Surgery 165 (2019) 44–49

Contents lists available at ScienceDirect

Surgery

journal homepage: www.elsevier.com/locate/surg

Epigenetic chromatin conformation changes in peripheral blood can detect thyroid cancer ☆

Huan Yan, MD[a,*], Ewan Hunter, PhD[b], Alexandre Akoulitchev, PhD[b], Patricia Park, BA[a], David J Winchester, MD[a], Tricia A Moo-Young, MD[a], Richard A Prinz, MD[a]

[a] NorthShore University HealthSystem, Evanston, Illinois
[b] Oxford Biodynamics, Oxford, United Kingdom

ARTICLE INFO

Article history:
Accepted 1 May 2018
Available online 28 October 2018

ABSTRACT

Background: Fine needle aspiration has been the traditional method for diagnosing thyroid cancer. Epigenetic chromatin conformation changes offer an alternative method of diagnosing cancer. The purpose of this study is to evaluate an EpiSwitch assay of epigenetic markers that can be used to diagnose thyroid cancer in blood samples.

Methods: From 2014 to 2016, adult patients with thyroid nodules having thyroidectomy were recruited and grouped based on benign, malignant, and atypia of undetermined significance or follicular lesions of undetermined significance fine needle aspiration cytology. Blood samples were collected before surgery. Final pathologic diagnosis was made from the thyroid specimens. Patients' blood samples were analyzed using the EpiSwitch assay, (Oxford Biodynamics, Oxford, UK), and the results were compared with surgical pathology to determine assay performance.

Results: In total, 58 patients were recruited: 20 benign, 20 malignant, and 18 atypia or follicular lesions of undetermined significance. An analysis of the malignant and benign fine needle aspiration groups found 6 epigenetic markers for thyroid. A total of 28 (48%) patients had thyroid cancer. The assay was able to correctly identify 25 of the 28 malignant nodules, showing sensitivity of 89.3% and specificity of 66.7%. The positive predictive value for the assay was 71.4%, whereas the negative predictive value was 87.0%.

Conclusion: An epigenetic assay of peripheral blood shows high sensitivity in detecting thyroid cancer and provides an additional method for its diagnosis.

Background

Thyroid nodules are common lesions with approximately 50% prevalence in the general population.[1–2] Only 5%–15% of thyroid nodules harbor malignancy. Fine needle aspiration (FNA) cytology has become the standard method for diagnosing cancer in thyroid nodules. However, it is an invasive procedure requiring a trained operator, and up to 25% of FNA cytology results are indeterminate.[3] The risk of malignancy for these indeterminate nodules ranges from 10%–30%, so repeat biopsy or thyroid lobectomy may be required to obtain a definitive benign or malignant diagnosis. The majority of patients who undergo diagnostic lobectomy turn out to have benign disease on final pathology. Another shortcoming of FNA cytology is its inability to diagnose follicular thyroid cancer because it can only examine cellular and not tissue architecture.

Molecular testing has emerged to improve diagnostic accuracy in thyroid nodules with indeterminate cytology results. This testing has been shown to be highly sensitive for detecting cancer with sensitivities at or above 90%. However, these tests are much less specific for cancer, which limits their positive predictive value (PPV). In addition, they still depend on obtaining a tissue sample.[2,4] As a result, the preoperative workup of indeterminate thyroid nodules remains a challenge.

Epigenetics is the study of the mechanisms that alter gene expression independent of genetic sequence. It has promising applications for enabling diagnosis and guiding treatment of both benign and malignant diseases.[5–6] The DNA in human cells wraps around histone proteins and organizes into complex 3-dimensional chromatin structures. The topographic conformation of chromatin brings together genetic sequences that are distant in the linear DNA sequence and allows for long-range genetic interactions.[7] Changes in chromatin conformation can affect genetic expression

☆ Presented at the American Association of Endocrine Surgeons 2018 annual meeting in Durham, North Carolina, May 6–8, 2018.

