THYROID
Volume 18, Number 11, 2008

DOI: 10.1089/thy.2008.0020

Iodine Content and Distribution in Extratumoral and Tumor Thyroid Tissue Analyzed with X-Ray Fluorescence and Time-of-Flight Secondary Ion Mass Spectrometry

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Background: The thyroid's ability to enrich and store iodine has implications for thyroid cancer genesis, progression, and treatment. The study objective was to investigate thyroid iodine content (TIC) in tumoral and extratumoral tissue in patients with papillary thyroid cancer (PTC) as opposed to thyroid healthy controls using two different techniques: X-ray fluorescence (XRF) and time-of-flight secondary ion mass spectrometry (TOF-SIMS).
Methods: Tissue samples from 10 patients with normal thyroids and 7 patients with PTC were collected. TIC was quantified with XRF, and the iodine stores were located on a histological level with TOF-SIMS.
Results: Mean TIC in controls was 0.6 mg/mL (range 0.3–1.2 mg/mL). For the cancer patients, the mean TIC was 0.8 mg/mL (range 0.2–2.3 mg/mL) in extratumoral thyroid tissue, but no iodine was detected in the tumors. TOF-SIMS investigation of the PTC patients showed significantly higher TIC in extratumoral tissue than in tumoral tissue. Iodine in the extratumoral tissue was predominantly located in the follicle lumen with a variation in concentration among follicles.
Conclusions: XRF and TOF-SIMS are two complementary methods for obtaining insight into content and localization of iodine in the thyroid. XRF can be used *in vitro* or *in vivo* on a large number of samples or patients, respectively. TOF-SIMS on the other hand provides detailed images of the iodine location. The combined information from the two methods is of value for further studies on iodine metabolism in thyroid malignancy.

Introduction

Thyroid carcinoma is the most common of the endocrine cancers, and the incidence in Sweden is just below 4 per 100,000. Sweden is considered to be iodine sufficient, and papillary thyroid cancer (PTC) is the histological type that accounts for the majority of cases of thyroid carcinoma. PTC is believed to be promoted by iodine excess, in contrast to follicular and anaplastic cancers, which are more common in iodine-deficient areas (1,2). It is therefore of interest to study iodine content and distribution in thyroid tissue from both tumor and extratumoral tissue in PTC patients as opposed to the situation in thyroid healthy patients.

The ability of the thyroid to store iodine is a prerequisite for radioiodine treatment. Iodine uptake and storage is among other factors affected by access to iodine and the amount of iodine already stored in the thyroid, in the iodine pool (3–5). Knowing the magnitude of the iodine pool and the iodine distribution among and within follicles can therefore be valuable for understanding the outcome of radioiodine treatment in thyroid cancer. Measurement of iodine content in the thyroid is difficult, though. Quantitative methods like computed tomography (CT) and neutron activation analysis (NAA) are time consuming. One chemical method involves measurement of the iodine content in a preparation of thyroglobulin (Tg) extracted from thyroid tissue (6). This quantitative method can, however, only be used *in vitro*. Other methods like iodine uptake measurements and urinary iodine, used in clinical practice, provide information of the iodine turnover rather than of the amount of iodine actually stored in the thyroid.

Quantitative information about the iodine pool can preferably be acquired with X-ray flourescence (XRF) analysis. XRF has been applied *in vivo* and *in vitro*, and thyroid iodine

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content (TIC) in normal thyroids is well documented. For malignant thyroid tissue, though, information regarding iodine content is scarce, and little work has been done using XRF to investigate the stable iodine pool in thyroid cancers (7). Time-of-flight secondary ion mass spectrometry (TOF-SIMS) is a more expensive and time-consuming method than XRF. The information is, however, more detailed because the analysis result is an image showing the iodine distribution within the thyroid tissue on a histological level. Regarding TOF-SIMS, no work has been done on malignant thyroid tissue in humans, whereas dynamic SIMS has been used in, for example, goitrous tissue (8). TOF-SIMS has higher mass resolution than dynamic SIMS has, thus improving the identification of stable iodine in matrices containing, for example, organic molecules with the same mass. Because high sensitivity in detection of iodine in the tissue is most important, TOF-SIMS should be the method of choice for these kinds of studies.

