Medizinische Fakultät

der

Universität Duisburg-Essen

Aus der Klinik für

Endokrinologie, Diabetologie und Stoffwechsel

Expression of PD-L1, CTLA-4, and LAG-3 in Human Thyroid Carcinoma

Inaugural – Dissertation

zur

Erlangung des Doktorgrades der Medizin

durch die Medizinische Fakultät

der Universität Duisburg-Essen

vorgelegt von

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2019

DOI: 10.17185/duepublico/70705
URN: urn:nbn:de:hbz:464-20191120-110257-3

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Tag der mündlichen Prüfung: 16.10.2019

CONTENTS

1 INTRODUCTION

1.1 Thyroid gland

The thyroid gland, which lies in the front of the trachea, wrapping around the cricoid cartilage and tracheal rings in human, is a two-lobed gland connected by an isthmus in the middle (Fig. 1A and Fig. 1B) (Hunt et al., 1968). The cricoid cartilage and thyroid cartilage are located above the thyroid gland, and two carotid arteries are situated behind the outer sides of the thyroid gland. The trachea, esophagus and larynx are all behind the thyroid (Fig. 1B).

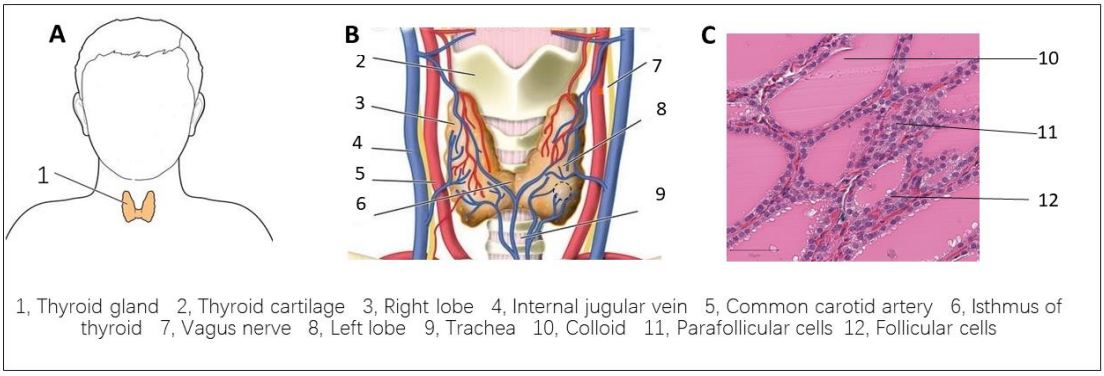

Figure 1. Location, anatomy, and microanatomy of thyroid gland. (Picture A modified from https://cdn.prod-carehubs.net/. Accessed September 15, 2018 and picture B modified from https://healthjade.com/thyroid-gland/. Accessed September 15, 2018).

The thyroid gland is supplied by inferior and superior thyroid arteries, drained via inferior, middle and superior thyroid veins and has an abundant lymphatic system (Hunt et al., 1968). Arteries and veins are paired on both the left and right thyroid lobe. Innervation of thyroid gland derives from the autonomic nerve, including parasympathetic fibers from the vagus nerves and sympathetic fibers from the ganglia of the sympathetic trunk (Kawagishi et al., 2008).

There are three elementary features of the thyroid gland: follicles, thyrocytes, and parafollicular cells (Fig. 1C). The main function of the thyroid gland is the production of

three hormones, triiodothyronine (T3), thyroxine (T4) and calcitonin. T3 and T4 are amino acid derivatives secreted by thyrocytes, whereas calcitonin is produced by parafollicular cells (Vadivelu et al., 1990). It is essential that T3 and T4 levels are within a normal concentration, effecting on all body cells and organs at all stages of life (Robbins, 1981).

1.2 Thyroid carcinoma

Thyroid carcinoma is the most common cancer of the human endocrine system. The incidence of thyroid carcinoma has been increasing dramatically over the last 20 years worldwide and is estimated to be the fourth most common cancer by 2030 (Rahib et al., 2014). As shown in Fig. 2, most of thyroid carcinomas arise from the thyrocytes of the thyroid gland. Thyroid carcinomas, depending on morphology and growth characteristics, have been classified into three categories: differentiated thyroid carcinoma (DTC), poorly differentiated thyroid carcinoma (PDTC), and undifferentiated or anaplastic thyroid carcinoma (ATC) (Alonso-Gordoa et al., 2015). One less common malignancy originating from parafollicular and perifollicular calcitonin-producing cells without the function of secreting thyroid hormone (TH) and taking up iodine, is named medullary thyroid carcinoma (MTC) (Pomorski et al., 2000). Because of PDTC and ATC accompanied with DTC in some thyroid carcinoma tissues, it is speculated that DTC maybe can develop into PDTC or ATC, and PDTC can deteriorate to ATC (Patel and Shaha, 2006).

