

# Value of Additional von Kossa Staining in Thyroid Nodules with “Suspicious for Malignancy” on Cytology

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**Background and Objectives:** We investigated the clinical value of additional von Kossa staining in thyroid nodules with “suspicious for malignancy” on cytology. **Materials and Methods:** From March 2010 to November 2010, 55 patients with 55 nodules which were diagnosed as “suspicious for malignancy” on cytology and had microcalcifications on ultrasound (US) underwent surgery and made up our final study population. We evaluated the role of the von Kossa stain as a preoperative diagnostic factor for thyroid cancer using histopathology as the “gold standard”. Diagnostic performances were calculated of the presence of psammoma bodies on both cytology and the von Kossa staining and of US in predicting thyroid cancers. **Results:** Of 55 nodules with microcalcifications on US and “suspicious for malignancy” on cytology, 53 (96.4%) were malignant and 2 (3.6%) were benign on histopathology. All pathologically benign nodules were negative on the von Kossa stain. The sensitivity, specificity, accuracy, positive predictive value (PPV), and negative predictive value (NPV) of the von Kossa stain were 28.3%, 100%, 30.9%, 100%, and 5% for diagnosis, respectively. **Conclusion:** Von Kossa staining can be a valuable diagnostic tool in a thyroid nodule with “suspicious for malignancy” on cytology and microcalcifications on US, objectively.

**Key Words:** Thyroid neoplasms, Fine-needle aspiration biopsy, Ultrasonography

## Introduction

Malignancy risk in thyroid nodules with “suspicious for malignancy” cytology has been reported to be 60–75%.<sup>1)</sup> Lobectomy is usually recommended in these nodules as an initial surgical approach.<sup>2)</sup> Total thyroidectomy can be performed when patients have an increased risk for malignancy such as large tumors (>4 cm), marked atypia on biopsy, a family history of thyroid carcinoma, and a history of radiation exposure.<sup>2)</sup> Sometimes, completion thyroidectomy is need-

ed in patients who initially undergo lobectomy and are confirmed with a malignancy after surgery.<sup>2)</sup>

There are several preoperative approaches to diagnose a malignancy in a thyroid nodule with “suspicious for malignancy” cytology.<sup>3–6)</sup> Suspicious ultrasound (US) features are associated with malignancy in thyroid nodules with “suspicious for malignancy” cytology.<sup>3,4)</sup> If no suspicious US features are found in the thyroid nodules, a frozen section may help surgeons decide the necessary extent of surgery.<sup>6)</sup> An additional BRAF<sup>V600E</sup> mutation test can help decide whether to perform therapeutic surgery in these no-

Received October 20, 2014 / Revised 1st December 23, 2014, 2nd February 3, 2015 / Accepted February 10, 2015  
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dules.<sup>5)</sup> Although the above additional methods can help surgeons determine appropriate treatment approaches, there are several limitations that occur when applying these various courses of treatment to daily practice. US assessment and frozen section are dependent on the performer and the molecular test is limited by its high expense.

Microcalcifications on US are a specific feature of thyroid malignancy<sup>7)</sup> and are associated with psammoma bodies (PBs).<sup>8)</sup> Psammoma bodies consist of microcrystals similar to calcium phosphate apatite crystals.<sup>8)</sup> The von Kossa staining is an exceedingly inexpensive analytic tool and can detect phosphates in the presence of acidic material without detecting the amount of calcium.<sup>9)</sup> Therefore, the von Kossa staining can detect malignant microcalcifications associated with PBs. Until now, there have been no reports evaluating the additional role of the von Kossa staining in the diagnosis of malignancies in thyroid nodules with “suspicious for malignancy” cytology. In this study, we investigated the clinical value of additional von Kossa staining in thyroid nodules with “suspicious for malignancy” cytology.

## Materials and Methods

The Institutional Review Board of Severance Hospital approved this study and required neither patient approval nor informed consent for our review of patients’ images and records. However, written informed consent was obtained from all patients for US-FNA prior to each procedure as part of daily practice.

### Study Population

Since 2006, our institutional registry has collected data on numerous patients including all patients with thyroid nodules who underwent US-FNAs at our institution. Using the registry, data was retrieved for patients with “suspicious for malignancy” cytologic results from March 2010 to November 2010 at our institution. There were 252 nodules in 247 patients which were diagnosed as “suspicious for malignancy” on cytology. Seventy four nodules in 74 patients had microcalcifications with or without macrocalcifications

on US. Among them, 55 patients with 55 nodules underwent surgery and made up our final study population. The mean age of the patients was 48.2 years (range, 17–76 years). The mean lesion size of the thyroid nodules was 8.9 mm (range, 3–50 mm).