* Corresponding author: NorthShore University HealthSystem, Surgical Oncology, 2650 Ridge Avenue, Walgreen Suite 2507, Evanston, IL 60201.
E-mail address: huanyan127@gmail.com (H. Yan).

https://doi.org/10.1016/j.surg.2018.05.081
0039-6060/

and serve as markers of malignancy. They have been used to diagnose a number of cancers, including melanoma.[7–9] These biomarkers offer an alternative method of diagnosing thyroid cancer that does not require tissue biopsy or antibody development. The purpose of this study is to develop an assay of chromatin conformation signatures in blood samples that can detect thyroid malignancy in patients with thyroid nodules.

Methods

After institutional review board approval was obtained, 60 adult patients who were scheduled for thyroid surgery were approached for the study. Written consent was obtained from each patient enrolled. Patient confidentiality was maintained in accordance with the Health Insurance Portability and Accountability Act guidelines. Identifiable patient information was kept in password-protected institution computers and was not shared with Oxford Biodynamics. Patients were grouped according to their preoperative FNA biopsy results: benign, malignant, or indeterminate (includes atypia/follicular lesion of undetermined significance and suspicious for follicular neoplasm). In total, 20 patients were enrolled for the benign group, 20 for the malignant group, and 18 for the indeterminate group. Power calculation was also not performed because this is a pilot study, and selection of the sample size was based on a prior study in which an epigenetic assay was developed for melanoma.[9] Demographic information, presence of thyroiditis, and ultrasound variables were collected for each patient. A patient was considered to have thyroiditis either based on positive tests for thyroid stimulation immunoglobulin, anti-thyroid peroxidase antibody, antithyroglobulin antibody, or findings of thyroiditis on final pathology. For each enrolled patient, 10mL of blood were collected preoperatively in plastic Vacutainer tubes with EDTA and stored in a −20°C to −80°C freezer within 60 minutes of collection. The blood specimens were sent to the laboratory of Oxford Biodynamics in Oxford, England, within 3 months of collection. None of the patient specimens in the indeterminate FNA group had molecular testing.

Identification of candidate biomarkers

Initial testing of the 20 malignant blood samples was done using proprietary EpiSwitch software (Oxford Biodynamics, Oxford, UK), which identifies loci of potential chromatin interactions using pattern recognition. The EpiSwitch software produced a list of potential loci in 10 genes: serpin gamily A member 1, telomerase reverse transcriptase, surfactant protein B, RET proto-oncogene, trefoil factor 3, MET proto-oncogene, LIM domain kinase 1, forkhead box E1, dual oxidase 2 (*DUOX2*), and melanocyte-specific protein 1 (*CITED1*). Primers were designed for each loci. Each primer was then tested against all other primers in the blood specimen of the malignant patient samples using the EpiSwitch chromatin conformation capture (3C) assays.

The EpiSwitch 3C assay detects the juxtaposition of distant DNA sites (also known as genetic loops) brought together by the topological structure of the chromatin. The assay is performed by first fixing chromatin with formaldehyde to capture intrachromatin associations. The fixed chromatin is then digested into fragments with TaqI restriction enzyme. Then the DNA strands are joined favoring cross-linked fragments. The cross-links are reversed and polymerase chain reactions (PCR) performed using the primer previously found by the EpiSwitch software. Each PCR requires 50μL of blood. In the PCR reaction, 2 validated genetic loops were used as positive controls. Each PCR reaction was performed 3 times per primer per sample. If 2 of the 3 gave a PCR product, this was deemed to indicate presence of a genetic loop. Each genetic loop was a candidate biomarker.