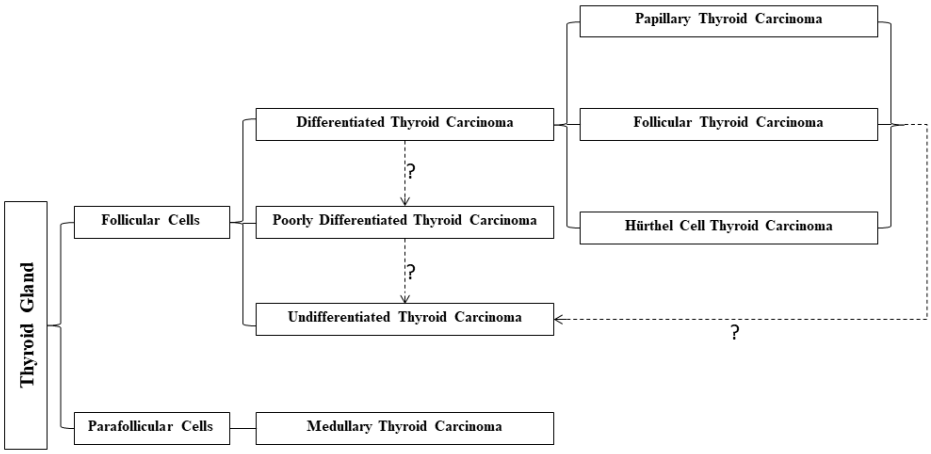

Figure 2. Histological derivation and classification of thyroid carcinoma.

1.2.1 Differentiated thyroid carcinoma

DTC comprises the majority and accounts for about 90% of all thyroid tumors (Jemal et al., 2006; Passler et al., 2004). As shown in Fig. 2, DTC has three sub-types: papillary thyroid carcinoma (PTC), follicular thyroid carcinoma (FTC), and Hürthel cell thyroid carcinoma. PTC is the most common DTC and classically accounts for 75-85% of all DTC. FTC is the second most common DTC with a reported incidence from 10% to 20% of DTC (De Crea et al., 2014; Passler et al., 2004), Hürthel cell thyroid carcinoma comprises the minority and only accounts for less than 5% of DTC (Fagin and Wells, 2016; Lim et al., 2017). DTC shows an indolent character and carries a favorable prognosis even though it frequently metastasizes or recurs to regional lymph node. The overall 10-year survival rate of DTC is about 85% (Eustatia-Rutten et al., 2006).

The therapy options include surgery, radioiodine therapy and levothyroxine substitution usually alone or in combination, according to the guidelines of the European Association of Nuclear Medicine (EANM) and American Thyroid Association (ATA) (Francis et al., 2015; Leenhardt et al., 2004). Surgery, including thyroidectomy with or without neck lymph node resection, has been shown to be the optimal treatment and the first choice for most of DTC (Francis et al., 2015; Leenhardt et al., 2004). Targeted therapy and immunotherapy have been considered for the treatment of advanced DTC in some clinical

trials (Tumino et al., 2017). However, the long-term effectiveness, indications, and side-effects need to be clarified with more patients' data.

1.2.2 Poorly differentiated thyroid carcinoma

PDTC is a thyroid malignant neoplasm showing an intermediate growth pattern between DTC and ATC (Volante et al., 2007). It was first described as "wuchernde Struma" by Langhans in 1907 due to its characteristic nesting pattern (Joll, 1941). In 2004, PDTC was incorporated in the World Health Organization (WHO) classification of thyroid tumors (Volante et al., 2007).

