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Midkine Concentrations in Fine-Needle Aspiration of Benign and Malignant Thyroid Nodules

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Abstract

Context—The primary preoperative method for distinguishing malignant from benign thyroid nodules is fine-needle aspiration (FNA) cytology, but it is frequently inconclusive. Midkine (MDK) is a heparin binding growth factor, which is overexpressed in papillary thyroid carcinoma (PTC).

Objective—We measured MDK concentrations in FNA samples from benign and malignant thyroid nodules to explore the possibility that MDK measurement might aid in the evaluation of thyroid nodules.

Design—35 subjects underwent pre-operative FNA of 45 thyroid nodules, followed by thyroidectomy, providing a histological diagnosis. FNA needle contents were first expressed for cytology and then the needle was washed with buffer for immunoassay. In 46 subjects without preoperative FNA samples, FNA was performed *ex vivo* on 62 nodules within surgically excised thyroid tissue.

Measurements—MDK was measured using a high-sensitivity sandwich ELISA and normalized to thyroglobulin (Tg) concentration in the sample to adjust for tissue content in the aspirate.

Results—The MDK/Tg ratio was higher in 18 PTCs than in 87 benign nodules (204 ± 106 vs 1.2 ± 0.3 ng/mg, mean $\pm$ SEM, $P < 0.001$). Using a threshold of 10 ng/mg, the sensitivity and specificity of the MDK/Tg ratio for diagnosis of PTC were 67 % and 99 %, respectively. All follicular variant PTCs had a MDK/Tg ratio < 10 ng/mg.

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Declaration of Interest: Y.H.J and J.B. are co-inventors on a patent application filed by the National Institutes of Health that covers the measurement of midkine in FNA samples. Otherwise, the authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Conclusions—The findings indicate that, in FNA samples, the MDK/Tg ratio in PTC is greater than in benign thyroid nodules, raising the possibility that this approach might provide adjunctive diagnostic or prognostic information to complement existing approaches.

Keywords

Midkine; washout; fine needle aspiration; thyroid nodule; papillary thyroid carcinoma

Introduction

Thyroid nodules are common in the adult population.¹ Approximately 5% of thyroid nodules are malignant.¹ The primary preoperative method for distinguishing malignant from benign lesions is fine-needle aspiration (FNA) cytology. Preoperative ultrasound guided FNA has been shown to accurately assign 62 to 85% of thyroid nodules as benign.² However, in up to 30% of FNA biopsies, the cytology is inconclusive, causing uncertainty about the need for surgery.³ Approximately 150,000 surgical procedures are performed for cytologically indeterminate nodules in the United States each year, and the majority of cytologically indeterminate nodules prove to have benign disease.⁴ There has been considerable research effort to identify better diagnostic tools to differentiate malignant from benign thyroid nodules, including, detection of high risk mutations, which typically yields high specificity but incomplete sensitivity^{12,13, 16} mRNA expression profiles, which typically yield greater sensitivity but lower specificity^{14,17}, immunohistological markers^{10, 11}, and microRNA expression profiles¹⁵. Thus, there remains a need for further improvements in the diagnostic approach to thyroid nodules. There is also a need to identify prognostic markers to help guide the extent of therapy needed.¹⁸

Midkine (MDK), a heparin-binding growth factor, is a basic, cysteine-rich polypeptide with a molecular weight of 13 kDa. MDK is expressed in multiple tissues in the mouse embryo and decreases to undetectable levels by adulthood in many tissues.^{19–21} High expression of MDK mRNA has been found in various human cancers such as breast, lung, stomach, colorectal, liver, ovary, urinary bladder, prostate, glioblastomas, neuroblastomas and Wilms' tumor.^{22–24} In malignant tissues, MDK is thought to promote cell proliferation, migration and angiogenesis.^{22–25} MDK is also expressed in papillary thyroid cancer (PTC) as assessed by immunohistochemical staining and in situ hybridization.²⁶ Approximately 90% of PTC express MDK, and the expression tends to be greater at the invading border of the tumor. MDK expression is reportedly greater in PTC that involves BRAF mutations.²⁷ Moreover, patients with PTC showed elevated circulating MDK levels, although the number of subjects studied was small.²⁸

We therefore hypothesized that MDK protein levels would be higher within papillary thyroid cancer than within benign thyroid nodules and consequently that measuring MDK concentration in the washout of FNA samples might help distinguish malignant from benign thyroid nodules. If that hypothesis is correct, then such measurements might provide useful diagnostic and/or prognostic information to supplement information from cytological analysis and from other molecular studies.

