Oncosuppressor-Mutated Cell-Based Diagnostic Platform for Liquid Biopsy Diagnoses Benign Head and Neck Masses and Predicts Malignancy in Thyroid Nodules: Results from a Consecutive Cohort of Patients

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Keywords

Liquid biopsy · Thyroid cancer · Head and neck lesions ·
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Metastatic And Transforming Elements Released Discovery

Abstract

Background: We reported that a novel oncosuppressor-mutated cell (OMC)-based platform has the potential for early cancer detection in healthy individuals and for identification of cancer patients at risk of developing metachronous metastases. **Objective:** Herein, we sought to determine the diagnostic accuracy of this novel OMC-based platform in a consecutive cohort of patients operated for suspicious head and neck masses. **Methods:** OMCs (*BRCA1*-deficient fibroblasts) were exposed to blood serum from patients with head and neck nodules before surgical removal. These cells were analyzed for their proliferation and survival. Treated OMCs were inoculated subcutaneously in NOD/SCID mice, and tumor growth was monitored over time. **Results:** OMCs exposed to serum from patients with malignant lesions displayed increased proliferation compared to those exposed

to serum from patients with benign lesions. Only OMCs exposed to serum from patients diagnosed with malignant thyroid neoplasia generated a cancerous mass. The sensitivity of the test was 92%, with only 1 false negative out of 34 patients. Immunohistochemical staining showed that the cancerous masses were poorly differentiated adenocarcinomas with high proliferative index. **Conclusions:** These data show that liquid biopsy combined with an OMC-based in vivo platform has the potential to diagnose benign head and neck masses and predict whether a thyroid nodule is malignant. These results strengthen the concept that OMCs can be used to detect circulating malignant factors in cancer patients.

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Introduction

Masses arising in the glands of the head and the neck regions are often seen in clinical practice, and the first priority is to determine whether they are benign or malignant. Malignancies of the head glands such as salivary

glands are more common in adult smokers older than 40 years [1]. Salivary gland tumors most often present as painless enlarging masses. Most are located in the parotid glands and most are benign. The principal hurdle in their management lies in the difficulty in distinguishing benign from malignant tumors. Investigations such as fine needle aspiration cytology and MRI scans provide some useful information, but most cases will require surgical excision as a means of coming to a definitive diagnosis. There is a rise in the incidence of salivary gland cancers; this group accounted for 6.3% of all head and neck cancers in 1974–1976, as compared to 8.1% in 1998–1999 [1].

The propensity of the thyroid gland to form nodules is demonstrated by the observation that up to 60% of randomly selected individuals have nodules, 95% of which are benign [2]. Most thyroid malignancies (>95%) are referred as well-differentiated thyroid cancers. They include papillary thyroid carcinoma and its variants (70–85%) and follicular thyroid carcinoma and its variants (15–20%). The remainder of malignancies (~5%) comprise medullary thyroid carcinoma, anaplastic thyroid carcinoma, and lymphomas [3, 4].

Thyroid cancer remains one of the least deadly human cancers (i.e., 2,170 deaths from thyroid cancer compared to 142,670 deaths from lung cancer in 2019) [5], with >98% of patients still alive after 5 years [6, 7]. During the last decade, the incidence of well-differentiated thyroid cancer has increased substantially [6–9], in part due to both detection of small, incidental microcancers (<1 cm) during neck imaging for other indications (i.e., workup for cancers, neuromuscular complaints, and ear/nose or throat discomfort) and to the liberal use of invasive ultrasound-guided fine needle aspiration cytology (USFNAC) [10–13].

The Bethesda classification for reporting thyroid cytopathology introduced in 2009 has proposed 6 cytological diagnostic categories to standardize the interpretation of USFNAC. Treatment for Bethesda categories II, V, and VI is well established and not subject to much discussion considering the extremely high rate of benign lesions in category II and of malignant lesions in group V and VI. Instead, the best treatment options for categories III and IV are subject of debate since their malignancy rates are 5–15% and 15–30%, respectively, in what are considered atypical, indeterminate, or inconclusive cytology [14]. Thus, a “diagnostic” surgery is often proposed to a subset of asymptomatic patients where the majority are expected to have benign disease.

