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# Evaluation of the Long-term Growth Potential of Benign Non-toxic Thyroid Nodules by the AgNOR Method; an 11-year Follow-up Study

**Tamas Solymosi[1]*, Istvan Gal[2], Miklos Bodo[3], Gyula Lukács[4] and Endre V Nagy[5]**

[1]Departmentof Thyroidology, Bugat Hospital, Gyöngyös, Hungary
[2]Departmentof Surgery, Robert Karoly Clinics, Budapest, Hungary
[3]Departmentof Pathology, Celldiagnostics, Budapest, Hungary
[4]Departmentof Pathology, Bugat Hospital, Gyöngyös, Hungary
[5]Division of Endocrinology, Department of Medicine, University of Debrecen Medical and Health Science Center, Debrecen, Hungary

## Abstract

**Objective:** The classification of benign thyroid nodules as stable or as fast growing may have clinical and economical relevance. The proliferative activity of thyroid cells determined by argyrophilic staining of the nucleolar organizer region (AgNOR) displays significant differences between benign and malignant thyroid lesions. We tested if that parameter can predict the growth potential of benign nodules.

**Methods:** On cytological samples of 106 benign nodules of 89 euthyroid patients, the mean AgNOR number per cell (AgNORc), the mean AgNOR area per cell (AgNORa) and the mean nuclear area were determined. The mean follow-up period was 11.9 years. The initial (V1) and the final (V2) nodule volumes and respective TSH values (TSH1 and TSH2) were determined.

**Results:** 66 nodules (62.2%) increased in volume. The median of V2/V1 was 1.31. V2/V1 correlated significantly with AgNORa, AgNORc, nuclear area and TSH-1. There was no significant correlation between V2/V1 and either age, duration of follow-up or TSH-2. No correlation was found between V2 and any of the parameters. We found on both forward and backward stepwise analysis that AgNORc values and initial TSH levels were significant determinates of the increase in volume size, i.e. both higher AgNORc values and higher initial TSH levels carry a greater risk for nodule growth.

**Conclusions:** The determination of AgNORc during FNA evaluation may contribute to the estimation of growth potential of thyroid nodules. Further studies using other enzyme techniques or new molecular methods are justified to achieve a better discrimination between stable and growing nodules.

**Keywords:** Thyroid nodule; FNAC; AgNOR; Prognosis; Ultrasound; Cytology

## Introduction

Nodular goiter is a frequent disease with an estimated prevalence of 4–7% by palpation and of 20-76% by ultrasonography (US) [1-5]. The prevalence of this cancer ranges from 5.0% to 7.7% in iodine sufficient regions [5] it is approximately half of this amount in moderately iodine-deficient regions [6]. Benign nodules below 3 cm in diameter in euthyroid patients rarely need surgery. Nodules deemed malignant by fine needle aspiration cytology (FNA) or those with a diameter more than 3 cm are candidates for surgery. Nodules, which are left in site because they have not met the FNA and size criteria for surgery, are often followed up for decades. Their size may remain stable or may grow over time. Recently, huge efforts have been made to find supplementary techniques, including sonographic parameters, enzyme and immune techniques, as well as novel molecular biological approaches to increase the diagnostic potential of FNA towards a better diagnostic accuracy. Less attention has been paid to the natural course of benign thyroid nodules, the few studies in this field are controversial [7]. The growth potential has sparsely been studied [8]. Thus, lifelong follow-up is the only choice in the majority of benign nodule cases. A predictive parameter of growth potential obtainable early during the course of the disease would be useful in this respect.

Earlier, we found that the proliferative activity determined by argyrophilic staining of the nucleolar organizer region (AgNOR) of thyroid cells displays significant differences between benign and malignant thyroid lesions [9] in FNA samples. In our present work, we tested if that parameter can predict the growth potential of benign thyroid nodules.

