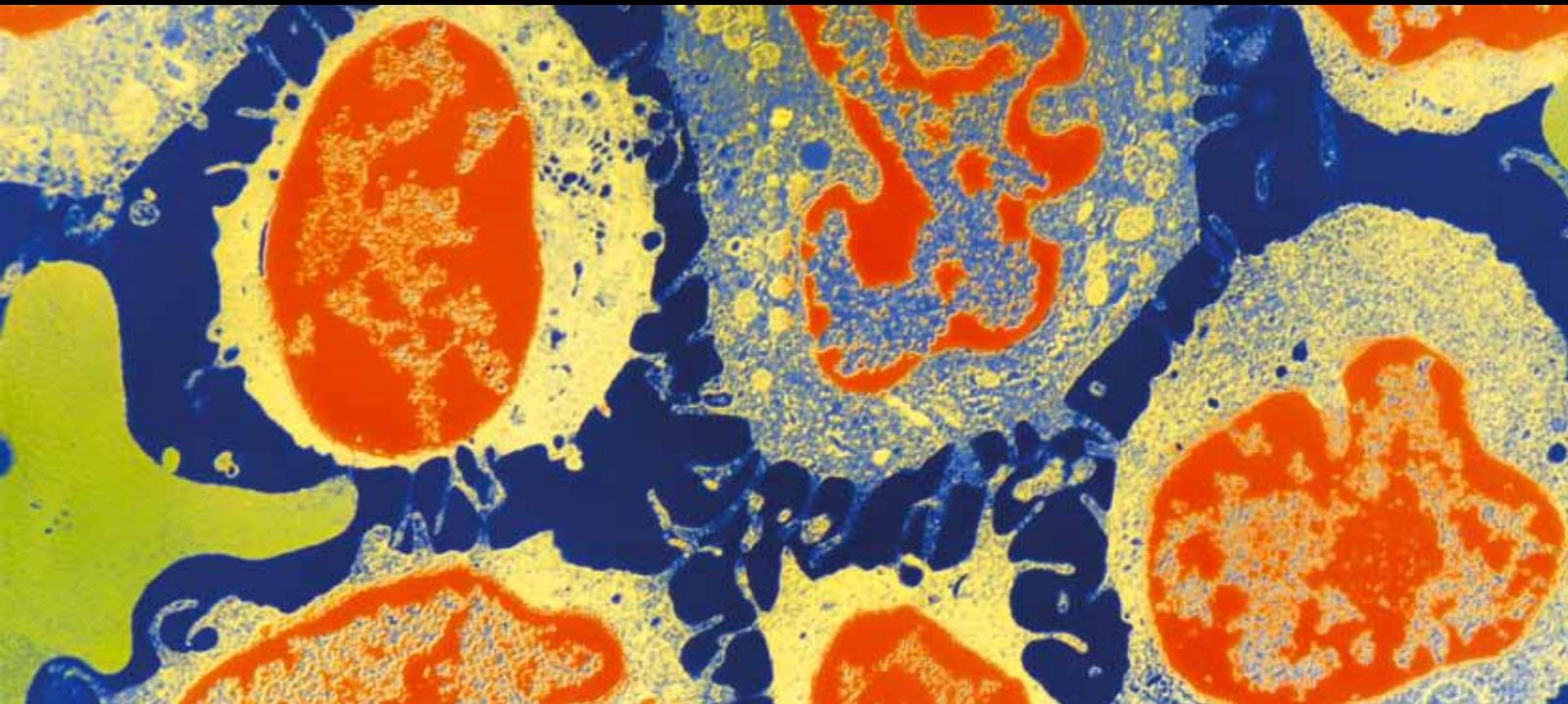


Thyroid Cancer: Molecular and Modern Advances

Guest Editors: Jennifer E. Rosen, Steven K. Libutti,
and Stephanie L. Lee



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Journal of Oncology

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Editorial

Thyroid Cancer: Molecular and Modern Advances

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Thyroid cancer is the most common endocrine malignancy and accounts for approximately 44,670 cases and 1,690 deaths in 2010 per the ACS. The purpose of this special issue is to provide an update on recent advances in the understanding of thyroid tumorigenesis and their implications in clinical practice and new surgical approaches to thyroid cancer and to the adoption of techniques used successfully in other tumor types for use in patients with thyroid cancer. Invited papers addressed several of the above topics.

Advances in surgery promise to allow expanded surgical treatment options and potentially make thyroid cancer surgery safer and better accepted by patients. Lang et al. provide a detailed overview of refinement in surgical techniques including endoscopic thyroidectomy techniques, the addition of the da Vinci robot, and the use of operative adjuncts in thyroid surgery such as intraoperative neuromonitoring and quick intraoperative parathyroid hormone. They also outline the clinical studies needed to better analyze the use of this new technology and its relative benefits and risks. Lombardi et al. present their review of a large series of patients with papillary thyroid cancer who underwent video-assisted thyroidectomy (VAT) for completeness of the surgical resection and short-to-medium term recurrence. Their results, while retrospective, seem to indicate that VAT is feasible and safe and may be a valid alternative to conventional surgery for small PTC. No surgical approach is without its concomitant risks—in thyroid surgery, this includes injury to one or both recurrent laryngeal nerves

which can result in poor voice quality and the potential for recurrent aspiration. Sanuki et al. discuss their findings of immediate reconstruction of the recurrent laryngeal nerve during thyroid cancer surgery in terms of voice outcomes using videostroboscopic, aerodynamic, and perceptual analyses. While their numbers were small, it addresses an important question often raised in surgery as to whether immediate or delayed reconstruction should be performed.

Advances in genomics have offered exciting insights into the biology of thyroid cancer. While much attention has been focused on the traditional nucleic acids, the study of proteins and their function has been relatively neglected, in part due to the difficulty of the techniques required. The paper by Miyoshi et al. provides a comprehensive overview of the vital role of glycosylation and functional glycomics in thyroid cancer.

As clinicians well know, an accurate cytological diagnosis is key to the treatment approach for patients with thyroid nodules; this diagnosis is based on distinctive cytological features in combination with immunocytochemistry. Pazaitou-Panayiotou and colleagues present here their study of 83 thyroid cancer fine needle aspirations using Thin Layer Cytology. They present data on a panel of immuno-markers (including Cytokeratin-19, Galectin-3, HBME1, CD-44, CD-56, and E-Cadherin) and describe this promising new technique to improve diagnostic accuracy.

Early detection is a key component of familial thyroid cancer; Bonora et al. provide us with an excellent review of

the familial syndromes, genetic abnormalities, and risk factors proposed to increase the likelihood of this type of neoplasia. Giovanella et al. present a very interesting case example of a rare functioning trabecular tumor of the thyroid gland which serves as a reminder to us that we must not overlook the potential for malignancy in the high-uptake pertechnetate positive nodule.

While the most extensively studied pathway for targeted therapy in thyroid cancer is RAS/RAF/MEK, clinical trials targeting therapies in this pathway are relatively new. The review by Poon and Tai provides us with a comprehensive update of known genomic changes in thyroid cancer and how this information is being used in clinical trials to improve targeted therapy.

Treatment of certain subtypes of thyroid cancer has been limited in part due to a dearth in therapeutic strategies. In a seminal paper, Dr. Steven Rosenberg proposed the concept that the body's immune system could be manipulated for cancer therapy. (Rosenberg SA (Jan 1984). "Adoptive immunotherapy of cancer: accomplishments and prospects". *Cancer Treat Rep* 68 (12): 233–55). Since then, a wide variety of immunotherapeutic approaches have been tested in clinical trials, in particular using either cell-based therapy or immunomodulators. These have led to fairly modest improvements in patient outcomes and a number of therapeutic challenges. This initial work has set the basis for a vigorous research effort to better understand the complex relationship and numerous intersecting pathways which regulate the body's response not only to cancer but to therapy for cancer as well. In their excellent review of dendritic cell-based immunotherapy for the subset of advanced, metastatic, or undifferentiated and anaplastic thyroid carcinoma, Papewalis et al. provide us with a focus on understanding the advances of antitumor immune response in thyroid carcinoma and well summarizes the rationale for adoptive dendritic cell transfer including the technical approach to this interesting new modality of treatment.

We hope that this special issue will educate, stimulate, and inspire support for participation in clinical trials and new research in the field of thyroid cancer. The development of new surgical approaches, effective targeted therapies, and better preventive interventions will assist in improving treatment and outcome in patients with thyroid cancer.

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Review Article

Technological Innovations in Surgical Approach for Thyroid Cancer

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Over the last decade, surgeons have witnessed dramatic changes in surgical practice as a result of the introduction of new technological advancement. Some of these changes include refinement of techniques in thyroid cancer surgery. The development of various endoscopic thyroidectomy techniques, the addition of the da Vinci robot, and the use of operative adjuncts in thyroid surgery, such as intraoperative neuromonitoring and quick intraoperative parathyroid hormone, have made thyroid cancer surgery not only safer and better accepted by patients with thyroid cancer but also offer them more surgical treatment options.

1. Introduction

New technologies have had a positive impact on our ability to diagnose and treat many surgical conditions [1]. Over the last decade, surgeons have witnessed dramatic changes in surgical practice as a result of the introduction of new technologies or technological advancement. Thyroid cancer is the commonest endocrine-related tumor. In our locality, its age-adjusted incidence has doubled over the last 25 years, and a similar trend has been reported elsewhere [2]. New technologies have had important influence in the management of this disease. In addition to improving the preoperative diagnostic accuracy and cancer staging with various imaging modalities, the techniques of thyroid cancer surgery have been refined and evolved in this era of technological advancement. In applying these new technologies, it is believed that surgical morbidity can be further reduced, hospital stay shortened, and patient satisfaction enhanced [3]. The present paper aimed at evaluating how some of these new technological innovations might improve patient outcomes and offer new surgical treatment options for patients diagnosed with thyroid cancer. These innovations include the development of various endoscopic thyroidectomy techniques, the addition of the da Vinci robot surgical system, as well as the use of operative adjuncts

such as intraoperative neuromonitoring (IONM) and quick intraoperative parathyroid hormone (IOPTH).

2. Endoscopic Thyroidectomy

The application of endoscopic visualization to thyroid surgery has allowed surgeons to perform thyroidectomy through incisions far smaller and less visible than the conventional Kocher's incision—the so-called "less is more." In general, these endoscopic techniques attempt to minimizing the extent of dissection, improving cosmesis, reducing postoperative pain, shortening hospital stay, and enhancing postoperative recovery. Michel Gagner was the first to apply endoscopic technique to neck surgery when he reported a totally endoscopic subtotal parathyroidectomy for a 37-year-old man suffering from familial hyperparathyroidism [4]. Although the endoscopic procedure took over 5 hours, it demonstrated the technical feasibility and safety. Over the turn of the last century, an increasing number of different endoscopic techniques have been described and may be categorized into namely cervical or direct and extracervical or indirect approaches [5]. The former is considered as truly minimally invasive since the skin incisions are small in the neck with direct access to the thyroid gland. On

the other hand, the extracervical approach is considered as an endoscopic instead of minimally invasive approach because incisions are made distant from the neck and so the procedure requires more extensive tissue dissections [6]. However, despite its invasiveness, it offers superior early cosmetic outcome because potentially unsightly scars can be hidden. This approach has been adopted more often in Asian countries where cosmesis seems to be of greater concern.

2.1. Cervical/Direct Approaches. These approaches include the endoscopic lateral cervical approach and the minimally invasive video-assisted thyroidectomy (MIVAT). In the endoscopic lateral cervical approach, two 2.5 mm and one 10 mm trocars are inserted under direct vision along the anterior border of the sternocleidomastoid muscle on the side of resection. Using endoscopic instruments, the dissection starts from the lateral aspect of the thyroid gland and moves medially with identification of the recurrent laryngeal nerve (RLN), parathyroid glands and skeletonisation of the superior and inferior thyroid vessels [7]. Excellent visualization of RLN and parathyroid glands is possible with magnification by the endoscope. However, this technique is limited to unilateral thyroid resection and its application in thyroid cancer surgery is restricted to subcentimeter papillary thyroid carcinoma (PTC) detected by high-resolution ultrasound machines. In contrast, the MIVAT would be preferred if bilateral thyroid resection is required because the incision is made in the middle instead of the lateral aspect of the neck. A 1.5 cm incision is made in the middle of the neck about 2 cm above the sternal notch. Blunt dissection is then carried out to separate the strap muscle from underlying thyroid lobe. A 5 mm 30 degree endoscope is placed inside the 1.5 cm wound for lighting and visualization. The procedure is performed under endoscopic view with the operating space maintained by external retraction. This technique was first applied for selected benign thyroid conditions by Miccoli et al. in 2000 [8]. However, with improvement in techniques, MIVAT has become increasingly adopted for low-to-intermediate risk differentiated thyroid cancer [9]. MIVAT has been shown to achieve similar completeness of resection [10, 11] and 5-year survival outcomes as those with low and intermediate risk PTC undergoing conventional thyroidectomy [9]. In addition, it has been shown that a concomitant central neck dissection is technically feasible in MIVAT during initial total thyroidectomy [12]. Also, for patients with low risk PTC with concomitant lateral lymph node metastases, a minimally invasive video-assisted functional lateral neck dissection through a small neck incision is also technically possible [13].

2.2. Extracervical/Indirect Endoscopic Approaches. Unlike the cervical approaches, these approaches involve making incisions either in the chest, breast, and/or axilla to hide the scars with clothing [14]. Ikeda et al. first described these approaches by placing three ports in the axilla with low-pressure gas insufflation for maintaining the operating space. Although cosmetic results were excellent, the procedure

was technically demanding and time consuming because of unintentional easy gas leakage and frequent interference of the 3 operating surgical instruments in the small available space in the axilla [15]. Kang et al. modified this technique by making this approach gasless with the space maintained by a specially designed skin-lifting external retractor [16]. In this approach, the procedure began with a 4 cm to 5 cm incision in the axilla and then a subcutaneous space was created from the axilla to the thyroid gland. To avoid the problem of interference of instruments, an additional 5 mm port was inserted in the chest area for medial retraction of the thyroid gland. Kang et al. recently reported their experience with this approach after performing 581 cases [16]. Among these patients, 410 patients had low-risk PTC. In their series, concomitant central neck dissection was performed and the rate of lymph node metastasis was 27.3% [16].

To further increase the degree of angulations and freedom of interference between instruments, a combined axillo-breast approach was developed utilizing 2 circumareolar trocars in the breast and a single trocar in the ipsilateral axilla. This approach was later modified by using bilateral axillary ports to allow better exposure to both sides of the thyroid compartment. This approach is now known as the bilateral axillo-breast approach (BABA). Despite the extensive tissue dissection, when compared with the conventional open approach, BABA has been shown to have similar results in terms of transient hypocalcemia, bleeding, permanent RLN paralysis and length of hospital stay [17]. More recently, a Korean group tried to eliminate wounds around the chest or breast areas all together by making incisions in the axilla and postauricular areas instead. They reported a small series of 10 patients using this approach and 7 underwent bilateral thyroid resection for low-risk PTC. They demonstrated the feasibility of this technique of scarless (in the neck) thyroid surgery [18].

3. Robotic-Assisted Thyroidectomy

The application and feasibility of the endoscopic approach was given a further boost with the availability of various robotic systems such as the da Vinci system (Intuitive Surgical, Sunnyvale, California). Unlike other cancers such as prostate cancer, the initial enthusiasm of using the robot in thyroid cancers was not great because of its relatively high cost, bulkiness of the robotic arm, and long operating time. However, since the publication of two large surgical series demonstrating the feasibility and safety of robotic-assisted thyroidectomy in differentiated thyroid carcinoma, an increasing number of specialized surgical centers worldwide are beginning to accept and perform this procedure. The theoretical advantages of using the robot over the endoscopic approach include the three-dimensional view offer to the operating surgeon, the flexible robotic instruments with seven degree of freedom and 90° articulation, the increased tactile sensation, and the ability to filter any hand tremors [19]. Kang et al. recently reported their experience of 200 robot-assisted total thyroidectomy using the gasless transaxillary approach for low-risk PTC with concomitant central

neck dissection and found excellent short-term results in terms of postoperative pain and patients' satisfaction [20]. This was followed briefly by another report of 338 benign and malignant cases using the same transaxillary [21]. To date, this group has performed over 1000 cases. A separate Korean group also reported similar results using the da Vinci robot via the BABA technique [22]. Although both techniques have been demonstrated to be feasible and safe, they have been limited to a few high-volume specialized centers. The surgeons performing these operations have had years of operating experience with the endoscopic approach and so the learning curve for a novel, nonendoscopic thyroid surgeon or someone who predominantly perform open thyroid procedures, remains undefined but is likely to be longer than one might think. Furthermore, better comparative studies such as a randomized controlled trial between robotic-assisted and endoscopic thyroidectomy are needed in order to better assess the added patient outcome benefits over the latter approach.

4. Surgical Adjunct: IONM

RLN injury is a leading cause of litigation in thyroid surgery [23]. To those with this injury, it not only affects the voice quality but also diminishes the overall quality of life because of communication, social and work-related problems [24]. Routine RLN identification is currently the gold standard of care in thyroid surgery. However, with the availability of IONM, the issues are whether this new technology could further enhance RLN preservation and reduce the risk of iatrogenic RLN injury in thyroid surgery or thyroid cancer surgery in particular.

Although IONM has been around for over 3 decades, its widespread usage in the surgical practice only dates back to 5–10 years. There has been an increased interest in applying this technique for thyroid surgery because of the introduction of new and user-friendly devices from technological advance [25]. Currently, there are two types of IONM systems, namely, those with electromyographic (EMG) documentation and those without EMG documentation. The former involves RLN stimulation with registration of the elicited laryngeal muscle activity through endoscopic insertion of electrodes into the vocal fold or with the use of endotracheal surface electrodes. The latter utilizes RLN stimulation with observation of posterior cricoarytenoid muscle contraction or palpation or intraoperative inspection of vocal cord function [26, 27]. To date, there is no consensus on which is the best system, and the choice depends on the availability of which system in your institution and the operator familiarity or experience. Regardless of which systems, there are potential flaws and pitfalls. In general, the positive predictive value (PPV) is proportionally low with this technology. That means that when a nerve has no signal during stimulation, it does not mean that it is injured. In fact, in our experience, the PPV was only 15% in low-risk thyroid surgery, that is, approximately only 1 out of 9 RLNs with no signals had an actual injury. This might be due to some technical errors such as detachment or displacement of

electrodes or poor contact of the probe with the nerve due to inadequate exposure [28]. Perhaps, direct vagal stimulation could possibly reduce some of these errors but need more unnecessary dissection. Even more intriguing is the fact that this technique is also associated with false negative results, albeit rarely. In our experience, among 271 nerves at risk, 15 (5.5%) ended with RLN palsy but of these, 7 still had a positive IONM signals. Therefore, it seems that IONM might not be able to detect "sublethal" injury to RLN. It is possible that the action potential could be propagated along the neural pathway, as detected by the IONM, but not to the extent of initiating laryngeal muscle contraction during the postoperative period [25, 28]. Fortunately, all these injuries would invariably recover.

On the other hand, although the objective of the use of this device is to avoid RLN injury during thyroid surgery, the evidence of supporting its routine use has been weak. The first multicenter study including 29,998 RLNs at risk confirmed that the incidence of RLN palsy was not significantly reduced by the additional use of IONM when routine RLN identification was performed [27]. There were more than 20 publications addressing this issue but majority of these studies were heterogeneous in terms of patients' characteristics (such as primary operations versus reoperations or benign versus malignant goiters), IONM techniques and the extent of resection (i.e., total versus subtotal lobectomy). A recent literature review could not definitely draw confirm conclusions or evidence on the effectiveness of IONM in reducing RLN injury in thyroid surgery [26]. Furthermore, most studies were either case-series with no control group or retrospective studies with inadequate statistical power to demonstrate a difference between those with or without using IONM. In fact, a randomized study utilizing approximately 7,000 patients in each arm of patients undergoing thyroidectomy with or without IONM will be required to have adequate statistical power to show a difference in outcome with reference to RLN paralysis [26, 27]. Interestingly though, the first prospective randomized study comparing IONM with routine RLN visualization only was recently published [28]. In this study, approximately 500 patients were randomized into each arm. The number of patients recruited in each arm was based on the principle of detecting a 2% difference in the incidence of transient RLN injury with a 90% probability at $P < .05$. This study did demonstrate a statistically significant difference in reducing transient RLN injury when IONM was adopted in comparison with RLN visualization only. However, as expected, the rate of permanent RLN injury was similar in the two study arms because of inadequate statistical power. Nevertheless, despite the inadequate power of most published IONM studies, there seemed to be a trend toward improved RLN protection with the use of this new technology [26]. In addition, the IONM may be of potential benefit for "difficult" cases such as reoperative thyroidectomy, locally advanced thyroid cancers or central neck dissection for cancer recurrence. Perhaps, for the novel and relatively inexperienced surgeons, the IONM might prove to be extremely invaluable for these difficult cases.

5. Surgical Adjunct: IOPTH or Quick Intraoperative Parathyroid Assay (qPTH) as an Assessment of Postthyroidectomy Hypoparathyroidism

Hypoparathyroidism is a common complication after bilateral thyroid resections or total thyroidectomy. Up to 30% of patients after total thyroidectomy develop temporary hypoparathyroidism [29]. There are many identifiable risk factors leading to postoperative hypoparathyroidism including thyroidectomy for thyrotoxicosis and thyroid cancer, thyroid reoperations, reduced stores of vitamin D, increased extent of thyroid resection, and need of concomitant central neck dissection [30, 31]. Patients undergoing thyroidectomy for thyroid cancer are particularly prone to hypoparathyroidism because they often need a more complete thyroid resection together with neck dissection. In fact, total thyroidectomy and routine concomitant central neck dissection has now been increasingly practiced worldwide for almost types of well-differentiated thyroid cancer to achieve lower recurrences, better disease-free survival, and enhanced postoperative thyroglobulinemia [32]. However, it has been shown that up to 60% of patients after concomitant central neck dissection could develop transient hypocalcemia secondary to the frequent occurrence of unintentional or incidental parathyroidectomy [33]. Therefore, in the presence of such a high incidence of postoperative hypoparathyroidism, the need of routine postoperative inpatient calcium monitoring remains questionable after thyroid cancer surgery while the early routine administration of oral calcium and/or vitamin D supplements seems to be relevant and can facilitate the early discharge from hospital shortly after surgery without developing unpleasant hypocalcemic symptoms [34]. In fact, a recent randomized study supported this strategy because routine administration of oral calcium was shown to markedly reduce the severity and symptoms of hypocalcemia [35]. However, the adoption of this strategy could lead to overtreatment in patients who do not have hypocalcaemia leading to rebound hypercalcemia and increased medication costs. On the other hand, this strategy might lead to inadequate treatment in patients with severe symptomatic hypocalcaemia as oral calcium alone may not fully correct the hypocalcemia and so vitamin D supplements is indicated in such situation [36].