Materials and Methods

Subjects and sample collection

Subjects (n=130) were adults with thyroid nodules who underwent preoperative FNA and/or thyroidectomy at the National Institutes of Health (NIH) Clinical Center between July 2011 and February 2014. Study protocols were approved by NIH Institutional Review Boards, and all patients provided written informed consent to participate in the study. Preoperative (*in vivo*) FNA samples were performed under ultrasound guidance. For each nodule, 1 to 4 FNA samples were obtained, using a 25-gauge needle without applying suction. After the content of each needle was expressed onto a microscope slide for conventional cytology, the needle was rinsed with 500 μ L of PBS containing 1% bovine serum albumin (BSA). The washout was aliquoted and stored immediately at -80°C until the assay was performed.

Additional FNA samples were obtained *ex vivo* immediately after thyroidectomy. In the excised thyroid, the selected nodules with surrounding tissues were bisected for procurement, and then FNA was performed by passing a 25-gauge needle without suction 10–15 times into a nodule with the intent of passing all the way through the half-nodule and into the surrounding tissue. The needle was washed with 500 μ L PBS containing 1% BSA. In contrast to the *in vivo* FNA sampling, the needle contents were not expelled prior to the buffer wash. The samples were aliquoted and stored immediately at -80°C until assay. 25 *ex vivo* FNA samples from normal thyroid tissue distant from any nodule were also obtained.

Peripheral blood (2.7 mL) was collected in a plastic citrate tube at the time of FNA from 39 subjects who subsequently had a histological diagnosis. The blood was centrifuged at 4°C for 15 minutes (3,000 g) within two hours of venipuncture. Plasma was aliquoted and stored at -80°C until MDK assay.

Midkine Sandwich ELISA Assay

MDK sandwich ELISA was performed using a commercial kit (Biovendor, Czech Republic) with modifications. In particular, poly-L-lysine was added to the biotin-labeled detection antibody solution provided with the kit to increase responsiveness of the ELISA assay (see Supplemental Information for assay method details). Intra-assay CV was 3.4 % at high concentration (0.7 ng/mL) and 5.2 % at low concentration (0.25 ng/mL). Inter-assay CV was 12.3%. The limit of detection was 8.7 pg/mL. The assay showed good parallelism (Supplemental Fig. 2).

Thyroglobulin Assay

50 μ L of FNA washout was diluted 10 fold in normal saline and the Tg concentration was measured with a chemiluminescent immunometric assay (Immulite 2000XPi, Siemens, UK) per manufacturer's instruction. If further dilution was needed, Tg reagent (Siemens healthcare diagnostics, USA) was used to dilute samples. Intra-assay CV was 5.8% for 5.6 ng/mL and 2.7 % for 173 ng/mL. Inter-assay CV was 6.2% for 5.5 ng/mL and 5.3% for 162 ng/mL.

Plasma Midkine Sandwich ELISA Assay

125 μ L of plasma was diluted in 125 μ L of TBSTA. The rest of the procedure was identical to the procedure described for assay of thyroid washout samples.

Statistical analysis

The relationship between MDK and Tg concentrations was analyzed by Pearson correlation after log transformation, including data from all individual passes. For all other analyses, all FNA samples from a single nodule were averaged. Histological groups were compared using the Kruskal-Wallis and the Mann-Whitney U test with Holm correction for multiple comparisons.

Results

Characteristics of subjects and nodules

In vivo or *ex vivo* FNA samples were obtained from 160 nodules in 130 subjects (median age, 48.5 years; 16 males). FNA samples were obtained percutaneously (*in vivo*) from 98 nodules (84 subjects). Of these, 45 nodules (35 subjects) were subsequently excised surgically. In an additional 62 nodules (46 subjects), FNA samples were instead obtained *ex vivo*, immediately after thyroidectomy. Histological diagnoses are shown in Fig. 1, cytological diagnoses in Table 1, and nodule sizes in Supplemental Table 1.