## Patients and Methods

Between 1993 and 1997, we performed AgNOR analysis and morphometry on FNA smears of 397 patients during their first visits as part of the evaluation for nodular goiter. TSH, aTPO, ultrasonography (US), scintigraphy and FNAs from both the largest nodule and, in the case of multinodularity, the largest hypoechogenic nodule were performed. AgNOR was performed on nodules meeting the following criteria: the nodule was solid, well-circumscribed and at least 10 mm in diameter on US. The physical character of the nodules by palpation have been graded from 1 to 5 and carefully recorded; 1 – soft and freely moveable, 2 - moderately firm and freely moveable, 3 - firm and freely moveable, 4 - hard, freely moveable, 5 – hard and not moveable.

Those patients, who did not require surgery, underwent regular

***Corresponding author:** Tamás Solymosi, H-3232 Mátrafüred, Fenyves u. 6., Hungary, Tel: 00-36-303480398; E-mail: mosolyis@t-online.hu

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follow-up examinations. US and TSH were performed every 1-3 years depending on the nodule size, growth tendency and complaints. FNA was repeated every 5 years after the initial examination in all patients, or even sooner if the nodule increased more than 25% in volume. Patients with a follow-up of more than 10 years (N =79) and as well as those who were operated on later than 6 years after the initial investigation (N=10) were entered in the study. Exclusion criteria are listed in Table 1.

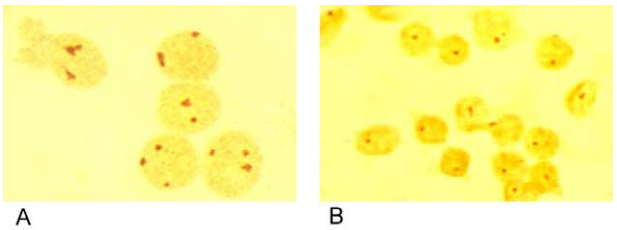
The AgNOR method and morphometry were performed on the FNA samples as described earlier [9,10]. We calculated the following parameters: the mean AgNOR count per cell (AgNORc), the mean AgNOR area per cell (AgNORa) and the mean nuclear area. These parameters were determined by the analysis of at least 100 nuclei in each case (Figure 1).

The volume of the nodule was measured on each US examination according to Brunn et al. [11]. We determined the volume of the nodule at the first and at each subsequent examination, as well. The initial (V1) and the final (V2) US volumes and respective TSH values (TSH1 and TSH2) were used for statistical analysis. All US examinations, FNAs and cytological analysis were performed by the same examiner (T.S.) with an intraclass correlation coefficient of 0.9868 (CI: 0.9804-0.9911). The results of the original AgNOR and the morphometric data arising between 1993 and 1997 were archived and we were not aware of these data on the follow-up investigations.

The SAS 9.2 (SAS Institute Inc., Cary, NC, USA) statistical software was used for statistical analysis. The examined parameters were characterized by descriptive statistics. Pearson and Spearman correlation analysis was used for the comparison of the various parameters. Logarithmic transformation was performed for skewed distributed variables. Multivariate regression analysis was applied to investigate the role and the extent of role of parameters in nodule volume increase (REG procedure). We stated that the nodule had increased in size when it exceeded the median increase in nodule volume (31%).

| AgNOR staining | <100 intact cells for investigation |
|---|---|
| Ultrasonography | Cystic component > 5% at any time |
| | Nodule in direct contact with an adjacent nodule |
| Scintigraphy | Autonomously functioning nodule in the thyroid at initial investigation |
| Laboratory | TSH > 4 mU/L at any time |
| | TSH < 0.2 mU/L at any time |
| | aTPO > 100 U/L |
| Medication | Thyrostatic and/or thyroid hormone and/or iodine containing drug at any time |
| Cytology | Suspicious or malignant and not operated on |
| | Lymphocytic thyroiditis |
| Histology | Malignant or diffuse lymphocytic thyroiditis |
| Follow-up period | < 10.years in non-operated patients |
| | < 6 years in operated patients |