### Imaging Evaluation

US images were obtained using a 5– to 12-MHz linear array transducer (iU22; Philips Medical Systems, Bothell, WA, USA) for evaluation of thyroid nodules by one of 8 board-certified radiologists specializing in thyroid imaging and were assigned arbitrarily according to the hospital’s daily schedule. Compound imaging was performed in all cases. All US-FNAs were performed by the same radiologist who performed the US examination. The nodule size was defined as the largest diameter on US. US features of all thyroid nodules that underwent US-FNAs were prospectively recorded by the previously described methods.<sup>10)</sup>

### US-Guided Fine Needle Aspiration (US-FNA)

US-FNA was performed by the same radiologist who performed US. US-FNA was performed with a 23-gauge needle attached to a 2 mL disposable plastic syringe and each lesion was aspirated at least twice with the freehand technique. Obtained samples were expelled on glass slides, smeared, and placed immediately in 95% alcohol for Papanicolaou staining. The remaining material in the syringe was rinsed in saline for cell block processing. Cytopathologists were not on site during the aspiration procedure and cytology slides were interpreted to confirm the cytologic diagnosis by an experienced pathologist. Based on the Bethesda System for Reporting Thyroid Cytopathology, FNA cytology results were classified as non-diagnostic, benign, atypia undetermined significance/follicular lesion of undetermined significance (AUS/FLUS), suspicious for follicular neoplasm or suspicious for a Hurthle cell neoplasm, suspicious for malignancy and malignancy.<sup>1)</sup>

### Cytology and Surgical Pathology

Cytology smears for each case were reviewed to assess the presence of calcifications by light micro-

scopy. Calcifications were divided into PBs and dystrophic calcifications (Fig. 1) by an experienced cytopathologist (H.J.K.) who was always unaware of the final pathologic results. When a nodule had dystrophic calcifications as well as PBs, we listed it as PBs. Subsequently, the cover slides were removed by xylene and rehydrated using xylene, decreased grades of ethanol and deionized water. The uncovered slides were stained by von Kossa. The slides were incubated with an aqueous solution of 5% silver nitrate for 60 minutes at room temperature under sunlight until they took on a dark brown color. Following a distilled water rinse, the slides were incubated with a 5% sodium thiosulfate solution for 5 minutes. Calcium salts were often stained black or brown-black.

Total thyroidectomy was performed in 35 patients, ipsilateral total and contralateral subtotal thyroidectomy in 2, and lobectomy in 18. In our institution, surgical specimens were fixed in 10% formalin and cut at 3-mm intervals.<sup>11)</sup> All suspicious lesions and surrounding tissues were embedded in paraffin wax and stained with Hematoxylin and Eosin for histological examination. The presence of calcifications, including the presence of PBs, dystrophic calcifications, or ossifications was determined from surgical slides. When a nodule had dystrophic calcifications or ossifications as well as PBs, we listed it as with PBs. All these processes were performed by an experienced cytopathologist (H.J.K.) who was always unaware of the final

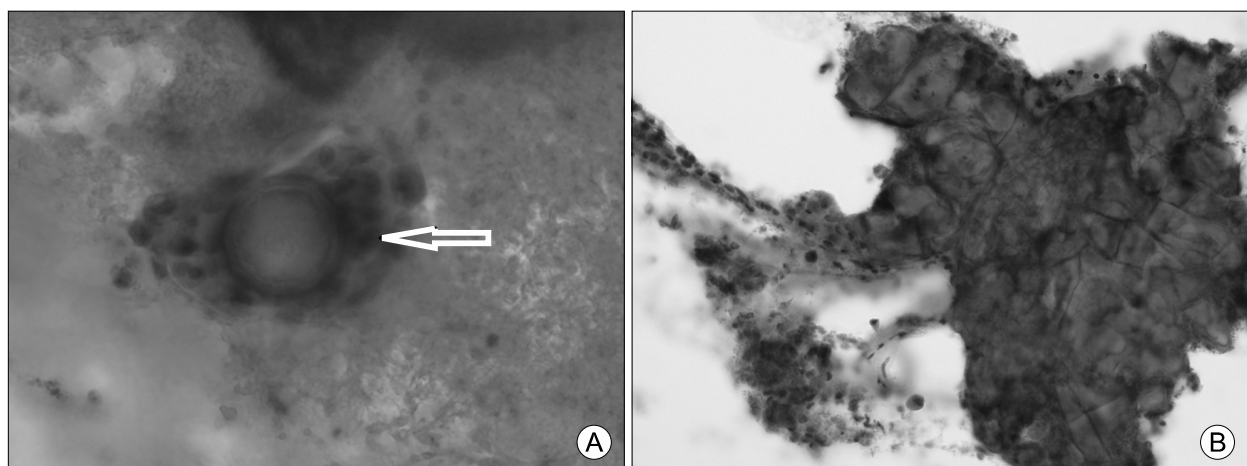
pathologic result of each case.