Biomarker selection and assay development

The presence of chromatin conformation markers in blood was compared between patients in the malignant and benign groups. Primers were designed for the previously identified candidate biomarkers, and each primer was tested 3 times on each blood sample with the EpiSwitch 3C assay. Gel electrophoresis was performed using the LabChip DNA 1K Version2 kit from Perkin Elmer (Perkin Elmer, Waltham, Massachusetts) to determine if each primer produced the expected PCR product. A biomarker was considered to yield a positive signal if its primer produced the expected PCR product in at least 2 of the 3 PCR reactions.

The data of every potential biomarker was entered into the machine learning algorithm of the Weka 3.6.7 classification software. The software uses pattern recognition to determine which biomarkers can be used to discriminate between malignant and benign samples. The markers are combined into an assay, which is refined and cross-validated on the patient sample. A stratified 10-fold cross-validation was performed, which involves randomly separating the data into 10 portions in which the proportion of malignant samples is kept approximately the same as in the full data set. The algorithm trains on 9 of the 10 portions and then tests on the tenth portion.

The EpiSwitch assay was then tested on the blood samples of all the enrolled patients. The assay gives a binary result of either malignant or benign, and the assay results were compared to the final pathologic diagnosis to determine accuracy. Sensitivity, specificity, negative predictive value (NPV), and PPV were calculated for the assay.

Results

The study group consisted of 58 patients: 20 with benign FNA, 20 with malignant FNA, and 18 with indeterminate FNA cytology results. Although 20 patients were recruited for the indeterminate group, 2 patients were excluded from the study because they did not have sufficient tissue for testing. Average patient age was 51 years, and 39 (67%) of the patients were female. The 3 groups were similar in age, sex, and nodule size (Table 1). The benign FNA group was significantly less likely to have thyroiditis when compared with the other 2. When comparing suspicious ultrasound findings, the malignant and indeterminate groups were more likely to have nodules with internal vascularity. Rates of hypoechoic nodules and microcalcifications were similar across the 3 groups.

Examination of the indeterminate FNA group showed that half of the patients had follicular neoplasms, whereas the other half had atypia or follicular lesions of undetermined significance (AUS/FLUS; Table 2). In total, 6 patients had invasive malignancies: 4 papillary and 2 follicular thyroid carcinomas. Of the patients, 2 had equivocal pathologic results. A lesion of uncertain malignant potential—a partially encapsulated nodule with follicular architecture and focally atypical nuclear features—occurred in 1 patient. Testing for *BRAF* mutation was negative, and the final pathologic opinion was that the lesion was borderline malignant. The other patient had a noninvasive follicular thyroid neoplasm with papillary-like nuclear features. For the purposes of this study, both patients were counted as having malignancy. Either Hurthle cell neoplasms or AUS/FLUS with predominantly Hurthle cells were found in 6 patients.

Initial testing of 634 chromosomes in the 20 malignant and 20 benign samples showed 10 loci of interest: serpin family A member 1, telomerase reverse transcriptase, surfactant protein B, RET proto-oncogene, trefoil factor 3, MET proto-oncogene, LIM domain kinase 1, forkhead box E1, *DUOX2*, and *CITED1*. From analysis of the 10 loci, 28 potential biomarkers were found; 6 biomarkers were ultimately selected for the assay (Table 3). The frequency for which

Table 1
Patient characteristics

	Benign	Malignant	Indeterminate	*P* value
Age, years (SD)	53.5 (±13.3)	44.6 (±12.1)	53.4 (±14.9)	.07
Nodule size, cm (SD)	2.4 (±1.5)	2.0 (±1.1)	2.2 (±1.6)	.64
Female sex	15 (75%)	11 (55%)	13 (72%)	.35
Thyroiditis	3 (15%)	10 (50%)	5 (28%)	.05
Ultrasound findings				
Multinodular	17 (85%)	12 (60%)	13 (72%)	.18
Microcalcifications	6 (30%)	6 (30%)	4 (22%)	.43
Hypoechoic nodule	5 (25%)	9 (45%)	7 (39%)	.40
Internal vascularity	5 (25%)	10 (50%)	13 (72%)	.02