This problem has prompted researchers to develop noninvasive tools to improve detection of thyroid cancer

in patients with indeterminate or inconclusive USFNAC. In such instances, molecular analysis of FNAC samples has recently gained in popularity as an adjunct to microscopic cytological evaluation. Many molecular markers, such as BRAF and RAS mutations and gene product aberrations, are promising as diagnostic and prognostic markers [15–17]. In general, molecular testing offers high specificity and positive predictive value, but sensitivity is low [18]. The role of molecular testing as a diagnostic adjunct is in evolution.

Recently, it was reported that human cancer cells deliver extracellular vesicles (EVs) that contain signaling molecules and genetic material able to hijack the replicative machinery of oncosuppressor-mutated cells (OMCs) and complete their malignant transformation [19–26]. In particular, we observed that EVs isolated from cancer patients increased the proliferation of OMCs such as Fibro-BKO (*BRCA1*-knockout fibroblast), HEK293, and MC-F10A PTEN-knockout and induced their transformation into malignant cells capable of forming cancer masses when injected subcutaneously into NOD-SCID mice [22–24, 26]. The phenotype of the tumors formed was compatible with the phenotype of the cancer cells from which the EVs were derived. EVs extracted from sera of healthy patients, used as control, were not able to transform OMCs into cancer cells, strengthening the conclusion that OMCs sense circulating malignant factors, uptake them, and become malignant. Based on these observations, we designed the world’s first biological platform for cancer screening, which was called the Metastatic And Transforming Elements Released Discovery (MATER-D) platform [23, 25, 26]. We previously tested the capability of this platform to diagnose different types of cancers, and excellent results were reported [23, 25, 26].

In the present study, we aimed to test the cancer predictive efficacy of our MATER-D platform in a consecutive cohort of patients operated for suspicious head and neck masses. The results of the MATER-D assay from the sera obtained preoperatively were correlated with the pathological analyses of the surgical specimens. Our study provides evidence that this in vitro-based platform can be used to diagnose benign head and neck masses and discriminate malignant from benign thyroid nodules.

Materials and Methods

Blood Collection and Serum Preparation

Blood samples were collected in vacutainer tubes containing clot-activation additive and a barrier gel to isolate serum (Becton Dickinson). After 60-min clotting at room temperature, tubes

Table 1. Final pathology of resected lesions

Cases	Disease	TP	Sex, age
A	Nodular hyperplasia	N	Female, 65
B	Benign parathyroid	N	Female, 45
C	Warthin's tumor (multifocal)	N	Male, 66
D	Nodular follicular hyperplasia	N	Female, 84
E	Benign parathyroid	N	Male, 64
F	Adenomatoid oncocytic follicular nodule	N	Female, 61
G	Benign submandibular gland	N	Male, 29
H	Oncocytic follicular adenoma	N	Female, 46
I	Follicular adenoma	N	Male, 73
J	Nodular hyperplasia	N	Male, 61
K	Benign parathyroid	N	Female, 56
L	Oncocytic follicular hyperplasia	N	Female, 65
M	Oncocytic follicular adenoma	N	Female, 69
N	Benign parathyroid	N	Female, 48
O	Parathyroid adenoma	N	Female, 34
P	Nodular hyperplasia	N	Female, 66
Q	Pleomorphic adenoma (salivary gland)	N	Female, 53
R	Tertiary hyperparathyroidism	N	Male, 66
S	Chronic sialadenitis	N	Female, 54
T	Hurthle (follicular) adenoma thyroid	N	Female, 78
U	Pleomorphic adenoma (salivary gland)	N	Male, 54
V	Pleomorphic adenoma (salivary gland)	N	Female, 57
W	Papillary thyroid carcinoma (pT1bN1a)	Y	Female, 51
X	Papillary thyroid microcarcinoma (pT1aN0)	Y	Female, 65
Y	Papillary thyroid carcinoma (pT2N1a)	Y	Male, 32
Z	Unifocal papillary thyroid microcarcinoma (pT1aNx)	Y	Female, 71
ZA	Multifocal papillary thyroid microcarcinoma (pT1bN1a)	N	Male, 43
ZB	Noninvasive follicular thyroid neoplasm (NIFTP)	Y	Female, 50
ZC	Papillary thyroid microcarcinoma (pT1aN0)	Y	Female, 41
ZD	Multifocal papillary carcinoma (T3N1)	Y	Female, 70
ZE	Micropapillary thyroid carcinoma (T1a)	Y	Female, 73
ZF	Papillary thyroid carcinoma (T2N1a)	Y	Female, 37
ZG	Micropapillary thyroid carcinoma (T1a)	Y	Female, 75
ZH	Micropapillary thyroid carcinoma (T1a)	Y	Female, 49