On the other hand, inpatient serial close monitoring of serum calcium is recommended after total thyroidectomy because most symptomatic hypocalcemia occurs around 24–28 hours after surgery [37]. A 24-hour or longer hospital stay is invariably required. Therefore, efforts are made to shorten hospital stays, decrease biochemical blood tests, and reduce hospital costs by adopting other strategies to achieve early prediction of postthyroidectomy hypocalcemia. With the availability of IOPTH and wide application in patients undergoing minimally invasive parathyroidectomy to predict postoperative cure, this new surgical adjunct has been applied to thyroid surgery to monitor parathyroid function and to predict the occurrence of postoperative normocalcaemia or hypocalcaemia. In our early prospective study of using IOPTH in predicting hypocalcemia

in 100 consecutive patients (including 33 patients with differentiated thyroid cancer) who underwent either total or completion thyroidectomy, we found that a normal level of IOPTH at 10 mins or a level less than 75% decline in IOPTH at 10 mins after excision of thyroid gland accurately identified normocalcemia [38]. It was suggested that intraoperative or early postoperative parathyroid hormone assay might be a sensitive tool to confirm postoperative normocalcaemia and identify patients at risk of developing postoperative hypocalcaemia. Since then, up to 30 different investigators have published their results of using various different IOPTH assays in predicting hypocalcemia after total thyroidectomy. The IOPTH levels and their rate of decline at various time points after surgery could be utilized for prediction of postoperative hypocalcaemia with variable sensitivity, specificity, and accuracy [39, 40]. However, based on two evidence-based reviews, it was recommended that the IOPTH level within a few hours after thyroid surgery could accurately predict postoperative normocalcaemia and identify patients at-risk of developing hypocalcemia, particularly severe, symptomatic hypocalcemia [34, 41]. It was suggested that patients could be stratified into high- or low-risk groups and PTH should be measured at 1–6 hrs after operation in comparison to preoperative PTH. A < or > 65% decline at 6 hours after operation should allow early discharge or facilitate the decision of early calcium supplement. On the other hand, a strategy of 2 cut-off points should be considered with a high accuracy. A <50% decline within few hours after surgery allowed early discharge while a >90% decline necessitated early calcium supplement because of the accuracy in predicting normocalcaemia and hypocalcaemia, respectively [41]. For those patients with 50%–90% decline, either serial calcium monitoring or routine treatment should be considered. In the AES guideline, one single serum PTH measurement is recommended at 4 hrs after operation [42]. A normal PTH can predict normocalcaemia, and patients can be discharged early with 7% subsequently developing mild hypocalcaemia. For patients with undetectable PTH level, oral calcium and vitamin D analogue should be administered early to avoid symptomatic hypocalcaemia. Intermediate or subnormal PTH level is a less accurate predictor of hypocalcaemia. In that case, oral calcium should commence or patients should be monitored with serial calcium levels for the need of calcium and/or vitamin D analogue [42]. Therefore, PTH assay can now be considered as a perioperative adjunct to predict normocalcaemia or hypocalcaemia with reasonable accuracy. It can facilitate early discharge, avoid routine calcium replacement, facilitate early calcium replacement to avoid symptomatic hypocalcaemia and decrease overall cost as well as increase patients' satisfaction.

6. Conclusions

New technologies have undoubtedly had a positive impact on the surgical management of thyroid cancer. The application of endoscopic visualization of the thyroid gland has allowed surgeons to perform safe surgery from extracervical skin

incisions. Although short-term outcome studies in various endoscopic techniques demonstrated comparative results as conventional open thyroidectomy for differentiated thyroid cancer, this particular operative approach is indicated for selected patients only and the benefit is still considered marginal with concerns of higher associated cost and longer operating time in performing these procedures. Although the robot procedures might offer some theoretical advantages over the endoscopic procedures, better-designed prospective comparative studies are required. Despite the lack of strong evidence for the benefit of routine use of IONM, there is a trend toward improved RLN protection and reduced iatrogenic RLN injury. As a surgical adjunct, IOPTH is being actively sought as a cost-effective tool for predicting postoperative hypocalcaemia in patients undergoing total thyroidectomy for thyroid cancer.

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Clinical Study

Video-Assisted Thyroidectomy for Papillary Thyroid Carcinoma

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Background. The results of video-assisted thyroidectomy (VAT) were evaluated in a large series of patients with papillary thyroid carcinoma (PTC), especially in terms of completeness of the surgical resection and short-to-medium term recurrence. **Methods.** The medical records of all patients who underwent video-assisted thyroidectomy for PTC between June 1998 and May 2009 were reviewed. **Results.** Three hundred fifty-nine patients were included. One hundred twenty-six patients underwent concomitant central neck node removal. Final histology showed 285 pT1, 26 pT2, and 48 pT3 PTC. Lymph node metastases were found in 27 cases. Follow-up was completed in 315 patients. Mean postoperative serum thyroglobulin level off levothyroxine was 5.4 ng/mL. Post operative ultrasonography showed no residual thyroid tissue in all the patients. Mean post-operative ^{131}I uptake was 1.7%. One patient developed lateral neck recurrence. No other recurrence was observed.

1. Introduction

Over the last decade several techniques for minimally invasive thyroid surgery have been described, including various endoscopic [1, 2] and video-assisted approaches [3–5] as well as minimal incision techniques [6, 7]. The primary aim of all these different approaches has been to improve the cosmetic results of conventional surgery [8]. Among all these techniques, video-assisted thyroidectomy (VAT) by the central access [3, 4] is one of the most diffuse worldwide and it has been adopted by several Centers, especially in Europe [9–11] and in USA [12].

Initial experiences published on VAT underlined the advantages of the procedure in terms of a better cosmetic result and less postoperative pain when compared with conventional surgery [13–15]. In relatively small series of patients and comparative studies, VAT has been demonstrated to be a reproducible, safe and effective technique [3, 4]. Larger multiinstitutional series have fully demonstrated its safety and efficacy in several clinical settings [9–12]. Several advantages of this technique have been clearly demonstrated: in particular less tissue trauma and less patient

discomfort [13–15]. Furthermore, in one recently published paper, we demonstrated that the incidence and the severity of early voice and swallowing postthyroidectomy symptoms are significantly reduced in patients who undergo VAT compared with those who undergo conventional surgery [16].

In the recent years, VAT has been applied successfully for the treatment of small papillary thyroid carcinomas (PTC) [17–20]. Findings of initial small comparative studies have supported the supposition that VAT allows for a surgical resection similar in terms of completeness to conventional surgery [17, 18] with no additional risk of cancer cell seeding [15]. In spite of these encouraging results, some experts consider PTC a contraindication for this approach [5, 8], and some have expressed doubts about its surgical radicality. Concern has been raised over whether this procedure is oncologically safe and adaptable beyond the few medical Centers where it has been developed and optimized [21]. Even though we and others [18, 19] have demonstrated that VAT allows to obtain the same completeness of the surgical resection as conventional thyroidectomy in patients with PTC, some surgeons are still very hesitant to treat PTC by a video-assisted approach. Additionally, one of the major

criticism was about the relatively low number of cases and the absence of an adequate follow-up. Recently Miccoli et al. [20] have demonstrated, after a quite long period of follow-up that minimally invasive video-assisted thyroidectomy can be safely employed in low and intermediate risk patients without negative impact on patients outcome.

Similarly, video-assisted central compartment lymph node dissection (VALD = video-assisted lymph node dissection) has been shown to be feasible with no additional risk of complications in patients with PTC [18, 22] and in RET-gene mutation carriers [23].

In this paper, we retrospectively evaluated the results obtained in a large series of patients who underwent VAT for PTC over a 10-year period, especially in terms of the completeness of the surgical resection and short-to-medium terms recurrence.

2. Materials and Methods

Between June 1998 and May 2009, 1356 patients underwent VAT at the Division of Endocrine Surgery of the Università Cattolica del Sacro Cuore, Rome, Italy. Eligibility criteria for VAT were thyroid nodules less than 35 mm on the largest diameter, an estimated thyroid volume within the normal range (less than 30 mL), and no previous conventional neck surgery and/or radiation therapy. Absolute contraindications were malignancies other than PTC and the presence of preoperatively demonstrated infiltrating tumors or lymph node metastases. All the patients who successfully underwent VAT for an histologically proven PTC were included in this study. The medical records of these patients were retrospectively reviewed.

The operative technique has been extensively described in previously published papers [9].

Postoperative serum calcium and phosphorus levels were measured in all the patients. Hypocalcemia was defined as a serum calcium level below 8.0 mg/dL. Laryngoscopy was performed preoperatively only in patients that had experienced voice changes and was performed postoperatively in all patients to check vocal cord motility.

All the patients underwent postoperative suppressive levothyroxine (LT4) treatment. All patients underwent serum thyroglobulin (sTg) and anti-Tg antibody measurements under suppressive LT4 treatment and an ultrasound (US) neck scan 3 to 6 months after surgery. This was the only follow up protocol adopted for patients with pT1 PTC ≤ 1.0 cm, in the absence of lymph node metastases and multifocality. ^{131}I ablation (RAI) was performed on the basis of stage and risk factors, according to the American Thyroid Association Guidelines [24]. Low-risk patients were evaluated by ^{131}I diagnostic whole body scan (DxWBS), quantitative ^{131}I neck uptake (RAIU) and TSH-stimulated sTg. All the high risk patients underwent RAI. Posttherapy whole body scan (TxWBS), RAIU and TSH-stimulated sTg levels were evaluated in this group of patients.

For the purpose of this paper, the completeness of the surgical resection was determined by neck ultrasound imaging, qualitative evaluation with DxWBS or TxWBS, RAIU, and TSH-stimulated sTg levels.

sTg was measured using a commercial electrochemiluminescence immunoassay (ECLIA) (Roche Diagnostic Co., Indianapolis, IN, USA) for Elecsys System 2010. The measuring range was 0.100–1000 ng/ml. RAIU was performed by administering a capsule of 3.7 MBq ^{131}I and 24-h isotope uptake was measured by an external probe equipped with a 2×2 cm NaI(Tl) crystal, attached to a series 35 multichannel analyser (ACN Monogamma, L'accessorio Nucleare S.r.l., Milano, Italy).

RAI required hospitalization and was administered 2–6 months after surgery in hypothyroidism condition (TSH levels above $30 \mu\text{UI}/\text{mL}$), obtained by withdrawing thyroid replacement hormone. Pregnancy was excluded before ^{131}I administration in all women of childbearing age. The ^{131}I activity was chosen on the basis of a system of “empiric adjusted doses”. By this system, for example, low activities (i.e., 1,850 GBq) were administered in patients with unifocal pT1 PTC, no lymph node metastases, and low RAIU values, while large activities (i.e., 5,550 GBq) were chosen for multifocal pT3 PTC, with lymph node metastases and high RAIU values [24, 25]. All patients were subjected to TxWBS before discharge, 2–5 days after administration of a therapeutic dose of ^{131}I , to visualize any thyroid remnants or metastases. Anterior and posterior whole body images were recorded using a large field-of view GE Starcam 3200i scintillation camera (General Electric Medical System, Milwaukee, WI, USA), fitted with a high-energy parallel-hole collimator.

Patients who did not receive RAI underwent long-term follow-up at 6–12 month intervals, at which time US scan was performed and sTg levels were measured while the patients were on LT4. Patients whose TxWBS revealed no thyroid remnants or metastases were also placed on this follow-up protocol. Patients whose TxWBS revealed thyroid remnants or metastases were subjected to DxWBS, RAIU and sTg off LT4 6–10 months after RAI. The criteria for successful thyroid ablation were defined as the disappearance of any visible area of uptake in the thyroid bed, a RAIU below 1% and undetectable sTg off LT4 ($\text{TSH} > 30 \mu\text{UI}/\text{mL}$) [25]. Patients with complete thyroid remnant ablation, as defined above, underwent long-term follow-up at intervals of 6–12 months with sTg measurements taken on LT4 and US neck scan. Patients with partial ablation were treated with additional therapeutic doses of ^{131}I . Tumor stage was defined according to the American Joint Committee on Cancer [26].

3. Results

Among the 1356 patients who underwent VAT, 370 (27.3%) were demonstrated by final histological examination to have PTC, and their medical records were reviewed for this paper. Two hundred and eighty-two out of these 370 patients have been already reported in a previously published paper [19].

Conversion to an open procedure was required in 2 patients because of intraoperative findings of gross central neck and upper mediastinum lymph node metastases. These patients underwent total thyroidectomy and central neck dissection (plus lateral neck dissection in one of them). Final histology showed the tumors to be pT3N1b PTC in both cases.

Video-assisted thyroid resection was successfully accomplished in 368 patients. Among them, 342 (92.9%) underwent video-assisted total thyroidectomy as the initial procedure and the remaining 26 (7.0%) underwent video-assisted thyroid lobectomy. Among patients in whom a lobectomy was performed, completion thyroidectomy was carried out by conventional procedures in 3 cases, which were operated at the beginning of the experience. Final histology showed a pT1 PTC in 2 of these cases and a pT3 in the third case. Six patients had small (<5 mm) pT1 PTC and were followed up after the initial lobectomy. None of them developed distant or local recurrence. The remaining 17 patients underwent subsequent video-assisted completion thyroidectomy. In 359 patients, total thyroid resection was achieved by the video-assisted approach (single procedure video-assisted total thyroidectomy in 342 cases; video-assisted thyroid lobectomy followed by video-assisted completion thyroidectomy in 17 cases). Follow-up data from these 359 patients were analyzed in this study. There were 323 women and 36 men with a mean age of 43.4 ± 11.2 years (range: 15–79). Preoperative diagnosis was: PTC in 104 cases (29.0%), suspicious nodule in 127 cases (35.4%), follicular (indeterminate) lesion in 111 cases (30.9%), toxic multinodular goiter in the remaining 17 (4.7%). The mean maximum diameter of the lesion evaluated preoperatively by US scan was 17.3 ± 6.3 mm (range: 6–35).

A video-assisted lymph node dissection was deemed necessary in 126 patients, 94 of whom were preoperatively diagnosed with small PTC or suspicious nodules and underwent a selective central node removal of enlarged lymph nodes; the remaining 32 of these patients received a complete video-assisted central compartment lymph node dissection (CCD = Central Compartment Dissection or level VI neck node dissection). The mean number of lymph nodes removed during VALD was 6 ± 4.06 (range: 1–19). The mean number of removed nodes in the CCD subgroup was 9.2 ± 3.7 (range: 6–19). Concomitant parathyroidectomy for a parathyroid adenoma was performed in 7 patients.

Mean operative time was 63.1 ± 27.3 min for lobectomy (range: 30–150), 66.9 ± 22.6 min for total thyroidectomy (range: 30–220), and 54.4 ± 24.9 min for completion thyroidectomy (range: 30–100). Mean operative time for CCD was 17.7 ± 3.4 min (range: 12–22).

Final histology revealed 285 pT1 PTC diagnoses (multifocal in 80 cases), 26 pT2 diagnoses (multifocal in 10), and 48 pT3 diagnoses (multifocal in 16). One hundred fifty six out of the 285 pT1 PTC had a maximum diameter <1 cm. Among them 94 were diagnosed preoperatively. The remaining 62 cases had an incidental diagnosis of microcarcinoma. Lymph node metastases were found in 24 of the cases in which CCD was performed, including 3 cases of micrometastases and also in three cases in which selective central node dissection was performed. Reactive changes were evident in the lymph nodes removed from the other 97 patients who underwent selective central node dissections. In patients with lymph node metastases (N1a), the primary tumor was pT1 in 15 cases and pT3 in 12 cases.

Post-operative complications included 11 transient recurrent nerve palsies (1.5% of the nerves at risk), 90 transient

hypocalcaemia cases (25%), 4 permanent hyperparathyroidism cases (1.1%) and 1 postoperative hematoma (0.4%). Mean postoperative stay was 3.1 ± 1 days (range: 2–7).

Complete follow-up data were available in 315 (87.7%) of the 359 patients included in the study; 246 of these had pT1 tumors, 22 had pT2 tumors, and 47 had pT3 tumors. The mean follow-up period was 21.5 ± 5.9 months (range: 5–40). In 137 of these patients (38.2%) with pT1 PTC <1.0 cm, in the absence of lymph node metastases and multifocality, follow-up evaluation included sTg measurements on LT4 and ultrasonography. sTg was undetectable (<0.1 ng/mL) and the US neck scan showed no thyroid remnants or lymph node involvement in all these cases.

For the remaining 178 patients postoperative ultrasonography showed no residual thyroid tissue; mean sTg after LT4 withdrawal was 5.4 ± 7.5 ng/mL (range 0.1–31.4); sTg was undetectable (<0.1 ng/mL) in 33 patients (18.5%); mean RAIU was $1.7 \pm 2.6\%$ (range: 0–18.2%). RAIU was <0.5% in 33 (18.5%) of the patients and <1% in 74 (41.5%) of the patients. In the 27 patients with lymph node metastases, sTg off LT4 was 4.6 ± 4.6 ng/mL (range: 0.44–13) and mean RAIU was $1.8 \pm 0.8\%$ (range: 1.2–3.4%).

Visual TxWBS evaluation demonstrated thyroid remnants and coexisting lymph node metastases in one high-risk patient. This last patient required a conventional lateral neck dissection after unsuccessful RAI two years after the initial surgery. TxWBS after the second operation showed no residual uptake.

4. Discussion

Among the indications for VAT, nodule size together with thyroid volume are the most important selection's limiting factors. For this reason, small suspicious or malignant nodules are, from a technical point of view, one of the best indications for VAT. However at the beginning of the experience there were some concerns about the feasibility and safety of VAT in case of malignancy. Nonetheless, after an adequate experience being achieved, this technique has now been proposed as a valid alternative to conventional surgery also for the resection of small PTC [17–20].

It has been supposed that manipulation and extraction of the thyroid gland through a small skin incision might increase the risk of thyroid capsule rupture, with possible cell seeding. However, we have previously demonstrated that thyroid gland manipulation does not differ between VAT and conventional surgery and that VAT does not confer additional risk of thyroid capsule rupture and cell seeding [15].

Another important concern about VAT is whether the surgical resection obtained with this approach is sufficiently complete. Previous studies suggested that the surgical radicality of VAT (as evaluated by postoperative sTg on and off LT4, ultrasound scan and RAIU) is comparable to that of conventional surgery [17–20]. The present study confirmed in a larger patient cohort that VAT allows for an adequate surgical resection in case of small PTC.

sTg on LT4 suppression therapy was undetectable in most of the patients. After LT4 withdrawal (TSH > 30 UI/L),

mean sTg level was low (5.4 ng/mL) and even undetectable in many patients.

Another point to take into account is the possibility to perform a central neck node clearance by the video-assisted approach. It is well known that even though up to 80% of patients with PTC could have at least microscopic metastatic spread to cervical lymph nodes, this does not seem to affect prognosis, at least in patients younger than 45 years. For this reason prophylactic neck dissection for this group of patients is not recommended [27]. We [19, 22] and others [23] have demonstrated that removal of unexpectedly enlarged central compartment lymph nodes is feasible and safe by video-assisted approach. The endoscope allows for meticulous exploration of the central compartment and quite easy identification of even slightly enlarged lymph nodes. At the same time, thanks to the magnification of the endoscope, good exposition of the operative field and of the neck structures permits careful dissection and safe preservation of the inferior laryngeal nerve and the parathyroid glands.

The mean number of lymph nodes removed in patients who underwent CCD demonstrates that it is possible to achieve satisfactory clearance of the central compartment. The incidence of central compartment node metastasis among the patients who underwent complete video-assisted CCD was comparable to the rates reported in the literature (11%–80%) [28].

Postoperative evaluation by sTg on and off LT4, US neck scan and RAIU tests confirmed that it is possible to obtain adequate surgical resection by the VAT approach, even in patients with central compartment lymph node metastases.

The results of the present study confirm our previous reports and are in agreement with a recently published paper. Indeed, Miccoli et al. [20] demonstrated that the completeness of the surgical resection is similar for both VAT and conventional surgery even in patient with minimal lymph node involvement ("intermediate risk").

Mean follow-up time was about 2 years, with the longest follow-up of about 3 years. This could be considered the main limitations of this paper, which is probably inadequate to assess the long-term recurrence rate of PTC, considering its indolent nature.

Indeed, depending on the initial treatment and on other prognostic variables, 30% of patients with PTC will have recurrence over several decades, with about two-thirds of the recurrences occurring within the first 10 years after initial therapy [29]. In the present series, we observed only one case of lateral neck metastases which appeared after two years after the initial video-assisted thyroidectomy. This further confirm the adequacy of VAT for the treatment of small PTC.

On the basis of the previous consideration, eligibility criteria for VAT are: thyroid nodules <35 mm; thyroid volume <30 mL; no previous conventional neck surgery and/or radiation therapy and small, low- to intermediate-risk, papillary thyroid carcinomas (PTC).

Concerns remain regarding the precise indications for lymph nodes dissection by video-assisted approach. It would be prudent to continue to perform central neck dissection only in case of enlarged central compartment lymph nodes, unexpectedly discovered during VAT for PTC or suspicious

nodules, or in case of prophylactic central neck node clearance, when required. Conversion to the conventional approach is mandatory if node clearance is not as accurate as in conventional surgery. Overt lymph node involvement still remains a contraindication for video-assisted procedures.

5. Conclusions

In conclusion, this study confirms that VAT is feasible and safe procedure for surgical treatment of small PTC. If selection criteria are strictly followed, it seems equivalent to conventional surgery in terms of completeness of the surgical resection. VAT can be considered a valid alternative to conventional surgery also for the treatment of small PTC, in absence of any clinical or instrumental sign of local invasion or lymph node involvement.

Nonetheless, longer follow-up evaluation could further validate its oncologic results, especially in terms of recurrence rates.

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Research Article

The Role of Immediate Recurrent Laryngeal Nerve Reconstruction for Thyroid Cancer Surgery

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Unilateral vocal fold paralysis (UVFP) is one of the most serious problems in conducting surgery for thyroid cancer. Different treatments are available for the management of UVFP including intracordal injection, type I thyroplasty, arytenoid adduction, and laryngeal reinnervations. The effects of immediate recurrent laryngeal nerve (RLN) reconstruction during thyroid cancer surgery with or without UVFP before the surgery were evaluated with videostroboscopic, aerodynamic, and perceptual analyses. All subjects experienced postoperative improvements in voice quality. Particularly, aerodynamic analysis showed that the values for all patients entered normal ranges in both patients with and without UVFP before surgery. Immediate RLN reconstruction has the potential to restore a normal or near-normal voice by returning thyroarytenoid muscle tone and bulk seen with vocal fold denervation. Immediate RLN reconstruction is an efficient and effective approach to the management of RLN resection during surgery for thyroid cancer.

1. Introduction

Unilateral vocal fold paralysis (UVFP) is one of the most serious problems in the management of thyroid cancer. The vocal folds may be paralyzed at the time of presentation, or the recurrent laryngeal nerve (RLN) may need to be sacrificed even when the RLN is functioning preoperatively. UVFP causes breathy voice, shortening of phonation, and aspiration. The negative impact of UVFP on a patient's quality of life has been confirmed by several outcome measurements [1, 2].

Different treatments are available for the management of UVFP including intracordal injection [3], type I thyroplasty [4], arytenoid adduction [5], and laryngeal reinnervations [6–9]. Laryngeal reinnervation has several advantages over other techniques. It has the potential of restoring a normal or near normal voice. RLN reinnervation can prevent the progressive loss of thyroarytenoid muscle tone and bulk [7, 9, 10] as seen with vocal fold denervation, which can limit the long-term results of the conventional static laryngoplasty procedure.

We report on cases involving the immediate reconstruction of the RLN during thyroid cancer surgery in patients with or without UVFP preoperatively and voice outcomes following the procedure with videostroboscopic, aerodynamic, and perceptual analyses.

2. Materials and Methods

During the period from 2000 to 2008, at Kumamoto University Hospital, Japan, we reconstructed the RLN in 12 patients who had UVFP or whose unilateral RLN needed to be sacrificed due to thyroid cancer (Table 1). In 12 patients with thyroid cancer involving unilateral RLN, we conducted direct anastomosis, free nerve grafting, and ansa cervicalis to RLN anastomosis in 1, 9, and 2 patients, respectively. There were 8 women and 4 men, and the ages at the time of reconstruction ranged from 18 to 82 years (mean 61.8). Six of the 12 patients (50%) had UVFP preoperatively and were classified as group I. The remaining six patients did not have UVFP before surgery. However, their RLNs were sacrificed

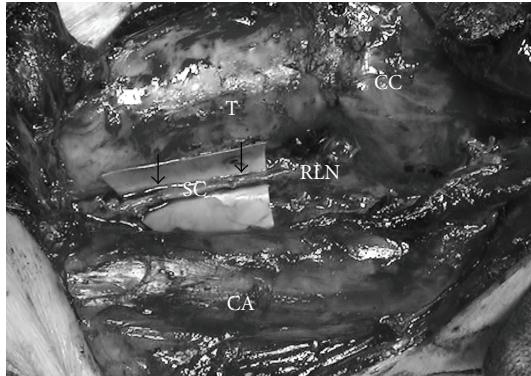


FIGURE 1: Free nerve graft. Supraclavicular nerves was used to fill the defect (arrow). T: trachea, CA: common carotid artery. SC: supraclavicular nerve, CC: cricoid cartilage.

due to invasion by cancer, and these 6 patients were classified as group II (Table 2).