SD, standard deviation

Table 2
EpiSwitch results for patients with indeterminate FNA

Patient number	FNA	Pathology result	EpiSwitch result
1	SFN, Hurthle cell	PTC, follicular variant	Benign
2	SFN	PTC, follicular variant	Malignant
3	SFN	PTC, follicular variant	Malignant
4	AUS/FLUS, Hurthle cell	PTC, classic (incidental)	Malignant
5	AUS/FLUS, Hurthle cell	FTC, Hurthle	Malignant
6	AUS/FLUS	FTC	Malignant
7	SFN	Follicular nodule	Benign
8	SFN	Follicular adenoma	Malignant
9	AUS/FLUS	Follicular adenoma	Malignant
10	AUS/FLUS, Hurthle cell	Follicular adenoma	Benign
11	SFN	Noninvasive follicular neoplasm	Malignant
12	SFN, Hurthle cell	Adenomatoid nodule	Malignant
13	SFN, Hurthle cell	Follicular adenoma, Hurthle cell type	Malignant
14	AUS/FLUS	Colloid nodule	Malignant
15	AUS/FLUS	Colloid nodule	Benign
16	SFN	Follicular neoplasm, uncertain malignancy	Malignant
17	AUS/FLUS	Follicular adenoma	Benign
18	AUS/FLUS	Nodular hyperplasia	Benign

SFN, suspicious for follicular neoplasm; *PTC*, papillary thyroid carcinoma; *FTC*, follicular thyroid carcinoma

Table 3
Frequency of biomarkers presence in blood samples

Biomarker	Benign ($n=30$)	Malignant ($n=26$)
CITED1 1/19	63%	57%
DUOX2 1/5	70%	88%
DUOX2 7/27	50%	73%
DUOX2 9/19	73%	88%
RET 15/19	43%	30%
MAYO 29/31	63%	77%

Table 4
Comparison of pathology and EpiSwitch results

	Pathology results	
EpiSwitch diagnosis	Malignant	Benign
Malignant diagnosis	25	10
Benign diagnosis	3	20

Table 5
Comparison of true positive and false negative malignant samples

	True positive ($n=25$)	False negative ($n=3$)	*P* value
Age, years (SD)	47 (±12)	54 (±22)	.40
Female sex	13 (52%)	1 (33.3%)	.54
Thyroiditis	10 (40%)	1 (33%)	.82
Histology			
Papillary Carcinoma	21 (84%)	3 (100%)	.45
Follicular Carcinoma	4 (16%)	–	
Tumor size, cm (SD)	2.1 (±1.3)	1.7 (±0.99)	.67
Nodal involvement	7 (29%)	1 (33.3%)	.882
Multifocal	12 (48%)	0	.11

SD, standard deviation

each biomarker was positive in the malignant and benign groups is shown in Table 3.

A total of 28 (48.3%) patients had thyroid cancer in their surgical specimens, and 30 patients had benign conditions. The assay was able to correctly identify 25 of the 28 malignant tumors, showing sensitivity of 89.3% (Table 4). Of the 30 patients with benign disease, 20 were correctly identified, yielding a specificity of 66.7%. The PPV for the assay was 71.4%, whereas the NPV was 87.0%. In the indeterminate group, the assay correctly identified malignancy in 2 patients with follicular carcinoma and 1 patient with an incidental 0.5cm papillary carcinoma that was not biopsied. The assay was less accurate with Hurthle cell lesions: correctly diagnosing 50% of these 6 patients. If these 6 patients were excluded from the analysis, sensitivity and NPV for the assay improves to 92% and 91%, respectively. The specificity and PPVs improve to 70% and 74%, respectively.

