tissues and reactive hyperplastic tissue had false positive expression of PAX8/PPARG fusion gene and this was accomplished by using the beta inverse function in the Microsoft Office Excel 2007 as it was stated in the chapter of method. This method permitted identification of the cut-off value of > 20.1% (approximately 10 cells per 50 counted cells) in order to consider a positive expression of the PAX8/PPARG fusion gene.

(Algeciras-Schimnich, et al., 2010)) Stated that samp-les were considered translocation positive if ≥ 10 cells showed the presence of the fusion (Gómez Sáez, (2011)). The cut-off in the study carried out was deter- mined after counting 100 nuclei. This level is much less than that considered in the present study. This difference in the cut-off may be due to the fact that considered the highest number of positive nuclei expressing PAX8/PPARG fusion gene in normal thyroid tissue as the minimum cut-off value to identify positive samples and ((Algeciras-Schimnich, et al., 2010)) did not use the beta inverse function in the Microsoft Office Excel program.

Another method for calculation of cut-off value in the present study was calculated using receiver operator characteristic (ROC) analysis in order to identify the level of expression of PAX8/PPARG fusion gene at which one can consider malignancy. Accordingly, the present study showed that a cut off value of number of cells expressing positive PAX8 per 50 cells was extremely sensitive and specific. The cutoff value was >19 cells / 50 cells with a sensitivity 96% of and specificity of 100%. Several studies estimated PAX8/PP ARG fusion gene re-arrangement in thyroid follicular adenoma and the rate of positive re-arrangement ranged from 3%-33.3% (Castro, et al. (2006)). In the present study the rate of positive PAX8/ PPARG fusion gene re-arrangement in thyroid follicular adenoma was 11.1%. On the other hand, the rate of positive PAX8/ PPARG fusion gene re-arrangement in thyroid follicular carcinoma was estimated to range from 25%-45%. (Dwight, et al., (2003)). In the present study, the rate of positive PAX8/PPARG fusion gene re-arrangement in thyroid follicular carcinoma was 41.2 %. Moreover, the rate of positive PAX8/PPARG fusion gene re-arrangement in follicular variant of papillary carcinoma (FVP-C) was estimated to range from 37.5% - 50%. In the present study the rate of positive PAX8/PPARG fusion gene re-arrangement in 44.4 %. So, the rate of positive PAX8/PPARG fusion gene re-arrangement recorded in the present study for follicular adenoma, follicular carcinoma and follicular variant of papillary thyroid carcinoma is within the range reported by other studies that used FISH as a method for estimation of this type of rearrangement. (Castro, et al., (2006)).

Follicular thyroid carcinoma patients with PPARγ fusion gene re-arrangement more frequently have vascular invasion, areas of solid/nested tumor histology, and previous non-thyroid cancers. Thus, PAX8/PPARG fusion gene re-arrangement typically correlates with the presence of malignancy (Jadhav, et al., 2012).

Generally speaking, the use of merely positive PAX8/ PPARG fusion gene rearrangement was proved unsatisfactory in segregating malignant thyroid tumor (follicular carcinoma and follicular variant papillary carcinoma) from benign follicular adenoma, since in the two situations the PAX8/PPARG fusion gene was identified in varying rates. Whereas choosing a much higher cut-off value based on receiver operator characteristic (ROC) curve analysis provided a highly sensitive and specific tool for segregating malignant from benign thyroid nodules. The level identified in the present study by this method was 13 cells per 50 counted cells (26%).

The mechanisms by which PAX8/PPARG may are not completely comprehended (Lui, et al., (2008)), however It has been demonstrated that the fusion protein increases cell development, cell suitability and induces anchorage independent growth while decreases apo-ptosis in vitro. There is no evidence however that this protein affect tumorigenesis *in vivo* (berhardt, et al., (2010)). In this way, it is still not clear whether additional events are needed to promote tumorigenesis.

Follicular carcinomas with PAX8/PPARG fusion gene rearrangement were found to express Galectin (LGA-LS3) while FTCs with RAS mutation were found to express HBME1. Moreover, the authors observed difference in the clinical-pathological features in the two groups, tumors harboring PAX8/PPARγ fusion gene rearrangement tend to present at a younger age be smaller in size have a solid/nested growth pattern and more frequently reveal vascular invasion.

Conclusion

Something that might be avoided with a preoperative test of adequate accuracy, unfortunately, the finding of a subset of PAX8/PPARG fusion gene revision positive adenomas reduces the negative predictive value of a preoperative PAX8/ PPARG rearrangement biopsy fin-ding this problem was avoided if the cut-off value, calcu-

lated in the present study by recipient administrator characteristic (ROC) analysis, was used.

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