### Data and Statistical Analysis

We evaluated the role of the von Kossa stain as a preoperative diagnostic factor for thyroid cancer using histopathology as the “gold standard”. Patients with benign or malignant thyroid nodules were compared according to gender, and the results of the von Kossa stain, using the Fisher’s exact test. Values with a skewed distribution were logarithmically transformed and analyzed. The normality of the variables was tested using the Kolmogorov–Smirnov test. An independent two-sample t test was used to compare patient age and size between benign and malignant nodules. When size was not normally distributed, a nonparametric method such as the Mann–Whitney U test was used. The sensitivity, specificity, accuracy, positive predictive value (PPV) and negative predictive value (NPV) of the presence of a PB on both cytology and von Kossa staining and US in predicting thyroid cancers were calculated. Statistical significance was assigned to p values < 0.05. Data were analyzed using SAS software (SAS System for Windows, version 9.1.3; SAS Institute, Cary, NC, USA).

## Results

Of 55 nodules with microcalcifications on US and suspicious for malignancy on cytology, 53 (96.4%)



**Fig. 1.** Two types of calcifications on cytologic slides. (A) “Psammoma bodies” showing calcified concentric rings (smear, Papanicolaou stain,  $\times 400$ ). (B) “Dystrophic calcifications” showing irregular plates (smear, Papanicolaou stain,  $\times 200$ ).

**Table 1.** Demographics of the 55 patients

Characteristics	Total (n=55)	Malignancy (n=53)	Benignity (n=2)
Age (years, mean±SD)	48.2±12.5	47.6±12.2	65.5±0.7
Size (mm, median, range)		9 (3–50)	8.5 (5–12)
Gender			
Male	6 (10.9%)	6 (11.3%)	0 (0%)
Female	49 (89.1%)	47 (88.7%)	2 (100%)
Cytology slide calcification			
Positive	17 (30.9%)	17 (32.1%)	0 (0%)
Negative	38 (69.1%)	36 (67.9%)	2 (100%)
PBs on cytology slides			
Positive	13 (23.6%)	13 (24.5%)	0 (0%)
Negative	42 (76.4%)	40 (75.5%)	2 (100%)
von Kossa stain			
Positive	15 (27.3%)	15 (28.3%)	0 (0%)
Negative	40 (72.7%)	38 (71.7%)	2 (100%)
PBs on pathology slides			
Positive	32 (58.2%)	32 (41.5%)	0 (0%)
Negative	23 (41.8%)	21 (41.5%)	2 (100%)

PBs: psammoma bodies, SD: standard deviation

**Table 2.** Final histopathologic diagnosis

	Final Pathology	No (%)	PBs on cytology slides	Positive on the von Kossa stain	PBs on pathology slides
Malignant (n=53, 96.4%)	Papillary carcinoma, conventional	49 (92.5%)	12	13	30
	Papillary carcinoma, FV	2 (3.8%)	0	1	0
	Papillary carcinoma, DSV	1 (1.9%)	1	1	1
	Medullary carcinoma	1 (1.9%)	0	0	1
Benign (n=2, 3.6%)	Adenomatous hyperplasia	2 (100%)	0	0	0