TP, transforming potential; N, no transforming potential; Y (yes), presence of transforming potential. Clinical stage is given in parentheses.

were centrifuged at 1,500 g for 15 min. Serum was collected and subjected to a second centrifugation at 2,000 g for 10 min to clear it from contaminating cells. Serum was aliquoted and stored at -80°C until use.

Patient Categorization

Recruitment of 34 consecutive patients that were supposed to undergo surgery for suspicious nodules of the head and neck region was conducted at the department of General Surgery at the Royal Victoria Hospital (Montreal, Canada), in accordance with an approved ethics protocol by the Ethics Committee of the McGill University Health Centre (MP-37-2018-3916 and SDR-10-057) (Table 1). Patients with history of cancer were excluded from the study. Blood samples were obtained upon written consent from all participants. The sera from these patients were tested in a double-blinded manner by using a biological platform based on *BRCA1*

knockout fibroblasts (Fibro-BKO), as previously described [22]. Blood samples were given an identifier before being delivered to the operator who proceeded to cell treatments. Both the operator and the pathologist involved in the analysis of the *in vivo* results from the animal experiments were blinded to the histology results of the masses excised from the patients. After obtaining the results from the pathology examination of the surgically removed nodules, patients were categorized into two cohorts: those patients whose results were compatible with benign lesions (cases A–V) and those patients whose results were positive for malignancy (cases W–ZH).

Cell Culture Conditions

Fibro-BKO cells were established as previously described [23]. Cells were maintained in DMEM-F12 medium supplemented with 10% fetal bovine serum and antibiotics (all from Wisent). When

cells reached 30% confluence, they were treated with 0.2- μ m filtered DMEM-F12 medium containing 10% of the sera obtained from the patients. Half of the media was changed every second day for the 3-week exposure duration. When cells reached 80% confluence, they were passaged (1 in 6) using 0.05% trypsin/2 mM EDTA (Wisent). To confirm that there was no contamination or carry-over of blood-derived cells, aliquots of the culture medium were placed in a culture plate and incubated at 37°C in an atmosphere of 5% CO₂ in air.

PDL Calculation

Cells were considered at population doubling zero the first time they were exposed to patient serum-containing culture medium. At every passage, cell number was determined, and population doubling was calculated using the following formula: population doubling level (PDL) = $\log(N_h/N_i)/\log_2$, where N_h is the number of cells harvested at the end of the incubation time and N_i is the number of cells inoculated at the beginning of the incubation time. Cumulative PDL was calculated by adding the previously calculated PDL.

In vivo Tumor Growth

Five-week-old female NOD-SCID mice (Jackson Laboratory) were used in accordance with guidelines and following a protocol approved by the McGill University Health Centre Animal Compliance Office (Protocol 2012-7280). Cells growing in the log phase were harvested and washed twice with HBSS. Mice were injected subcutaneously with 2×10^6 cells in 200 μ L HBSS/Matrigel mixture. Mice were followed up daily for any sign of discomfort and tumor growth monitoring. Once a palpable subcutaneous mass was detected, its size was recorded using a caliper. The mass volume was estimated using the following formula: $V = a \times b^2 \times (\pi/6)$ (where a = major diameter, b = minor diameter, and V = volume) [21]. Four weeks after injection, all mice were euthanized.