**Table 1:** Exclusion criteria.



A B

**Figure 1:** Nuclei of benign thyroid cells from two nodules (A and B) stained by the AgNOR method (100x) The mean AgNOR counts were 3.11 and 1.06, mean AgNOR areas 3.98 μm² and 1.96 μm², and mean nuclear areas 49.4 μm² and 18.1 μm² in A and B, respectively. The volume of nodule A increased by 118% while the volume of nodule B decreased by 28% over 10 years.

| Parameter | Mean | Standard deviation | Median | Min | Max |
|---|---|---|---|---|---|
| Age of patients (ys) | 53.6 | 10.3 | 52 | 32 | 77 |
| Follow-up (ys) | 11.9 | 2.41 | 11.5 | 6 | 18 |
| Initial volume (V1) (mL) | 3.51 | 4.18 | 2.01 | 0.29 | 20.0 |
| Final volume (V2) (mL) | 5.53 | 7.64 | 3.09 | 0.28 | 45.1 |
| V2/V1 (%) | 203.1 | 235.4 | 131.4 | 15.0 | 1706 |
| AgNOR count | 2.07 | 0.73 | 1.98 | 1.06 | 5.23 |
| AgNOR area (μm²) | 3.03 | 1.36 | 2.79 | 1.08 | 6.65 |
| Nuclear area (μm²) | 33.4 | 14.0 | 29.7 | 13.1 | 63.9 |
| TSH initial (mU/L) | 1.11 | 0.80 | 0.84 | 0.21 | 3.22 |
| TSH final (mU/L) | 1.20 | 0.83 | 1.02 | 0.21 | 4.00 |

**Table 2:** Basic parameters of the nodules (N = 106)

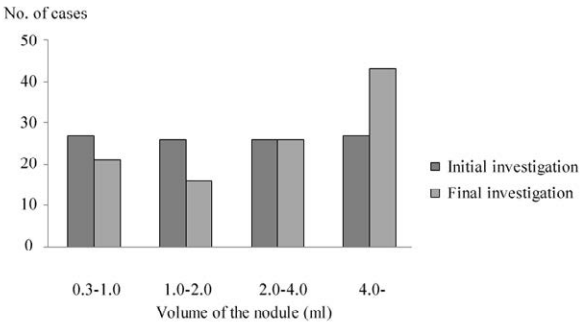



**Figure 2:** Distribution of nodule volumes at the initial and final investigations (n=106).

The study followed the tenants of the Declaration of Helsinki. Patients gave oral informed consent.

## Results

### Proliferative activity and nodule volume changes

The characteristic data of the patients and nodules are shown in Table 2. The median age of patients was 53.6 years. There were 8 men and 81 women included in the study. The number of nodules analyzed was 1 in 73 patients, 2 in 15 patients and 3 in only one patient. The distribution of nodule volumes is presented in Figure 2. The mean follow-up time was 11.9 years. We found that 66 nodules (62.2%) increased in volume. The median of the V2/V1 was 1.31 meaning that 50% of the nodules increased in size by at least 31% over time.

### Correlation between TSH, AgNOR nuclear area and the initial size of the nodule

There was no significant correlation between the V1 and AgNOR or nuclear parameters. However, there was a significant negative correlation between the V1 and TSH-1 (Table 3).

### Determinants of nodule increase

As demonstrated in Table 4, V2/V1 correlated significantly with AgNORa, AgNORc, nuclear area and TSH-1. There was a negative correlation between V1 and V2/V1 (r=0.16) which did not reach the level of statistical significance (p=0.10). There was no significant

correlation between V2/V1 and either age, duration of follow-up or TSH-2. No correlation was found between V2 and any of the parameters.