1.2.2.1 Pathogenesis of PDTC

As one of the aggressive thyroid malignancy subtypes, PDTC occurs often in the older patients. Asioli and co-worker reported, the mean age of PDTC is 60.6 years and the prevalence is about 0.5 - 7.0% of all thyroid carcinomas (Asioli et al., 2010). Some studies suggest that risk factors such as iodine deficiency and radiation exposure play a role in PDTC disease development (Asioli et al., 2010). However, LiVolsi and co-workers pointed out that iodine deficiency may be a risk factor for PDTC, but no evidence of the association with radiation exposure and PDTC occurrence has been observed (LiVolsi et al., 2011). Recently the genomic and transcriptomic landscape of PDTC has been studied with a next generation sequencing (NGS) approach (Cha and Koo, 2016). Some studies intend to demonstrate that PDTC is characterized by several oncogenic alterations, such as in telomerase reverse transcriptase (TERT), tumor protein p53 (TP53), proto-oncogene v-Raf murine sarcoma viral oncogene homolog B (BRAF) and phosphatidylinositol-4, 5-bisphosphate 3-kinase, catalytic subunit alpha (PIK3CA) gene (Xu and Ghossein, 2016). However, no genetic mutations or chromosomal rearrangements have been demonstrated to be unique for PDTC, and genetic heterogeneity was less frequent in PDTC than in ATC (Molinaro et al., 2017).

1.2.2.2 Diagnostic criteria of PDTC

PDTC represents intermediated characteristics between DTC and ATC (Patel and Shaha, 2006). The diagnostic criteria of PDTC were heterogeneous, controversial, and hardly applicable in the diagnostic practice until its classification as a separate entity in terms of architecture and high-grade features by the 2004 WHO classification and the 2006 Turin proposal (Volante et al., 2007). More and more pathologists set the cytomorphological characteristics for refining the diagnostic criteria of PDTC. It includes the presence of a solid/trabecular/insular growth pattern, lack of nuclear features of PTC, and presence of one of the following features: (a) convoluted nuclei, (b) tumor necrosis, (c) three or more mitoses per 10 high-power field (Volante et al., 2007).

1.2.2.3 Treatment and prognosis of PDTC

Because of its rarity, biologic aggressiveness and no available standard guidelines, knowledge regarding the optimal management of PDTC remains limited. It is generally accepted that surgery is the most effective therapeutic strategy (Wachter et al., 2018). Beside a total thyroidectomy with lymph node dissection, there are tracheal resections, pharyngo-esophagectomy with reconstruction, and tracheotomy utilized for PDTC because of a higher prevalence of laryngotracheal, esophageal or prevertebral space infiltration and airway obstruction (Volante et al., 2007). Nonetheless, the 5 years survival rate of PDTC is still poor (Walczyk et al., 2010). Some researchers suggested that external beam radiation therapy (EBRT) is typically reserved for these patients with aggressive forms of PDTC (Xue et al., 2017). However, the results of retrospective studies involving EBRT in PDTC patients showed no statistical improvement in overall survival (OS) rates (Kiess et al., 2016). Sanders Jr. et al. recommended, considering the potential for iodine-uptake and very few side effects, ^{131}I therapy in all postoperative PDTC patients who accepted a total thyroid resection (Sanders et al., 2007). Yet, over 15% of patients with

PDTC are associated with a decreased capacity of ^{131}I uptake, resulting in limited radioactive iodine (RAI) ablation in this population (Nikiforov, 2008).

In this ten years, with the development of target multiple kinase pathways, endocrinologists initiated to focus on novel therapeutic agents for PDTC (Grande et al., 2012). Some reports about immune checkpoints and ligand expressions in PDTC with a limited patients number were emerged within two years (Ahn et al., 2017; Antonelli et al., 2018). Unfortunately, the progressions of clinical results are still not enough until now. PDTC exhibits the aggressive characters of malignant behaviors with a limited advance in its prognosis.

1.2.3 Anaplastic thyroid carcinoma

ATC is the most aggressive thyroid malignancy (McIver et al., 2001). About 90% of ATC are accompanied with extra-thyroid spread at the time of diagnosis and about 75% of the patients develop pulmonary or mediastinal metastases (McIver et al., 2001). Because of the aggressive character of ATC, all of its stages were defined as stage IV by the American Joint Committee on Cancer (AJCC) (Kebebew et al., 2005). Relying on the size of the tumor, the region of lymph node involvement, and metastatic status, ATC stage is divided into three levels: stage IVa, IVb, and IVc (Table 1) (Kebebew et al., 2005).

Table 1. Staging of anaplastic thyroid carcinoma.

Classification	Tumor	Lymph nodes	Metastases
Stage IVa	T4a (Tumor does not extend beyond the thyroid capsule)	Any N	M0
Stage IVb	T4b (Tumor extends beyond the thyroid capsule)	Any N	M0
Stage IVc	Any T	Any N	M1

T= Tumor, N= Lymph nodes, M= Metastases, 0= Without, 1= With.