Moreover, analysis with both XRF and TOF-SIMS utilizes the stable iodine, ^{127}I, naturally present in the thyroid tissue; thus, there is no need for, for example, radioiodine for detection and localization of iodine in the thyroid.

The study objective was to use both XRF and TOF-SIMS as complementary methods to determine total iodine content and distribution in tumoral and extratumoral tissue from PTC patients compared to tissue from thyroid healthy controls.

Materials and Methods

Thyroid tissue samples were collected at surgery from 17 nonselected, consecutive patients who agreed after informed consent that thyroid biopsies could be taken for scientific investigation during surgery. All 17 patients were recruited in Gothenburg, Sweden, an area that has previously been reported as iodine sufficient (9).

Control patients for XRF analysis

Ten of the 17 patients had the diagnosis of laryngeal carcinoma and underwent total laryngectomy. These patients—eight men and two women, age ranging between 57 and 80 years (mean age 69 years)—served as controls for XRF measurements. During laryngectomy, the thyroid lobe of the tumor side in the larynx always is removed to get enough radicality. All these patients had no earlier history of thyroid disease and were clinically euthyroid at the time of surgery. None of them had received external radiation of the neck. Preoperative X-ray examination showed normal structure of the thyroid parenchyma, and the biochemical analyses were normal (free T4 and thyroid-stimulating hormone). The pathological reports of the extirpated thyroids showed no pathological changes, and the tissue obtained from these patients is referred to as normal thyroid tissue.

PTC patients for XRF and TOF-SIMS analysis

Seven of the 17 patients underwent primary surgery for PTC. None of the seven patients with PTC had previously been treated with radiation to the neck, and all were otherwise healthy except one who had Hepatitis B and another who had inflammatory bowel disease (colitis). All seven PTC patients underwent total thyroidectomy with central or central and lateral lymph node dissection (Table 1). Maximum tumor size varied between 8 and 65 mm. At surgery none of the patients had signs or symptoms indicating the presence of distant metastases, but four of them had regional lymph node metastases. The pathological tumor-node-metastasis (pTNM) stages (10) for the tumors are given in Table 1. For all cancer patients, samples were taken from both the tumor and the thyroid tissue that was not part of the tumor, here referred to as extratumoral tissue. According to the pathological report, the extratumoral tissue was normal.

All thyroid specimens were reexamined by an experienced histopathologist (J.M.). At reexamination, the histopathologist was blinded to the results from the XRF and TOF-SIMS investigations.

XRF analysis

XRF is based on the emission of characteristic, element-specific X-rays from a sample irradiated with ionizing radiation. The method has been verified to give results in close agreement with those measured with the independent technique NAA (11). The energy of the ionizing radiation must be higher than a threshold value (the binding energy of the innermost electrons), and with irradiation of tissue sections containing stable iodine, fluorescent radiation at 28.6 keV will be emitted with an intensity proportional to the amount of iodine in the sample. The XRF measurements were done on a system with an 11.1 GBq ^{241}Am radiation source. Detection was made with a planar high-purity germanium (HPGe)

Table 1. Patients, Age and Gender, Thyroid Weight, Tumor Size, pTNM Stage, Inflammation, Radioiodine Remnant Ablation Using 4000 MBq ^{131}I, and Results from Scintigraphy after Postoperative Radioiodine Ablation Treatment

Patient no.	*Age (years) and gender*	*Thyroid weight (g)*	*Tumor size (mm)*	*pTNM*	*Inflammation*	*^{131}I Remnant ablation*
11	26 M	119	65×45×36	T3N1a + bM0	None	Yes, no uptake
12	30 F	40	34×28×26	T2N1a + bM0	Abundant	Yes, no uptake
13	21 F	42	8×8×8	T1N1a + bM0	None	Yes, no uptake
14	54 F	16	20×18×17	T2N0M0	None	n.d.
15	58 M	47	15×14×13	T4aN1M0	None	Yes, no uptake
16	40 F	18	25×20×15	T2N0M0	None	n.d.
17	24 M	42	26×23×21	T2N1M0	Abundant	Yes, no uptake