### Multivariate regression analysis of various parameters

We found on both forward and backward stepwise analysis that AgNORc values and initial TSH levels were significant determinants of the increase in volume size, i.e. both higher AgNORc values and higher initial TSH levels carry a greater risk for nodule growth (Table 5).

## Discussion

As of now, there are no reliable predictions of the growth potential of benign thyroid nodules. Therefore, patients with benign nodules require lifelong follow-up. Considering the fact that the majority of the nodules are benign, both the inconvenience of the follow-up visits for the patient and health care costs for the community are considerably large. Our current knowledge on the natural course and the molecular mechanisms of benign non-toxic nodular goiter formation is surprisingly limited [12]. The early classification of detected thyroid nodules as stable or fast growing may have clinical and economical relevance. Stable/slow growing lesions are subjects for follow-up examinations, while fast growing nodules need early intervention.

| Variable | Pearson correlation coefficient | P |
|---|---|---|
| Age | 0.105 | 0.284 |
| Initial TSH | -0,236 | **0.015** |
| AgNOR count | -0.090 | 0.357 |
| AgNOR area | -0.073 | 0.457 |
| Nuclear area | -0.100 | 0.308 |

**Table 3:** Correlation of the initial nodule volume with other parameters.

| Variable | Pearson correlation coefficient | P |
|---|---|---|
| Age | -0.094 | 0.336 |
| Initial TSH | 0.198 | **0.042** |
| AgNOR count | 0.307 | **0.001** |
| AgNOR area | 0.288 | **0.003** |
| Nuclear area | 0.240 | **0.013** |

**Table 4**: Correlation of the change in nodule volume (V2/V1) with other parameters.

| Variable | Parameter estimate | Standard error | Type II SS | F value | P value |
|---|---|---|---|---|---|
| V1 | -0.00241 | 0.01982 | 0.00900 | 0.01 | 0.9036 |
| Age | -0.00496 | 0.00764 | 0.25738 | 0.42 | 0.5177 |
| Duration of follow-up | 0.03794 | 0.03356 | 0.77987 | 1.28 | 0.2611 |
| Log AgNOR count | 0.67394 | 0.26423 | 3.97058 | 6.51 | **0.0123** |
| Log AgNOR area | 0.65981 | 0.39223 | 1.72716 | 2.83 | 0.0958 |
| Log nuclear area | -0.54217 | 0.41899 | 1.02201 | 1.67 | 0.1988 |
| Log initial TSH | 0.33269 | 0.13635 | 3.63356 | 5.95 | **0.0165** |
| Log final TSH | -0.20691 | 0.13315 | 1.47383 | 2.41 | 0.1235 |
| Nodule character by palpation | 0.12548 | 0.08565 | 0.94847 | 2.15 | 0.1473 |

**Table 5**: Multivariate regression analysis: dependence of the nodule volume change (V2/V1) on other parameters.

The conclusions of the few studies published in this field are controversial. There are retrospective studies which state that most of the nodules do not increase over time [13,14] while other studies draw an opposite conclusion (Table 6) [7,13-18]. We have found that 50% of the nodules had grown in volume during the follow-up period; this value had been in the range of the 11% to 61% in previous studies. Differences in environmental factors as well as different genetic backgrounds may be taken into account; however, differences may also be explained by methodological causes. In the present study, the initial and final US were performed by the same examiner. The presence of lymphocytic thyroiditis or significant cystic components was among our exclusion criteria as they may interfere with the growth ratio [7]. In addition, the follow-up period was markedly longer than in any of the previous studies.