2.1. Method of RLN Reconstructions. The immediate RLN reconstruction was done at the time of surgery for primary or recurrent thyroid cancer. The ends of the severed RLN were anastomosed directly when possible. When the defect was longer than 5 mm, a free nerve graft taken from the transverse cervical nerve, supraclavicular nerve, or ansa cervicalis was used to fill the defect (Figure 1). When the proximal stumps of the RLN could not be utilized for nerve repair, ansa cervicalis to RLN anastomosis was performed. The ipsilateral ansa cervicalis was identified on the surface of the internal jugular vein, and its branches to the sternohyoid muscles were dissected. The major branch or usually the branch common to these branches was transected, and the proximal end was anastomosed to the distal stump of the RLN. Most commonly, the ipsilateral ansa cervicalis was used for reinnervation. In one case (Patient no. 7) suffering from recurrent disease, due to loss of the ipsilateral ansa cervicalis nerve in the excessive cicatricial tissue, the contralateral ansa cervicalis was used. In Patient no. 2, thyroid cancer has invaded the distal portion of the RLN at the Berry ligament. We resected the RLN at the entrance of the larynx. The inferior pharyngeal constrictor muscle was divided along the lateral edge of the thyroid cartilage in order to find the distal stump of the RLN. The stumps were anastomosized with the supraclavicular nerve. The anastomosis was usually made with three, or sometimes four, stitches of 8–0 or 9–0 nylon thread using microsurgical instruments with an operation microscope or a surgical magnifying glass.

2.2. Voice Outcome Measurements. The patients received videostroboscopic, aerodynamic, and perceptual analyses pre- and postoperatively. In group I, some patients were not analyzed preoperatively. The postoperative assessment was done no less than 6 months after operation in all patients.

For videostroboscopic evaluation, each patient performed a sustained phonation of the vowel /e/ or /i/ at his

or her habitual pitch and loudness. Images were recorded using a videoendoscopic (VNL-1171; Pentax, Tokyo, Japan) and stroboscopic unit (LS-3A; Nagashima, Tokyo, Japan) onto a digital videocassette recorder (DVCPRO; Panasonic, Yokohama, Japan) to assess the mucosal wave and glottal closure. We rated the mucosal wave of vocal fold vibration and glottal closure using a four-point grading scale (0 = worst, 3 = the best).

For aerodynamic evaluation, each patient was asked to produce a sustained vowel /a/ at a comfortable pitch and loudness for as long as possible. The maximum phonation time (MPT) and mean airflow rate (MFR) were measured using a phonation analyzer (PS-77E, Nagashima, Tokyo, Japan).

Perceptual voice evaluations were conducted using the GRBAS (Overall Grade: G, Roughness: R, Breathiness: B, Asthenia: A, and Strain: S) rating scale [11]. Three of 5 parameters (G, R, and B) were analyzed because UVFP causes breathy dysphonia. S and A were not analyzed due to limited application in the measurement of UVFP. Each parameter was rated by a speech language pathologist according to a 4-point scale (0 = normal; 1 = slight disturbance; 2 = moderate disturbance, 3 = sever disturbance).

Perceptual analyses were performed following standard procedures in cooperation with a trained speech therapist.

Statistical analyses (SigmaStat 3.5 for windows, San Joe, CA) were performed using the Wilcoxon signed-rank test and unpaired *T* test: *P*-values less than .05 were considered statistically significant.

3. Results

The voices of the patients who underwent the immediate RLN reconstruction began to improve after a certain period postoperatively. Table 2 summarize the results of the videostroboscopic, aerodynamic, and perceptual examinations.

The follow-up period in this study (Table 1) ranged from 7 to 103 months (average of 34.6 months). We show the data on the periods of vocal cord paralysis depending on the referral form and or the patients' history in group I.

3.1. Videostroboscopic Findings. No visible vocal fold movement was detected during the follow-up period. The post-operative score of mucosal wave (2.5 ± 0.5) in group I was significantly greater than the preoperative score (1.2 ± 1.0). The postoperative glottal closure (2.7 ± 0.5) in group I was significantly greater than the preoperative score (1.5 ± 0.8). Nine of the 12 patients had complete glottal closure during phonation. The small glottal gap remained in 2 patients (Table 2). In one of the 12 patients (Patient no. 10), the vocal folds could not be observed during phonation because of bilateral arytenoidal overhang on the glottis. Despite this, the patient's voice quality was quite good as demonstrated by other measurements. All patients recovered the mucosal wave after the operation with the exception of one patient (Patient no. 9) who also suffered from a polypoid vocal fold.

TABLE 1: List of twelve cases of immediate RLN reconstruction in the present series.

Patient No.	Age	Gender	Side	UVFP before surgery	Methods of reconstruction	Follow-up, mo
1	39	F	L	3 mo	FNG	27
2	64	F	L	2 mo	FNG	36
3	82	F	L	2 mo	FNG	12
4	69	F	R	—	FNG	103
5	35	F	L	13 mo	FNG	68
6	72	M	L	3 mo	FNG	18
7	74	F	L	—	ARA	7
8	71	F	R	3 mo	ARA	40
9	61	M	R	—	FNG	38
10	86	F	L	—	DA	36
11	18	M	R	—	FNG	22
12	71	M	R	—	FNG	9

UVFP: unilateral vocal fold paralysis, FNG: free nerve grafting, ARA: ansa cervicalis to RLN anastomosis, and DA: direct anastomosis.

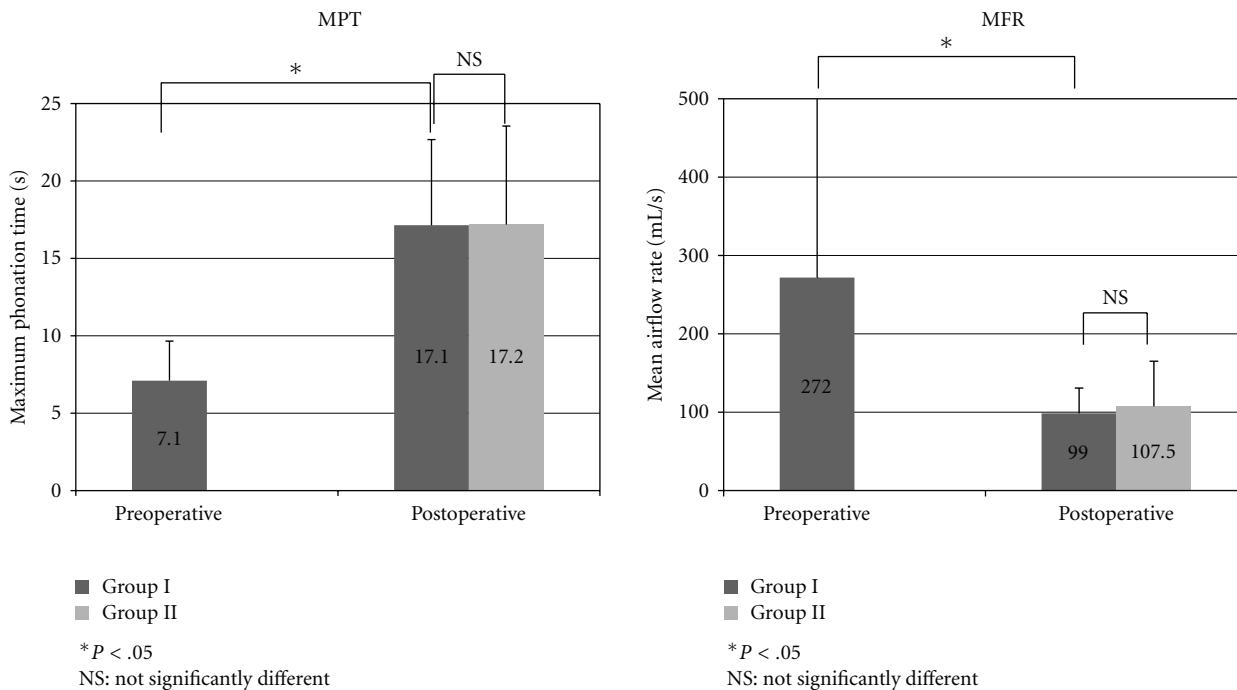


FIGURE 2

3.2. Aerodynamic Findings. Normal speakers usually have an MPT of more than 10 seconds and an MFR between 100 ml/sec and 200 ml/sec. The postoperative recordings of MPT ($16.2 \text{ sec} \pm 6.2$) in group I were significantly greater than the preoperative levels ($7.1 \text{ sec} \pm 2.55$). The postoperative MFR ($110.3 \text{ ml/sec} \pm 38.4$) in group I was significantly reduced compared with the preoperative levels ($271 \text{ ml/sec} \pm 325.1$) (Figure 2). In group II, three of the 6 patients did not receive a preoperative examination. Therefore, it was not possible to make a statistical comparison of preoperative and postoperative data. However, the postoperative results of MPT and MFR showed that patients' voices were returned to a normal condition. There were no significant

differences between the postoperative data of both groups (Figure 2).

3.3. Perceptual Analysis. The comparative perceptual analyses of each scale in group I and II are shown in Figure 3.

In group I, the mean score for G was 1.2 ± 0.8 preoperatively and 0.3 ± 0.8 postoperatively. The mean scores for B were 0.7 ± 0.8 preoperatively and 0.2 ± 0.4 postoperatively. The mean score for R was 1.0 ± 0.6 preoperatively and was reduced to 0.2 ± 0.4 postoperatively. All three characteristic scores were significantly reduced postoperatively.

In group II, half of the cases did not receive preoperative examinations. Also, Patient no. 9 had a polypoid vocal cord

TABLE 2: Preoperative and postoperative voice data.

	Patient No.	Mucosal wave		Glottal closure		MPT (sec)		MFR (ml/sec)		Grade		Roughness		Breathiness	
		Preop	Postop	Preop	Postop	Preop	Postop	Preop	Postop	Preop	Postop	Preop	Postop	Preop	Postop
Group I	1	1	3	1	3	8.9	26.5	184.0	86.0	1	0	0	0	1	0
	2	1	2	2	2	6.2	19.9	109.0	70.0	2	0	1	0	1	0
	3	3	3	2	3	9.6	13.5	116.0	104.0	0	0	0	0	0	0
	5	1	2	2	2	8.7	16.2	176.0	168.0	1	0	1	0	1	0
	6	0	2	0	3	2.7	10.6	932.0	146.0	2	1	2	1	2	1
	8	1	3	2	3	6.5	10.4	114.0	88.0	1	0	0	0	1	0
	Mean ± SD	1.2±1.0	2.5±0.5	1.5±0.8	2.7±0.5	7.1±2.6	16.2±6.2	271.8±325	110.3±38.4	1.2±0.8	0.2±0.4	0.7±0.8	0.2±0.4	1.0±0.6	0.2±0.4
Group II	4	3	2	3	3	22.2	25.4	61.0	62.0	0	0	0	0	0	0
	7	NA	3	NA	3	NA	14.9	NA	64.0	NA	0	NA	0	NA	0
	9	1	1	3	3	22.4	24.3	60.0	102.0	2	1	1	1	2	1
	10	IV	IV	IV	IV	11.3	11.5	41.0	78.0	0	0	0	0	0	0
	11	NA	2	NA	3	NA	12.0	NA	168.0	NA	0	NA	0	NA	0
	12	NA	3	NA	3	NA	17.7	NA	168.0	NA	0	NA	0	NA	0
	Mean ± SD	2.0±1.4	2.2±0.8	3.0±0.0	3.0±0.0	18.6±6.4	17.6±6.0	54.0±11.3	107±49.4	0.7±1.2	0.2±0.4	0.3±0.6	0.2±0.4	0.7±1.2	0.2±0.4

NA: not assessed, IV: invisible glottis during phonation, MPT: maximum phonation time, and MFR: mean airflow rate.

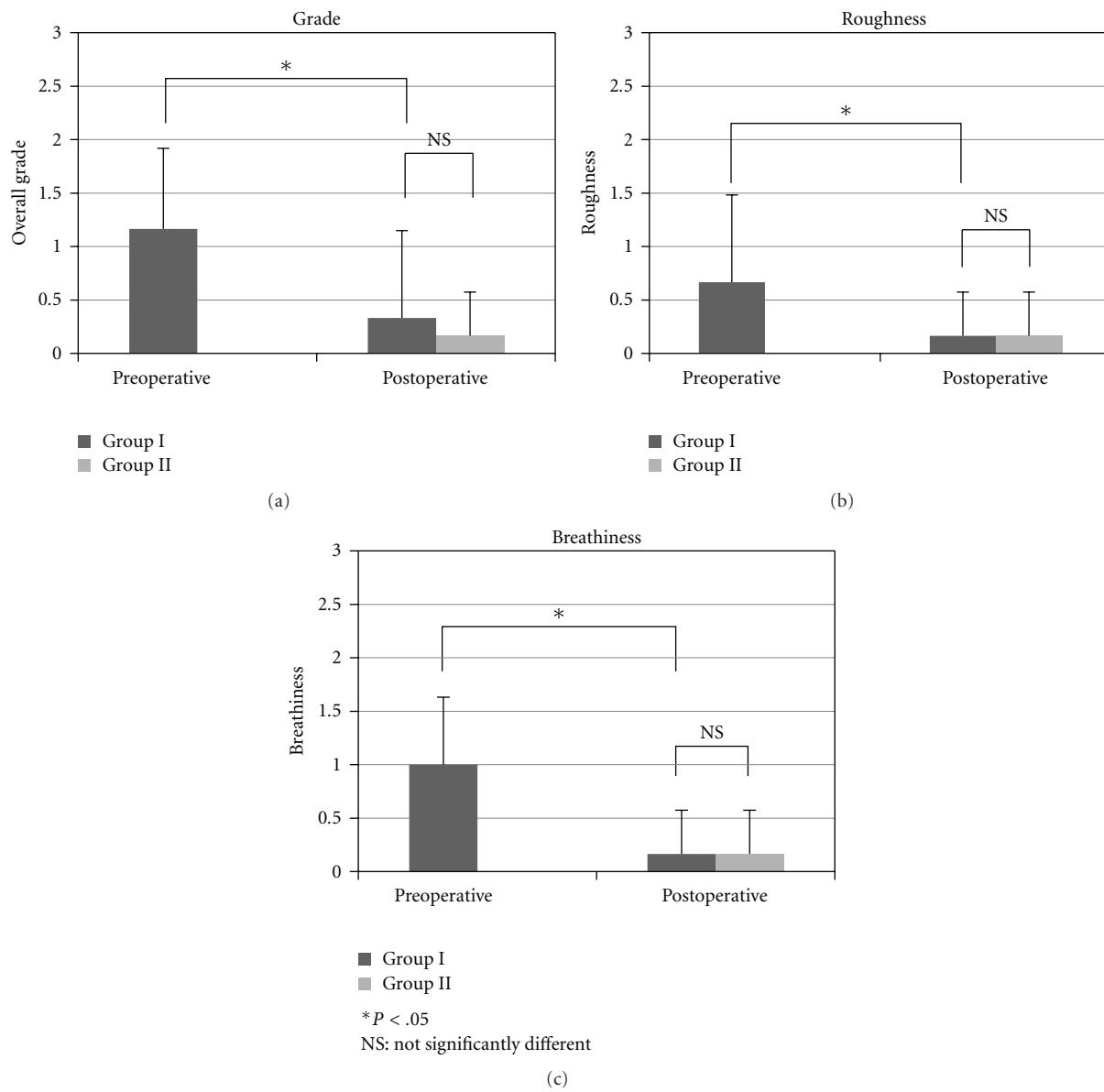


FIGURE 3

which caused an elevated score of the perceptual analysis before the surgery. However, the postoperative perceptual scores were within normal ranges.

4. Discussion

A major morbidity associated with thyroid surgery is injury to the RLN, resulting in poor voice quality and the potential for recurrent aspiration.

Reconstitution of vocal fold thickness and median position can be accomplished by vocal fold augmentation [3], vocal fold medialization with type I thyroplasty [4], arytenoid adduction [5], or RLN reinnervation [6–8]. These techniques can be used alone or in combination to achieve improved vocal and swallowing functions. RLN reinnervation has several advantages over other techniques. It has

the potential of restoring a normal or near-normal voice by returning thyroarytenoid muscle tone and bulk [6, 7, 9, 12, 13] in contrast with the conventional laryngoplasty procedure [7]. Reconstruction of the RLN in the management of UVFP during the thyroid cancer surgery is rarely reported [9, 14–18]. Some disadvantages of this technique are the requirement of a delayed time to voice improvement, the requirement of intact donor and recipient nerves, and the possible delay or failure of reinnervation in elderly patients.

In this study, we assessed the outcome of immediate RLN reconstruction during thyroid cancer surgery with or without UVFP before the surgery. In particular, the aerodynamic analysis showed that all measured values entered normal ranges in both groups I and II. It should be noted that as patients in group II had a normal voice preoperatively, phonatory function was not analyzed in some cases. As such,

aerodynamic analyses were employed to measure changes in phonatory function postoperatively. These results coincided with the glottal closure and mucosal wave assessed with videostroboscopy (Table 2). These results were achieved with no bias as to the method of RLN reconstruction such as direct anastomosis, nerve grafting, and ansa cervicalis and RLN anastomosis. Nerve anastomosis under microscopic control was important for precise neuronal repairs.

Several techniques of peripheral nerve repair exist, including microsuturing, gluing [19, 20] and grafting [21]. Cyanoacrylate synthetic glue has therefore been proposed because its application for nerve repair is relatively easy, it maintains the anastomosis even while under tension, and it avoids all risk of viral transmission [22, 23]. In spite of this, this adhesive has received criticism due to its toxicity, excessively slow resorption, as well as the possibility of the induction of an inflammatory reaction in the perineural tissues [24, 25]. In the present study, the standard microsuturing technique was employed for RLN reconstruction. RLN reconstruction requires surgical precision; however, most surgeons involved in the treatment of thyroid cancer and RLN should possess the requisite proficiency to conduct the procedure.

In group I, all patients who had UVFP with vocal fold atrophy before the surgery experienced restored phonatory function after the immediate RLN reconstruction during thyroid cancer surgery. According to other studies, clinical improvements have been noted 2–4 months after the reinnervation [10, 15, 16, 26], which corresponds with our findings of 3–4 months (not shown). Green et al. [7] demonstrated in a canine model that at 5–6 months, evoked electromyography (EMG) indicated some degree of reinnervation. These EMG findings in animal experiments were also reported in clinical patients who underwent serial EMG studies after laryngeal reinnervation for UVFP [13, 26]. Miyauchi et al. [18] reported phonatory function improvement after reconstruction of RLN in 88 patients with nerve resection and also in 51 (58%) patients who had UVFP preoperatively. Reconstruction of RLN may provide partial or full recovery from vocal fold atrophy and the returning thyroarytenoid muscle tone during phonation.

Feasibility of the GRBAS scale for assessment of subjective voice outcome with laryngoplasty has been reported [11, 27, 28]. This study also indicated a G change from 1.2 to 0.2, an R change from 0.7 to 0.2, and a B change from 1.0 to 0.2 in group I patients with reinnervation RLN. In group II, mean values of G, R, and B reached the same values as Group I postoperatively. All G, R, and B scores were under 0.5 scales after the surgery. Perceptual analysis showed that our patients' voices were returned to normal or near normal after the surgery.

In this study, thyroid cancer was only limited to invasion of the unilateral RLN. There are some advanced invasions, such as to the trachea, larynx, or esophagus. In such advanced cases it may not be possible to reserve distal stumps of RLN at the entrance of the larynx. If there is no distal portion of the RLN left below the Berry's ligament, the inferior pharyngeal constrictor muscle should be divided along the lateral edge of the thyroid cartilage to find a distal

stump of the RLN. The thyroid cartilage is retracted, and cricothyroid joint is opened. Behind the thyroid cartilage the RLN forms several branches. The abductor and adductor branches of the RLN are identified, and the adductor branch is dissected superior to ensure sufficient length for anastomosis. When adductor branches cannot be found and opposite RLN can be preserved, arytenoid adduction may be performed to keep phonatory function.

5. Conclusion

In this case series we reported favorable patient outcomes as measured by aerodynamic and perceptual analyses as well as videostroboscopic findings after immediate RLN reconstruction for severed RLN during thyroid cancer surgery. Also, we reported that our patients experienced a normal or improved voice postoperatively, regardless of the length of time they had suffered from UVFP. Despite these favorable results, the small sample size utilized in this study limits the conclusions that may be drawn. Further research could help to confirm the results and expand the application of this procedure.

The immediate RLN reconstruction for severed RLN during the thyroid cancer surgery is highly effective in preventing the loss of phonatory function.

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Review Article

Involvement of Aberrant Glycosylation in Thyroid Cancer

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Glycosylation is one of the most common posttranslational modification reactions and nearly half of all known proteins in eukaryotes are glycosylated. In fact, changes in oligosaccharides structures are associated with many physiological and pathological events, including cell growth, migration and differentiation, and tumor invasion. Therefore, functional glycomics, which is a comprehensive study of the structures and functions of glycans, is attracting the increasing attention of scientists in various fields of life science. In cases of thyroid cancer, the biological characters and prognosis are completely different in each type of histopathology, and their oligosaccharide structures as well as the expression of glycosyltransferases are also different. In this review, we summarized our previous papers on oligosaccharides and thyroid cancers and discussed a possible function of oligosaccharides in the carcinogenesis in thyroid cancer.

1. Introduction

Oligosaccharides are mostly found on the cell surface and extracellular matrix (ECM), and also in various organelles such as the Golgi, ER, lysosome, cytosol, and nuclei. As compared to research on DNA, RNA, and proteins, study on glycans is rather difficult and the research in this field has been neglected for a long period, the same being true for glycomics as compared to proteomic research. In order to characterize the structures of glycans, glycobiology including glycomics is essential for understanding of the structures and functions of proteins. In the last couple of years, most of the glycosyltransferases (over 180 glycosyltransferase genes) have been identified, based on the genome sequence data and bioinformatics approach. Glycosylation reactions are catalyzed by the actions of glycosyltransferases; sugar chains are added to various complex carbohydrates. Modified oligosaccharides have the ability to interfere with carbohydrate-protein or protein/glycoprotein-glycoprotein interactions and, as a result, regulate many physiological and pathological events, including cell growth, migration and differentiation, and tumor metastasis. Cell surface

carbohydrates contribute to a variety of interactions between a cell and its extracellular environment, since they are located on the outermost layer of the cell. Carbohydrates are the first molecules to be encountered and recognized by other cells, antibodies, invading viruses, and bacteria. Many secreted molecules such as hormones and toxins have also been reported to bind to carbohydrate receptors on the cell surface. Most receptors on the cell surface are N-glycosylated, including epithelial growth factor receptor (EGFR), integrins, and transforming growth factor β receptor (TGF β R). Increasing evidence indicates that sugar chains on glycoproteins are involved in the regulation of cell-cell communication, signal transduction, and protein folding and stability [2–5]. During the carcinogenesis in thyroid cancer, the biological characteristics of tumor cells dramatically changed with malignant transformation into undifferentiated thyroid cancer. In this period, the expression of glycosyltransferases as well as their target proteins would also be altered. The alteration of oligosaccharide structures on the cell surface could control cellular differentiation and biological characteristics. Also, aberrant expression of glyco-related genes could be a marker of thyroid cancer.