Among the 28 malignancies, the assay correctly identified 18 of the 20 malignant FNA samples and 7 of the 8 malignant tumors from the indeterminate FNA group. The true positive patients tended to be younger and female and more frequently had multifocal tumors when compared with the false negative group. However, none of the comparisons reached statistical significance as would be expected with such small numbers (Table 5). Tumor size, lymph node involvement, and rates of microcarcinoma were also similar between the groups. All 3 false negative samples were papillary carcinoma: 1 follicular variant, 1 classic variant, and 1 tall cell variant. In the true positive group, 2 of the samples were follicular carcinomas.

The EpiSwitch assay correctly identified 20 of the 30 benign lesions: 15 of the 20 benign FNA group and 5 of the 10 benign

Table 6
Comparison of true negative and false positive for malignant samples

	True negative ($n = 20$)	False positive ($n = 10$)	P value
Age, years (SD)	52 (±11)	55 (±15)	.61
Female sex	15 (75%)	10 (100%)	.08
Nodule size, cm	2.6 (±1.5)	1.9 (±1.3)	.24
Multinodular	15 (75%)	9 (90%)	.10
Thyroiditis	2 (10%)	5 (50%)	.02

SD, standard deviation

lesions from the indeterminate FNA group. The patients with false positive results were 5 times as likely to have thyroiditis compared with the patients with true negative results (Table 6). Age, gender, nodule size, and rates of multinodularity were similar between the 2.

Discussion

This study demonstrates the feasibility of a relatively noninvasive assay that can be developed into a diagnostic tool for thyroid nodules. Each individual biomarker lacks sufficient power to discriminate malignant from benign samples, but when combined into an assay, the six biomarkers in the EpiSwitch assay demonstrate 89.3% sensitivity for detecting malignancy in patients with thyroid nodules by testing for chromatin conformation changes in peripheral blood. For the indeterminate FNA group alone, the assay was able to correctly identify 7 of the 8 patients with malignancy. The malignancy rate for the indeterminate FNA group was 44% (8/18) for the present study and is significantly higher than the 25% malignancy rate for the institution. The specificity and PPV are lower at 66.7% and 71.4%, respectively, and positive assay results may still have a substantial proportion of patients with benign disease. Thyroiditis is associated with a false positive result among the benign lesions. Further refinement and validation of the assay will be needed to improve its diagnostic accuracy.

This pilot study of the EpiSwitch assay suggests that it can be applied in several ways in the diagnosis and management of thyroid nodules. The assay can serve as an adjunct to FNA for nodules with indeterminate or nondiagnostic cytology results. In these patients, the assay does not require a repeat biopsy, is not affected by the quality of the initial biopsy, and can additionally detect malignancy in patients with follicular thyroid cancer. It can also serve as the diagnostic test of choice when FNA is not desired or not practical. This includes patients who refuse invasive biopsy or have multiple nodules for which biopsy of each nodule would be impractical and onerous. Once the specificity improves, the assay can guide the clinician in deciding on the correct surgery to perform and avoid reoperation after lobectomy.

The chromatin conformation changes are located in genes for which altered expression has been shown to be associated with thyroid carcinogenesis. The RET proto-oncogene, located at 10q11.2, codes for a tyrosine kinase receptor. Gain-of-function mutations in this gene have been shown to be associated with papillary and medullary carcinomas.[10] Specifically, chromosomal rearrangements in chromosome 10 cause fusion of RET to heterologous genes and form chimeric oncogenes called *RET/PTC*, and these chimeric oncogenes have been shown to induce thyroid cell hyperplasia and neoplasia in mouse models.[11–12]. At the *DUOX2* gene, 3 of the chromatin conformation changes were found. Located at 15q21.1, this gene encodes for the protein dual oxidase 2, which produces hydrogen peroxide for thyroid hormone formation.[13] Lacroix et al showed that *DUOX2* expression was altered in papillary and follicular thyroid cancer cells with its protein product more frequently found in neoplastic tissue.[14] The *CITED1* gene encodes for a transactivator protein more frequently associated with melanoma cells, but Huang et al.[15] showed that its gene product was overexpressed in 10 out of 10 patients with papillary carcinoma. The Mayo 29/31 chromatin signature is found in the interleukin 12B gene. This gene encodes for a subunit of IL-12, a cytokine that drives differentiation of CD4+ T cells into T helper cells when the body is in a proinflammatory state.[16]