Histological Analysis

Mice xenotransplants were collected, fixed in 10% buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin according to standard protocols or processed for immunohistochemistry. In brief, 5- μ m tissue sections were dewaxed in xylene and rehydrated with distilled water. After antigen unmasking and blocking of endogenous peroxidase (3% hydrogen peroxide), the slides were incubated with primary antibody: rabbit monoclonal Ki67, mouse monoclonal Vimentin, rabbit monoclonal TTF1, mouse monoclonal HBME-1 (all from Ventana), mouse monoclonal CK7, mouse monoclonal CK20, mouse monoclonal thyroglobulin (all from DAKO), rabbit monoclonal CDX-2 (Cell Marque), and mouse monoclonal BRAFV600E (Abcam). Labeling was performed using iView DAB Detection Kit (Ventana) on the Ventana automated immunostainer. Sections were counterstained lightly with hematoxylin before mounting.

Statistical Analysis

Statistical differences were analyzed using an ANOVA test. A p value <0.05 was considered statistically significant. A Fisher's exact test was used to compare the rates of tumor presence at 30 days between the cohorts of animals implanted with Fibro-BKO exposed to serum from patients with neoplasia versus the cohort of animals implanted with Fibro-BKO exposed to serum from patients with nonneoplastic nodules.

Results

Serum from Patients with Thyroid Malignant Lesions Increased the Proliferation of Fibro-BKO Cells

Patient's serum was collected before surgery and exposed to Fibro-BKO cells for 3 weeks. The culture medium was changed every second day. Every week, cells were collected, counted, and passaged. Cell proliferation was analyzed by measuring PDL at successive cell passages (Fig. 1). Cells treated with serum of patients with thyroid malignancy displayed higher growth rate measured as cell population doubling time compared with cells treated with serum from patients with non malignant lesions (Fig. 1a). We observed a 1-cycle gain at passage 3 (i.e., at the end of in vitro exposure) in cancer serum cultures (Fig. 1b). We ranked all the PDL obtained at day 21 of the in vitro culture, and we performed receiver operating characteristic analyses which gave an excellent test quality (i.e., AUC = 0.9417) (see online suppl. Fig. 1; see online Supplementary Materials). We found that at a cutoff of 14.25 PDL, we could differentiate between the growth behavior of cancer cases-exposed from benign cases-exposed cells with a sensitivity of 100% and a specificity of 90% (online suppl. Fig. 1). Cell viability, independently of the serum used (benign or malignant), was not affected during the treatment period (Fig. 1c). These data show that thyroid cancer patients' serum as opposed to healthy patients' serum significantly enhanced the proliferation of Fibro-BKO cells in vitro without affecting their viability.

Fibro-BKO Cells Accurately Sense Cancer Circulating Factors and Become Tumorigenic

At the end of the 3-week exposure to patient's serum in vitro, the Fibro-BKO cells were injected subcutaneously into NOD-SCID mice. Tumor growth was monitored, and tumor volumes were calculated (Fig. 2; online suppl. Fig. 2). The results of the test were then cross-checked with the final pathology of the resected lesions. The patients ($N = 34$) were categorized, based on the pathological analysis of the excised lesions, in two cohorts: malignant ($N = 12$; cases W-ZH) versus benign ($N = 22$; cases A-V) (Table 1).

Serum from all patients with benign lesions (cases A-V) did not transform Fibro-BKO cells (0 out of 22 patients, specificity 100%) (Table 1; Fig. 2a, b). In contrast, serum from all patients diagnosed with thyroid cancer except one (case ZA) transformed Fibro-BKO cells into cancer (11 out of 12 patients, sensitivity 92.3%), and the difference was statistically significant (Fisher's exact test,

Fig. 1. Thyroid cancer patients' serum increased the proliferation of Fibro-BKO cells during in vitro treatment. Fibro-BKO cells were cultured for 3 weeks in the serum from patients with thyroid carcinoma or benign lesions. **a, b** Cell growth was analyzed by counting the number of viable cells at every passage. Line graph shows the population doublings capability (**a**); column graph represents

cumulative population doublings at the end of the in vitro treatment periods (**b**). **c** Cell viability was calculated as the percentage of viable cells over total counted cells using Trypan blue exclusion dye. Data are presented as mean $\pm$ SD. p values were given, and differences were considered statistically significant at $p < 0.05$.