Midkine and thyroglobulin concentrations in FNA washout

MDK concentrations were low in samples from normal thyroid tissue (0.03 ± 0.01 ng/mL, Table 1) and in benign nodules obtained *in vivo* (0.02 ± 0.01 ng/mL, Fig 2A). MDK was higher in samples from benign nodules obtained *ex vivo* (0.14 ± 0.03 ng/mL, Fig 2A), presumably because these samples included the entire needle contents whereas the *in vivo* needles were first expelled for cytology. Among the benign samples, MDK concentrations were low in adenomatoid/colloid nodules, follicular adenomas and benign nodules with underlying chronic lymphocytic thyroiditis (0.05 ± 0.01 , 0.2 ± 0.1 and 0.05 ± 0.02 ng/mL, respectively, $p=NS$). In contrast, MDK concentrations in samples from PTC showed higher levels than in benign nodules, whether obtained *in vivo* (0.12 ± 0.05 vs 0.02 ± 0.01 ng/mL, $p=0.011$) or *ex vivo* (1.2 ± 0.5 vs 0.14 ± 0.03 ng/mL, $p=0.003$) (Fig. 2A and Table 1).

Tg concentration was also measured in the FNA washout samples. Of 280 FNA washout samples, 38 samples, all collected *in vivo*, had Tg concentration less than 100 ng/mL and were therefore considered to contain inadequate thyroid tissue and were excluded from further analysis. For 4 nodules, Tg was inadequate in all passes obtained. For these nodules, the cytology was reported as “insufficient cells.” They did not undergo repeat FNA because of their low-risk characteristics or because they were lost to follow up. Tg concentrations were lower in malignant than in benign nodules sampled *in vivo* ($3,900 \pm 2,500$ ng/mL vs $77,000 \pm 21,000$ ng/mL, $P=0.005$) and *ex vivo* ($74,000 \pm 25,000$ ng/mL vs $730,000 \pm 120,000$ ng/mL, $P=0.003$, Fig. 2B).

The majority of subjects (125 of 130 subjects) were tested for anti-Tg antibodies. 7 subjects had elevated anti-Tg antibodies. However, FNA Tg levels were not significantly different in

subjects with elevated anti-Tg antibodies compared to those without anti-Tg antibodies ($100,100 \pm 60,700$ ng/mL vs $370,000 \pm 66,000$ ng/mL, $P = 0.18$).

Midkine to thyroglobulin ratios in FNA washout

MDK concentration was positively associated with Tg concentrations in FNA washout samples from benign nodules (analysis included all individual passes, $R^2 = 0.35$, $p < 0.001$). This correlation likely occurred because both MDK and Tg concentrations in the washout fluid were dependent on the amount of thyroid tissue present in the sample. Therefore, to correct for the amount of thyroid tissue, we normalized MDK levels to Tg levels by calculating the ratio of MDK concentration to Tg concentration (MDK/Tg, ng/mg).

Samples from benign nodules including adenomatoid nodules, follicular adenomas, and Hurthle cell adenoma showed low MDK/Tg ratios (Table 1). The MDK/Tg ratio was significantly higher in PTC samples than in benign nodules (204 ± 106 vs 1.2 ± 0.3 ng/mg; $P < 0.001$, combined *in vivo* and *ex vivo* samples; Fig. 3A, B; Table 1). However, all 4 follicular variant PTCs had low MDK/Tg ratio (2.2 ± 1.1 ng/mg, Table 2). The MDK/Tg ratio showed no significant correlation with nodule size in PTC. Interestingly, PTCs with lymph node metastases tended to have higher MDK/Tg ratios than PTC without metastases, although the association did not reach statistical significance (494 ± 312 vs 91 ± 52 ng/mg, $p = 0.056$). The MDK/Tg ratio, subject characteristics, and nodules characteristics for individual subjects with PTC are shown in Table 2. In one additional subject with a history of previous PTC, *in vivo* FNA was performed on a neck mass, which yielded high washout MDK levels and MDK/Tg ratio (0.09 ng/mL and 74.1 ng/mg, respectively); subsequent postsurgical histological examination demonstrated recurrent PTC in a lymph node. Because the lesion was metastatic, the subject was not included in any aggregate analyses. Two medullary thyroid cancers also showed elevated MDK/Tg ratio (Fig. 3A, Table 1).