This study has several limitations. First, some of the patient samples (the benign and malignant groups) that were used to develop the assay were also used to test it. This retrospective cross-validation was necessary because of the small number of enrolled patients, but it may lead to an overestimation of the assay's accuracy. Follow-up studies with higher patient enrollment would be essential for prospective validation. Second, the biomarkers were developed from samples of differentiated thyroid cancer, and it is unknown if the assay can be used for the diagnosis of other histologic types, such as medullary or anaplastic thyroid cancer. It is highly possible that a completely different assay will need to be developed for these other histologies. Third, the chromatin conformation changes are unlikely from circulating tumor cells because only 50μL of blood is required for each assay and the concentration of tumor cells in differentiated thyroid cancer is quite low. A recent study by Xu et al on 14 patients with differentiated thyroid cancer showed that only 1 patient had 3 tumor cells in 7.5mL of blood, whereas the remaining 13 patients had 0 or 1 detectable tumor cell in 7.5mL of blood.[17] The material being assayed is either free genetic compounds released from tumor cells in the thyroid gland into circulation or from circulating white blood cells. We are currently conducting a validation study of 20 patients with known malignant thyroid nodules to compare the chromatin conformation signatures in blood with those found in the primary tumor. The results of this study will help determine if the signatures in blood mirror those found in tumor cells.

In conclusion, we have demonstrated the feasibility of developing a diagnostic tool for detecting thyroid cancer using patient blood samples. It can provide an additional tool for clinicians to guide decision-making in patients with suspicious thyroid nodules, particularly when tissue biopsy is not acceptable or unavailable. Future studies will focus on prospective validation and assay refinement to improve accuracy.

Conflicts of interest

Dr Ewan Hunter and Dr Alexandre Akoulitchev are both employees of Oxford Biodynamics, which owns the EpiSwitch technology used in the study. Dr Hunter is head of statistics and director of business development and Dr Akoulitchev is chief science officer. They were involved in developing and running the assay. With respect to the manuscript, they assisted in making sure the Methods section is accurate. The final pathology results were done at NorthShore University Hospital. Oxford Biodynamics did not provide any financial assistance to NorthShore or any of the other authors. Funding and patient recruitment were both done using NorthShore staff and Department of Surgery funds. The remaining authors have no financial disclosures or conflicts of interest.

References

1. Davies L, Welch HG. Increasing incidence of thyroid cancer in the United States, 1973–2002. *JAMA*. 2006;295:2164–2167.
2. Bernet V, Hupart KH, Parangi S, Woeber KA. AACE/ACE disease state commentary: molecular diagnostic testing of thyroid nodules with indeterminate cytopathology. *Endocr Pract*. 2014;20:360–363.
3. Witczak J, Taylor P, Chai J, Amphlett B, Soukias JM, Gautam D, Tennant BP, Geen J, Okosieme OE. Predicting malignancy in thyroid nodules: feasibility of a predictive model integrating clinical biochemical, and ultrasound characteristics. *Thyroid Research*. 2016;9:4.
4. Zhang M, Lin O. Molecular testing of thyroid nodules: a review of current available tests for fine-needle aspiration specimens. *Arch Pathol Lab Med*. 2016;140:1338–1344.