1.2.3.1 Pathogenesis of ATC

ATC mainly occurs in the patients with ages more than 50 years old and with an aggressive histopathologic growth pattern (McIver et al., 2001). ATC dedifferentiation is related with gains and losses in some chromosomal regions and includes the disturbances of signal transduction and cell cycle derangement (Smallridge et al., 2009). Different mutations were described in DTC, several of these mutations have also been found in ATC (Romei et al., 2018). The TP53 tumor suppressor gene mutation, as a late character in undifferentiation, has been found almost exclusively in ATC (Moretti et al., 2000). BRAF is a cytoplasmic serine-threonine protein kinase that plays a critical role in the mitogen-activated protein kinase (MAPK) signaling pathway. Latteyer and co-workers even reported that a higher accumulation of BRAF mutations was found in ATC that arose from PTC, comparing with those without evidence to arose from PTC (Latteyer et al., 2016). Because of lack of sufficient evidence for their clinical utility, the use of these molecular studies is not recommended for diagnosis or management of ATC in most clinical management guidelines presented by the ATA and the European Thyroid Association (ETA) (Leite, 2018; Russ et al., 2017).

1.2.3.2 Clinical presentation and diagnosis of ATC

No specific symptoms and signs can be used for ATC diagnosis. Only locoregional symptoms, such as neck pain, dyspnea, dysphagia and a rapidly growing neck lump are present in most ATC patients (McIver et al., 2001). Other symptoms, including Horner's syndrome, hoarseness, and locoregional hematoma, often are used for a diagnosis of invasion of parasympathetic system, the recurrent laryngeal nerve, or carotid arteries (Broome et al., 2009).

Cytologic examination of fine needle aspiration (FNA) specimen with or without gene analysis is the only method to definitely diagnose ATC preoperatively. If FNA results are suspicious, core or surgical biopsy should be performed to establish the diagnosis of ATC

(Haddad et al., 2015). Although some ATC can be diagnosed with FNA preoperatively and frozen pathological section during operation, most of ATC were diagnosed postoperatively by hematoxylin and eosin (H&E) staining.

1.2.3.3 Treatment and prognosis of ATC

The traditional treatment options for ATC include surgery, chemotherapy and radiotherapy. Multimodality treatment combining with surgery, chemotherapy and radiotherapy is generally recommended (Pezzi et al., 2017). According to the 2012 ATA guidelines, surgery is recommended in ATC patients in stage IVA and IVB and when gross tumor resection is possible (Wendler et al., 2016). Complete resection has been identified as a positive prognostic factor (Sugitani et al., 2001). Unfortunately, the surgery cannot prolong survival as expected, except for the few patients whose cancers are small in size and confined entirely to the thyroid gland, although theoretically there is a potential benefit for ATC morbidity (Wendler et al., 2016). EBRT has been used widely in many carcinomas to increase survival, improve local control and also has been used for palliation (Hodolic et al., 2015; Jain et al., 2007). However, it is not certain beneficial for ATC. Some previous investigations used a variety of chemotherapeutic agents in thyroid carcinomas, but only a few studies compared different chemotherapy regimens in ATC patients (Wendler et al., 2016). Until now, the study suffers from its limited number of ATC patients with radiotherapy or/and chemotherapy, and the OS rate for ATC patients undergo radiotherapy or/and chemotherapy is still poor with commonly less than one year (Pezzi et al., 2017).

1.3 Immune system and cancer immunology

1.3.1 Immune system and cancer

The immune system is a defense system for an organism and plays a crucial role in the maintenance of the integrity of biological organism structures and functions (Li et al.,

2009). In humans, the immune system can be classified into different subtypes, such as humoral immunity and cell-mediated immunity, or innate immune system and adaptive immune system (Choy et al., 2017). One important function of the immune system is the detection of a wide variety of pathogens, such as viruses, bacteria, fungi and parasitic worms, and cancer cells or even abnormal cells (Li et al., 2009).

Cancer is a cluster of diseases disturbing the organ's structure and function, involving the aberrant cell growth with the potential to invade normal cells or spread to other organs of the body. About 90–95% of cancers are due to genetic mutations. The remaining 5–10% of cancers, are due to inherited genetics (Anand et al., 2008). Typically, mutations or alterations in multiple genes are necessary to transform a normal cell into a cancer cell for most malignancies. However, besides providing protection against pathogens, the immune system is also strongly involved in abnormal cells attacking, normal microenvironment maintaining and cancer prevention (Suen et al., 2018). This process is referred to as tumor immune surveillance. Normally, the immune system can target and destroy cancer cells with genetic mutations or molecular alterations (Dunn et al., 2002). However, tumor cells can evade themselves from being detected by T cells through downregulating major histocompatibility complex (MHC) class I molecules, making it difficult for T cells to recognize and kill them. This process is named tumor immune escape (Terry et al., 2017). The immune interactions involved in tumor surveillance and tumor progression have been called "immunoediting" (Dunn et al., 2002).