Sensitivity, specificity, positive predictive value and negative predictive value

A threshold value of 10 ng/mg for the MDK/Tg ratio was selected based on data from nodules with a histological diagnosis (87 benign and 18 PTC), yielding a sensitivity, specificity, positive predictive value, and negative predictive value of 67, 99, 92 and 93% (Fig 3B). The area under the receiver operating characteristic (ROC) curve was 0.91 ($P < 0.0001$, Fig 3C).

Pass to pass variability

For the preceding analyses, values from multiple passes from each nodule were averaged. We next analyzed the variability among individual passes taken from the same malignant nodule (Fig 4). For 2 of the malignant nodules, only a single pass with sufficient Tg was obtained. For 14 of 16 malignant nodules with multiple passes, MDK/Tg ratios showed sufficient agreement that all passes from each nodule had MDK/Tg ratios either all below or all above the threshold of 10 ng/mg for malignancy (Fig 4). However, for two subjects (subject 7 and 16), individual values spanned the diagnostic threshold. For benign nodules, only one nodule had MDK/Tg values from individual passes that spanned the cut-off value of 10 ng/mg (data not shown).

Plasma midkine concentrations

Plasma MDK concentrations did not differ between subjects with benign nodules and with PTC (0.22 ± 0.01 ng/mL vs 0.22 ± 0.03 ng/mL, $p = \text{NS}$).

Discussion

The current study is the first to assess MDK concentrations in FNA samples from malignant and benign thyroid nodules. We found that MDK levels in FNA washout samples were higher in PTC than in normal thyroid tissue or benign nodules. In contrast, Tg levels were lower in malignant than in benign samples, which indicates that Tg is not simply a measure of tissue content. However, from a practical standpoint, this tendency is fortuitous because, as a result, normalization of MDK to Tg improved the separation between malignant and benign samples. The elevated MDK/Tg ratio in PTC, compared to benign nodules, raises the possibility that such MDK measurements might provide an adjunctive diagnostic tool to supplement information from cytological analysis and from other molecular studies. In addition, PTCs with lymph node metastases tended ($p = 0.056$) to have higher MDK/Tg ratios than PTC without metastases, raising the possibility that MDK levels in thyroid malignancies might serve as one prognostic marker to help guide the extent of therapy needed.¹⁸ The MDK/Tg ratio was also elevated in both medullary thyroid cancers studied and in a single lymph node containing metastatic PTC.

In contrast to a previous study,²⁷ we did not find significant differences in plasma MDK concentrations between subjects with PTC and those with benign nodules. The discrepancy in findings might be due to the use of different assays, which might, for example, detect different variants of MDK, such as truncated MDK.³⁰ Also, in our study, no blood samples were obtained from subjects who had recently received any heparin including heparin solutions used to maintain patency of intravenous lines, because heparin administration has been shown to markedly elevate circulating MDK concentrations.³¹

The measurement of MDK/Tg concentration ratios in FNA washout has several advantages over other methods that are currently under evaluation. The approach requires only straightforward immunoassays for two proteins that are easily measurable and thus is likely to be less expensive and more easily standardized than methods that involve immunohistochemistry or measurement of multiple RNA levels. In addition, MDK can be measured in the washout of the FNA needle after expression of material for cytology and thus does not require an additional, dedicated needle pass. For most nodules, we obtained multiple FNA samples and then averaged the results. In general, the MDK/Tg ratios among different passes from the same nodule showed reasonably good agreement, such that, for most malignancies, a single pass would have yielded the correct diagnosis. However, for two malignant nodules, the variability among different passes was sufficiently large such that the MDK/Tg ratios spanned the diagnostic threshold, suggesting that obtaining multiple passes might give greater diagnostic accuracy compared to a single pass.