5. Verma M, Srivastava S. Epigenetics in cancer: implications for early detection and prevention. *Lancet Oncol*. 2002;3:755–763.
6. Dawson MA, Kouzarides T. Cancer epigenetics: from mechanism to therapy. *Cell*. 2012;150:12–27.
7. Crutchley JL, Wang XQD, Ferraiuolo MA, Dostie J. Chromatin conformation signatures – ideal human disease biomarkers? *Biomarkers Med*. 2010;4:611–626.
8. Bastonini E, Jeznach M, Field M, Juszczyk K, Corfield E, Dezfouli M, et al. Chromatin barcodes as biomarkers for melanoma. *Pigment Cell Melanoma Res*. 2014;27:788–800.
9. Jakub JW, Grotz TE, Jordan P, Hunter E, Pittelkow M, Ramadass A, et al. A pilot study of chromosomal aberrations and epigenetic changes in peripheral blood samples to identify patients with melanoma. *Melanoma Res*. 2015;25:406–411 PubMed PMID26225582.
10. Santoro M, Carlomagno F. Central role of RET in thyroid cancer. *Cold Spring Harbor Perspectives in Biology*. 2013;5:12.
11. Kim DW, Hwang JH, Suh JM, Kim H, Song JH, Hwang ES, et al. RET/PTC (rearranged in transformation/papillary thyroid carcinomas) tyrosine kinase phosphorylates and activates phosphoinositide-dependent kinase 1 (PDK1): an alternative phosphatidylinositol 3-kinase-independent pathway to activate PDK1. *Mol Endocrinol*. 2003;17:1382–1394 PubMed PMID12738763.
12. Wang J, Knauf JA, Basu S, Puxeddu E, Kuroda H, Santoro M, et al. Conditional expression of RET/PTC induces a weak oncogenic drive in thyroid PCCL3 cells and inhibits thyrotropin action at multiple levels. *Mol Endocrinol*. 2003;17:1425–1436 PubMed PMID12690093.
13. Ohye H, Sugawara M. Dual oxidase, hydrogen peroxide and thyroid diseases. *Exp Biol Med (Maywood)*. 2010;235:424–433.
14. Lacroix L, Nocera M, Mian C, Caillou B, Virion A, Dupuy C, et al. Expression of nicotinamide adenine dinucleotide phosphate oxidase flavoprotein DUOX genes and proteins in human papillary and follicular thyroid carcinomas. *Thyroid*. 2001;11:1017–1023.
15. Huang Y, Prasad M, Lemon WJ, et al. Gene expression in papillary thyroid carcinoma reveals highly consistent profiles. *Proceedings of the National Academy of Sciences of the United States of America*. 2001;98:15044–15049.
16. Hiromatsu Y, Fukutani T, Ichimura M, Mukai T, Kaku H, Miyake I, et al. Interleukin-12B gene polymorphism does not confer susceptibility to Graves' ophthalmopathy in Japanese population. *Endocr J*. 2006;53:753–759.
17. Xu JY, Handy B, Michaelis CL, Waguespack SG, Hu MI, Busaidy N, et al. Detection and prognostic significance of circulating tumor cells in patients with metastatic thyroid cancer. *J Clin Endocrinol Metab*. 2016;101:4461–4467.

Discussion

Dr Mark Cohen (Ann Arbor, MI): I applaud the NorthShore group for their efforts in this area.

We are moving toward an era of precision medicine and liquid biopsies. I think that as we advance and improve our technological abilities that it's going to become much more of a reality in endocrine cancers as well as all other types of cancer.

When we think about this as a group, we have to be very cognizant about what thresholds we need to achieve to have a meaningful impact on decision-making for patients.

So when you look at your data, a positive predictive value of 71% means that about 30% of the time you have a positive test that's actually negative, and a negative predictive value of 13% means that 13% of the time the patient with the negative test really has cancer, right?

So if those are within the same circle on a Venn diagram, that's great. But if they are not overlapping and mutually exclusive in 2 separate groups, then 43% of the time you have an inaccurate test.

My question is whether you think this assay is good enough to make any meaningful changes in diagnostic practices? And, if not, what is good enough?

What numbers should we really be shooting for with an assay to think about changing the approach to how we diagnose and treat patients?