$p < 0.001$) (Table 1; Fig. 2a, b). Histological analyses of the tumors excised from mice displayed features of poorly differentiated adenocarcinoma with a high proliferative index. Independently of the malignant serum used, we observed over 90% positivity for Ki67 (Fig. 2c).

However, attempts to characterize these tumors immunohistochemically failed to show any more differentiating features as they were negative for thyroid-specific markers (i.e., HMBE-1, thyroid globulin, and TTF1) (Fig. 2c). Instead, we confirm our previous findings that, independent of the cancer serum analyzed, these cells changed their fate by losing vimentin expression and displayed a unique pattern of markers expression (CK7-/CK20+/CDX2+) (Fig. 2c) [26]. As half of thyroid cancer lesions display mutated BRAF-V600E phenotype [16], we labeled the mouse xenografts for this epitope (online suppl. Fig. 3). However, we did not detect any positive staining. These data strengthen the hypothesis that the ability to differentiate in a specific cancer lineage depends on the type of cell that uptakes the oncofactors rather than the oncofactors themselves as shown in a previous publication [26]. These data suggest that Fibro-BKO cells are able to sense cancer-derived circulating oncogenic factors, uptake them, and

turn malignant and confirm that this in vitro cell-based platform has the potential to detect malignancy in patients with thyroid nodules.

MATER-D Test Accurately Discriminates Benign from Malignant Lesions in Bethesda III/IV Patients

To verify the accuracy of the MATER-D test in the group of patients with thyroid lesions, we performed a subgroup analysis of patients with thyroid nodules only. Serum from all patients with benign thyroid lesions (cases A–T, $n = 10$) did not transform Fibro-BKO cells (0 out of 10 patients, specificity 100%) (Table 2). In contrast, serum from all patients diagnosed with thyroid cancers (cases W–ZH, $n = 12$) except one (case ZA) transformed Fibro-BKO cells into cancer (11 out of 12 patients, sensitivity 92.3%), and the difference was statistically significant (Fisher's exact test, $p < 0.001$). MATER-D was able to discriminate malignant lesions in 2 (Z and ZG) out of 4 patients that despite being classified as Bethesda II (D, J, Z, and ZG) were operated on because of enlarging nodules and suspicious features on US. Furthermore, all lesions categorized as Bethesda III and IV were correctly diagnosed by the MATER-D test (Table 2). Serum from all Bethesda III/IV patients with pathological confirmation

(For legend see next page.)

of benign thyroid lesions did not transform Fibro-BKO cells (0 out of 8 patients, specificity 100%). In contrast, serum from all the Bethesda III/IV patients diagnosed with thyroid cancer on final pathology, transformed Fibro-BKO cells into cancer (4 out of 4 patients; Table 2). These data indicate that the MATER-D platform can precisely detect cancer in indeterminate FNAC of thyroid lesions (Bethesda III/IV category) with a sensitivity of 100%.

Discussion

The problem of distinguishing benign from malignant masses of the head and neck region as well as overdiagnosis of thyroid malignancies in patients with thyroid nod-

ules requires the development of noninvasive tools to correctly diagnose malignancy, thus decreasing the number of avoidable surgeries and reducing morbidity and high costs for the healthcare system [10–13]. In the present study, we assessed the sensitivity of the MATER-D platform in diagnosing cancer in head and neck lesions and in discriminating malignant from benign lesions of the thyroid. We found that this test was able to distinguish malignant from benign lesions with a sensitivity of 92.3%.