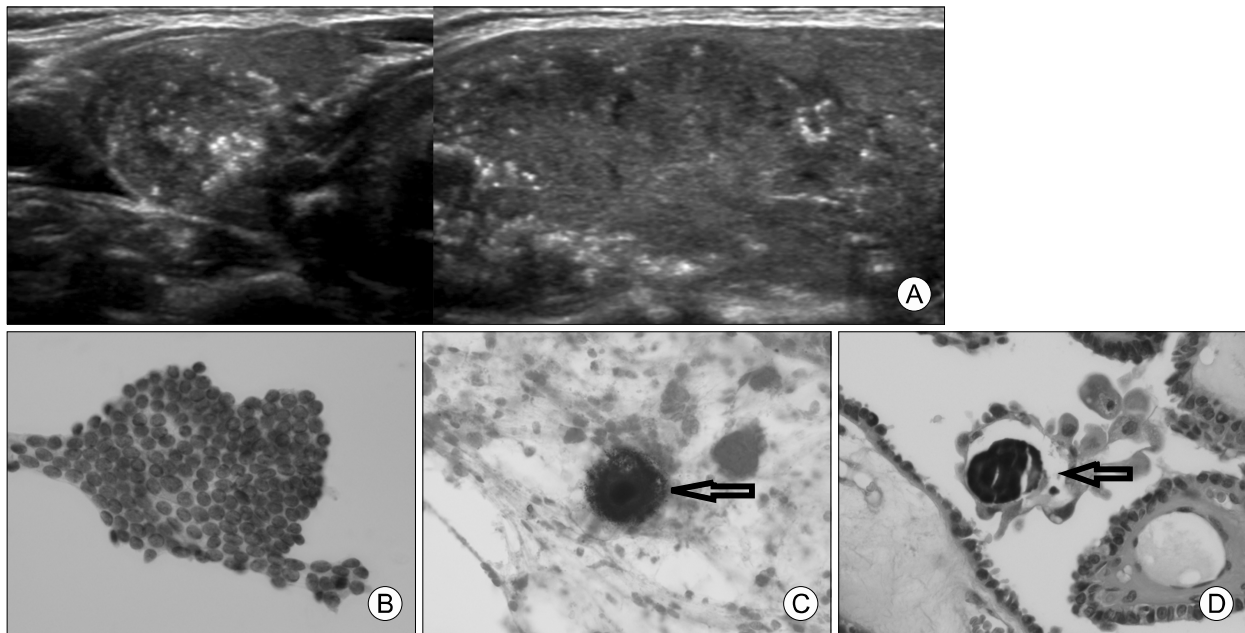
DSV: diffuse sclerosing variant, FV: follicular variant, PBs: psammoma bodies

were malignant and 2 (3.6%) were benign on histopathology (Table 1). The mean age was 48.2 (range, 23–72) years. Patients with benign thyroid nodules were significantly older than ones with malignant thyroid nodules ( $p<0.001$ ). There was no significant difference in nodule size and gender between the benign and malignant groups. The histopathology results are summarized in Table 2. The 12 papillary thyroid cancers (PTCs) (24.5%, 12/49) showed PBs on cytologic slides and none of the 2 cases of the follicular variant of PTCs showed PBs on cytologic slides.

On cytologic slides, 17 (30.9%, 17/55) nodules showed calcifications, of which 11 nodules had PBs, 4 had dystrophic calcifications, and 2 had both PBs and dystrophic calcifications. The sensitivity, specificity, accuracy, positive predictive value (PPV), and neg-

ative predictive value (NPV) of PBs on cytology slides for predicting malignancy were 24.5%, 100%, 27.3%, 100%, and 4.5%, respectively. There were 15 nodules which were positive on the von Kossa stain that were also confirmed as malignant (13 PTCs) 1 follicular variant type of PTC, and 1 diffuse sclerosing variant type of PTC) on histopathology (Fig. 2). All pathologically benign nodules were negative on the von Kossa stain. The sensitivity, specificity, accuracy, positive predictive value (PPV), and negative predictive value (NPV) of the von Kossa stain for diagnosis were 28.3%, 100%, 30.9%, 100%, and 5%, respectively.

We examined the surgical specimens, specifically searching for the presence of calcifications. Calcifications were found in 44 (80%) of 55 nodules, including PBs (n=32), dystrophic calcifications (n=9), and heter-



**Fig. 2.** A 23-year-old female diagnosed with papillary thyroid carcinoma. A 5-cm hypoechoic mass with internal microcalcifications was seen on (A, left-transverse scan, right-longitudinal scan) the right-longitudinal scan. On the cytology smear, there was a crowded group of follicular epithelial cells with powdery chromatin, nuclear grooves and micronucleoli, which were interpreted as “suspicious for papillary thyroid carcinoma” (B, smear, Papanicolaou stain,  $\times 400$ ). Psammoma bodies were seen in cytologic slides and the von Kossa stain was positive (C, arrow, smear,  $\times 400$ ). On the surgical slide, psammoma bodies were associated with tumor cells (D, arrow,  $\times 400$ ).

otopic ossifications ( $n=3$ ). Among nodules with PBs on pathologic slides which were all confirmed as cancers on pathology, 17 (46.9%, 15/32) showed calcifications on cytologic slides and 13 (40.6%, 13/32) were positive on the von Kossa stain. Among 9 nodules with dystrophic calcifications on surgical slides, 2 (22.2%, 2/9) showed calcifications on cytologic slides. One showed both a PB and dystrophic calcifications and 1 showed dystrophic calcifications on cytologic slides. These were positive on the von Kossa stain. All 3 cancers (2 PTCs and 1 follicular variant type of PTC) with heterotopic ossifications on surgical slides were negative on the von Kossa stain. There were no calcifications found in 11 nodules (20%) positive on the von Kossa stain even though preoperative US showed microcalcifications.