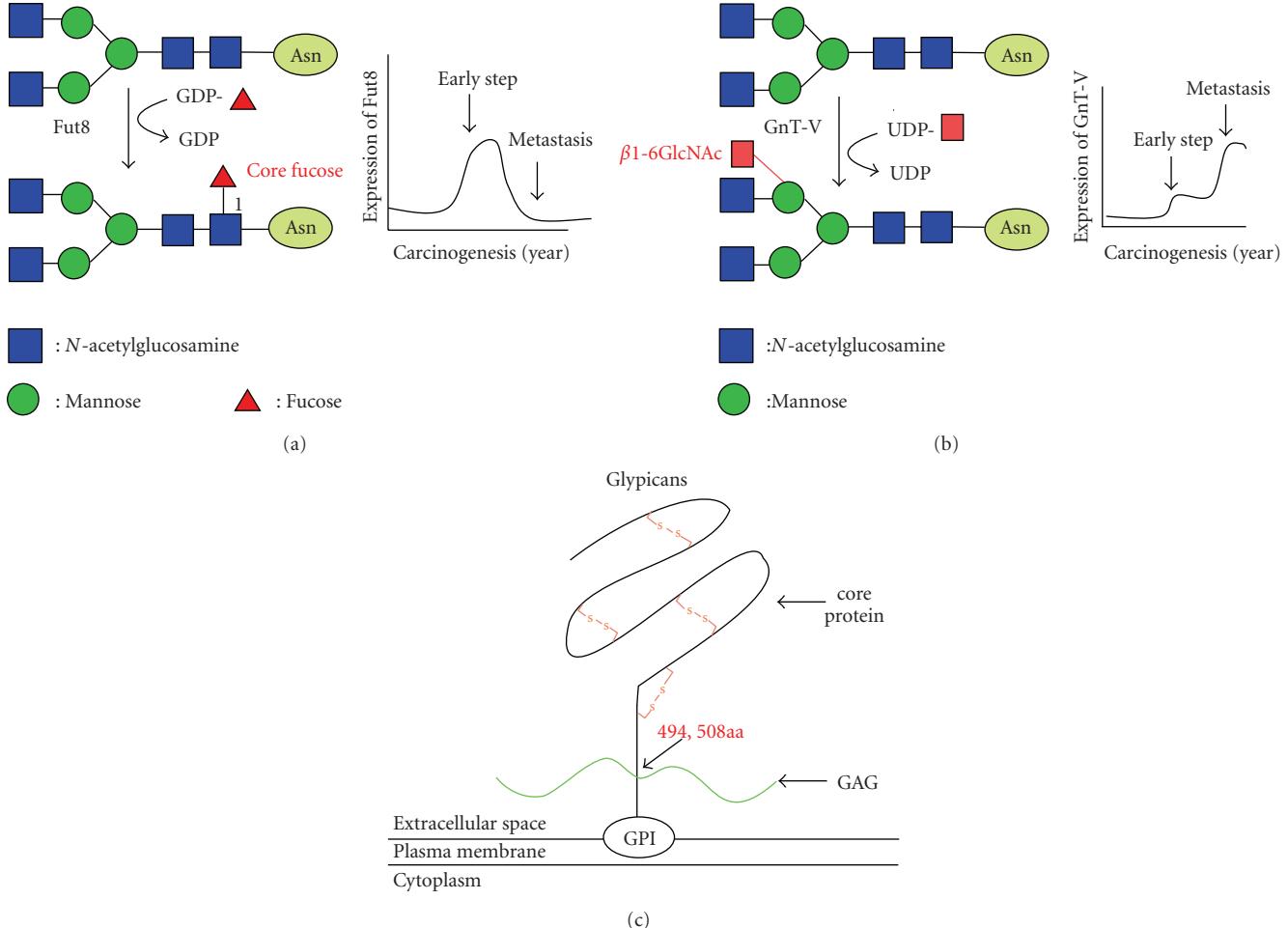


FIGURE 1: Oligosaccharide-related molecules in this review. (a) Reaction pathway catalyzed by *Fut8*. Expression of *Fut8* was increased at the early phase of carcinogenesis and decreased at the advanced stage. (b) Reaction pathway catalyzed by *GnT-V*. Expression of *GnT-V* was up-regulated in a two-step manner. (c) Structure of GPC3. GPC 3 is a proteoglycan with two oligosaccharides at the 494 and 508 amino acid residues and binds to cell membranes through a GPI-anchor. GPC3-deficient mice showed overgrowth at 14-day embryo stage.

In this review, we mainly focus on the importance of oligosaccharides in thyroid cancer, according to our previous reports.

2. Why Did We Start a Glycomics Project?

Glycobiology is one of the most difficult research areas in life science because oligosaccharides exhibit a variety of aspects and their functions often differ with the organ, species, or type of cancer. In terms of cancer, prominent changes in oligosaccharide structures on glycoproteins are dependent on sialylation, fucosylation, and branching formation. Both sialylation and fucosylation modify the charges on total oligosaccharide structures and thus control receptor and adhesion molecules on the cell surface. Sialylation of IgG oligosaccharides can regulate allergic reactions and this minor IgG group suppresses autoimmune reactions [6, 7]. Fucosylation is one of the most important oligosaccharide modifications, being linked to cancer and inflammation [8].

Many reports have implied the involvement of branching formation of *N*-glycans and *N*-acetylglucosaminyltransferase V (*GnT-V*) which is key enzymes producing the branching [9]. Regarding many biological phenomena, Dr Taniguchi's group have succeeded in the purification and characterization of glycosyltransferases, which are involved in the branching formation for *N*-glycans [10]. Involving cDNAs or antibodies for these glycosyltransferases, many studies have revealed the relationship to carcinogenesis and/or tumor metastasis. Figure 1 shows three targets of glyco-related proteins in this review. Basically, the expression of *Fut8* and *GnT-V* is relatively low and increases with malignant transformation. Expression of *Fut8* increases in the early phase of carcinogenesis, but decreases at the stage of metastasis. In contrast, two-step increases in *GnT-V* expression are observed in certain kinds of cancer. Expression of glycan 3, which is a proteoglycan belonging to the glycan family, increases/decreases in an organ-specific manner.

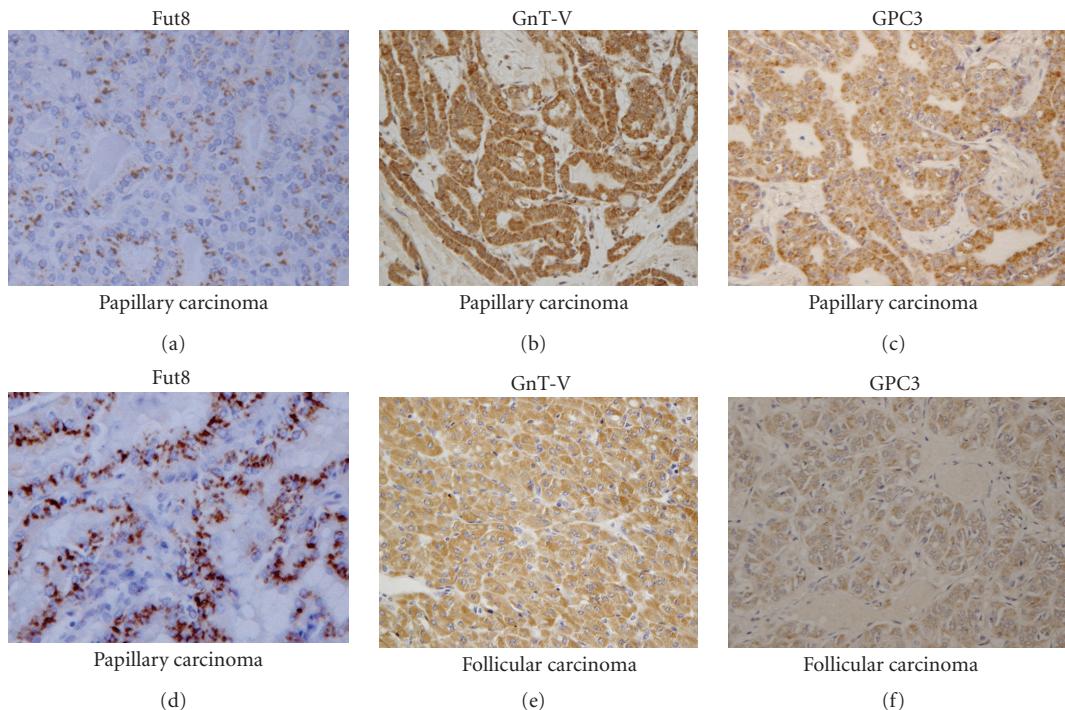


FIGURE 2: Immunohistochemical studies on Fut8, GnT-V, and GPC3 in thyroid cancer. The staining of Fut8 was located in the Golgi apparatus, while GnT-V staining was observed throughout a cell. Staining of GPC3 was heterogeneous and showed a membrane pattern.

3. Fut8 and Thyroid Cancer

Fucosylation is one of the most important modes of glycosylation in cancer. Fucosylation is regulated by several kinds of fucosyltransferases, the GDP-fucose synthetic pathway and GDP-fucose transporters. Before these complicated mechanisms of fucosylation were clarified, fucosylated target proteins were found and used as tumor markers. Increases in fucosylated alpha-fetoprotein (AFP) were reported by Drs Breborowicz et al. [11] and Dr Taketa et al. [12]. They first found microheterogeneity of AFP in several liver conditions and then found increases in α 1-6 fucosylation (core-fucosylation) of AFP on lectin affino-electrophoresis. AFP is a well-known tumor marker for hepatocellular carcinomas (HCCs), but it is sometimes also increased in benign liver diseases such as chronic hepatitis and liver cirrhosis. In contrast, AFP with core-fucosylation is a very specific marker of HCCs [13, 14]. AFP with core-fucosylation was called AFP-L3, because it was detected in the L3 fraction on LCA (Lens culinaris agglutinin) lectin-electrophoresis. Core-fucosylation comprises the attachment of fucose to the innermost *N*-acetylglucosamine in *N*-glycans. α 1-6 fucosyltransferase (Fut8) catalyzes this core-fucosylation reaction. Dr. Uozumi et al. succeeded in the purification and cDNA cloning of Fut8 from porcine brain [15]. Studies on cancer and fucosylation have moved to the second stage since that time. Fut8 knockout mice show emphysema-like lesions in the lungs because core-fucose is important for the functions of reactions such as EGF-R and TGF β -R [5, 16]. When we established a monoclonal antibody for Fut8, we performed the first immunohistochemical study on thyroid cancer [17].

This is because there are various types of thyroid cancer and the prognosis differs for each pathological condition. As a result of immunohistochemical studies, involving 133 cases of thyroid cancer, the expression of Fut8 was found to be quite low in normal follicles. As shown in Figure 2, positive staining of Fut8 was observed in papillary carcinomas. This staining pattern could be called Golgi localization. The number of Fut8-positive cases of follicular carcinomas was relatively low. We concluded that expression of Fut8 might be a key factor for the progression of thyroid papillary carcinomas, but not of follicular carcinomas. High expression of Fut8 was observed in 33.3% of papillary carcinomas and the incidence was directly linked to tumor size and lymph node metastasis. In contrast, this phenomenon was less frequently observed in cases of follicular carcinomas and anaplastic (undifferentiated) carcinomas. Furthermore, decreases in Fut8 expression in papillary carcinomas might be linked to anaplastic transformation. These results seem to be similar to the results in the case of colon carcinogenesis that is, “fucosylation and defucosylation” [18]. Further studies should determine the biological function of core-fucose in thyroid cancer.

4. GnT-V and Thyroid Cancer

N-Acetylglucosaminyltransferase V (GnT-V) is one of the most important glycosyltransferases in tumor metastasis [9]. So far, more than 80 papers on GnT-V and tumor metastasis have appeared, with this number being quite high as compared to other glycosyltransferases. One mechanism

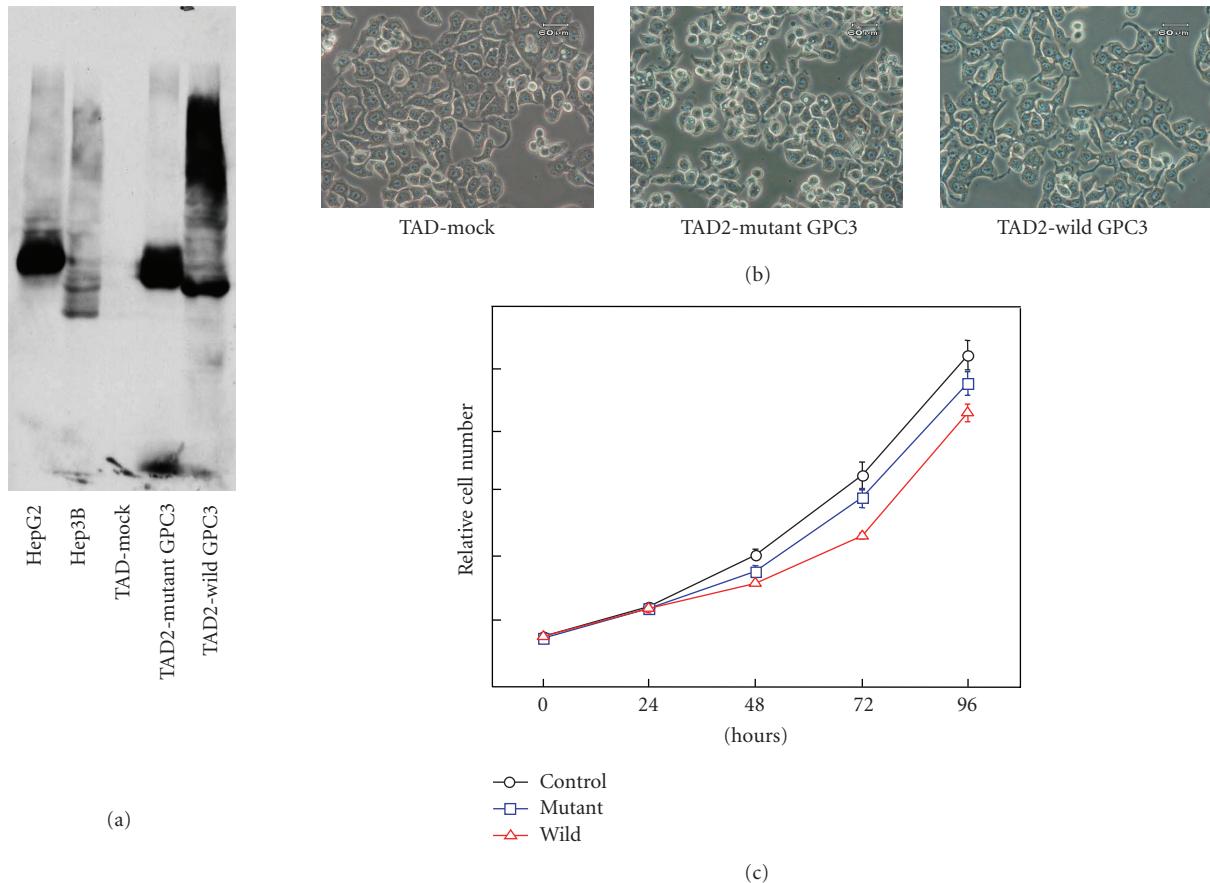


FIGURE 3: The effect of overexpression of GPC3 with/without oligosaccharides on thyroid cancer cell lines. Western blot analysis of GPC3 was performed using cellular proteins derived from HepG2 cells, Hep3B cells, parental TAD2 cells, mutant GPC3-transfected TAD2 cells, and wild type GPC3-transfected TAD2 cells. (b) Microscopic observation of parental TAD2 cells, wild type GPC3-transfected TAD2 cells, and mutant GPC3-transfected TAD2 cells. Morphological changes were observed for wild type GPC3-transfected TAD2 cells, as compared to the other two types of cells. (c) Cell proliferation was evaluated by means of WST-1 assaying.

underlying GnT-V and tumor progression is beta1-6 GlcNAc branching formation, a product of GnT-V, up-regulating cell surface growth factor receptors such as EGF-R [19]. The other mechanism is suppressing of the degradation of glycoproteins, resulting in an increase in protease expression [20]. Matriptase is a tumor-associated type II transmembrane serine protease that positively regulates carcinoma metastasis by activating the latent forms of hepatocyte growth factor (HGF) and urokinase-type plasminogen activator (uPA) [21]. The overexpression of GnT-V in gastric carcinoma cells enhances the degradation of matriptase and accelerated the peritoneal dissemination of these cancer cells in athymic mice [20]. Matriptase purified from GnT-V-transfected gastric cancer cells is resistant to trypsin and this resistance is dependent on the oligosaccharides linked to the 772 Asn residue of matriptase [22]. These findings indicate that GnT-V modifies the oligosaccharide structure of matriptase, thus altering the function of proteases. According to the results of these *in vitro* studies on GnT-V and matriptase, we performed immunohistochemical studies, using 132 cases of thyroid cancers [23]. While neither GnT-V nor matriptase was expressed in normal thyroid tissue, positive staining for

matriptase and GnT-V was observed in 52/68 and 66/68 cases of papillary carcinomas, 3/23 and 10/23 cases of follicular carcinomas, 5/13 and 9/13 cases of follicular adenomas, and 11/28 and 6/28 cases of anaplastic carcinomas, respectively. Immunohistochemistry, as well as Western blotting, showed that the expression of matriptase paralleled the expression of GnT-V. However, the expression of matriptase mRNA was not correlated with its protein level, suggesting that the enhancement of matriptase expression could be caused by a posttranslational modification such as glycosylation through GnT-V-mediated glycosylation. In the case of papillary carcinomas, the levels of expression of both GnT-V and matriptase were significantly higher in tumors of 1 cm or less in size (microcarcinomas) and in cases without poorly differentiated lesions, and the two proteins were significantly correlated. In contrast, the prognosis of thyroid carcinomas after surgery was correlated with the expression of neither GnT-V nor matriptase, because the levels of their expression were quite low in anaplastic (undifferentiated) carcinomas. These results suggest that prolonged stabilization of matriptase is stabilized through GnT-V-mediated glycosylation *in vivo*, thus extending its halftime and permitting it to play

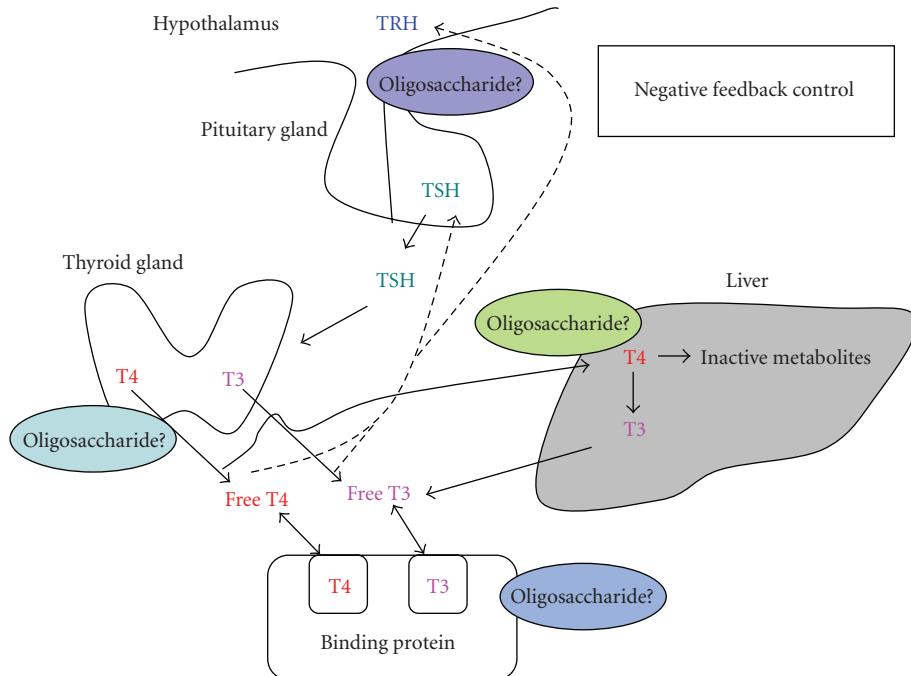


FIGURE 4: Oligosaccharides involve in the regulation of thyroid function. The production of thyroid hormone by the thyroid gland is regulated by the hypothalamus and the pituitary gland. Hypothalamic thyrotropin-releasing hormone (TRH) induces the pituitary gland to release thyroid-stimulating hormone (TSH) into the general circulation, whereby it reaches the thyroid gland and stimulates the production and release of thyroid hormone [1]. T4 is the predominant secretory product of the thyroid gland, with peripheral deiodination of T4 to T3 in the liver and kidneys supplying roughly 80% of the circulating T3. Both circulating T3 and T4 directly inhibit TSH synthesis and are released independently; T4 via its rapid conversion to T3. Circulating T4 and T3 are bound predominantly to serum proteins. FT3 is the metabolically active form of thyroid hormone, whereas protein-bound T3 and T4 may be considered reservoirs of the hormone in equilibrium with the metabolically active free hormone.

a role in the early phases of papillary carcinomas, but not in the later phase of their progression. The staining pattern of GnT-V was different from that of Fut8 although GnT-V and Fut8 are glycosyltransferases located on the Golgi apparatus. GnT-V is cleaved by gamma secretase, resulting in its secretion into the serum or conditioned medium [24]. The secreted type of GnT-V acts as an angiogenesis cofactor [25].

5. Glypican 3 and Thyroid Cancer

Glypican 3 (GPC3) is one of the heparan sulfate proteoglycans (HSPGs) that are attached to the cell surface through a glycosylphosphatidylinositol (GPI) anchor [26]. While high expression of GPC3 is observed in fetal organs, it is scarcely detected in adult tissues. Interestingly, the serum levels of GPC3 measured by means of an enzyme-linked immunosorbent assay were increased in patients with HCC at 40 ~ 53%. Since there was no correlation between the GPC3 and alpha-feto protein levels, 82% of HCC patients were positive for at least 1 of these 2 tumor-markers. GPC3 was also identified as a tumor marker for melanomas [27]. GPC3 is involved in several kinds of cell signaling such as Wnt/Wingless, Hedgehog, TGF- β , and fibroblast growth factor, resulting in the stimulation of HCC growth [28]. When we performed immunohistochemical analysis of

GPC3 in thyroid cancer [29], GPC3 was scarcely expressed in normal thyroid glands, but was dramatically enhanced in certain types of cancers including 100% of follicular carcinomas (20/20 cases) and 70% of papillary carcinomas (48/69 cases). Expression of GPC3 in follicular carcinomas was significantly higher than that of follicular adenomas ($P < .0019$). In contrast, no expression of GPC3 was observed in 20 cases of anaplastic carcinomas. When expression of GPC3 was investigated in 69 cases of papillary carcinomas according to their clinical background, it was found to be expressed at an early stage. These data prompted us to perform experiments on transfection of GPC3 into thyroid cancer cell lines to determine the biological function of GPC3. When the GPC3 gene was transfected into a human thyroid cancer cell line, TAD2, cell growth was dramatically suppressed in the wild type of GPC3 transfectants, but not markedly suppressed in the GPC3 oligosaccharide mutants (Figure 3). This oligosaccharide of GPC3 is heparan sulfate and the mutants were established by point mutation of the amino acid sequences of GPC3 expression vectors. The cell morphology dramatically changed with the transfection of GPC3 with oligosaccharides but not GPC3 without oligosaccharides. In the case of HCCs, oligosaccharides of GPC3 were not linked to cell signaling or cell growth [28]. Further studies are required to characterize GPC3-mediated cell signaling in thyroid cancer.

6. Perspective

The differential diagnosis of follicular adenocarcinoma from follicular adenomas is the most important issue. In certain cases, even pathological examination is not perfect. Galectin 3 has been reported in one of the candidate pathological markers for a differential diagnosis [30]. Recently, we established a quantitative ELISA assay for GPC3 and investigated its clinical usefulness for the diagnosis of thyroid cancer [31]. Galectin 3 binds to polylactosamine structures on N-glycans and controls the function of cell surface receptors. The polylactosamine structures are synthesized through the reaction of GnT-V and the molecular mechanisms underlying GnT-V-related cell signaling would be due to galectin 3 [19]. It is important to determine the biological function of galectin-3 in thyroid cancer. Basically, the thyroid is an endocrine organ that produces thyroid hormone. Many factors control the release of thyroid hormone via receptor-ligand mediated signaling (Figure 4). In this system, oligosaccharides seem to be deeply involved, and therefore control of thyroid function via oligosaccharides would be a promising research target. Many hormones including thyroid hormone could be regulated through glycosylation [32]. In this review, we did not describe sialic acids in thyroid carcinoma. Sialic acids are a terminal oligosaccharide on N-/O-glycans and have a variety of biological functions [33]. While the biological significance of sialic acids in thyroid cancer remains unknown, changes in cellular sialylation have been changed in malignant formation [34]. Transgenic or knockout mice as to glycosyltransferases including sialyltransferases would be a powerful tool for determining the oligosaccharide function in thyroid carcinogenesis directly. Such mice should be mated with model mice for thyroid cancer, if any.

Abbreviations

Fut8:	α 1-6 fucosyltransferase
EGF-R:	epithelial growth factor receptor
TGF β R:	transforming growth factor β receptor
AFP:	α -fetoprotein
HCC:	hepatocellular carcinoma
(GnT-V):	N-acetylglucosaminyltransferase V
GPC3:	Glypican 3.

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Research Article

The Immunocytochemistry Is a Valuable Tool in the Diagnosis of Papillary Thyroid Cancer in FNA's Using Liquid-Based Cytology

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Introduction. Papillary thyroid carcinoma (PTC) is the most common malignancy of the thyroid. An accurate cytological diagnosis is based on distinctive cytological features in combination with immunocytochemistry. **Methods.** A number of 83 fine needle aspirations, positive for papillary thyroid cancer (44 from thyroid nodules and 39 from cervical lymph nodes), were studied using Thin Layer Cytology. A panel of the immunomarkers Cytokeratin-19, Galectin-3, HBME1, CD-44, CD-56, and E-Cadherin was performed. **Results.** Positive expression of CK-19 was observed in 77 cases (92.7%), of Galectin-3 in 74 cases (89.1%), of HBME1 in 65 (78.3%), and of CD-44 in 72 cases (86.7%). Loss of expression of CD-56 was observed in 80 cases (96.4%) and of E-cadherin in 78 (93.9%). **Conclusions.** Our data suggest that Thin Layer Cytology increases the diagnostic accuracy in papillary carcinoma and seems to be a promising technique for further investigation of thyroid lesions permitting the possibility to use archive material. Positive immunoexpression of CK-19, Galectin-3, HBME-1, and CD-44 improves the diagnostic accuracy of papillary thyroid cancer. Furthermore, loss of E-cadherin and of CD-56 expression is a feature of malignancy.