No uptake on scintigraphy means that no abnormal uptake was found outside the thyroid bed. Patient no. 14 and no. 16 had negative serum thyroglobulin tests postoperatively, and hence there was no indication for postoperative radioiodine remnant ablation treatment.
pTNM, pathological tumor-node-metastasis; n.d., not done.

detector (EG&G, ORTEC, Oak Ridge, TN; thickness 10 mm, $\phi = 25$ mm) connected to a linear amplifier (Fast Spectroscopy Amplifier 2024; Canberra, Meriden, CT), an A/D converter (ND582; Nuclear Data, Meriden, CT), and an multichannel analyzer with a PC-based spectroscopy program (PCA II; Tennelec/Nucleus, Oak Ridge, TN) (12).

The measured samples were considerably smaller than the thyroids or tumors and weighed 0.25 mg on average. Calibration of the XRF system was therefore conducted with iodine solutions of varying concentrations in different droplet volumes, covering the many possible combinations of size and iodine content of the tissue samples. Both the calibration and the measurements were performed in the same geometry, that is, the tissue or calibration droplet volume on an air-equivalent surface, positioned at the intersection of the source and detector collimator openings (12).

Tissue samples from all patients were analyzed with XRF. The samples were frozen to −20°C or −30°C directly after surgical excision. XRF analysis could have been performed just after the surgical excision, but for practical reasons, the samples were frozen until more than one specimen had been collected, at most for 3 months. Earlier evaluation of iodine loss by our group has shown that this freezing procedure does not alter the iodine content (13).

Sample preparation for TOF-SIMS analysis

Fragu *et al*. (14) have presented a sample preparation method with chemical fixation that is suitable for retaining elements bound to macromolecules, such as iodine bound to Tg. The thyroid tissue in this study was treated in close accordance with their protocol: at surgery, the tissue was put in modified Karnovsky fixative (2% paraformaldehyde, 2.5% glutaraldehyde, and 0.05 M sodium cacodylate buffer pH 7.2) for storage at 4°C for 1–6 days. The samples were then rinsed and postfixed in OsO_4 and 0.1 M Na-cacodylate before being dehydrated in a series of ethanol solutions and embedded in Agar 100 resin. Histological controls (1 μm) for light microscopy and sections for TOF-SIMS analysis (2 μm) were cut (Reichert Ultracut E, Depew, NY). The light microscopy sections were stained according to Richardson *et al*. (15) with a solution containing 0.5% methylene blue, 0.5% Azur II, and 0.5% borax.

TOF-SIMS analysis

TOF-SIMS analysis was performed on material collected from four of the patients with PTC (patients 11 through 14). Both extratumoral tissue and tumoral tissue from each PTC patient were prepared and analyzed. The 2-μm sections were placed on silicon plates and analyzed with a TOF-SIMS spectrometer (TOF-SIMS IV; IONTOF GmbH, Münster, Germany). TOF-SIMS is based on high-resolution mass spectrometric analysis of secondary ions (positively or negatively charged) emitted from a sample surface during irradiation with a beam of energetic primary ions. The resulting secondary ion mass spectrum contains peaks representing element ions, molecular fragment ions, and often also intact molecular ions that provide information about the chemical composition of the sample surface. The principle is the same as the one applied in dynamic SIMS analysis, but the advantage of TOF-SIMS compared to SIMS is the higher mass resolution. This may have implications for the results because TOF-SIMS better separates stable ^{127}I from, for example, organic compounds with the same mass.

All TOF-SIMS analyses were done using 25 keV Bi_3^+ primary ions at an average beam current of 0.2 pA. Spectra of positive or negative secondary ions were collected by scanning the primary ion beam over tissue areas ranging from $100\times100\ \mu m^2$ to $500\times500\ \mu m^2$. Data acquisition times ranged from 100 to 500 seconds. The collected data were stored in raw data files with the analysis area divided into 128×128 pixels or 256×256 pixels, each of which contained a full mass spectrum. The raw data files were used for retrieving mass spectra representing the total analyzed area or smaller selected areas (regions of interest). Peaks of interest in the spectra were identified, and their relative intensities were calculated and normalized, either against the total intensity of the spectrum or against a selected reference peak in the spectrum. The raw data files were also used for constructing images that showed the intensity distribution of selected peaks like iodine in the analyzed area.