The high mean concentration of MDK in the FNA washout from PTC raises the possibility that this approach might have diagnostic utility. Although the approach shows 99% specificity for PTC, the sensitivity was only 67%, due in large part to the fact that low

MDK/Tg ratios were observed in follicular variant PTC. Therefore, this approach is unlikely to be helpful for follicular variant, a malignancy which often yields inconclusive cytology. Whether or not this approach might be useful as an adjunctive diagnostic tool in other cases of indeterminate cytology is unclear at present. In this pilot study with limited statistical power, sensitivity and specificity were calculated including all lesions with a histological diagnosis. However, the adjunctive diagnostic utility of this test would primarily depend on the sensitivity and specificity based only on samples with indeterminate cytology. In addition, in this pilot study, we included samples that were obtained *ex vivo*. Although the results obtained *ex vivo* and *in vivo* were highly consistent, a future definitive determination of diagnostic value should involve only *in vivo* samples. In our study, there were only 5 nodules with indeterminate cytology sampled *in vivo*; thus a much larger study would be needed to evaluate definitively the adjunctive diagnostic utility of this approach. If the current findings were confirmed in such subsequent studies, then the finding of high MDK/Tg ratio in a nodule with indeterminate cytology might provide useful evidence of malignancy. However, a low MDK/Tg would not be sufficient in isolation to exclude malignancy, but instead would need to be combined with clinical, ultrasound, cytological, and other biochemical/molecular data.

The measurement of the MDK/Tg ratio has several potential limitations as a clinical tool. One limitation is that sufficient thyroid tissue must be present in the washout sample to evaluate the MDK to Tg ratio in both malignant and benign lesions. To address this limitation, we excluded from analysis samples that contained a Tg concentration less than 100 ng/mL. As a result, 4 % of nodules had no sample with adequate Tg to calculate MDK/Tg ratio. A second possible limitation of this approach is that thyroglobulin measurement in the FNA samples might be artifactually low in subjects with elevated anti-Tg antibodies,²⁹ thus causing a false elevation in the MDK to Tg ratio, although, in our study, we did not find evidence for this effect. A third limitation is that this method does not overcome the inherent sampling error of FNA related to the path of the needle and the location of sampling in a nodule. For example, if the FNA needle passes through the benign component of a nodule and misses the malignant component, both the cytology and the MDK/Tg ratio will presumably fail diagnostically.

In conclusion, our findings indicate that the MDK to Tg ratio in FNA washout samples is higher in PTC than in benign lesions. Whether or not this approach is useful as an adjunctive diagnostic tool for nodules with indeterminate cytology or as a prognostic tool will require additional, larger studies to determine.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Flowchart of thyroid nodules evaluated

Tg, thyroglobulin concentration; PTC, papillary thyroid cancer; MTC, medullary thyroid cancer.

Figure 2. Midkine (A) and thyroglobulin (B) concentrations in FNA washout from histologically confirmed benign nodules and PTC

FNA was performed either percutaneously before thyroidectomy (*in vivo*) or immediately after thyroidectomy (*ex vivo*). MDK concentrations were higher in malignant than in benign samples, whereas Tg concentrations were higher in benign samples than in malignant samples. Both MDK and Tg concentrations were higher in *ex vivo* than *in vivo* samples, presumably reflecting greater tissue content in the samples.

Figure 3. Midkine/thyroglobulin ratio (MDK/Tg, ng/mg, mean $\pm$ SEM) in FNA washout from benign nodules and PTC

The MDK to Tg ratios of multiple passes were averaged for each nodule. (A) Normal thyroid tissue was obtained *ex vivo* distant from any nodule. Nodules that were found to be benign by cytology only (Benign Nodules-C) are shown separately from those found to be benign by histology (Benign Nodules -H). (B) MDK/Tg ratios are shown for individual nodules with a histologically confirmed diagnosis, including those sampled by FNA both *in vivo* and *ex vivo*. Benign nodules (open circles), PTC (closed circles). Horizontal line indicates proposed diagnostic threshold value of 10 ng/mg. (C) The diagnostic ability of the MDK/thyroglobulin ratio was evaluated by constructing a receiver-operating characteristic (ROC) curve, using data from all nodules (87 benign, 18 PTC) with a histological diagnosis

including nodules sampled both *in vivo* and *ex vivo*. The area under the ROC curve was 0.91 ($P < 0.0001$), suggesting good diagnostic utility. PTC, papillary thyroid cancer. MTC, medullary thyroid cancer.