1. Introduction

Papillary thyroid carcinoma (PTC) is the most common malignancy of the thyroid gland, and fine needle aspiration cytology (FNAC) is the only preoperative diagnostic method used in its detection. The cytological diagnosis of PTC is considered particularly reliable and allows for a definitive diagnosis and treatment planning at the time of FNA [1, 2] as it is based on specific cytomorphological characteristics. However, these characteristics can occur in other thyroid lesions, both benign and malignant. In such cases, immunocytochemistry seems to play an important role in order to facilitate the uncertain results and helps to avoid diagnostic pitfalls.

Although FNAC is widely accepted as the primary diagnostic procedure for thyroid nodules [1], few reports have been published regarding immunocytochemistry mainly on liquid-based Thin Layer technique [3]. According to recent

research, several diagnostic and prognostic markers have been proposed in improving the diagnostic accuracy of papillary thyroid cancer such as Cytokeratin-19 (CK-19), HBME-1, Galectin-3, CD44, CD56, E-cadherin, c-erbB-2, p21^{cip1}, p27^{kip1}, and p16^{ink4a} [4–13].

The purpose of our study was the evaluation of immunocytochemistry in combination with cytomorphology in the diagnosis of papillary cancer in FNA smears using liquid-based cytology.

2. Materials and Methods

Cytopathological files of primary papillary thyroid cancer as well as metastatic ones were reviewed from our cases during the last five years. A total number of 83 FNA cases were included; 44 were from thyroid nodules and 39 from cervical lymph nodes. In addition, 30 benign cases that belonged

mainly in thyroid adenomatous nodules were used as control. The majority of the cases concerned ultrasound-guided aspirations of thyroid nodules or lymph nodes performed in collaboration with an experienced endocrinologist and cytopathologist on site. All malignant cases and controls were histologically confirmed.

The cytological material was prepared using, mainly, liquid-based cytology. The conventional method was used in 13 cases. Immediately after the aspiration, one or two conventional smears were prepared, and the remaining sample was rinsed immediately in a Preservcyt (Cytoc Corporation, Boxborough, MA). A thin evenly dispersed monolayer of cells was dispersed from the filter onto the slide in a cycle of 20 mm in diameter. ThinPrep slides were prepared in the laboratory using the TP2000 instrument, and both conventional and ThinPrep slides were stained with a modified Papanicolaou method. Immunocytochemistry was performed only in slides prepared by thin layer method. All samples were stained in an automated immunostainer (Ventana ES, Ventana medical system, Inc., Tucson, AZ, USA) and the related Ventana reagents were used, using standard manufacturer's instructions. Our panel included Cytokeratin 19 (Dilution 1:50, Clone b170, IgG-1, Ylem), Galectin-3 (Dilution 1:50, Clone 9C4, Novocastra), HBME-1 (Dilution 1:50, monoclonal HBME, Ylem), CD 44 (Dilution 1:50, Clone DF1485, IgG1, Dako), CD-56 (Dilution 1:50, Clone Moc-1, Dako), and E-cadherin (Dilution 1:25, Clone 36B5, Novocastra).

According to manufacturer's suggestions, the samples were immersed in a citrate buffer solution and heated for 20 minutes at 350 W. The slides were rinsed in tap water for 5 minutes, and then they were incubated with 3% H₂O₂ for 4 minutes to quench the endogenous peroxidase activity. We used the primary antibodies diluted in an appropriate dilution with Ventana antibody diluent in a Venatana user fillable dispenser. A standard avidin-biotin method was applied. The primary antibody was bound by a biotin-conjugated mouse secretory antibody formulation and next an avidin enzyme conjugate bound to the biotin present on the secondary antibody followed. The primary antibody-secondary antibody-avidin enzyme complex was then visualized utilizing a precipitating enzyme product. The staining intensity was assessed in a semiquantitative way, independently by two experienced cytopathologists. A scale from negative (0) to weak (1), moderate (2), and strong (3) was applied to each slide while the pattern of stain was classified as membranous or cytoplasmic, diffuse or nuclear. A positive stain was defined by the presence of either a moderate (2) or a strong stain (3) in at least 10% of tumor cells, and the percentage of the remaining positive cells, was evaluated.

3. Statistical Analysis

A standard independent-samples *t*-test was used for the evaluation of differences in the expression between each immunomarker in cancer material and controls, respectively. Calculations were carried out using SPSS version 14.0.

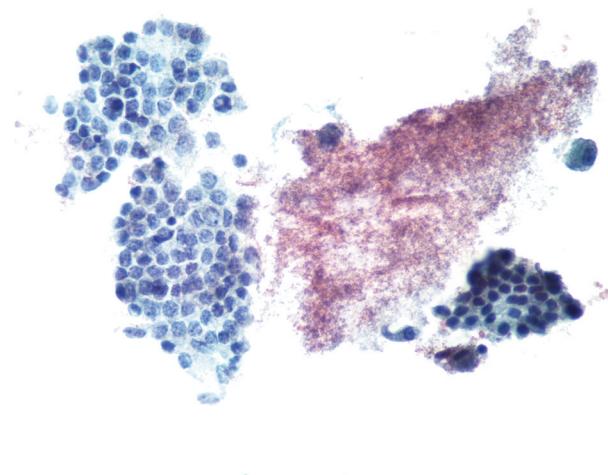


FIGURE 1: Papillary thyroid carcinoma (left) and normal thyroid tissue (right) (Pap stain $\times 400$).

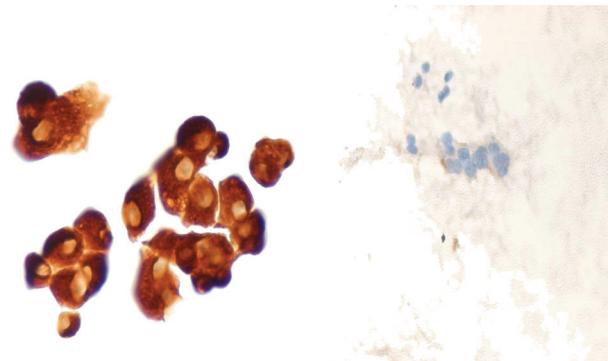


FIGURE 2: CK-19 expression in a PTC case (left) and negative normal control (right) (ThinPrep $\times 400$).

Results were considered statistically significant when *P*-value was less than .05.

4. Results

Our cases included 44 FNAs of thyroid nodules, diagnosed as papillary thyroid cancers, and 39 FNAs from cervical lymph nodes with metastases from papillary thyroid cancer too (Figure 1). CK-19 was positive in 77 out of 83 cases (92.7%), with strong diffuse membranous and cytoplasmic staining (Figure 2). Galectin-3 was positive in 74 out of 83 cases (89.1%) with a strong cytoplasmic immune expression (Figure 3). HBME1 showed a predominantly strong membranous pattern and was positive in 65 out of 83 cases (78.3%) (Figure 4). CD-44 was positive in 72 out of 83 cases (86.7%), and the staining was intense membranous and diffuse cytoplasmic (Figure 5). No sample of the control group showed positivity in the above four controlled immunomarkers (Table 1(a)).

A week positive stain to CD-56 was observed in 3 (3.6%) cases, while 80 (96.4%) cases showed loss of expression. E-cadherin was positive in 5 out of 83 (6%) cases, and

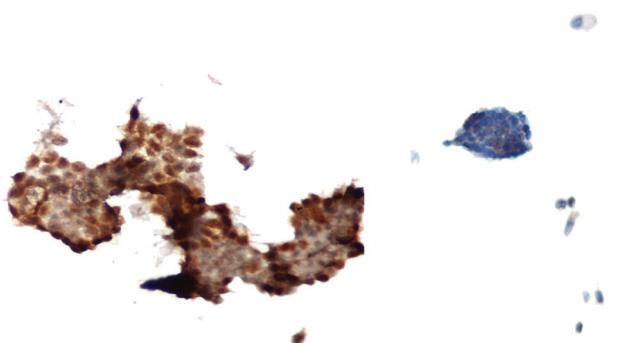
TABLE I

(a) Results of Immunocytochemistry in FNAs, cases, and controls, (*n* is the number of samples)

Immunomarker	Cases		Cases		<i>P</i>	Controls	
	<i>n</i>	Expression %	<i>n</i>	Loss of expression %		<i>n</i>	Expression %
CK-19	77	92.7	6	7.3	<.005	—	—
Galectin-3	74	89.1	9	10.9	<.005	—	—
HBME1	65	78.3	18	21.7	<.005	—	—
CD-44	72	86.7	11	13.3	<.005	—	—

(b) Expression of CD-56 and E-Cadherin in FNAs, cases, and controls. *n* is the number of samples

Immunomarker	Cases		Cases		<i>P</i>	Controls	
	<i>n</i>	Expression %	<i>n</i>	Loss of expression %		<i>n</i>	Expression %
CD-56	3	3.6	80	96.4	<.005	30	100
E-Cadherin	5	6.0	78	94.0	<.005	30	100

FIGURE 3: Galectin-3 expression in a PTC case (left) ($\times 400$) and negative normal control (right).

78 (93.97%) cases showed loss of expression. All controls retained their normal expression in the above two immunomarkers (Table 1(b)).

The immunoexpression of all markers was similar in both thyroid and lymph node FNAs. The microscopic evaluation of each immunomarker expression is summarized in Tables I(a) and I(b) and Figure 6.

5. Discussion

It is well known that FNA is usually the first choice for the preoperative diagnostic evaluation of thyroid nodules in everyday clinical practice [1, 2]. A preoperative accurate diagnosis of papillary thyroid cancer is important in determining the clinical management of these patients. Acquiring a good aspirate is the first step toward a correct diagnosis. The preoperative diagnosis of PTC with FNAC does not present difficulties, as PTC presents distinctive features including nuclear grooves, papillary fronds, monolayered sheets of cells, psammoma bodies, multinucleate giant cells,

FIGURE 4: HBME-1 expression in a PTC case ($\times 400$).

and intranuclear cytoplasmic inclusions. However, these cytomorphic criteria are not always possible to observe. Furthermore, follicular and papillary patterns are often overlapping between benign and malignant lesions. For these reasons, diagnostic pitfalls may be noted [14]. Based on our experience and according to the literature data, the follicular variant of papillary carcinoma is thought to be one of the most common causes of false negative cytologic diagnosis of PTC [15]. On the other hand, occult and cystic papillary carcinoma may be a source of error. Therefore, the distinction of true papillary thyroid carcinoma from lesions that share some cytologic features with PTC is of clinical importance. We suggest that immunocytochemistry can play an important role in the differential diagnosis of these uncertain or borderline cases [4, 9, 15].

In this study, we found that CK-19, Galectin-3, CD-44, and HBME1 were highly expressed in papillary carcinomas, a finding that is in agreement with other data reported in the literature [5, 8, 10, 16–19]. Moreover, we demonstrated that the immunoexpression of CD-56 and E-cadherin was absent in almost all cases of this study and this coincides with literature data.

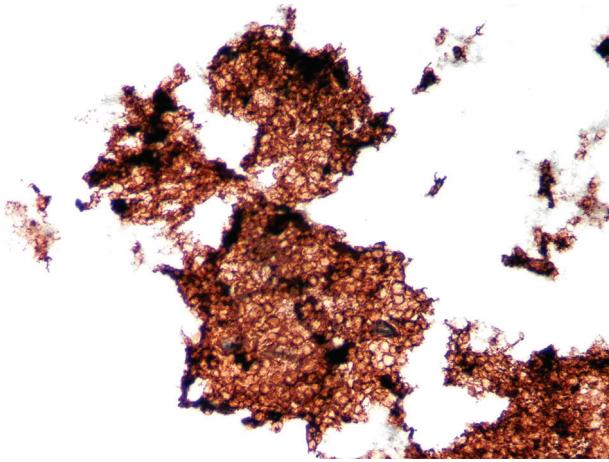


FIGURE 5: CD-44 expression in a PTC case ($\times 100$).

It is reported that CK-19, a cytoskeletal protein, is significantly increased in papillary thyroid carcinoma and is helpful in distinguishing papillary thyroid cancer from benign or other malignant thyroid carcinomas [4]. A strong diffuse or membranous immunoexpression stain, as we have found in our cases, is considered to be in favor of PTC, but focal CK19 staining may be found in benign lesions as well. Galectin-3 is a glucoprotein that plays an important role in organogenesis. It belongs to the family of lectins, is localized mainly in the cytoplasm, and is involved in regulating cell-cell and cell-matrix interactions. Galectin-3 staining is considered positive when cytoplasmic membranous or nuclear staining is present. Many series, as well as the present study, have showed that Galectin-3 is useful as a marker of malignancy in thyroid nodules although some studies have produced conflictive results [20]. The standard form of CD-44, an adhesion molecule, has been associated with extracellular matrix adhesion and lymphocyte homing. Variable expression of CD-44 in PTCs has been demonstrated, and these carcinomas were found to express intense cell membrane or diffuse cytoplasmic staining. CD-44 was expressed in 86.7% of our cases, and the staining was intense membranous and diffuse cytoplasmic. HBME1 is a marker of mesothelial cells and is expressed in malignant thyroid follicular tumors. It is recently applied as an immunomarker in PTCs with high expression. However, this positive immunoexpression does not exclusively indicate papillary differentiation [21]. Our results showed that HBME1 is expressed in a high percentage of PTCs cases.

CD-56, a neural cell adhesion molecule, is present in follicular epithelial cells of normal thyroid. The expression of CD-56 protein was found to be strong within all nonmalignant thyroid cells, but not in cases of PTCs [5]. 96.4% of our cases with PTC showed loss of its expression. On the contrary, all our controls expressed CD-56. E-cadherin, a 120-Kda glucoprotein with a transmembrane domain, is a calcium-dependent homophilic cell adhesion molecule which plays a central role in epithelial integrity, in cell adhesion and differentiation, as well as in the maintenance of cell polarity and tissue architecture. Its impairment correlates

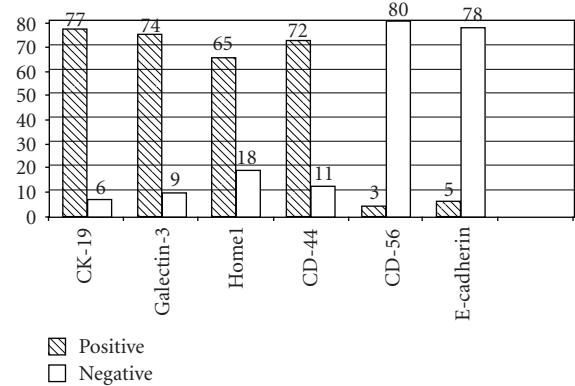


FIGURE 6: Schematic presentation of the expression of each of the six immunomarkers in our 83 cases. Positive means cases with expression of the immunomarker. Negative means cases with no expression or loss of expression, (in the case of CD-56 and E-Cadherin).

with tumor invasion and metastasis [22]. 94% of the cases in this study showed loss of the expression of E-cadherin and all control aspirates expressed it. We suggest that loss of expression of CD-56 and E-cadherin may provide an objective diagnostic tool, and they may be extremely useful in the diagnosis of PTCs, especially in equivocal cases.

Cytologic material used by the conventional method is in most cases unavailable for additional investigation either due to the inadequacy of cellularity or the presence of excess blood mucus or inflammation. This gap is filled by liquid-based cytology and thin layer techniques which may be effective innovations [23]. The quality of the immunocytochemical reaction on the thin layer prepared slides as far as the morphologic details, and the purity of background is considered to be better than conventional smears. Liquid-based cytology offers the possibility of creating archival material and applying new techniques, such as immunocytochemistry in the same sample, and this is significant for further and future revaluation. The gold standard of immunocytochemistry, in order to lead to an accurate diagnosis, is the use of a panel with at least two or three markers in combination with cytomorphology [17, 24]. Attention should be made to the extent, intensity, and pattern of staining. Liquid-based cytology has been validated extensively for the preoperative diagnosis of thyroid nodules [23, 24]. This method is fully reproducible, performed by minimum training of personnel, is safe, time effective, and offers the possibility of an accurate application of immunocytochemistry for the study of neoplastic thyroid lesions. To our knowledge, immunocytochemistry in the diagnosis of papillary thyroid cancer in FNA's using liquid-based cytology is for the first time used in such an extensive material.

6. Conclusions

Our results suggest the following. (1) A precise diagnosis of PTC in fine needle aspiration material is practicable

and credible with the use of contemporary liquid cytology techniques and immunocytochemistry, which is the key for a correct and accurate diagnosis. (2) Positive immunoexpression of CK-19, Galectin-3, CD-44, and HBME1 contributes the most in PTCs diagnosis, and these antibodies can be considered as first-line immunomarkers. Although HBME1 is not necessarily a marker of papillary differentiation, it serves as an indicator of thyroid papillary malignancy especially in combination with the above markers. (3) CD-56 can assist in decision making about the benign or malignant nature of the aspirated material. Loss of expression seems to agree with the presence of papillary thyroid cancer. (4) Loss of the expression of E-cadherin seems to serve as a good indicative marker of malignancy. (5) Thin layer cytology is a promising technique for the investigation of thyroid lesions and increasing the diagnostic accuracy in papillary carcinoma. (6) There is a clear need for the development of additional molecular markers in order to improve the diagnostic capabilities and thereby advance the clinical management in patients with borderline FNA results.

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Review Article

Genetic Predisposition to Familial Nonmedullary Thyroid Cancer: An Update of Molecular Findings and State-of-the-Art Studies

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Familial thyroid cancer has become a well-recognized entity in patients with thyroid cancer originating from follicular cells, that is, nonmedullary thyroid carcinoma. The diagnosis of familial thyroid cancer provides an opportunity for early detection and possible prevention in family members. Understanding the syndromes associated with familial thyroid cancer allows clinicians to evaluate and treat patients for coexisting pathologic conditions. About five percents of patients with well-differentiated thyroid carcinoma have a familial disease. Patients with familial non-medullary thyroid cancer have more aggressive tumors with increased rates of extrathyroid extension, lymph node metastases, and frequently show the phenomenon of "anticipation" (earlier age at disease onset and increased severity in successive generations). So far, four predisposition loci have been identified in relatively rare extended pedigrees, and association studies have identified multiple predisposing variants for differentiated thyroid cancer. This suggests that there is a high degree of genetic heterogeneity and that the development of this type of tumor is a multifactorial and complex process in which predisposing genetic variants interact with a number of incompletely understood environmental risk factors. Thus, the search for the causative variants is still open and will surely benefit from the new technological approaches that have been developed in recent years.

1. Thyroid Cancer: Epidemiology and Clinicopathologic Features

Thyroid cancer is divided into two types, based on the cells of origin: differentiated thyroid cancer of follicular cell origin (NMTC) and medullary thyroid cancer (MTC) originating from parafollicular cells [1]. The molecular mechanisms underlying MTC development, mainly due to gain-of-function mutations of the proto-oncogene RET have been studied in depth and are out of the scope of this work. Readers are referred to [2] for an updated review of the subject. Thyroid cancer is the most common form of neoplasia of the endocrine system, accounting for about 1%–3% of all cancers, with an annual incidence in various parts of the world ranging from 0.5–10 per 100 000 [3]. The incidence of thyroid cancer is increasing, with one of the fastest rates of increase among common human cancers. Today, non-medullary thyroid cancer is the seventh most

common tumor in women [4]. Improvement in cancer diagnosis can partly explain the phenomenon. However, early diagnosis alone is unlikely to account for this increase, and environmental factors must also play a role [5]. Several risk factors have been identified for thyroid carcinoma, such as radiation exposure, iodine deficiency and excess, and previous history of benign thyroid disease, such as nodules and autoimmune thyroid diseases. NMTC also has a significant gender bias, with a ratio of affected women : men of 2.6 : 1 [6], suggesting that the hormonal environment may play an important role. The vast majority of patients with sporadic NMTC have well-differentiated tumors, papillarythyroid carcinoma (PTC), or follicular thyroid carcinoma (FTC). Papillary thyroid carcinoma accounts for approximately 80% of NMTC and is characterized by distinctive nuclear alterations including pseudoinclusions, grooves, and chromatin clearing. PTCs smaller than 1 cm are referred to as papillary microcarcinomas [7]. These tumors have been identified in

TABLE 1: Familial syndromes with associated non-medullary thyroid cancer.

Syndrome	Clinical Features	Inheritance pattern	Locus	Gene
Gardner's syndrome (FAP)	gastrointestinal adenomatous polyps, osteomas, epidermoid cysts, hypertrophy of the retinal epithelium, desmoid tumors, PTC (cribiform-morular variant)	Autosomal dominant	5q21	APC
Cowden disease	hamartoma, breast cancer, PTC, FTC	Autosomal dominant	10q22	PTEN
Carney complex	pituitary, gonadal, and adrenal gland cancer, PTC, FTC	Autosomal dominant	17q23-24	PRKAR1a
Werner syndrome	Premature aging, soft tissue sarcomas, osteosarcoma, FTC/PTC	Autosomal recessive	8p11-p12	WRN

up to 35% of individuals at autopsy, suggesting that they may be extremely common, although they rarely become clinically relevant. PTC can be also multifocal but is typically slow growing, with a tendency to spread to lymph nodes and usually has an excellent prognosis. Activation of the mitogen-activated protein kinase (MAPK) pathway as a result of mutually exclusive BRAF or RAS mutations or of somatic recombination of RET (RET/PTC rearrangement) or NTRK1 (TRK rearrangement) genes is found in the majority of PTCs [8, 9]. Follicular thyroid carcinoma accounts for approximately 15% of NMTC and is defined by its invasive features that result in infiltration of blood vessels and/or full penetration of the tumor capsule, in the absence of the nuclear alterations of papillary carcinoma. FTC is rarely multifocal does not usually metastasize to the regional lymph nodes but tends to spread via the bloodstream to the lung and bones. An important histologic variant of FTC is the oncocytic (Hürthle cell, oxyphilic) follicular carcinoma composed of eosinophilic cells replete with mitochondria. Activating mutations of RAS genes, activation of the PTEN/AKT pathway (due to activating mutations of PIK3CA or loss-of-function mutations of PTEN) [10, 11], or rearrangements of the genes PAX8 and PPAR yon chromosome 2q24 are often found in sporadic FTC [12]. The least common form of NMTC, accounting for less than 5% of cases, is poorly differentiated and anaplastic (undifferentiated) thyroid carcinomas, the latter representing one of the most lethal forms of human tumors. Mutations in the tumor suppressor gene p53 are typically identified in this type of carcinoma [13].

2. Familial Syndromes

Several syndromes are associated with non-medullary thyroid cancer such as Gardner's syndrome (familial adenomatous polyposis, FAP), Cowden disease (multiple hamartoma), Carney complex, and Werner syndrome. A summary of the clinical features and genes involved (when known) in familial syndromes associated with thyroid cancer is reported in Table 1 [14]. Gardner's syndrome or FAP results from loss-of-function mutations in the APC (adenomatous polyposis coli) tumor-suppressor gene on chromosome 5q21 and is associated with gastrointestinal adenomatous polyps, osteomas, epidermoid cysts, congenital hypertrophy of the retinal epithelium, desmoid tumors, and thyroid cancer. FAP is associated with a "cribiform-morular variant"

of PTC, so called because of its unique histopathologic features. However, fewer than 2% of patients with FAP develop PTC, suggesting that PTC development is probably related to increased susceptibility rather than a specific mutation. Cowden disease, also known as "multiple hamartoma syndrome," is associated with an increased risk of developing both benign and malignant breast tumors, and well-differentiated thyroid tumors. Eighty percent of patients have germline mutations of the PTEN (phosphatase and tensin homologue) tumor-suppressor gene. Carney complex involves both endocrine and nonendocrine organs and can affect pituitary, thyroid, gonadal, and adrenal glands. Both PTC and FTC have been associated with Carney complex type 1. The adrenal gland pathology includes a primary pigmented micronodular hyperplasia, which is the only known familial cause of adrenal Cushing's syndrome, and additional pathologies include myxomas, nevi, and schwannomas. Carney complex type 1 is associated with a mutation in the PRKAR1α (protein kinase A regulatory subunit type 1-alpha) gene. Werner syndrome is a disease of connective tissue characterized by premature aging and is often referred to as "progeria." Patients have a predisposition to cancer, notably soft tissue sarcomas, osteosarcoma, and thyroid cancer (FTC and also PTC) which may develop as the result of genomic instability. Mutations in the RecQ helicase WRN gene have been identified, but the exact role of WRN is not known. It is possible that RecQ helicase acts as a tumor suppressor and may be responsible for genome stability.