## Discussion

We found that all thyroid nodules with “suspicious for malignancy” cytology and with positive von Kossa stains were confirmed as cancers. All nodules with

positive von Kossa stains were cancers, suggesting a 100% PPV. Of the 38 malignant thyroid nodules, 15 nodules had positive von Kossa stains, resulting in a 28.3% sensitivity. The von Kossa stain was first described as a “silver nitrate method for calcium deposits” by von Kossa,<sup>12)</sup> and was modified afterwards.<sup>13,14)</sup> In the staining process, silver ions react with phosphate in the presence of acidic material and do not react with calcium.<sup>9)</sup>

Psammoma bodies are 50–70  $\mu\text{m}$  round-shaped calcific concretions which are concentrically laminated.<sup>15)</sup> They have been thought to arise from thickening of the base lamina of the vascular stalk of neoplastic papillae, followed by vascular thrombosis, calcification, and tumor cell necrosis and necrosis and calcification in intralymphatic tumor thrombi.<sup>15)</sup> Psammoma bodies can be seen in various conditions such as papillary neoplasms of various organs, endometriosis, hyperplastic nodular goiter, or lymphocytic thyroiditis.<sup>16–18)</sup> For diagnosing a thyroid malignancy, PBs are not common findings (0–25%) on cytology and PBs alone cannot be a reliable characteristic of malignancy with–

out other typical cytologic features.<sup>19)</sup> Also, it's not easy to differentiate PBs from other calcifications in daily practice due to their small size, natural continuum, and the two-dimensionality of cytology slides.<sup>20)</sup> In this study, 13 nodules which had "suspicious for malignancy" cytology results and had PBs observed on cytologic slides were confirmed as cancer. This result is similar to a previous report which concluded that thyroid cancers can be reliably diagnosed when cytologic features included PBs with other cytologic features such as papillary folds, nuclear grooves, and intranuclear inclusions.<sup>20)</sup>

Microcalcifications on US can be correlated with pathologic concretions including PBs, dystrophic calcifications, and inspissated colloids. Therefore, all nodules with microcalcifications on US were not confirmed as cancers although the risk of malignancy may be high in these nodules.<sup>3)</sup> In this study, the malignant rate was exceedingly high at 96.4% in thyroid nodules with microcalcifications on US and "suspicious for malignancy" on cytologic results, compared with the risk of malignancy found for thyroid nodules with "suspicious for malignancy" on cytologic results according to the Bethesda System for Reporting Thyroid Cytopathology (BSRTC).<sup>1)</sup> Pathologically, PBs are lamellated structures which sets them apart from dystrophic calcifications and inspissated colloids. Also, dystrophic calcifications are of irregular shape and inspissated colloids do not have calcifications.<sup>20)</sup> Although PBs on cytology slides showed a 100% PPV for diagnosing malignancy in this study, inherent limitations of cytologic interpretations might result in different diagnostic performances in other situations. Therefore, we evaluated the role of additional von Kossa staining which can detect phosphate in the presence of acidic material without detecting calcium.<sup>9)</sup> The specificity and PPV of the von Kossa staining for diagnosing malignancy were both 100%, in contrast to lower sensitivity and NPV. Considering the subjective nature of interpreting cytologic slides and its inherent limitation, the von Kossa staining can lead to a definite diagnosis in thyroid nodules with "suspicious for malignancy" cytology and microcalcifications on US, objectively.

Some limitations might be an issue in this study.

First, this is a retrospective study and included 55 patients who underwent surgery, which is a relatively small sample size, resulting in inevitable selection bias. Second, US and cytology results were interpreted by several radiologists and cytopathologists. Therefore, there may be interobserver variabilities in the assessment of US findings and cytology of thyroid nodules, respectively.<sup>21,22)</sup> Future studies should be performed in other institutions to verify our results.

## Conclusion

From the preceding discussion, we conclude that the von Kossa staining can be a valuable diagnostic tool in a thyroid nodule with "suspicious for malignancy" cytology and microcalcifications on US, objectively.

## Conflict of interest

None of the authors has any conflict of interest, financial or otherwise.

## Acknowledgments

This study was supported by a faculty research grant from Yonsei University College of Medicine for 2012 (No. 6-2012-0140). This research was also supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) by the Ministry of Education (2013R1A1A-2058817). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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