The study was approved by the Ethics Committee at Göteborg University.

Results

XRF analysis

The iodine concentration in thyroids from the laryngectomized control patients analyzed with XRF varied between 0.3 and 1.2 mg/mL with a mean of 0.6 mg/mL. For the patients with PTC, there was a significant difference in TIC between tumoral and extratumoral thyroid tissue. The mean iodine concentration in extratumoral tissue was 0.8 mg/mL, and compared to the laryngectomized patients, there were larger inter-individual variations (range 0.2–2.3 mg/mL) and also higher concentrations for some patients. The tumor tissue, however, contained too little iodine to reach the detection level of 0.1 mg/mL (Fig. 1).

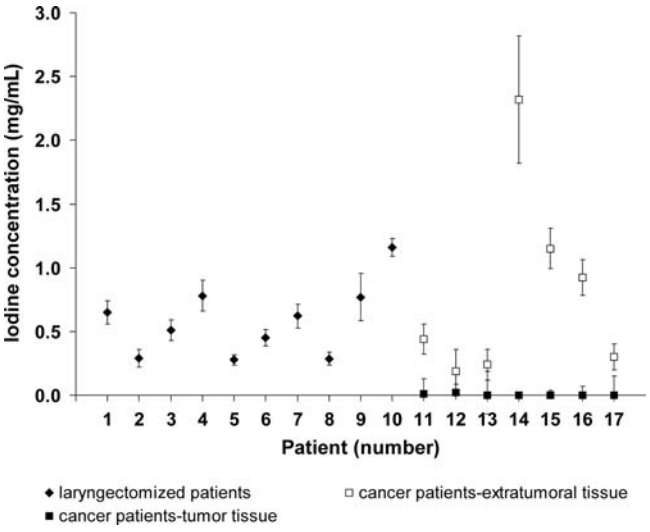

FIG. 1. Iodine concentration in thyroid tissue samples from 10 laryngectomized patients and from 7 patients with papillary thyroid cancer measured with XRF. For all cancer patients, samples were taken from extratumoral and also tumor thyroid tissue.