3. Familial Nonmedullary Thyroid Carcinoma (FNMTC): Risk Factors and Genetic Predisposition

Patients with familial NMTC may have more aggressive tumors with increased rates of extrathyroid extension, lymph node metastases, and larger tumors in younger patients [4], underscoring the importance of accurate diagnosis. Establishing the diagnosis of a familial thyroid carcinoma may provide the opportunity for its early identification and possible prevention in family members. About 5% of patients who have well-differentiated thyroid carcinoma show a familial occurrence. The clinico-pathological features included in the analysis on familial NMTC are as reported in [15]; the mean tumor size in familial PTC/FTC is ~1.7 cm

diameter. When the primary cancer site is considered in epidemiological studies, the thyroid gland shows the highest estimate of familial relative risk among all organs (between 5 and 10.4 folds as compared to ~1.8 and 2.7 for breast and colon cancer, resp., [16]). This indicates the presence of a strongly inherited component of the disease [6, 16, 17]. As for all diseases, the identification of a relevant familial recurrence risk in NMTC is complicated by the argument that families share the same environment as well as a common genetic background and it is therefore difficult to distinguish between environmental and genetic contributing factors. Although the initial familial aggregations reported over a period of time were seen as chance occurrences due to a shared environment, familial occurrence has more recently been recognized as a significant risk factor in NMTC [18]. Since the first observation of a familial case of PTC by Robinson and Orr [19] who reported PTC in twins, a number of pedigrees have been identified. It has been estimated that a family with three affected members with clinical NMTC due to environmental causes would occur by chance less than once in every 100 years. Therefore, the existence of pedigree collections of the size presented by Lesueur et al. [20] cannot occur by chance, but rather are associated with inherited predisposition. Similarly, Malchoff et al., who presented a large pedigree with PTC with 5 clinically significant cases, noted that the chance of 5 PTC occurring in a family of 23 individuals by chance would be 1 in two billion [21]. A population-based study in Norway of 5673 patients who had a first-degree relative with an index thyroid cancer, confirmed that these patients had an increased risk of developing thyroid cancer [22]. The incidence of PTC, primary pigmented nodular adrenocortical disease, and its associated conditions increased sixfold in men and fourfold in women if a first-degree relative had NMTC. Patients who have two first degree relatives with well-differentiated thyroid cancer and do not have a familial syndrome are currently classified as having familial NMTC [23]. Those who have more than two direct relatives with FNMTC have a decreased survival compared with patients who have one or two affected direct relatives [14]. In order to differentiate between FNMTC and familial syndromes associated with thyroid cancer, it is important to detect extra-thyroidal disease in patients affected with a familial syndrome. Patients who have familial NMTC have more aggressive tumors with increased rates of extra-thyroid extension, lymph node metastases, and larger tumors in younger patients. A recent work by Capezzone et al. compared the features of patients with sporadic and familial NMTC patients and tested whether FNMTC patients with parent-child relationship exhibit an "anticipation" phenomenon (earlier age at disease onset and increased severity in successive generations) [15]. In fact, significant differences between sporadic PTC and FNMTC patients included more frequent tumor multifocality ($P = .001$) and worse final outcome in FNMTC patients ($P = .001$). In addition, among FNMTC cases with parent-child relationships, an earlier age at disease presentation ($P < .0001$), diagnosis ($P < .0001$), and disease onset ($P = .04$) were found in the second generation when compared with the first generation. Patients in the second generation had a

higher rate of lymph node metastases at surgery and worse outcome when compared with the first generation.

4. Familial Risk in FNMTC

Considering these findings, NMTC does appear to be an inherited trait. The prevalence of FNMTC has been estimated to be between 2.5% and 8% of the total NMTC patients. The majority of FNMTC pedigrees are small [20]; the pattern of inheritance is usually autosomal dominant, but since a large number of pedigrees present a significant lack of penetrance, polygenic inheritance cannot be ruled out [20, 24]. The variable expression of FNMTC suggests that the responsible gene(s) may lead to predisposition or susceptibility to thyroid cancer. In addition to NMTC, members of FNMTC pedigrees may present a spectrum of benign thyroid diseases including follicular adenoma, diffuse and multinodular goitre, and autoimmune thyroiditis. As mentioned above, these diseases are considerably more common in the general population than NMTC. However, the high occurrence of these diseases in FNMTC pedigrees suggests that they are a part of FNMTC, and pedigree members are therefore considered to be at increased risk of benign thyroid disease [18, 23].

5. FNMTC Susceptibility Loci

In order to identify the gene(s) responsible for thyroid cancer predisposition, several groups have undertaken genome wide linkage analysis using microsatellite markers evenly distributed across the genome and informative large pedigrees with multiple affected members. To date, four different loci have been identified for genetic predisposition to familial NMTC. The first locus to be implicated in familial PTC was MNG1, which maps to chromosome 14q31. In this case, the authors studied a family from Montreal, Canada, with 18 affected with multinodular goitre (MNG), two of whom presented with PTC and one with follicular adenoma. The predisposing locus was identified by linkage analysis with a maximal LOD (Logarithm Of Odds, a genetic measure of the significance of linkage) score of 4.88 distal to marker D14S1030, the region of interest spanning around 1 Mb [25]. The locus was confirmed in other families with MNG [26], but no evidence of linkage was found in additional FNMTC pedigrees suggesting that only a small percentage of FNMTC can be attributed to this locus [20, 27]. A second locus, PRN1, was identified by Malchoff et al. on chromosome 1q21 in an American family with five members affected by PTC, one by colon cancer and two by papillary renal carcinoma [28]. The susceptibility locus was identified at 1q21 with a maximum LOD score of 3.58 in a critical region of 20 cM. This region encompasses the NTRK1 gene, but no mutation segregating with the disease was found among FNMTC pedigrees [27]. To date, linkage to this locus has not been confirmed in any other independent study. The existence of a susceptibility locus for familial NMTC (NMTC1) on chromosome 2q21 was first identified in a large Tasmanian pedigree with recurrence of papillary thyroid

carcinoma (PTC) [29]. In the same study, linkage analysis of 80 FNMTc pedigrees gave a significant LOD score of 3.07 at marker D2S2271. Stratification based on the presence of at least one case of the follicular variant of PTC, the phenotype observed in the Tasmanian family increased the corresponding LOD score for chromosome 2, suggesting that this locus may be strictly correlated to the follicular variant of PTC. Finally, the fourth susceptibility locus is TCO (Thyroid Carcinoma with Cell Oxyphilia) mapped on chromosome 19p13.2 by Canzian et al. [30]. The study analysed a large French pedigree with six patients affected by MNG and three by PTC. A review of the histology indicated that all cases examined presented oncocytic features. Oncocytic tumors, also known as oxyphilic tumors and in the thyroid gland as Hürthle cell tumors, are made of cells replete with mitochondria with a “swollen” (i.e., “oncocytic,” from the Greek word onkoustai, to swell) appearance. Oncocytic tumors may occur at various sites (see [31], for a review) but they are more frequently observed in the thyroid gland. Thyroid oncocytic tumors originate from follicular cells, with the exception of the rare oncocytic variant of medullary carcinoma. They can be benign (oncocytic adenomas) or malignant (oncocytic carcinomas). Oncocytic tumors in the thyroid are regarded as special subtypes, since their features are distinct enough to set them apart from corresponding neoplasms lacking mitochondrial accumulation. Accordingly, oncocytic thyroid carcinomas are classified as variants of FTC (more commonly) or of PTC (less commonly) [1]. Using this distinctive phenotype for data analysis led to the successful identification of a predisposing locus for thyroid cancer with oncocytic features on chromosome 19p13.2, with a significant LOD score at marker D19S916 (3.01). The TCO locus extends up to ~2 Mb and has been further refined to a 1.6 Mb interval by adding more markers and more families with affected individuals showing oncocytic tumors [32]. It is worth noting that linkage to the TCO locus has been subsequently confirmed in independent studies [18, 27]. Furthermore, analysis of additional families has provided evidence for the genetic interaction between the TCO at 19p13 and NMTC1 at 2q21 loci, resulting in a significantly increased risk of NMTC in patients that carry both susceptibility loci [32]. Several groups have undertaken a positional candidate gene approach to identify the causative mutation underlying TCO predisposition, taking into account the specific phenotype of thyroid oncocytic tumors, that is, the abnormal proliferation of mitochondria and the presence of a severe bioenergetic defect [33–37]. Our group recently defined the bioenergetic defect in vitro in the only existing thyroid oncocytic tumor cell model, the XTC.UC1 cell line. Two mutations were identified in mitochondrial DNA (mtDNA) which affect complex I and III of the OXPHOS respiratory chain [38]. One of the mutations is a single base pair insertion in the ND1 complex I gene, causing a premature stop codon and the absence of the protein as evaluated by western blot analysis. The second is a missense mutation in the CYTB complex III gene. These two mutations markedly decrease the activity of both respiratory complexes and explain the defective ATP synthesis previously reported [33, 34]. Subsequently, the

high occurrence of pathogenic mutations impairing complex I has been confirmed by our group *in vivo* in sporadic thyroid oncocytic tumors, leading to the conclusion that disruptive complex I mutations may well be considered to be the hallmarks of the oncocytic phenotype [39]. The close association between disruptive complex I mutations and the oncocytic phenotype has also been confirmed in other types of oncocytic tumors [40–43]. Since the human complex I is composed of 45 subunits, only 7 of which are mtDNA-encoded, our search for the defective nuclear gene on chromosome 19p13.2 began with the analysis of genes encoding mitochondrial complex I subunits and mapping to the TCO locus. So far, somatic and germline missense mutations have been reported in the *NDUFA13* gene (previously known as GRIM19) [44], a member of complex I and a key regulator of cell death. Germline mutations have been reported by our group in *TIMM44*, a component of the mitochondrial translocon complex for importing nuclear-encoded proteins into the mitochondria [45]. However, the effect of these mutations in TCO predisposition is still uncertain, since either no functional studies have been presented (at least for *NDUFA13*) or they did not reveal a clear negative effect in functional molecular studies [45]. Moreover, the family in which the locus was originally mapped [30] did not carry any coding mutation in *NDUFA13* and *TIMM44*, suggesting either that these genes are not directly involved in the TCO etiology or at least the presence of genetic heterogeneity in TCO. Several other genes mapping to the region encode proteins involved in mitochondrial functions and/or with a known role in tumor development, and these have been subsequently screened. A list of all the genes so far analysed without identifying etiological variants is reported in Table 2 (our unpublished results). Thus, the search for the TCO predisposing variants is still open. The application of next-generation sequencing technologies capable of sequencing of ~2 megabases of genomic DNA in a single experiment, will allow the entire TCO region to be resequenced, and not just the coding region of genes, a limit of the traditional candidate gene approach. Identification of the relevant mutation(s) will surely benefit the characterization of TCO.

6. Association Studies in Thyroid Cancer

FNMTc has been identified as a clinically distinct entity from its sporadic counterpart, and although the mutated gene(s) have not yet been identified, the genetic basis underlying its predisposition has been recognized by the scientific community. However, several studies have recently reported the identification of predisposing common variants in association-based case-control studies using single nucleotide polymorphism (SNP) high throughput genotyping [46–49]. One of the most significant studies was a genome-wide association scan in a large cohort of Icelandic cases with thyroid cancer and respective controls, followed by a replication study in individuals of European descent [49]. The authors demonstrated that two common variants, located on 9q22.33 and 14q13.3, are associated with the disease. Overall, the strongest association signals were observed for rs965513 on 9q22.33 (OR = 1.75; $P = 1.7 \times 10^{-27}$)

TABLE 2: New changes in cDNA of TCO candidate genes on 19p13.2.

PCR product	Change in cDNA	Type of aa change	Het frequency in TCO patients (%)	Het frequency in controls (%)
<i>TIMM44x4</i>	G344A C925A	silent P308Q	12.5 12.5	na 0 0 na na
<i>TIMM44x9</i>	G1274A G509A	silent silent	12.5 12.5	na na 22.5 3.0
<i>TIMM44x13</i>	T971C G868T	silent 3'UTR	12.5 12.5 8.0	na na na na
<i>ELAVL1x3</i>	C591A G1111C	silent R219P	42.9 13.0 25.0 14.2	na na na na
<i>ELAVL1x5</i>	Δ(CTTCCACTTCA	3'UTR 5'UTR	12.5 12.5	na na na na
<i>CCL25x5</i>	AC) bp1267-1280	T289M	37.5 50.0 37.5 50.0	na na 7.3 na na
<i>LASS1x3</i>	C182T A866T	K1266N	37.5 37.5	
<i>MARCH2x5</i>	A3798C C4996T	silent Y1897D	37.5 12.5 37.5 37.5	
<i>MARCH2x5</i>	T5692G G9569C	S3189T	12.5 50.0	
<i>GRIM19x1</i>	A21814G T21858A	I7271V silent	12.5	
<i>EDG5x2</i>	G26790A C25572T	silent silent		
<i>MUC16x1</i>	T25638G A27791C	D8546E		
<i>MUC16x1</i>	C31905T G31721A	N9263T		
<i>MUC16x1</i>	G36955A G39400A	silent R10573H		
<i>MUC16x2</i>	C41705T	E12318L		
<i>MUC16x3</i>		V13133M		
<i>MUC16x3</i>		P13901L		
<i>MUC16x3</i>				
<i>MUC16x3</i>				
<i>MUC16x3</i>				
<i>MUC16x5</i>				
<i>MUC16x5</i>				
<i>MUC16x5</i>				
<i>MUC16x15</i>				
<i>MUC16x39</i>				
<i>MUC16x63</i>				

na= not available. The changes cosegregating are reported. Only for *TIMM44* missense change, the variant was not found in a large group of controls. The other genes screened so far but with no new changes identified are *NDUFA7*, *PPAN*, *FBLX12*, *ICAM1*, *ICAM4*, *LASS1*, *LASS4*, *SMARCA4*, *DNM2*, and *ANGPL4*; *RAB11B*; *ADAMTS10*; *PIN1*; *UBL5*; and *KEAP1*.

and rs944289 on 14q13.3 (OR = 1.37; $P = 2.0 \times 10^{-9}$). The gene closest to the 9q22.33 locus is *FOXE1* (TTF2), and *NKX2-1* (TTF1) is among the genes located at the 14q13.3 locus. Both variants contributed to an increased risk of both papillary and follicular thyroid cancer. Approximately 3.7% of individuals were homozygous for both variants, and their estimated risk of thyroid cancer was 5.7-fold greater than that of noncarriers. In a study using a large sample set from the general population, both risk alleles are associated with low concentrations of thyroid stimulating hormone (TSH), and the 9q22.33 allele was associated with low concentration of thyroxin (T(4)) and high concentration of triiodothyronine (T(3)). Another interesting study, although not specific for thyroid cancer as such, in an isolated population of Sardinians identified a strong association ($P = 1.3 \times 10^{-11}$) between rs4704397 and circulating TSH levels [48]. Serum TSH is a sensitive indicator of thyroid function, and overt abnormalities in thyroid function lead to common endocrine disorders. Each additional copy of the minor A allele was associated with an increase in TSH. The marker is located in intron 1 of *PDE8B*, encoding a high-affinity cAMP-specific

phosphodiesterase. The association was replicated in genetically distant cohorts from Tuscany and Old Order Amish (overall P value = 1.9×10^{-20}). It will be very interesting to analyse the association of the above-mentioned SNPs with the familial form of non-medullary thyroid carcinoma.

7. Conclusions

Familial NMTC represents 3%–7% of all thyroid tumors and is associated with some of the highest familial risks among all cancers, with a risk of developing this type of neoplasia for first-degree relatives of 5–10-fold compared to the general population. Several predisposing loci have been identified in the recent years but the mutated gene has not been identified yet. However, the new technologies already available have rendered the resequencing of large genomic regions from defined linkage intervals reasonably possible in single experiments, and the application of these methods will greatly speed up the search for the NMTC gene(s). It will then become possible for NMTC families to receive careful genetic counseling for all at-risk members similarly to what

is already happening for FMTC. In addition, the study of FNMTC genetics, and in particular familial TCO represents a key model for the study of nuclear and mitochondrial crosstalk in thyroid tumor development. The thyroid gland is characterized by a high energetic metabolism and any genetic defect (either of nuclear or mitochondrial origin) leading to bioenergetic deficits may lead to an alteration of this crosstalk and to an alteration of the mitochondrial phenotype, as seen in TCO. Identifying the causative mutations involved in these energetic defects will also provide a powerful tool to design new and specific treatments for such tumors.

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Case Report

Hyperfunctioning Solid/Trabecular Follicular Carcinoma of the Thyroid Gland

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A 68-year-old woman with solid/trabecular follicular thyroid carcinoma inside of an autonomously functioning thyroid nodule is described in this paper. The patient was referred to our clinic for swelling of the neck and an increased pulse rate. Ultrasonography showed a slightly hypoechoic nodule in the right lobe of the thyroid. Despite suppressed TSH levels, the ^{99m}Tc -pertechnetate scan showed a hot area corresponding to the nodule with a suppressed uptake in the remaining thyroid tissue. Histopathological examination of the nodule revealed a solid/trabecular follicular thyroid carcinoma. To the best of our knowledge, this is the first case of hyperfunctioning follicular solid/trabecular carcinoma reported in the literature. Even if a hyperfunctioning thyroid carcinoma is an extremely rare malignancy, careful management is recommended so that a malignancy will not be overlooked in the hot thyroid nodules.

1. Introduction

Hyperthyroidism due to thyroid carcinoma is an extremely rare phenomenon. It is commonly believed that the diagnosis of a solitary autonomously functioning thyroid nodule (AFTN)—a solitary “hot” nodule in radionuclide imaging—can almost always rule out malignancy in the nodule [1]. In this paper, we present the rare case of follicular carcinoma manifesting as an AFTN.

2. Case Report

A 68-year-old female, affected by a long-standing asymptomatic normally functioning nodule in the right lobe of the thyroid, developed symptoms of neck swelling and palpitations. The patient presented a resting pulse rate of 108 and blood pressure of 145/90 mmHg. A large painless, well-defined, hard nodule was palpable in the right lobe of the thyroid; the left lobe was normal, and there were no cervical lymphadenopathies. Ultrasonography (US)

of the thyroid revealed a large and slightly hypoechoic nodule (diameters: 33 × 38 × 53 mm). Thyroid function tests showed elevated free triiodothyronine (fT3) of 7.60 pmol/L (reference range 2.30–6.30 pmol/L) and undetectable thyroid-stimulating hormone (TSH) of 0.006 mIU/L (normal 0.4–4.0 mIU/L). The free thyroxin (fT4) was normal at 11.4 pmol/L (reference range 7.5–21.1 pmol/L), and both thyroperoxidase and thyrotropin-receptor autoantibodies were negative (<60 UI/mL and <1 U/L, resp.). A ^{99m}Tc -pertechnetate scan demonstrated a large hot area with inhomogeneous uptake and no cold areas inside corresponding to the nodule, with a suppressed uptake in the remaining thyroid tissue (Figure 1(a)). Six and 24 hours radioiodine uptake (RAIU) values were 10% (normal: 5–15%) and 32% (normal: 10–35%), respectively. The nodule was considered an AFTN, but radioiodine treatment (that is the first-line treatment for AFTN in our centre) was ruled-out based on the symptoms (i.e., neck swelling) and the volume of the nodule. Right lobectomy was performed (Figure 1(b)) and the histological examination

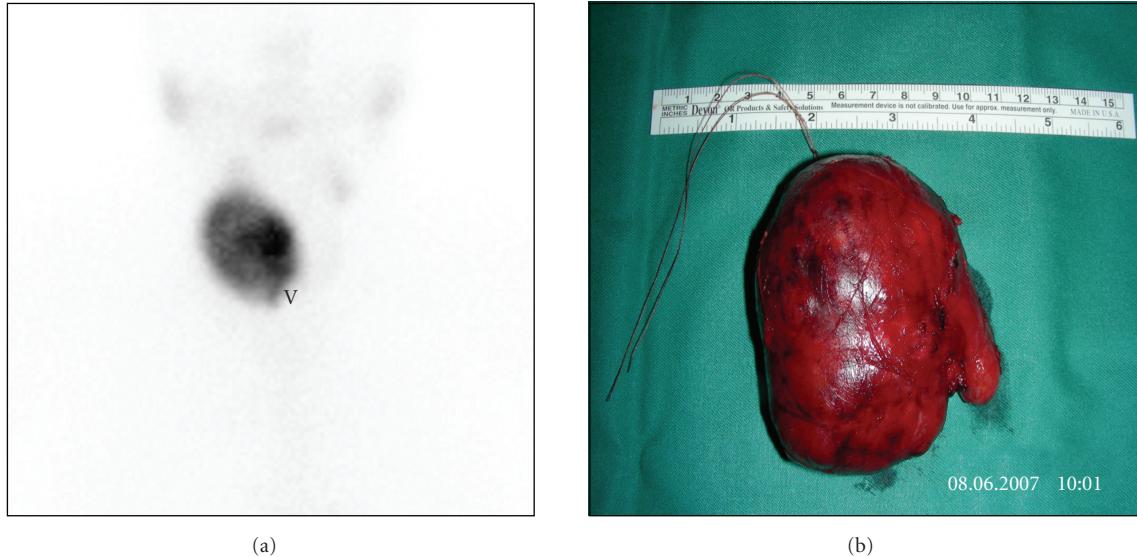


FIGURE 1: $^{99\text{m}}\text{Tc}$ scan: hot thyroid nodule in the right thyroid lobe with suppressed extranodular thyroid tissues (a). Surgical specimen from right lobectomy and isthmectomy (b).

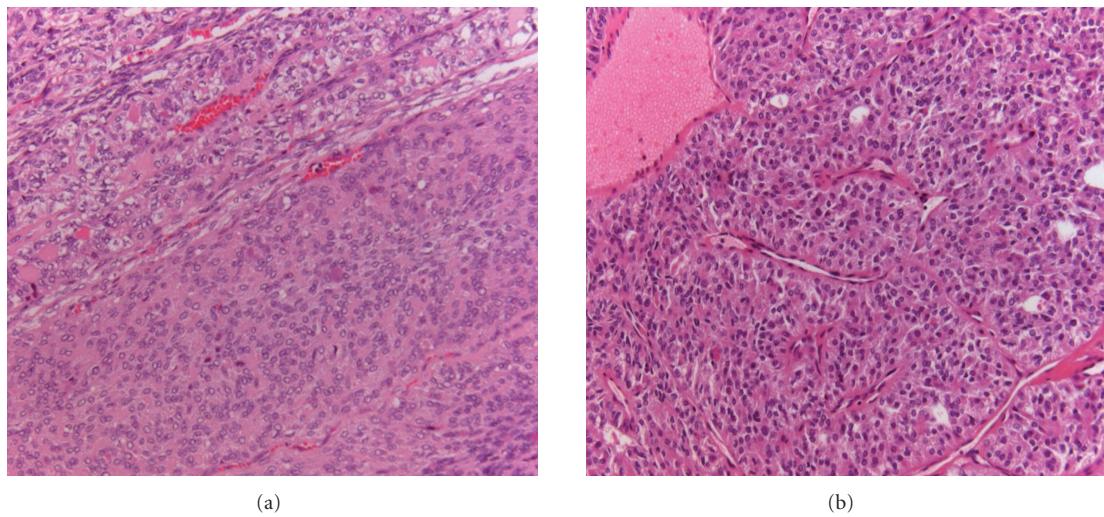


FIGURE 2: Hematoxylin/eosin histological stains: follicular carcinoma with solid (a) and solid-trabecular (b) features.

revealed a follicular carcinoma with solid and trabecular parts and focal signs of angioinvasivity (Figures 2(a), (b)). The surrounding thyroid tissue showed a follicular architecture with no signs of tumour infiltration or spreading. Since the patient declined further surgery, a radioiodine ablation was directly performed by administering ^{131}I (2.5 GBq). Serum thyroglobulin was 9.4 ng/mL before ^{131}I treatment, with a corresponding TSH level of 36 mUI/L. Six months after thyroid ablation, a ^{131}I whole-body scanning after recombinant human TSH administration was negative with a corresponding undetectable serum Tg (i.e., <0.2 ng/mL). Further followup by clinical examination, including neck US and Tg measurement, every 6 months, is negative up to now (3.4 years follow-up).