A lot of patients and colleagues I talk to would not tolerate a cancer risk of more than 10%. So what do you feel is "good enough"?

Dr Huan Yan: In terms of the sensitivity and specificity, you would want approximately 95% or better for clinical use. You don't want to inaccurately diagnose patients who have malignancy, when you are telling them that they don't. Sensitivity is something that needs to be improved with this assay.

Dr John Yim (Pasadena, CA): I would like to add a comment and a question.

The negative predictive value needs to be higher and the positive predictive value needs to be higher, and obviously there's potential for this. It's not ready to be used as a diagnostic test, I would say.

Is it my understanding that the switch is with chromatin conformation? Are you saying there was an inherent conformation of those particular genes?

And my second question is, what cells are you actually seeing this in? Do you have any methods to see if you are getting this DNA from white blood cells in the plasma or just free DNA?

Dr Huan Yan: This test is looking for those interactions between distant genetic sequences brought about by the different conformations of the gene. So what the assay does is this: We first use restriction enzymes to divide the DNA into fragments. We run the fragments through a PCR and use gel electrophoresis to separate the fragments. Most fragments will be very short. The portions of the genetic sequences that have cross-links will be larger, and that's what we are detecting with this assay.

This test uses whole blood which is not centrifuged. The source of the DNA in the blood is presently unknown. Most likely, the genetic material is derived from white blood cells. We looked at the presence of free-floating genetic material in serum and did not find a detectable amount. In terms of whether or not these are circulating tumor cells, there's 1 study from MD Anderson that shows that for differentiated thyroid cancers, the concentration of tumor cells is exceedingly low in blood, approximately 1 cell for every 7.5 milliliters. For this assay, each run uses only 50 microliters of whole blood. Therefore, the DNA is unlikely to be from circulating tumor cells. Most likely the conformational genetic changes seen in the assay are derived from white blood cells.

Dr Mira Milas (Phoenix, AZ): Thank you for a very lovely study and thank you to you and your colleagues for not giving up on the concept of finding a better biomarker in blood for differentiated thyroid cancer patients. I would encourage you to keep on with this research. It is hard work. Everybody in this audience is familiar with what has been tried before, and only if you persist on studying it will you find whether it can help in our patient population.

What were the rates of detecting this marker in patients without any thyroid disease, with benign thyroid disease, or thyroid cancer?

I noted with curiosity that most of the false positives were from patients with thyroiditis, which is also interesting because that has been borne out in previous work. I would be interested to hear your thoughts on that.

Dr Huan Yan: Individual markers have low sensitivity and specificity for diagnosing thyroid cancer. The current combination of 6 markers has increased the accuracy of this assay.

We also noticed that patients with thyroiditis had an increased false positive rate. This led us to investigate the Mayo 29-31 marker. This is a marker from the interleukin 12B gene, which codes for the inflammatory cytokine IL12B. Future studies may not include this marker.

Dr Roger Tabah (Montreal, Canada): How did this behave in the FLUS/AUS category?

Dr Huan Yan: Eight of the 18 patients with FLUS on FNA were subsequently diagnosed with malignancy. Of these 8, 7 had a positive epigenetic assay. Although these numbers are small, this is an 88% sensitivity.

In the 10 patients with benign disease, 5 had a negative epigenetic assay, giving a specificity of 50%.

Dr Roger Tabah (Montreal, Canada): These are cancer-naive patients? In other words, they haven't been treated for another malignancy?

Dr Huan Yan: That's correct.

Dr Emad Kandil (New Orleans, LA): So what's next? Are you trying to see if you can adjust your panel to account for thyroiditis? Are you trying to add something? What's the next step?

Dr Huan Yan: We agree that this must be considered a pilot study. More patients from other centers will need to be evaluated. Other markers will need to be considered in this larger patient population. Additional data and modifications will hopefully improve the accuracy of this assay.