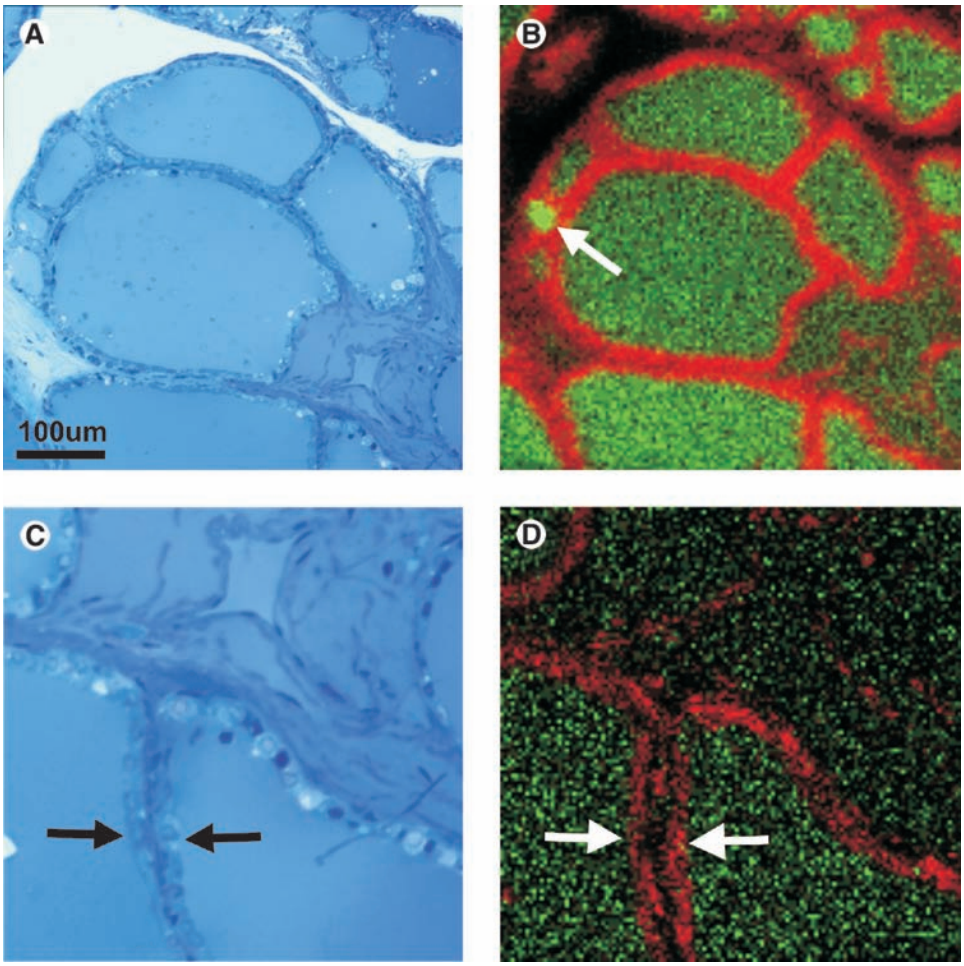

FIG. 2. Extratumoral thyroid tissue from papillary thyroid cancer patient no. 11. Panels (**A**) and (**C**) are light microscopy images corresponding to the areas analyzed with time-of-flight secondary ion mass spectrometry (TOF-SIMS) shown in panels (**B**) and (**D**). The signals from iodine and PO_3^- are presented in the same image. The red in the TOF-SIMS images is the signal from PO_3^-, presumed to be from cell membranes, and the green marks iodine. Iodine signals were found inside follicles. One follicle in (**B**) had a higher amount of iodine (↑). (**C, D**) Magnifications of the lower right corners of (**A, B**), respectively. In (**C, D**), the two rows of follicular epithelium are clearly visible (→←).

TOF-SIMS analysis

The positive secondary ion mass spectra showed strong signals from ammonium (NH_4, 18 u [atomic mass unit]), sodium (23 u), and potassium (39 u), but organic ions otherwise dominated. Among the latter, the phosphocholine ion at 184 u, a characteristic fragment ion from the membrane lipid phosphatidylcholine, could be detected. Traces of iron were also present. The negative spectra showed strong O–, OH–, CN–, and CNO– signals, as well as signals from phosphate (PO_3^-, 79 u). Clear iodine signals at 126.97 u were detected for all extratumoral tissue samples and in a few areas in the tumor tissue samples. Cluster ions of the type $Os_xO_y^-$, representing the OsO_4 staining, were also detected.

Previous TOF-SIMS studies on tissue samples have demonstrated that phospholipids in cell membranes give rise to strong PO_3^- signals (16). A clear co-localization between the PO_3^- signals and OsO_4 signals was observed in the ion images from the tissue slides. Because the latter stains lipid membranes, the PO_3^- signal was used here as an indicator for cell membrane lipids. Ion images for iodine and PO_3^- in combination with light microscopy were thus used to map the distribution of iodine in relation to the cell membrane.

Light microscopy images on tissue from the PTC patients showed a structural difference between the extratumoral and tumor thyroid tissue. Extratumoral tissue presented the typical follicle structure, whereas tumor tissue contained few follicles. The TOF-SIMS images of extratumoral tissue (Figs. 2 and 3) showed that iodine was predominantly located in the follicle lumen but was also seen within other tissues (Fig. 2A, B). Moreover, the ion images indicated differences in the amount of iodine between individual follicles (Fig. 2B). In Figures 2C and 2D, which are magnifications of the lower right corner of Figures 2A and 2B, the thyrocytes surrounding adjacent follicles are clearly visible as separated rows. Another part of the same tissue section displayed a different type of uneven iodine distribution among follicles in the same thyroid (Fig. 3). In all four tumor tissue samples that were studied with TOF-SIMS, the follicles were less frequent and the iodine signal was weak (Fig. 4).