There was only 1 patient in our study group (case ZA; multifocal papillary thyroid microcarcinoma [PTMC] with a lymph node positive for micrometastasis) who gave a false-negative result. A variety of factors, which range from test-related technical issues to the pathophysiology of the disease itself, can explain this false negative. Thus, it is known that

Table 2. Correlation of final pathology of thyroid lesions with the MATER-D test result

Case ID	Bethesda	Final pathology	MATER-D, cm ³
A	III	Nodular hyperplasia	0.000±0.000
D	II	Nodular follicular hyperplasia	0.000±0.000
F	IV	Adenomatoid oncocyctic follicular nodule	0.000±0.000
H	IV	Oncocyctic follicular adenoma	0.000±0.000
I	III	Follicular adenoma	0.000±0.000
J	II	Nodular hyperplasia	0.000±0.000
L	III	Oncocyctic follicular hyperplasia	0.000±0.000
M	IV	Oncocyctic follicular adenoma	0.000±0.000
P	III	Nodular hyperplasia	0.000±0.000
T	IV	Hurthle (follicular) adenoma	0.000±0.000
W	VI	Papillary thyroid microcarcinoma (pT1bN1a)	0.634±0.048
X	III	Papillary thyroid microcarcinoma (pT1aNO)	0.023±0.002
Y	VI	Papillary thyroid carcinoma (pT2N1a)	0.051±0.001
Z	II	Papillary thyroid microcarcinoma (pT1aNx)	0.036±0.004
ZA	VI	Multifocal papillary thyroid microcarcinoma (pT1bN1a)	0.000±0.000
ZB	III	Noninvasive follicular thyroid neoplasm (NIFTP)	0.008±0.004
ZC	III	Papillary thyroid microcarcinoma (pT1aNO)	0.048±0.012
ZD	VI	Multifocal papillary carcinoma T3N1	0.009±0.003
ZE	IV	Micropapillary thyroid carcinoma	0.062±0.007
ZF	VI	Papillary thyroid carcinoma (T2N1a)	0.064±0.009
ZG	II	Micropapillary thyroid carcinoma (T1a)	0.085±0.005
ZH	V	Micropapillary thyroid carcinoma (T1a)	0.145±0.004

MATER-D, cm³: Fibro-BKO cells were cultured for 3 weeks in the serum from patients with thyroid carcinoma or benign lesions. Exposed cells were injected subcutaneously in NOD/SCID mice, and tumor growth was monitored for 4 weeks. At euthanasia, tumor volumes were calculated ($n = 4$ xenotransplants per treatment).

Fig. 2. Thyroid cancer patients' serum induced the transformation of Fibro-BKO. **a, b** Fibro-BKO cells treated as described in Figure 1 were injected subcutaneously in NOD/SCID mice, and tumor growth was monitored for 4 weeks. **a** Once tumors were palpable, their diameters were measured to determine the time course of the xenotransplant growth. **b** At euthanasia, mean volumes of tumors obtained with cells exposed to sera from the 2 cohorts of patients

were calculated. Values are mean $\pm$ SD ($n = 4$ xenotransplants per treatment [2 mice per treatment injected at 2 sites]). **c** Formalin-fixed paraffin-embedded xenotransplant samples were processed for H&E staining and immunolabeled with antibodies for cell proliferation or specific to cancer markers. Representative pictures are shown. Scale bar, 50 μ m.

PTMC ranges from precancerous lesions treated with conservative approach to more aggressive forms of cancer with a poorer prognosis [27–32]. Although this patient was node positive, it is currently unknown which course the disease would take even in the presence of lymph node micrometastasis. Although we cannot exclude technical issues with the MATER-D platform, the evidence that this test was able to detect papillary microcarcinomas in other patients, even at an earlier stage (Z, ZC, ZE, ZG, and ZH) than the false-negative case, suggests that the disease in this patient could have a silent course, as reported in many cases of patients who died for reasons unrelated to thyroid cancer and were later found to have PTMC at autopsy [30]. In this context, OMCs failed to transform because of absence of circulating oncogenic factors, which would reflect a benign course of a locally invasive disease. However, at the moment, this hypothesis remains only speculative.