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Figure 4. Pass to pass variability in the midkine to thyroglobulin (MDK/Tg) ratio in nodules with PTC

MDK/Tg ratios are shown for all individual FNA samples (passes). For 14 of 16 malignant nodules with multiple passes, MDK to Tg ratios showed sufficient agreement that all passes from each nodule had MDK to Tg ratios either all below or all above the threshold of 10 ng/mg for malignancy. However, for two subjects (subject 7 and 16), individual values spanned the diagnostic threshold.

Table 1

Midkine concentrations and midkine to thyroglobulin ratios (MDK/Tg) in FNA washout

Histology	Cytology	N ^a	MDK (ng/mL)	MDK/Tg (ng/mg)
Normal thyroid ^b	-	25	0.03 ± 0.01	0.4 ± 0.1
Graves' disease	-	2	0.06 ± 0.01	0.54 ± 0.34
AN ^c	AN	70	0.03 ± 0.01	1.3 ± 0.5
	AUS ^d /FLUS ^e	6	0.06 ± 0.04	0.2 ± 0.1
	Insufficient ^f	1	0.04	1.0
Hurthle cell adenoma	AUS	1	0	0
	Insufficient	1	0.1	9.6
Follicular adenoma	AUS/FLUS	8	0.2 ± 0.1	2.5 ± 1.5
PTC	AN	3	0.5 ± 0.4	1.8 ± 1.4
	AUS	5	0.5 ± 0.3	27 ± 17
	PTC	9	0.7 ± 0.4	388 ± 198
	Insufficient	1	0.04	51
MTC	MTC	2	1.8 ± 1.8	22 ± 6.5
N/A ^g	AN	42	0.05 ± 0.03	4.3 ± 1.1
	AUS	4	0.01 ± 0.005	4.8 ± 4.4
	Insufficient	3	0.01 ± 0.01	5.2 ± 2.7

^aNumber of nodules^bSamples from normal thyroid tissue, obtained distant from any nodule.^cAdenomatoid nodule^dAtypia of undetermined significance^eFollicular lesion of undetermined significance^fNo cytological diagnosis due to insufficient cells^gThyroidectomy not performed

Table 2
Clinical and pathological data for the 18 subjects with papillary thyroid carcinoma

Subject	Diagnosis	Sex	Age	Tumor size (cm)	Metastases ^a	MDK/Tg (ng/mg)	Sampling Method
1	PTC, tall cell variant	M	45	3	None	92.5	<i>Ex vivo</i>
2	PTC, tall cell variant	F	20	2.4	5/6 LN	58	<i>Ex vivo</i>
3	PTC, classic	M	58	1	None	2.4	<i>Ex vivo</i>
4	PTC, follicular variant	F	42	1.1	None	0.8	<i>Ex vivo</i>
5	PTC, classic	F	20	4.7	None	728	<i>Ex vivo</i>
6	PTC, classic	M	49	0.9	None	51	<i>Ex vivo</i>
7	PTC, classic	F	39	1	None	4	<i>Ex vivo</i>
8	PTC, follicular variant	F	48	0.5	None	4.5	<i>Ex vivo</i>
9	PTC, tall cell	F	28	1.8	6/8	22.4	<i>Ex vivo</i>
10	PTC, classic	F	28	1.6	None	37	<i>Ex vivo</i>
11	PTC, classic	F	42	3.1	None	254	<i>In vivo</i>
12	PTC, tall cell variant	F	58	5	4/33 LN	1816	<i>In vivo</i>
13	PTC, classic	F	37	0.5	None	21.1	<i>In vivo</i>
14	PTC, follicular variant	F	38	1.7	None	4	<i>In vivo</i>
15	PTC, follicular variant	F	25	3	None	0	<i>In vivo</i>
16	PTC, unclassified ^b	F	38	6	None	55	<i>In vivo</i>
17	PTC, tall cell	F	37	3	2/7 LN	534	<i>In vivo</i>
18	PTC, classic	F	28	0.7	None	17	<i>In vivo</i>

^aLN, lymph node; (number of positive lymph nodes/total lymph nodes examined).

^bHistology was inconclusive but c. 1801A>G (K601E) BRAF mutation was detected.