Discussion

Thyroid cancers exhibit multiple biochemical abnormalities leading to alterations in thyroid iodine metabolism. Compared to normal thyroid tissue, most thyroid cancers have reduced iodine uptake, lowered Tg iodination, and a slower thyroid hormone synthesis rate (17). Less Tg-bound iodine is thus expected to be stored in the remaining follicle lumen, an effect that would greatly affect the iodine retention time in the thyroid and hence has implications for radioiodine treatment in thyroid cancers.

XRF and TOF-SIMS proved to be valuable methods in the study of thyroid iodine. Whereas XRF gives quantitative in-

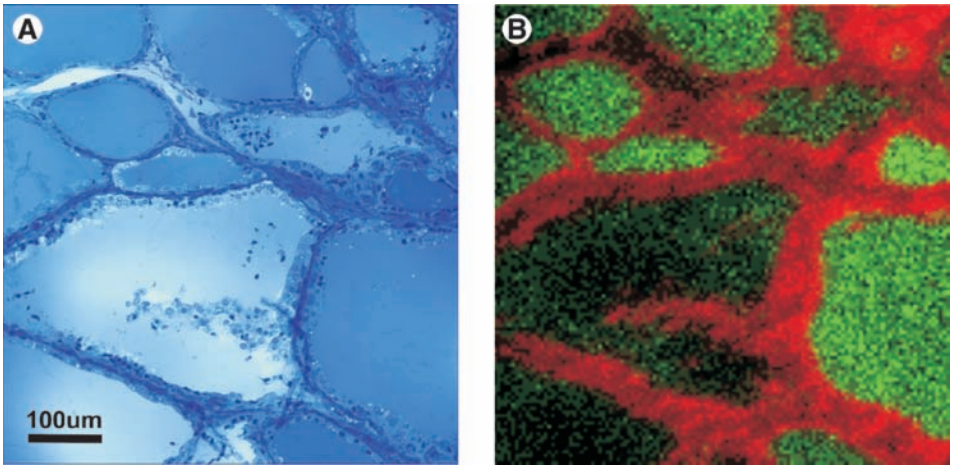

FIG. 3. Extratumoral thyroid tissue section from cancer patient no. 11, showing large variations in follicular iodine content. The red in the time-of-flight secondary ion mass spectrometry (TOF-SIMS) images is the signal from PO_3^-, presumed to be from cell membranes, and the green marks iodine. The brighter green areas in (**B**) represent higher iodine concentrations. Panel (**A**) is the corresponding light microscopy image of the tissue section analyzed with TOF-SIMS (**B**).

formation of the iodine in a sample, TOF-SIMS provides information of where the iodine is located on a follicular level. Due to the low cost and short measurement time, XRF can be applied on a large number of samples. This is in contrast to the more time-consuming TOF-SIMS, which preferably is used with high signal location specificity on a smaller number of samples. Together, these methods may thus be employed for increasing the knowledge of thyroid iodine metabolism, information that is difficult to obtain with the same accuracy using other methods.

For normal thyroid tissue from thyroid healthy controls, XRF analysis of extratumoral tissue from the PTC patients showed a normal TIC compared to the scarce iodine in tumor tissue. This outcome agreed with the TOF-SIMS results which revealed that iodine in extratumoral thyroid tissue in PTC patients was mostly stored in the follicle lumen, but that there was a marked reduction in the number of follicles in tumor tissue, and the iodine signal was weak. This has not previously been shown with TOF-SIMS, and few studies have reported on iodine in thyroid cancers analyzed with XRF.

Our results showing lower iodine stores in PTC demonstrate the effect of the dysfunctions associated with thyroid cancer. The present TOF-SIMS analysis showed no iodine accumulation in the tumor tissue, neither in the follicle lumen nor in the thyrocytes, thus indicating a dysfunction of sodium/iodide symporter (NIS). If the thyroid cancer tissue was lacking only in iodide oxidation and Tg iodination capacity, accumulation of iodine in the thyroid cells would have been expected.