As shown in Fig. 3, immunoediting is composed of 3 phases: (a) Elimination: Immune cells distinguish and destroy transformed abnormal cells to prevent the development of cancer cells; (b) Equilibrium: The immune system recognizes and kills tumor cells, some tumor cells evade detection and elimination by the immune cells. When tumor and immune system keep in a dynamic balance, the tumor cells appear to be clinically dormant; (c) Escape: Immunosuppressive mechanisms or genetic mutations allow tumor cells to escape from detection and control by the immune system and grow in an unrestricted way.

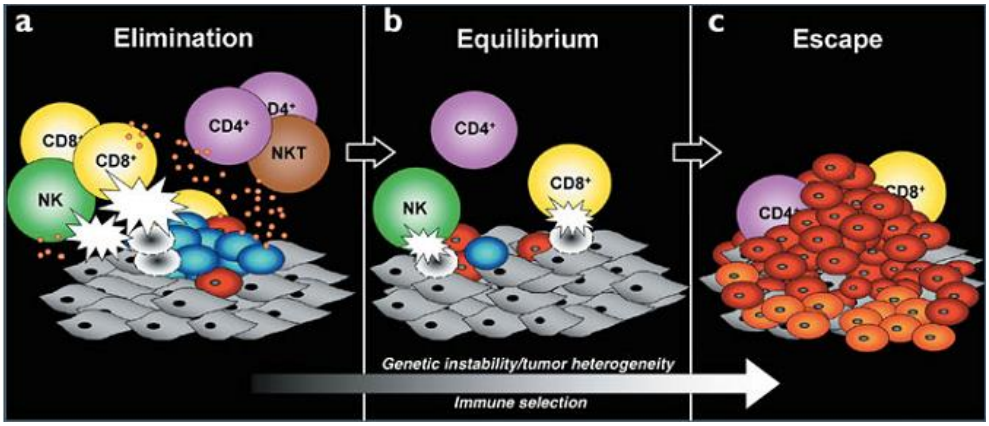

Figure 3. Mechanisms of immunoediting (Dunn et al., 2002). Cancer immunoediting encompasses three processes. (a) Elimination corresponds to immunosurveillance. (b) Equilibrium represents the process by which the immune system iteratively selects and/or promotes the generation of tumor cell variants with increasing capacities to survive immune attack. (c) Escape is the process wherein the immunologically sculpted tumor expands in an uncontrolled manner in the immunocompetent host. Blue cells: developing tumor cells. Red cells: variants tumor cells. Gray cells: underlying stroma and non-transformed cells. Orange cells: additional variants tumor cells. NK=Natural killer cells.

1.3.2 Cancer immunotherapy

Generally, cancer immunotherapy (CI) is the treatment which relates to the immune system to fight cancer. CI has two subcategories: passive immunotherapy and active immunotherapy (shown in Table 2) (Zhang and Chen, 2018). Passive immunotherapy is the administration of agents such as monoclonal antibodies (MAbs) and lymphocytes that enhance existing anti-cancer response. Active immunotherapy is the process of promoting self-immune system to attack tumor cells via vaccination or non-specific immunomodulation (Zhang and Chen, 2018). CI is rapidly advancing and considered to be the most positive therapy for some lethal malignancies in the future.

Table 2. Classification of cancer immunotherapy (Zhang and Chen, 2018).

Passive immunotherapy		Active immunotherapy	
Immunomodulating antibodies -Checkpoint inhibitors -Immune co-stimulatory antibodies	Adoptive immunotherapy -Tumor-infiltrating lymphocytes -T-cell receptor (TCR) gene-modified lymphocytes -Chimeric antigen receptors (CARs)	Specific -Vaccines	Non-specific -Immune adjuvants -Cytokines

1.3.2.1 Cytokines and immune adjuvants

Cytokines are immunomodulating agents secreted by immune-related cells and can mediate endocrine, paracrine or autocrine signaling with non-specific activity of immunotherapy (Dinarello, 2007). Cytokines present a instantly responding reaction, promoting the immune cells to communicate with each other, to mediate a coordinated, rapid and efficient response to a target antigen (Lee and Margolin, 2011). Several cytokines, including granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon-α (IFN-α), interferon-γ (IFN-γ), interleukin-2 (IL-2), IL-8, IL-12, IL-15 and IL-18, are reported with the activity of anti-tumor or resisting the malignancies growth (Vacchelli et al., 2016). However, partial overall clinical response and high toxicity of both IL and IFN lead to a limited option for CI (Ni and Lu, 2018).