Another interesting result of our study is that 1 patient with noninvasive follicular thyroid neoplasm (NIFTP) tested positive (case ZB), even though NIFTP is considered a borderline or premalignant lesion [33]. This result confirms the notion that MATER-D can detect dysplastic lesions, premalignant lesions, and cancer in situ, as demonstrated in our previous studies [26, 34]. Furthermore, the evidence shown here that OMCs turn malignant when exposed to the serum of a patient with NIFTP indicates that oncogenic factors are produced by these types of lesions and circulate in the blood. This entity therefore should not be regarded as completely benign and confirms that patients with this diagnosis should be monitored closely for cancer recurrence [35].

The MATER-D test has shown to sense oncogenic factors present in the serum even years after removal of the primary tumor [25, 26, 34]. The evidence that the serum of a patient already treated for cancer still carries oncogenic factors up to 10 years after its removal and is able to transform target cells in vitro is intriguing and paves the way to fascinating hypotheses that are being actively studied in the field of horizontal transfer of malignant traits and genomestasis [20, 21, 36, 37]. At the same time, this notion highlights one of the contraindications to perform the MATER-D test, which is previous history of cancer of any type or stage. To perform MATER-D in these cases would lead to positive results regardless of the pathology of the thyroid nodule and would lead to unnecessary treatments. It is important to note that MATER-D at the moment lacks the ability to determine cancer histotypes, and as such, patients with thyroid lesions who had a positive test and a benign histology on a thyroid nodule specimen should undergo extensive screening for other types of cancers.

Although the MATER-D test shows a very high sensitivity with respect to other liquid biopsies based on molecular signatures [38] and shows intriguing features possessed by circulating oncogenic factors, its potential to determine cancer histotypes could further improve by the development of new markers to identify circulating cell-free moieties and molecules packed in circulating EVs specific to different types of cancers [19–21, 24, 25, 39, 40]. Furthermore, although the platform and the data are very interesting, the small number of patients being tested makes this study a proof-of-principle pilot study that still requires extensive validation.

It is our opinion that a proper understanding of the mechanisms that drive the OMCs to sense and uptake those circulating cancer messengers and turn cancerous might also improve the sensitivity of the liquid biopsies based on cancer gene sequencing. The identification of the specific circulating factors responsible for the malignant transformation of the OMC might help narrow the number of genes that needs to be targeted and sequenced in conventional liquid biopsies and increase their precision, sensitivity, and cancer specificity [26].

The results herein presented suggest that liquid biopsy combined with an OMC-based diagnostic platform has the potential to improve the sensitivity of cancer diagnosis in patients presenting with head and neck masses arising from the salivary glands and thyroid lesions. The sensitivity is 100% for the most difficult thyroid lesions such as the ones belonging to the Bethesda III/IV category. Further studies with a larger number of patients are warranted to confirm the excellent data obtained in this specific subset of patients.

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Statement of Ethics

Patients were recruited in accordance with an approved ethics protocol by the Ethics Committee of the McGill University Health Centre (MP-37-2018-3916 and SDR-10-057). Patient's signed consent was obtained before enrollment in the study. Animal study was conducted in accordance with guidelines and an approved protocol by the McGill University Health Centre Animal Compliance Office (Protocol 2012-7280).

Conflict of Interest Statement

The authors declare no conflicts of interest.

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Author Contributions

Conceptualization: Mohamed Abdouh, Vincenzo Arena, Manuel Arena, Aurelio Lorico, and Goffredo Arena; formal analysis: Mohamed Abdouh, Roger Tabah, and Zu-hua Gao; funding acquisition: Goffredo Arena; investigation: Mohamed Abdouh, Roger Tabah, and Zu-hua Gao; methodology: Mohamed Abdouh and Goffredo Arena; resources: Zu-hua Gao and Goffredo Arena; supervision: Goffredo Arena; writing – original draft: Mohamed Abdouh; writing – review and editing: Mohamed Abdouh, Roger Tabah, Aurelio Lorico, and Goffredo Arena.

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