It is not yet known whether individual variations in TIC have implications for the development of thyroid disease, for example thyroid cancer. The mean normal tissue TIC found in this study is comparable to those found in earlier studies. A Swedish study by Heedman and Jacobson reported a mean iodine content of 12.9 mg (18), giving a TIC of 0.6 mg/mL, with the assumption of a mean thyroid volume of 20 mL. A more recent *in vivo* study in Mölnlycke, Sweden, identified a mean TIC of 0.4 mg/mL (9). Of note is that in our study we found a wider TIC range in the extratumoral tissue from patients with PTC (0.2–2.3 mg/mL) than in the samples from thyroid healthy patients (0.3–1.2 mg/mL). However, comparable and wider ranges have been reported in other studies investigating normal thyroids (7,11,18). These variations are somewhat surprising but can be explained by the numerous factors affecting thyroid metabolism; in addition, they reflect the intricate mechanisms of thyroid iodine homeostasis. It should also be noted that the sample size in the present study was small.

Apart from individual variations in iodine content, there are also small-scale heterogeneities. Large variations in iodine concentration among individual follicles have been discovered in normal thyroid tissue (19,20). More recent findings of

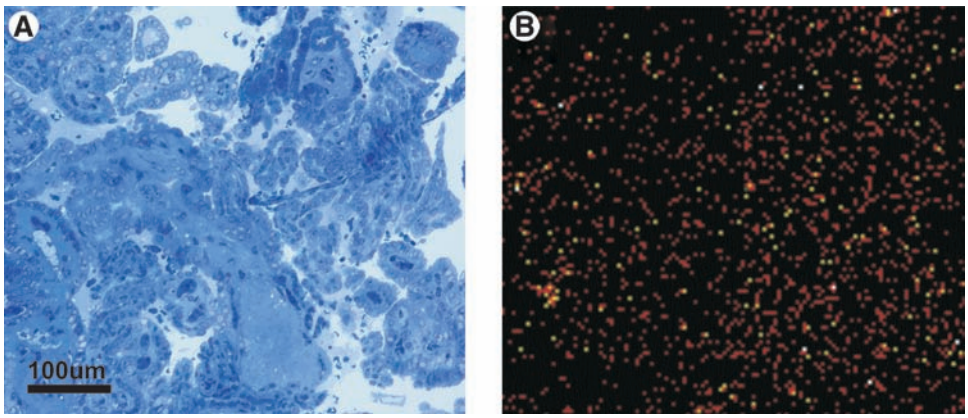

FIG. 4. Tumor tissue from patient no. 13. No follicles and little iodine were seen in the light microscopy image (**A**) and in the time-of-flight secondary ion mass spectrometry (TOF-SIMS) image (**B**). Because of the scarce iodine, the iodine signal was not to be distinguished among the stronger signals from PO_3^-. The TOF-SIMS image (**B**) in this figure therefore shows the signal from iodine alone, where increasing intensity is represented by the colors red, green, and white, in that order.

heterogeneous distribution of NIS protein even within a given follicle also imply that the iodine distribution may be inhomogeneous within the thyroid (21). Our TOF-SIMS findings showing differences in iodine content between follicles within the same thyroid provide further evidence of interfollicular variance.

It is of outmost importance that the preparation methods used preserve the iodine content and distribution. Our group (13) and Rognoni *et al*. (22) evaluated glutaraldehyde, a major component of the Karnovsky fixative used for the TOF-SIMS analysis, with respect to iodine loss. They found that the iodine loss was relatively small, 5%, compared to sectioning that could lead to a random iodine loss of 10%. The iodine loss due to fixation in Karnovsky was not expected to interfere with the results interpretation. Possible iodine loss in the samples analyzed with XRF could also be disregarded because freezing as a sample preservation method has been verified as preserving the iodine content (13). The samples taken for XRF analysis were relatively large; thus, the small-scale variations in iodine distribution should not have strongly affected the results.

Conclusion

It was found that tumor tissue contained little or no iodine, but the iodine concentration measured in extratumoral thyroid tissue and in thyroid tissue from thyroid healthy controls was within the expected range. XRF and TOF-SIMS are two complementary methods fully applicable for *in vitro* quantitative and qualitative analysis of iodine in thyroid tissue samples. Both methods may be further developed in this area and are also relevant in other applications.

Acknowledgment

This study was supported by King Gustav V Jubilee Clinic Cancer Research Foundation, Göteborg, Sweden.

Disclosure Statement

No competing financial interests exist.

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