Immune adjuvants are compounds or macromolecular complexes that can regulate and enhance the ability of immune responses (de Brito et al., 2009). They are also able to improve lymphocytes role, boost the function of antitumor cells and even enhance antigen-specific reactions (de Brito et al., 2009). But most of them are still nonspecific immune adjuvants with a limited anti-tumor response (Lim, 2015). Thus, immune

adjuvants and cytokine-based immunotherapy can be recognized as a helpful but not optimal option for thyroid carcinoma treatment for its non-specific response to tumor.

1.3.2.2 Monoclonal antibodies

MAbs are modified proteins used to combine with a specific molecule of signal transduction pathways in cancer or act with immunological regulating procedures (Teillaud, 2012). By engineering MAbs against functional targets, anti-cancer effects have been achieved in some preclinical or clinical malignancy models (Maleki et al., 2013). The biological response to MAbs depends on different variables including the function of its target, the antibody avidity and effector function. Some efficient compounds with various functions such as cytotoxic chemotherapeutic agents, radionuclides or toxins can be added to MAbs for producing immunoconjugates with a corresponding function for fighting tumor (Teillaud, 2012). Food and Drug Administration (FDA) has already approved MAbs for the treatment of some malignancies, and also several new MAbs clinical trials are now ongoing every year (Henricks et al., 2015). Naked MAbs are the most common type of MAbs for resisting malignant tumors and can promote the immune response against malignancies (Cartron and Watier, 2017). However, until now, no investigations for the application of MAbs in ATC and PDTC were reported.

1.3.2.3 Adoptive T cell therapies

Adoptive cell transfer (ACT) of tumor-associated antigen-specific T cells is a very attractive strategy of immunotherapy for human malignant tumors. Some studies of ACT utilizing tumor-infiltrating lymphocytes (TILs) have affirmative clinical results in metastatic melanoma patients (Schmitt et al., 2015). This approach has a bright future for most malignancies. However, the utility is limited by the difficulty in expanding viable TILs (Hawkins et al., 2010). Another problem with the application of ACT in thyroid carcinomas is that it is a highly personalized treatment and it is not easy to fit into

appropriate modes of oncological treatment in thyroid carcinomas (Schmitt et al., 2015). The strategy is labor-intensive and requires laboratory expertise. Besides that, a new reagent needs to be created for each thyroid carcinoma patient and this makes it less economic benefits and difficult to commercialize.

1.3.2.4 Cancer vaccines

Cancer vaccines, as one of the most prospective strategies of immunotherapy, take effects by restoring or stimulating the capability of the immune system to fight human malignancies (Gilboa, 2016). It consists of therapeutic vaccines and preventive vaccines (Speiser and Flatz, 2014). In this decade, therapeutic cancer vaccines achieved remarkable preclinical and clinical advances, for the progression of personalized cancer vaccines (Gouttefangeas and Rammensee, 2018). The mechanism of preventive vaccine is recognizing antigens in infectious agents for the immune system and preventing cancer from developing in organism (Speiser and Flatz, 2014). Some cancers caused by oncoviruses seem to have the preferable progress, such as cervical cancer and some liver cancers were respectively caused by human papilloma virus (HPV) and hepatitis B virus (HBV). It is more helpful for cancer vaccines to against those viruses and prevent those types of cancer (Hussein et al., 2016). One cancer vaccination is to separate antigens from cancer cells and immunize patients against those antigens, by stimulating the immune system to destroy the tumor cells (Li et al., 2018). Another method is to induce an immune response to attack cancers in situ using oncolytic viruses (Li et al., 2018). It is still difficult to produce a tumor-specific therapeutic vaccine with optimal treatment effects for most malignancies. Until now, no evidence was shown that cancer vaccines are available for thyroid carcinoma.

1.3.2.5 Immune checkpoint proteins and inhibitors

With the development of immunosurveillance and tumor immune evasion theories, there are various immune checkpoint molecules that have been detected on the membrane of

cancer or T cells, such as programmed cell death receptor 1 (PD-1)/ programmed death-ligand 1 (PD-L1), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), and lymphocyte-activation gene 3 (LAG-3) (Marcq et al., 2017b). It is well known that only when checkpoints have been blocked by inhibitors, T cells can be activated and begin to attack and kill abnormal or tumor cells (shown in Fig. 4) (Sweis and Luke, 2017). Because there are more and more dramatic effects in many malignancy treatments with immune checkpoint inhibitors, immunotherapy has started to revolutionize the therapy of several malignancies in the past five years (Yan et al., 2018).