3. Discussion

Our patient presented with a palpable thyroid nodule and hyperthyroidism with the absence of TRAb and TPOAb. The nodule was proved to be functionally autonomous by $^{99\text{m}}\text{Tc}$ -pertechnetate imaging and RAIU. However, a follicular solid/trabecular carcinoma was finally proved by histological examination. Hyperthyroidism due to thyroid carcinoma is a rare, but well-recognized phenomenon. This situation has been generally described as resulting from excessive production of thyroid hormone by extensive functioning metastases, usually from follicular carcinoma [2, 3]. The incidence of thyroid carcinoma in a hot nodule is reported to be very low by most authors [4–6], but the incidence

is somewhat higher in other retrospective studies [7, 8]. Actually, thyroid carcinoma in a hot nodule has been described in numerous case reports prior to ours. However, unlike our case, most of these cases show a cold area within a hot nodule, indicating that the thyroid carcinoma itself did not produce thyroid hormone [9]. Women are far more often affected than men, but no significant peak with regard to age was noted [10]. Interestingly, the histological features of these tumors correspond in principle to the papillary carcinoma, as opposed to the metastatic functioning carcinomas, essentially being of follicular type [11–15]. Classical follicular histology is described in the few reported cases of hyperfunctioning follicular carcinoma while only one case with a clear-cell variant histotype is described [16–18]. To the best of our knowledge, we are the first to report a case of hyperfunctioning aggressive follicular carcinoma with solid and trabecular features. This case underlines the clinical importance of predicting the incidence of malignancy in hot thyroid nodules. However, reports in the literature indicate significant difficulty in determining the risk that AFTN will undergo malignant degeneration. Some clinical findings set forth the risk factors for malignancy in thyroid nodules: age <20 or >60 years, male sex, the family history of differentiated or medullary thyroid carcinoma or of familial adenomatous polyposis (Gardner's syndrome), past history of head and neck radiation, rapid tumor growth, irregular outline, fixation to adjacent structures, and symptoms of tumor invasion [1, 15, 19]. In actual practice, however, few patients have these symptoms, and most nodules are nearly asymptomatic [1]. The classical benign AFTN presents itself as a smooth, well-defined, round or ovoid mass that moves freely and occurs in patients aged 40 or over with a history of long-standing and slowly expanding mass in the neck [19]. The US pattern, as well as the vascular signals in power or color-Doppler samplings, is largely overlapped in malignant nodules and AFTN, as occurred in our patient [1]. An incomplete suppression of radionuclide uptake in extranodular thyroid tissues was reported as a risk factor of malignancy, but this did not occur in our patient [20]. Differentiating a benign follicular adenoma from a malignant follicular carcinoma is challenging by cytology, and a thyroid scan is advocated in these cases, considering functioning nodules as being benign [1, 21]. Hot nodules outside the thyroid can be helpful in diagnosis of malignancy in the case of metastatic thyroid carcinoma, but this is rare in practice [22]. In our patient, surgical treatment was preferred to radioiodine ablation considering her symptoms and the nodule's size. However, ^{131}I could be administered in patients with similar clinical presentation; a progressive increase in the nodule size after ^{131}I treatment was signalled as a suspicious sign for malignancy in these cases and should be promptly evaluated [23]. A possible reason for why thyroid carcinomas occasionally produce excessive hormone without extensive metastases could be due to gene mutations reported to occur in AFTN, including mutations of G protein α chain gene and TSH-receptor gene with associated elevated intracellular cAMP [19]. By contrast, Bourasseau et al. denied TSH-receptor and G protein α chain gene mutation in hyperfunctioning thyroid carcinoma [24].

Consequently, further studies are needed to clarify this issue. The clinical course of a nonmetastatic hyperfunctioning thyroid carcinoma depends on its histological features and on the patients' age and tumor stage at the time of diagnosis [25]. It seems that the prognosis of metastatic follicular carcinoma does not differ with the presence or absence of hyperthyroidism [15, 16, 26]. However, the prognosis of nonmetastatic hyperfunctioning papillary and follicular thyroid carcinoma was not fully described in the literature. In conclusion, our case showed that an aggressive follicular thyroid carcinoma with solid/trabecular features without metastases could produce hyperthyroidism, suggesting that malignancy (even if very rare) cannot always be excluded in a hot thyroid nodule. Consequently, careful management is recommended so that malignancy is not overlooked in the scintigraphically hyperfunctioning nodules.

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Review Article

Molecular and Other Novel Advances in Treatment of Metastatic Epithelial and Medullary Thyroid Cancers

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An understanding of the mutations of the proto-oncogenes and tumor suppressor genes that occur in thyroid cancers should eventually explain the diverse clinical characteristics of these tumors and also direct therapy. Some insights have already emerged in the last decade; some abnormalities in tumor genes are consistently associated with specific clinical and pathologic findings. These genetic abnormalities usually represent somatic mutations in tumors of follicular epithelial origin, as opposed to inherited mutations in medullary thyroid cancers of parafollicular C cells origin because most thyroid tumors are sporadic and not familial. This is different from the multiple endocrine neoplasia syndromes in which the primary tumorigenic gene mutations are inherited. This improved understanding of the molecular basis of these diseases has led to the development of novel targeted therapeutic approaches which will be discussed in this paper.

1. Introduction

Thyroid cancer is the most common form of malignant endocrine tumor. In 2002, the estimated incidence of thyroid cancer worldwide was reported to be in excess of 141 000 [1] with a female predilection (more than 75% being women). The thyroid gland is composed of two main parenchymal cell types. Differentiated thyroid cancers arise from follicular cells giving rise to 3 separate subgroups, papillary, follicular, and hurtle cell. The 3 different subtypes have different clinical behavior. Papillary thyroid cancer (PTC) forming 80% of all thyroid cancers has a 10-year survival rate of more than 90%. PTC displays a spectrum of differentiation from well differentiated, tall cell variant, to poorly differentiated. Follicular thyroid cancer (FTC) constitutes 10% of all thyroid cancers with a wider range of 10-year survival between 43%–94% indicating great heterogeneity within the same disease entity [2–4]. Hurthle cell carcinoma is the most infrequent differentiated carcinoma arising from the follicular cells with incidence estimated to be between 3%–10%. The overall survival rates for Hurthle cell carcinoma are similar or worse compared to follicular carcinoma. One

study showed disease-free survival of 40.5% at 10 years [5].

Anaplastic thyroid cancers (ATCs) are undifferentiated tumors of the thyroid follicular epithelium. In marked contrast to differentiated thyroid cancers, anaplastic cancers are extremely aggressive, with a disease-specific mortality approaching 100%. Fortunately, anaplastic thyroid cancer accounts for only 2%–5% of all thyroid cancers [2–4, 6]. Medullary thyroid carcinoma (MTC) is a neuroendocrine tumor which arises from parafollicular or C cells of the thyroid gland. MTC accounts for 3%–5% of all thyroid cancers and portends a higher mortality rate [2, 3].

The prognosis of thyroid cancer as a whole is generally favourable representing only 0.5% (35 000) of all cancer deaths [7]. However, prognosis for patients with metastatic, iodine nonavid, differentiated thyroid cancer and anaplastic cancer remains poor. This paper reviews the therapeutic options available for this subset of patients. Better understanding of molecular pathogenesis of thyroid cancer coupled with significant advances made in cancer therapy guarantees promising and exciting therapeutic options in years to come.

2. Differentiated Thyroid Cancer (DTC)

Papillary and follicular thyroid cancers are often treated similarly albeit numerous biologic differences. Standard of care of differentiated thyroid cancer consists of total thyroidectomy often followed by radioactive iodine (RAI) when indicated, aiming for remnant ablation and adjuvant therapy. High-risk patients (age >45, male gender, presence of metastatic disease, extra-thyroidal tumour extension, and tumour diameter >4 cm) should also receive thyroid stimulating hormone (TSH) suppression therapy with thyroid hormone [8]. Up to 25%–50% of patient's tumor loses ability to take up iodine. This is attributed to downregulation of sodium iodide (NaI) symporter (NIS). Promoter methylation of genes required for NaI metabolism leads to decreased expression and is linked to increased signaling through the MAPK pathway [9].

Overall survival (OS) rapidly falls once thyroid cancer metastasize, and is no longer amenable to radioiodine therapy making chemotherapy the only option for systemic treatment. Doxorubicin has been recognized as the standard of care since 1970s based on response rate of 30% in a population of 30 patients [10]. This perhaps is an overestimate of the efficacy of doxorubicin as subsequent trials did not achieve similar response rates. A phase two trial evaluating the efficacy of doxorubicin combined with cisplatin yielded a response rate of less than 10%. The progression-free survival (PFS) time of the patients was estimated at 2 months, and median overall survival was 8 months [11]. Clearly, novel strategies are needed in view of limitations of conventional cytotoxic therapy. Increasing knowledge on cancer thyroid biology has provided an impetus to identifying novel therapeutics and rational trial designs.

Thyroid cancers are highly vascular and express high levels of vascular endothelial growth factor (VEGF) [12]. Inhibition of VEGF receptor signaling alone or in combination has been shown to inhibit growth of thyroid tumors in orthotopic nude mouse [13]. VEGFR therefore seems an attractive and plausible target in iodine refractory metastatic thyroid cancer in humans.

PTC frequently carries gene mutations and rearrangements that lead to activation of the mitogen activated protein kinase (MAPK). Rearrangements of RET and NTRK1 tyrosine kinases, activating mutations of BRAF and RAS are sequential components leading to activation of MAPK. These gene alterations are mutually exclusive given any PTC [14, 15]. PTC is associated with rearrangements of two different transmembrane tyrosine kinase genes: RET and NTRK1. These rearrangements result in the production of chimeric proteins with tyrosine kinase activity that contributes to the development of the malignant phenotype. The chimeric genes resulting from RET rearrangements are referred to as RET/PTC and those resulting from NTRK1 rearrangements as TRK. Of note, TRK is an extremely rare occurrence. On average, no more than 20% of PTCs have such rearrangements with at least 6 different chimeric RET/PTC and TRK genes shown. TRK may be associated with a more aggressive clinical behavior [16] whilst RET/PTC possibly

has a more favorable prognosis [17]. The RAF proteins are serine-threonine kinases that activate the RAF/MEK/MAPK signaling pathway. The T1799A mutation of the BRAF gene, occurs in 29%–69% of papillary thyroid cancers [14, 18–20]. The predicted protein product BRAFV600E in PTC is associated with reduced expression of key genes governing iodine metabolism, specifically the impairment of both NIS expression and targeting to membrane leading to dedifferentiation [21, 22]. BRAF mutations have also been shown to be associated with extrathyroidal invasion, lymph node metastasis, and advanced tumor stage hence conferring a worse clinical prognosis compared to BRAF wild-type [23]. Finally, Ras has also been reported in follicular variant of PTC in which no clear evidence of aggressiveness has been found [24]. It is worthy to note that the genetic changes linked with pathogenesis of PTC are mutually exclusive.

The translocation t(2;3)(q13;p25) has been identified in FTC which results in fusion of part of the DNA-binding segment of the PAX8 gene and the peroxisome proliferator-activated receptor gamma 1 (PPAR-gamma-1) gene; PAX8 is a thyroid transcription factor, and PPAR-gamma-1 is a transcription factor that stimulates cell differentiation and inhibits cell growth. The product of the fusion gene blocks the action of the PPAR-gamma-1, an effect that might inhibit cell differentiation and stimulate cell growth [25]. Overexpression of normal c-myc and c-fos genes, as well as mutations of H-ras, N-ras, and K-ras proto-oncogenes, is found in follicular cancers. These abnormalities may confer a growth-promoting effect that may act additively with other oncogene or tumor suppressor gene mutations [26, 27].

The above discovery over the last 5–10 years allowed clinical trials of new therapies for well-differentiated thyroid cancer.

Motesanib diphosphate is an oral tyrosine kinase inhibitor (TKI) targeting the VEGFR 1–3, PDGFR, and cKIT. A multicentre, open-label phase 2 trial was initiated testing the efficacy of this drug in a cohort of 93 patients with progressive DTC. Of the 93 patients, 30% were still on drug after 48 weeks. The partial response (PR) rate was 14% with another 35% maintaining stable disease for at least 24 weeks. The median progression-free interval was 40 weeks. The most common side effects included fatigue, nausea, diarrhea, hypertension, and unexpectedly increased requirements in thyroxine replacement [28].

Axitinib is another oral TKI that blocks VEGFR, PDGFR and cKIT. A multicentre phase II trial examined the efficacy of axitinib in advanced or metastatic thyroid carcinoma of which 75% had DTC. There was 31% PR rate among the DTC patients. Median PFS was 18 months. Common side effects were all typical of this class of drug [29].

Sorafenib is an oral TKI targeting VEGFR 2 and 3, RET and BRAF, cKIT, PDGFR, and FLt-3. Two phase II trials were performed in patients with metastatic PTC. Kloos et al. [30] investigated the use of sorafenib among 36 evaluable patients, and PR was seen in 8%, with a minor response (defined as 23%–29% reduction in tumor diameter) in another 19%. Gupta-Abramson et al. [31] showed that out of 30 patients with metastatic iodine refractory thyroid carcinoma who received sorafenib 400 m twice a day, 23% had a PR

lasting up to 84 weeks and 53% achieving stable disease (SD). The median PFS was 79 weeks. In the latest update in abstract form [32], out of 50 evaluable patients, 36% achieved PR and 46% SD with clinical benefit rate of 82%. PFS measured was 84 weeks. The median OS of the initial cohort of 30 patients was 140 weeks. This compares favorably with the historic OS of patients treated with doxorubicin. Patients who harbor BRAFV600E mutation which was a poor prognostic marker in the prekinase era trended towards better outcome when treated with sorafenib compared with patients with wild-type BRAF. Sorafenib is now recognized as a potential therapeutic agent in the NCCN guidelines and is FDA approved for treatment of advanced hepatocellular carcinoma and advanced renal cell carcinoma.

Sunitinib an oral small molecule TKI which demonstrates VEGFRs, RET, and RET/PTC subtypes 1 and 3 inhibition was tested in 31 patients. 13% of the patients achieved PR whilst 68% had documented disease stabilization [33]. Severe adverse effects were similar to that of sorafenib. In another phase II trial where sunitinib was administered on a continuous basis at 37.5 mg every day to 33 patients (26 had well-differentiated thyroid carcinoma), complete response was seen in 2 patients (7%), 25% had PR, and 48% had SD with a clinical benefit rate of 83% [34]. Thus, sunitinib is also available for use in selected thyroid cancer patients with metastatic disease outside of clinical trial. Despite early promise of gefitinib demonstrating efficacy in preclinical models on the premise of many papillary thyroid cancers displaying activated EGF receptor signaling, the phase II study was met with disappointing results. There was no documented objective response with median progression of only 4 months [35].

Two phase 2 studies have just been reported in ASCO 2010 in refractory thyroid carcinoma. AZD6244, an oral inhibitor of mitogen-activated protein kinase, MEK-1/2, was found to result in median progression-free of 32 weeks [36]. Aflibercept, a VEGF-trap, was evaluated in 21 patients culminating in stable disease rate of 83% [37].

In a phase II trial, 29 patients with metastatic differentiated thyroid cancer were treated with increasing doses of thalidomide. PR was reported in about 20 percent of patients, SD was seen in about 35 percent, and there were no complete responses. Most common and serious toxicities included fatigue, infection, and neuropathy, and half of patients discontinued therapy due to side effects [38]. Twenty one patients were treated with 25 mg on lenalidomide in an open label phase two trial with PR of 22% and SD 44%. Treatment was well tolerated with mainly grade 3 hematological toxicities [39].

There are clearly multiple agents which have been tested at phase II levels which have tremendous results warranting validation in phase 3 clinical trials. Future trials should perhaps investigate efficacy of combination targeted therapy with chemotherapy in first line treatment of advanced thyroid cancer. There are currently a few phase I/II trials underway investigating other targets such as BRAFV600E selective kinase inhibitor, proteasome inhibitor, and PPAR-gamma agonist. Everolimus, an mTOR inhibitor, has shown preclinical activity and warrants further testing [40].

3. Anaplastic Thyroid Carcinoma (ATC)

ATC is uncommon and comprises 1%-2% of thyroid malignancies. ATC typically affects elderly patients with median age of 68 at diagnosis. It has a fulminant course with almost 100% disease-specific mortality. The median survival is approximately 4 months with one year overall survival of 16%-19% [41].

ATC may originate de novo or from preexisting PTC or FTC. A number of gene mutations have been identified. Mutation of the p53 tumor suppressor gene has been identified in up to 70% of anaplastic thyroid cancers [42]. Production of inactive p53 protein leads to dysregulation of both apoptosis and the cell cycle. Mutations within the β -catenin gene occur in up to 65% of ATC [43]. The beta catenin protein plays a role in cell adhesion and in signaling through the Wnt pathway. The exon 3 mutation is associated with increased nuclear localization of the beta catenin resulting in increased nuclear signaling and hence tumorigenesis or tumor progression.

It seems likely that in some PTC, dedifferentiation may lead to ATC. This is highlighted by studies showing between 15%-50% of ATC containing BRAF mutation [44, 45]. Interestingly, the incidence of BRAF mutation has been reported to be as high as 62% in radioactive iodine-refractory thyroid cancers [46]. Mutations involving RAS (regulates the RAS-RAF-MEK-ERK and PI3K/AKT1 pathways) are found to affect between 6%-50% of ATC [47, 48]. Somatic mutations of the catalytic subunit of the phosphatidylinositol 3'-kinase (PIK3CA) have been identified in about 23 percent of anaplastic thyroid cancers [49].

Clinical management of anaplastic thyroid cancer hinges on retrospective series of major centres owing to its rarity and thus lack of randomized studies.

For ATC localized to the thyroid, complete resection should be attempted in conjunction with postoperative adjuvant therapy. At Roswell Cancer Institute, the 5-year overall survival of the patients who achieved complete resection was 60% [50]. In contrast, Kobayashi et al. [51] reported a 1-year survival rate of 20% for patients who had complete resection whilst no one with residual disease was alive. Haigh et al. [52] managed 33 patients and found surgical resection with curative intent in eight which resulted in median survival of 43 months and 5-year survival of 50%. Most received adjuvant chemoradiotherapy. Meanwhile, De Crevoisier [53] reported high long-term survival when radio-chemotherapy was administered after complete resection. These patients received 6 cycles of doxorubicin (60 mg/m^2) and cisplatin (120 mg/m^2) with 40 Gy of external beam radiotherapy (EBRT). At the end of treatment, complete local response was seen in 19 patients. With a median followup of 45 months, 7 patients were still in complete remission of which 6 had previously achieved complete resection. All the above together with SEER analysis lends support to adjuvant radio/chemotherapy in improving survival outcomes. However this is confounded by the fact that patients who received adjuvant treatment usually have less extensive disease.

There is no effective therapy for advanced or metastatic anaplastic thyroid cancer, and the disease is uniformly fatal.

The median survival from diagnosis ranges from three to seven months, and the one-year survival rate ranges 20%–35% [50, 52].

ECOG conducted a prospective trial from 1976 to 1982 whereby 21 patients were randomized to doxorubicin versus 18 randomized to doxorubicin plus cisplatin. The single agent arm had one partial response while the 2-drug regimen produced 3 complete responses and 3 partial responses. Median survival however was only 2.7 months. There were however 2 prolonged CRs lasting 35 and 41 months [54]. Ain et al. conducted a phase II clinical trial exploring efficacy of paclitaxel as a 96-hour infusion. The objective response rate was 52%. Overall survival was 6 months with a higher median survival of 32 weeks in the 10 responders [55].

Like its differentiated counterpart, understanding of the molecular pathogenesis has allowed studies on novel targeted agents to be carried out. Combretastatin A-4 phosphate (CA4P), a tubulin binding disrupting agent, showed some interesting findings in the study of Dowlati et al. [56]. One of the 3 patients with ATC achieved complete response and was alive at 30 months. However, in a later phase II trial, out of 18 patients, no objective responses were seen; 6 had stable disease, and 12 patients progressed with median survival of 20 weeks. It appears that CA4P has limited efficacy as a single agent, and perhaps combination with either chemotherapy or another targeted agent may be beneficial [57]. Combination of high-dose intermittent gefitinib was evaluated in a phase I trial, and one patient with ATC had a 4-month partial remission [58]. Cohen et al. evaluated axitinib in phase II trial. Two patients with ATC were enrolled. One had a PR of 4 months whilst the other patients progressed [29].

Several agents are currently being tested in phase I or II settings for advanced anaplastic thyroid cancer including CS7017, an oral PPAR inhibitor, in combination with paclitaxel, imatinib and bavituximab in combination with doxorubicin and pemetrexed with paclitaxel.

4. Medullary Thyroid Carcinoma (MTC)

Medullary thyroid cancers (MTCs) are tumors of thyroid parafollicular C cells which are part of the amine precursor uptake decarboxylation (APUD) system rather than thyroid epithelial cells. MTCs do not concentrate iodine. They may occur as sporadic tumors, as a component of multiple endocrine neoplasia type (MEN) 2, or as a familial MTC syndrome without MEN association. The gender distribution is equal. The tumor is unilateral in majority of sporadic forms but mostly bilateral and multifocal in familial cases. They secrete calcitonin and sometimes carcinoembryonic antigen (CEA), both of which can serve as tumor markers [59]. Calcitonin production is stimulated by both calcium and pentagastrin. This forms the basis of the stimulation tests using calcium infusion [60, 61]. The primary treatment for MTC is extensive and meticulous surgical resection. Total thyroidectomy is frequently needed in addition to complete resection of lymph nodes in the central neck, paratracheal and upper mediastinal region. Fortunately, mutations in the

RET proto-oncogene have now been established in MEN-2. Family members can be screened at birth, obviating yearly provocative testing and allowing affected members to be offered early thyroidectomy [62].

Among patients with recurrent local or distant disease, the rate of progression is variable, and some patients survive for years despite obvious metastatic disease. This fact also makes assessment of the efficacy of any therapy very difficult; about all that can be done is to determine whether a therapy leads to tumor shrinkage. Despite these limitations, it seems clear that external beam radiotherapy is rarely effective, and chemotherapy has only occasionally been effective.

The treatment of MTC using cytotoxic drugs has mirrored the historical development of chemotherapy use in neuroendocrine tumors. Single agent regimens have been characterized by low response rates (<10%) and/or lack of durable response. Doxorubicin, aclarubicin, mitoxantrone, and streptozocin were the agents investigated previously as monotherapy [63–65]. Dacarbazine in combination with other drugs is recommended for patients with metastatic MTC in guidelines from the National Comprehensive Cancer Network [66]. Dacarbazine plus 5-fluorouracil (5-FU) is an active combination. In one series, three of five patients had a partial response, while in a second study, one patient had a complete remission lasting ten months. In preclinical studies, the novel tubulin-binding agent combretastatin A-4 phosphate, in combination with doxorubicin, was active in a xenograft model of MTC in nude mice. Preclinical evidence from the same centre also suggested activity for irinotecan in MTC cell lines [67].

Other modalities evaluated including interferon alpha, long-acting somatostatin analog, yttrium-90-labelled somatostatin analogs, and immunotherapy have largely found to confer marginal clinical benefit [68–74].

A marine cyclodepsipeptide, aplidin (plitidepsin), extracted from a Mediterranean tunicate was investigated in a phase I trial involving six patients with MTC out of 67 with thyroid cancers. Four of the six had stable disease for over six months [75]. Since many MTC patients have spontaneous disease stabilization, this is not definitive results; however this drug merits further investigation. A small molecule inhibitor, arylidene-2-indolinone (RPI-1), abolishes the constitutive tyrosine phosphorylation caused by the specific RET proto-oncogene mutation seen at cysteine residue 634 in the syndrome multiple endocrine neoplasia type 2A (MEN2A). Hopefully, this agent will have future applications both in inherited MTC as well as in papillary cancers associated with the RET/PTC1 gene rearrangement [76].