Only a few studies have focused on the natural course of benign nodules. Up till now, there have not been any reliable techniques to predict the stable or growing character of a nodule. Viacava et al. have demonstrated with MIB-1 marker that the proliferative activity of adenomas is greater than that of the surrounding extranodular normal tissue [19] Cornianu et al. have found similarly higher proliferative activity in thyroid nodules compared with non-nodular tissue using the PCNA method but not with the Ki-67 technique [20]. Ferraz et al. have demonstrated greater proliferative activity in benign nodules than in extranodular tissue with microRNA technique [21]. Increased expression of cell cycle-associated genes has been found, and a special relevance has been attributed to the protein kinase C pathway, whereas no evidence of RAS-MAPK signaling was found [12]. Sapio et al. [8] investigated the RET/PTC oncogene in cytological samples of benign nodules, and found a 4.3-fold difference in volume increase in the RET/PTC positive group (15.2% of patients) compared with the RET/PTC negative group. To the best of our knowledge, the study by Sapio et al. has been the only one focusing on the predictive value of proliferative markers in relation to the growth potential of benign thyroid nodules.

Lymphocytic thyroiditis has been demonstrated to increase the proliferative activity of benign nodules either by Ki-67 [20] or by the AgNOR method [9]; for this reason, patients with lymphocytic thyroiditis on FNA were excluded from the present study.

A major limitation of our work is the relatively low number of nodules studied. The strict inclusion criteria may account for this, and, in addition, patients were progressively lost for follow up with time. However, in this rather small sample, we were able to show that AgNORc and initial TSH were significant determinants of volume increase.

| Study | Country | No. of nodules | Mean follow-up (months) | Criteria of increase | Increased | Decreased | No change |
|---|---|---|---|---|---|---|---|
| | | | | | % of nodules | | |
| Papini et al. [18] | Italy | 41 | 60 | volume ≥ 11.7% | 56 | 22 | 22 |
| Rago et al. Java script: new show content ('active', 'references'); [15] | Italy | 27 | 36 | volume ≥ 30% | 11·1 | - | - |
| Quadbeck, et al. [16] | Germany | 139 | 59 | volume ≥30% | 61·2 | 23 | 15·8 |
| Alexander, et al. [18] | USA | 330 | 20 | volume ≥ 15% | 39 | - | - |
| Erdogan et al. [7] | Turkey | 531 | 39 | volume ≥ 30% | 24·1 | 20·7 | 55·2 |
| Present study | Hungary | 106 | 143 | volume ≥ 30% | 51.2 | 16.0 | 32.8 |

**Table 6:** Previous studies on the natural course of benign thyroid nodules.

We were the first ones to demonstrate on FNA samples that AgNORc and AgNORa are significantly higher in malignant thyroid lesions than in benign ones fifteen years ago. Since then, this finding has been confirmed by several other groups [22-30]. The widespread application of the technique has been hindered by the relatively wide overlap between AgNOR values of the benign and malignant groups [31]. In the present study, we show that there is a close correlation between the proliferative activity of single cells determined by AgNOR and the growth potential of the whole nodule. The best parameter was the mean AgNORc. It seems self-evident that cells from a carcinoma present higher proliferative activity than benign cells and it is also not surprising that nodules containing cells with higher proliferative activity tend to grow, or tend to grow more rapidly compared to nodules containing cells with less proliferative activity. In addition to AgNORc, TSH-1 was also associated significantly with further nodule growth on multivariate logistic regression analysis. A significant negative correlation was found between V1 and TSH-1. The apparent contradiction of this finding with the former may be explained by the fact that smaller nodules increase greater than larger nodules. The practical relevance of AgNOR as a predictive parameter in individual cases is limited.

The measurement of AgNOR has the advantage that instead of a qualitative (yes or no) result, as in the case of presence or absence of a RET/PTC mutation, a more gradable result can be obtained. A quantitative marker is more realistic and reflects the differences in the proliferative character of the cells. One of the limitations of AgNOR is its time-consuming procedure. More simple methods like the mean nuclear area per cell, which can be determined automatically, may have also practical significance. Other enzyme techniques or new molecular methods may arise, which will give even better differentiation between growing and stable nodules. Nevertheless, we tested the AgNOR method as we performed AgNOR test on initial examinations 20 years earlier.

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