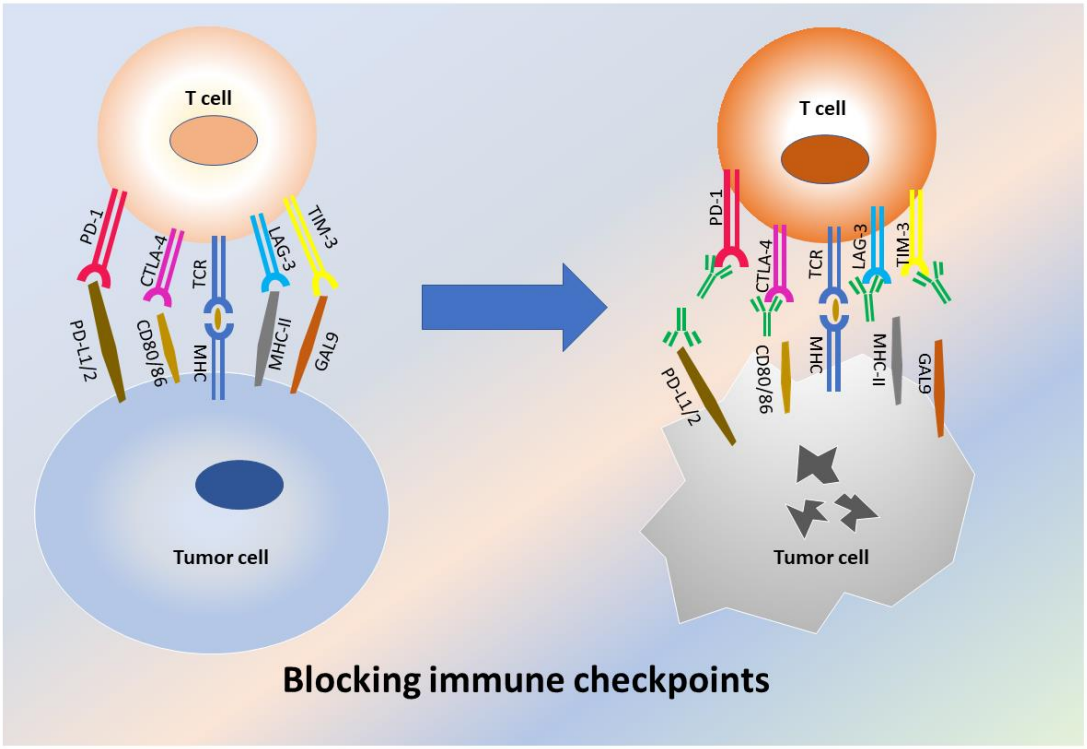

Figure 4. Immune checkpoints and blocking mechanism. PD-L1= Programmed death-ligand 1, PD-1= Programmed cell death receptor 1, CTLA-4= Cytotoxic T-lymphocyte-associated protein, LAG-3= Lymphocyte-activation gene-3, TIM-3= T-cell immunoglobulin and mucin-domain containing-3, GAL9= Galectin-9, MHC= Major histocompatibility complex, TCR= T-cell receptor.

1.3.2.5.1 PD-1/PD-L1

PD-1 is a type of transmembrane protein with a structure of an intracellular domain and an extracellular part connected by a transmembrane region (Ishida et al., 1992). The intracellular domain contains two phosphorylation sites located in an immunoreceptor tyrosine-based inhibitory motif and an immunoreceptor tyrosine-based switch motif (Ishida et al., 1992). PD-1 is expressed mainly on the surface of activated T cells, B cells, and macrophages, as an immune checkpoint receptor, negatively regulating immune responses (Reiss et al., 2014). PD-1 has two ligands, PD-L1 and programmed death-ligand 2 (PD-L2), which both are members of the B7 family (Ghiotto et al., 2010). PD-L1, known as cluster of B7 homolog 1 (B7-H1), is a membrane protein that in humans is encoded by the CD274 gene (Ghiotto et al., 2010). PD-1/PD-L1 pathway can negatively regulate human immune response in malignancies. As one of well-known ligands, PD-L1 was also detected as a receptor of immune checkpoint expressed in variety cancer cells (Pai et al., 2016).