Another novel therapeutic approach includes modulation of angiogenesis considering that VEGF is elevated in 75% of metastatic MTC [77]. Several phase 2 studies have been conducted to evaluate the efficacy of this strategy. 91 patients with advanced/metastatic medullary thyroid cancer were treated with motesanib diphosphate in a multicentre trial. Although the objective response rate was only 2%, almost half of the patients experienced prolonged stable disease [78]. Similarly, sunitinib was noted to confer an overall response rate of 35% and clinical benefit rate of 91% in another study involving 25 patients [79]. Building

on the knowledge that germline or somatic mutations of RET account for 75% of MTC, a randomized, double-blind phase 3 trial involving 331 patients was conducted utilizing vandetanib an oral inhibitor of RET, VEGFR, and EGFR. Although the overall survival analysis is immature it is assuring to note that patients treated with vandetanib had a significantly longer progression-free survival compared to the placebo group (HR 0.45, $P = .0001$) [80]. Antitumor activity has been noted in XL184, an oral inhibitor of RET, MET, and VEGFR2. Hepatocyte growth factor (FGF) receptor tyrosine kinase (MET) has also been detected in MTC and transduction of normal human thyroid cells with mutant RET results in upregulation of MET. Moreover preclinical model suggests that MET and VEGFR2 play synergistic roles in promoting tumor angiogenesis and dissemination [81]. A phase 1 dose finding study involving 37 patients with MTC yielded PR rate of 29% and prolonged SD of 68% [82]. The efficacy of XL 184 is currently being evaluated in a randomized phase 3 trial.

5. Conclusion

The improved understanding of the molecular basis of pathogenesis and development of thyroid cancers of follicular epithelial origin and medullary thyroid cancers has vastly increased the armamentarium of therapeutic options available to the patient with metastatic disease. A continued rational approach to the development of these therapeutic modalities will further define their future clinical applications.

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Review Article

Advances in Cellular Therapy for the Treatment of Thyroid Cancer

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Up to now, there are no curative therapies available for the subset of metastasized undifferentiated/anaplastic thyroid carcinomas. This review describes the possible use of immunocompetent cells which may help to restore the antitumor immune recognition for treating an existing tumor or preventing its recurrence. The most prominent experimental strategy is the use of dendritic cells (DCs) which are highly potent in presenting tumor antigens. Activated DCs subsequently migrate to draining lymph nodes where they present antigens to naïve lymphocytes and induce cytotoxic T cells (CTL). Alternatively to DC therapy, adoptive cell transfer may be performed by either using natural killer cells or ex vivo matured CTLs. Within this review article we will focus on recent advances in the understanding of anti-tumor immune responses, for example, in thyroid carcinomas including the advances which have been made for the identification of potential tumor antigens in thyroid malignancies.

1. Introduction

The most frequently occurring forms of thyroid cancer have a good prognosis. The neoplasms originate from two distinct endocrine cell types of the thyroid gland: thyroid hormone producing follicular epithelial cells and calcitonin-producing parafollicular cells (C-cells). The most prevalent thyroid cancer is that of papillary origin (PTC; about 60–80%) it differs from follicular (FTC; 15–25%) and anaplastic carcinomas (ATC; 2–5%) but all are derived also from follicular epithelial cells. Only about 3–10% of the thyroid tumors derive from parafollicular C-cells. These tumors are termed as medullary thyroid carcinoma (MTC) [1, 2].

The good prognosis of PTC and FTC is originally attributed to the steady growth rate and the competence of taking up iodide. This leads to the potential classical therapeutic option of radioiodide (^{131}I) treatment after a surgical revision of the tumor mass [3]. The varieties of the different tumors of follicular origin reveal in their distinct morphology, their kind of metastatic spreading, and their molecular alterations leading to different pathways involved in the boost of cell proliferation [4].

The carcinoma bearing the worst prognosis is ATC which nearly almost derive *de novo* or rarely from pre-existing

PTC or FTC from which it may dedifferentiate sometimes revealing transitional stages [5, 6] and, however, may develop into highly malignant ATC with low survival rates. ATC have a high mitotic rate with both hematological and lymphovascular invasions and do not retain any of the biological features of the original follicular cells, such as uptake of iodine and synthesis of thyroglobulin [7]. Due to their molecular alterations and biological inoperable feature these tumors are inaccessible to classical treatment options such as radio- and chemotherapy [8]. New drugs, such as fosbretabulin [9], bortezomib, and TNF-related apoptosis induced ligand (TRAIL) [10] are now introduced and trialed in clinical labs and human clinical studies.

Beside, MTC might also have a good prognosis when it is restricted to the thyroid gland itself [11]. In case of distant metastases moieties of the patients develop a rapidly progressing disease leading to death. Clinical management of this disease is possible only by monitoring the location and expansion of the tumor mass and metastases followed by an extensive surgery and by observing antigens like calcitonin or carcinoembryonic antigen during the follow up [12].

Especially for the noncurative thyroid tumors such as ATC and all invasive and metastasized PTC and FTC it is of substantial interest to develop new approaches for the

treatment of these cancers. Here, we can learn from immunological interventions which have already been applied to patients with other nonendocrine and endocrine cancers including MTC.

2. Advances in Cellular Therapy

An alternative approach for the treatment of cancers may represent the use of cell vaccines aiming to activate the immune system. Antitumor immunity is coordinated by both innate and adaptive immunity, and mainly mediated by cytotoxic T cells (CTLs), natural killer (NK), and natural killer T (NKT) cells. The induction and coordination within this context is arranged by dendritic cells (DCs) [13, 14]. DCs are highly potent antigen-presenting cells with the ability of taking up and processing tumor antigens in the peripheral blood and tissues. They subsequently migrate to the draining lymph nodes to present antigens to naïve T lymphocytes and induce a cellular immune response by direct priming of CD8+ CTLs and a cross-presentation involving CD4+ helper cells. Moreover, DCs are also important in inducing humoral immunity as explained by their capacity to activate naïve and memory B cells [15] and NK [16] as well as NKT cells [17]. Thus, DCs can modulate the whole immune repertoire they represent an excellent tool for treating an existing tumor or preventing its recurrence.

In vivo, two main pathways of DC differentiation have been described depending on their cell lineage and the tissue where they are activated [18]. Under *in vitro* conditions, myeloid DCs may be generated from monocytes by activating with granulocyte/macrophage colony-stimulating factor (GM-CSF). The monocytes which encounter interleukin-4 (IL-4) become DCs known as IL-4-DCs [19–21]. On the other hand, monocytes differentiate into different DC subsets after costimulation with other cytokines such as IFN- α , TNF or IL-15 [22–28]. One essential precondition for the success is the priming of DC with special tumor associated antigens (TAA). These TAA must bear high immunogenic properties against the background of HLA restriction. In this context, the broadest experience has been gained for the therapy of malignant melanoma [29–33] as well as in renal cell carcinoma [34–38]. Even though only limited success has been achieved from the clinical point of view, DC vaccinations are superior compared to other antitumor vaccination strategies [39]. Importantly, DC vaccination strategies have steadily been improved by the number of immunocompetent tumor antigens identified thus far [40–43], even in endocrine malignancies [44]. One way of assessing tumor antigens is based on the use of computer-based algorithm software namely SYFPEITHI or BIMAS [45–48] which helps to predict immunocompetent tumor epitopes. Examples of classical tumor epitopes are those of MAGE, GAGE, and NY-ISO1 in malignant melanoma [49–55].

Alternatively to DC therapy, new strategies were introduced using antigen-specific CTLs or potent NK cells to burst the immune tolerance. Adoptive T-cell immunotherapy is mainly performed by the generation of large numbers of

antigen-specific CTLs. But both tumor-specific CD4+ helper 1 (Th1) cells and cytotoxic CD8+ T cells might be generated *in vitro* and administered directly to patients [56, 57]. Maturation of specific lymphocytes were performed by *ex vivo* exposure to cytokines, HLA-restricted antigen epitopes, and either *in vitro* generated DCs [57, 58] or with CD3 and/or CD28-specific mAb [58, 59].

On the other hand, NK cells are the major representatives of the innate immune system that is regulated by positive and negative mechanisms. NK cells interact with tumor cells through activating receptors of the immunoglobulin superfamily (NKp30, NKp44, NKp46, NKG2D) and the lectin-like type II transmembrane proteins exhibiting a C-type lectin domain (CD94, CD161), and on the other hand, through inhibitory receptors (KIR, e.g., NKG2A) which primarily bind to MHC class I antigens on target cells [60, 61]. Moreover, NK cells express CD16 which serves as a receptor for antibodies to home on target cells performing the antibody dependent cellular cytotoxicity [62, 63].

Within the last decade a multitude of different studies and clinical trials have been performed using cell therapies [64–67]. Cancer immunotherapy using DC or adoptive CTLs has much promise because malignant cells can be affected by the immune system without damaging healthy tissue and without dangerous side effects. Nevertheless, careful monitoring of the elicited T-cell response and quality assurance is mandatory to establish a rationale for specific immunotherapy and to bring it from bench to bedside [68, 69]. In any case, the identification of tumor cell specific antigens is crucial for establishing clinically effective tumor immunotherapies and monitoring the induced immune response, including quantification of antigen-specific CTLs. Other approaches might use NK or NK-T cells, respectively, however with less experiments compared to DC vaccinations [70, 71].

3. Experience in Cellular Therapies for Medullary Thyroid Carcinoma

The idea of using polypeptide hormones as tumor antigens for cancer therapy resulted from the observation in autoimmunity, where responses to self tissue antigens led to tissue damage. The most intensively investigated autoimmune disease is T1DM, which is characterized by infiltration of pancreatic islets by self-reactive lymphocytes, leading to destruction of insulin-secreting β -cells. Insulin itself is probably the most important autoantigen described thus far [72]. This is supported by the fact that many reactive T cells invading pancreatic islets are specific for immune insulin epitopes and are capable of adoptively transferring diabetes in non-obese diabetic (NOD) mice [73, 74].

This violability of the immune system leads to the option to reconstitute immunity by antitumor immunotherapy. One goal was scored by the work of Bradwell and Harvey identifying the use of polypeptide hormones as tumor antigens [75]. They were first to apply a combination of synthetic human and bovine parathyroid hormone (PTH) peptides for vaccination of one patient with metastatic parathyroid

cancer. They demonstrated increased PTH-specific antibody titers, resulting in a notable decrease in serum calcium levels and a relief of clinical symptoms. Nonetheless, no association with reduced tumor mass was observed. Thereafter, Betea et al. [76] performed an immunization trial with bovine and human PTH fragments and with intact full-length human PTH. The effect of this treatment was a specific antibody production to all PTH fragments, resulting in largely diminished PTH and serum calcium levels. Most importantly, they observed a remarkable decrease in tumors of pulmonary metastases, indicating a PTH specific cytotoxic immune response.

Based on this knowledge, the polypeptide hormone calcitonin has been proposed as tumor antigen for immunotherapy in MTC. Since then, several vaccination trials have been performed in murine models and as well in man. Vaccination studies with CT-loaded DCs were performed in a transgenic mouse model for MTC mice displaying the identical mutation (substitution of Cys for Arg) within the *RET* protooncogene at codon 634 as most patients with multiple endocrine neoplasia type 2A [77, 78]. As in patients with hereditary MTC, Ret/Cal mice develop diffuse C cell hyperplasia and MTC with increased serum CT levels [77]. Depending on the CT epitopes used epitope specific CD8+ CTLs were visualized via tetramer analyses and by functional lysis assays. These results were accompanied by a largely diminished tumor outgrowth [79, 80].

In humans, several studies used full-length CT for priming DCs [81, 82]. Importantly, in one patient, a remarkable transient regression of pulmonary and liver metastases was seen. Detailed *in vitro* analyses revealed a CT-specific T-cell reactivity, which was Th1-driven, in some patients, as determined by a large increase in IFN- γ production [83]. Thereafter, a new protocol with interferon- α generated DCs with direct tumor lysis activity was performed [84]. These cells were also loaded with full-length CT [85]. After a long-term follow-up of more than 48 months, two of five MTC patients showed stable disease with changes in tumor size and tumor marker of less than 25%. This is important because it shows a direct connection between induction of cytotoxic immunity and clinical response [85].

4. Potential Tumor Antigens in Poorly Differentiated Thyroid Carcinomas

Tumor-associated antigens (TAA) are surface-associated molecules such as receptors, transmembrane proteins or secreted/membrane-attached peptides that are mostly cancer specific, often overexpressed and recognized by the immune system [120]. Therefore, identifying specific TAA is of key importance for developing new options for immunotherapy for incurable cancers. Up to now, however, no single TAA for primary thyroid carcinomas have been proven but there are a couple of candidates which might have the potential to become one.

Potential tumor antigens which might represent a distinct tumor association can be divided into the groups of

classical cancer testes antigens, specific receptors, functional-associated proteins, and metastases-associated proteins. Find an assembly of potential TAA and of already performed experiments in Table 1.

The most prominent tumor antigens are certainly the *cancer testes antigens*, which have already been identified in many malignancies and which have intensively used in the context of immunotherapy [31–33, 40–42, 50–52]. These antigens belong to a gene family which has been reported to be expressed in tumor cells but not in normal tissues aside from the testicular germ cells where the absence of MHC class-I molecules protect the cells from testicular autoimmunity, as the antigenic peptides are not be displayed at the cell surface [121]. This makes these TAA so attractive for immunotherapy since no side effects are expected. In PTC and FTC the cancer testis genes *MAGE* and *GAGE* were identified in human thyroid carcinomas [86–88]. For instance, *MAGE-3* is detectable in 29% of follicular tumor tissue and in 80% in papillary thyroid carcinomas. This observation explores the possibility of specific immunotherapy using these TAA for vaccination trials.

Another group of potential tumor antigens represent the large group of *receptors*. The main player are the IGF-I receptor in thyroid carcinomas [89, 90, 122] and the receptor tyrosine kinases as EGF-R, PDGF-R α & β , VEGF-R 1&2, c-KIT especially in FTC and some ATC [91, 93, 94]. Whether these receptors are likely to be used as tumor antigens has still to be proven. IGF-I is known to have significant effects on cell proliferation and differentiation, it is a potent mitogen, a powerful inhibitor of programmed cell death, and has a well-established role in the transformation of normal to malignant cells. So, especially the overexpression of the IGF-I receptor might have a possible target function while its presence is important for the development of a malignant phenotype [89, 123]. Up to now, however, only antibody-based therapies were performed [90, 124].

Other receptors untypically expressed in thyroid carcinomas might also be considered. For example, CD10 a common antigen for acute lymphoblastic leukemia has been found to be useful in the differential diagnosis of malignancy. Moreover, it has been shown to be expressed in a group of PTC as well as in papillary microcarcinomas [95] and in some FTC [96]. Several reports implicated the chemokine receptor CXCR4 in thyroid tumor aggressiveness. The target for CXCR4 is the chemokine CXCL12/SDF-1 α & β involved in both embryonic and tumor angiogenesis [97]. This receptor might be also a potent target for a direct CTL offense.

In 2007, characteristic biomarkers were described for PTC lymph node metastasis [98]. By real-time reverse transcription-PCR and immunohistochemistry three genes were discovered consistently overexpressed in lymph node metastasis. Especially, LIM (kinase) domain containing 2 (LIMD2) and the protein tyrosine phosphatase receptor type C (PTPRC also known as CD45) were significantly different expressed in tumor samples versus metastatic samples. Additionally, lymphotxin beta (LTB), a type II membrane anchored protein of the TNF family had borderline significance. Since there are no antibodies for LIMD2, only

TABLE 1: List of possible thyroid tumor associated antigens.

Tumor	Potential TAA	Methods			in vivo	Clinical trial	
		RT-PCR	Biochemical	IHC			
<i>Cancer antigens</i>							
PTC	MAGE	×	[86, 87]	×	[86–88]		
FTC	GAGE	×	[87]	×	[87]		
<i>Receptors</i>							
	IGF-I R	×	[89]	×	[89, 90]		
	EGF-R			×	[91, 92]		
	PDGF-R	×	[93]				
	VEGF-R	×	[93, 94]	×	[94]		
	c-KIT	×	[93]				
	CD10			×	[95, 96]		
	CXCR4			×	[97]		
	LIMD2	×	[98]	×	[98]		
	CD45	×	[98]	×	[98]		
	LTB	×	[98]	×	[98]		
<i>Functional associated</i>							
	Thyroglobulin	×	[99–101]				
	HBME-1			×	[102, 103]		
	CK19	×	[101]	×	[102]		
	Galectins			×	[103]		
	Fibronectin-1	×	[101]	×	[102]		
	Survivin			×	[104]		
	TERT	×	[105–107]	×	[108]		
<i>Metastases associated</i>							
	MMPs			×	[109–111]		
	CD147			×	[109]		
	u-PA/u-PA-R	×	[112, 113]				
	Fascin			×	[114]		
ATC	Autotoxin	×	[115, 116]				
	CD133	×	[117]	×	[117, 118]		
MTC	CT				×	[84]	
	PPCT				×	[80, 85]	
	CEA					×	[81, 82]
	NY-ESO-1		×	[119]			

ATC: anaplastic thyroid cancer; FTC: follicular thyroid cancer; MTC: medullary thyroid cancer; PTC: papillary thyroid cancer.

MAGE/GAGE: melanoma antigens, also known as cancer testis antigen; IGF-I R: insulin growth factor-receptor; EGF-R: epidermal growth factor-receptor; PDGF-R: platelet-derived growth factor-receptor; VEGF-R: vascular endothelial growth factor-receptor; c-KIT: hematopoietic stem cell receptor; CD10: common acute lymphocytic leukemia antigen; CXCR4: stromal cell-derived factor 1 receptor; LIMD2: LIM kinase containing receptor-2; CD45: protein tyrosine phosphatase receptor type C; LTB: lymphotoxin beta membrane anchored protein; HBME-1: human mesothelial cell marker 1; CK19: cytokeratin 19; TERT: telomerase reverse transcriptase; MMPs: matrix metalloproteinases; CD147: basigin, a subunit of the monocarboxylate transporter; u-PA and u-PA-R: urokinase-type plasminogen activator and its receptor; CD133: hematopoietic stem cells antigen; CT: calcitonin; PPCT: preprocalcitonin; CEA: carcinoembryonic antigen; NY-ESO-1: a cancer testis antigen.

RT-PCR: real-time PCR; IHC: immunohistochemistry.

PTPRC and LTB could be tested by immunohistochemistry. All samples tested, showed both proteins in metastases and little or no expression in primary tumor.

Moreover, there are the so-called *functional-associated antigens*. PTC and FTC derive from thyroid epithelial cells resulting in a large expression of thyroglobulin which also

represent a classical tumor marker [99, 100, 125, 126]. Whether thyroglobulin also represents a tumor antigen needs, however, to be proven.

Beside, a couple of other antigens have recently been described for thyroid carcinomas. One of those is a biomarker for the malignancy status of PTC and FTC. The

human mesothelial cell marker 1 (HBME-1) have originally been described in mesotheliomas. Meanwhile, it is known that it is expressed in 90 to 100% of the carcinomas with a strong membrane staining [102, 103]. Additionally, several other antigens are on debate like human type I cytokeratin 19 (CK19), galectin-7, and probably galectin-3 which are overexpressed in papillary and follicular malignancies [102, 127, 128]. CK19 is an acidic protein of 40 kDa that is part of the cytoskeleton of epithelial cells and is highly expressed by differentiated thyroid carcinomas, mainly of the papillary subtype. The soluble fragments of CK19 can already be measured by immunometric assays [129]. Galectins are a structurally related family of lectin proteins that bind specifically to beta-galactoside in a calcium-independent manner; originally they are cytosolic proteins involved in growth regulation and internal processes such as pre-mRNA splicing [130] but they are able to translocate into vesicles due to participate in cell-cell and cell-matrix adhesion [130, 131]. Moreover, elevated levels of fibronectin-1 might be a good target due to its alternative splicing during tumorigenic process which leads to different isoforms of extracellular domains or connecting segments [101, 102]. Likewise, two other TAA which have a broad expression pattern in many types of human malignancies are now described in thyroid carcinomas as well. One is survivin which is overexpressed in poorly differentiated thyroid cancers inducing antiapoptotic processes [104] and the telomerase reverse transcriptase (TERT), that is, concomitant in cancer cells responsible for the stabilization of the telomeres receiving an immortalization of the respective cells [105–108]. Both antigens were already used as targets for several vaccination studies in melanomas and breast cancer [132–137].

Finally, there is the group of *metastases-associated proteins*. The growth of a neoplasm and its ability to metastasize is a multistep process dependent on angiogenesis and immunological reactions of the organism. In this process, adhesive factors like soluble intercellular adhesion molecules (sICAM-1) and vascular cellular adhesion molecules (sVCAM-1) are involved. The serum of peripheral blood of patients with thyroid cancer before surgery revealed these factors in a significant higher concentration compared to controls [138]. Since these soluble factors are egested by the tumor itself and might also be associated to the membrane exhibiting a potent tumor antigen.

Aside, several other factors are described being involved in invasion and metastasis. One main component is the group of matrix metalloproteinases (MMPs) since they disrupt extracellular matrix proteins. An increase of circulating MMP-2 [109, 110, 139, 140], MMP-7 [110, 111], and MMP-9 [110, 112, 141] was affirmed manifold. In this context, CD147 one of the molecules involved in regulating the expression MMP-2 was described to be expressed more frequently in PTC and ATC patients and is associated with their clinicopathologic features [109]. Additionally, the urokinase-type plasminogen activator (u-PA) and its specific receptor (u-PA-R) are involved in the disruption of the extracellular matrix which relies on the activation of plasminogen and an interaction with MMPs [112, 113]. One report advised on an antigen, namely, fascin which is

markedly upregulated in more than 60% PTC. It is displaying an actin-bundling protein, however, associated with high-grade extensive invasion [114].

5. Special Situation in Anaplastic Thyroid Carcinoma

In ATC, a multitude of molecular alterations are found [142, 143] but only a small number of potential antigens have been described. Nonetheless, some potential antigens discovered for FTC and PTC are also found in ATC although molecular variances may also lead to a downregulation of those. The most prominent loss is described for c-Kit which was monitored to be absent in most ATC [144, 145].

Two additional proteins were recently found in ATC. One is called autotaxin which has a nucleotide pyrophosphatase/phosphodiesterase and lysophospholipase D activity. It is usually secreted but also membrane-associated and is a highly bioactive enhancer for motility of thyroid tumors [115, 116]. Beside, expression of CD133 displaying a hematopoietic stem cells antigen was described in tumor derived cells lines [117, 118].

6. Future Perspectives

The research for cellular cancer therapy has bred some promising approaches but until now no single vaccination regimen tested is indicated as a standard anticancer therapy. In order to circumvent the escape of thyroid tumor cells under T-cell pressure, polyvalent vaccination strategies may help to overcome this situation. This goal can be achieved by either loading DCs with a pool of peptide antigens which might be individually identified as TAAs or by adoptive CTL or NK/NK-T cell transfer. The major drawback in many human malignancies including thyroid tumors is, however, the lack of established tumor antigens which, in addition, have already been applied in clinical context. As mentioned above, a multitude of proteins and receptors have been described to be overexpressed in a certain percentage of these thyroid tumors. Whether some of those are true TAAs recognized from cells of the adaptive immune system is still elusive and needs to be clarified. Not till then, they could be used in clinical trials in humans. Nonetheless, it is necessary to search for other potential tumor antigens. Within this context, novel technologies, that is, high-throughput gene microarray, should further be implemented in order to identify new antigens.

Another way of improving present treatment concepts is to use a combination therapy by which tumor cells are selectively affected and the tumor escape mechanisms are accessory blocked or decreased [146]. In this context, conventional chemotherapy has already been supported by a combination with DC vaccination showing some clinical benefits in nonendocrine tumors [147–149]. In endocrine (e.g., thyroid) tumors such data does, however, not exist. More relevant, however, might be a combination of cellular immunotherapy and tyrosine kinase inhibitors (TKIs) affecting target-specific agents. In thyroid malignancies different

TKIs particularly sorafenib, motesanib, vatalanib, and so forth, have already been applied in clinical studies [150–152]. Although TKIs have been described to deplete immunoregulatory, for example, regulatory T cells they also have an effect on all T cells and DCs [153, 154] but interestingly not on NK cells [155]. On the other hand, TKIs' affect DCs to activate NK cells [156–158]. The depletion of T cells by TKIs also resulted in a reconstitution with a predominant expansion of antigen-specific T cells [159] and the higher binding capacity of CTLs to MHC presenting antigens [160]. So, the combination of cellular therapies with targeted molecules including TKIs hold promise for successful cancer therapies in the future.

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