Many scientists and physicians have accumulated extensive knowledge and clinical experiences regarding hPD-1 gene and PD-1/PD-L1 inhibitors (Su et al., 2018). There are abundant of PD-1 and PD-L1 inhibitors applied for clinical malignancy therapy after the approval of FDA of America (Fig. 5).

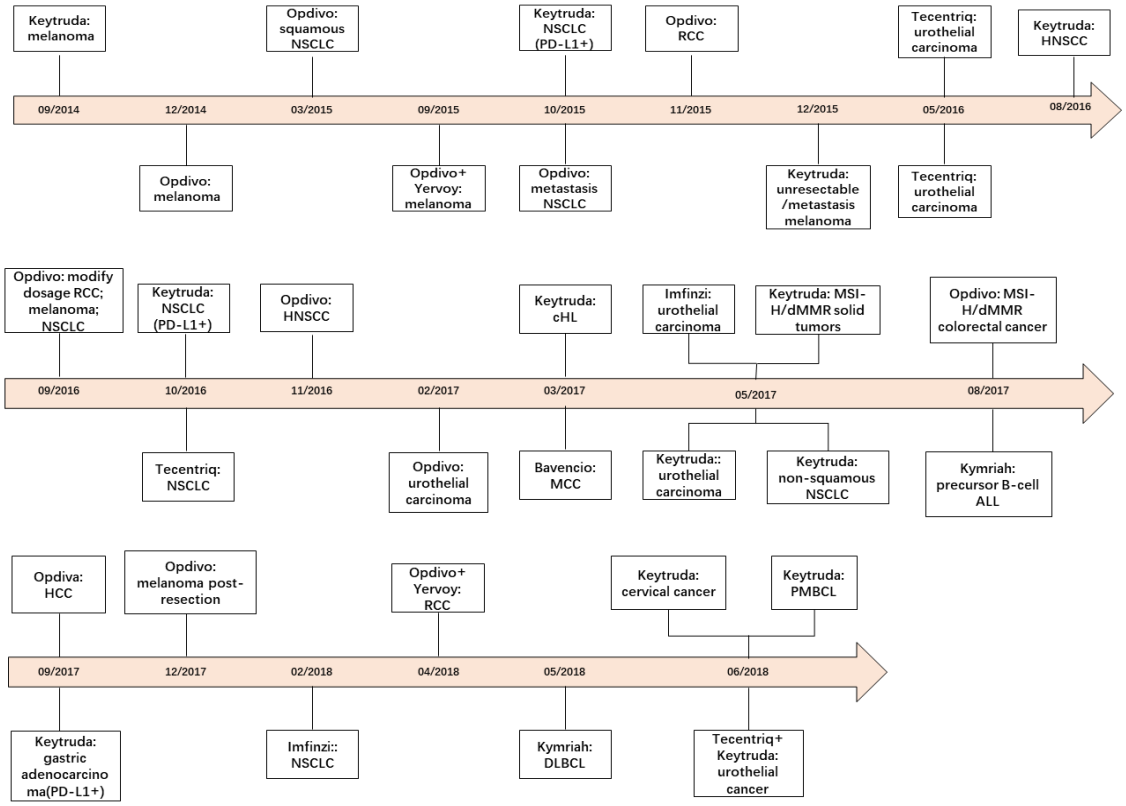

Figure 5. A timeline of FDA PD-1/PD-L1 inhibitors approvals. FDA= Food and Drug Administration, ALL= Acute lymphoblastic leukemia, NSCLC= Non-small cell lung cancer, PD-L1+= Programmed death-ligand 1 positive, RCC= Renal cell carcinoma, cHL= Classical Hodgkin lymphoma, HNSCC= Head and neck squamous cell carcinoma, MCC= Merkel cell carcinoma, MSI-H= Microsatellite Instability-High, dMMR= Mismatch Repair Deficient, HCC= Hepatocellular Carcinoma, DLBCL= Diffuse large B-cell lymphoma, PMBCL= Primary mediastinal large B-cell lymphoma. (Materials from https://www.fda.gov/. Accessed September 20, 2018.)

One retrospective study of thyroid carcinoma shows that PD-1/PD-L1 expression is associated with features of aggressiveness and poor prognosis of patients (Ahn et al., 2017). Because of the paucity of therapies and poor prognosis, PDTC and ATC are also detected by PD-1/PD-L1 targets as the novel targets for a prospective immunotherapy in these malignancies (Rosenbaum et al., 2018; Zwaenepoel et al., 2017).

1.3.2.5.2 CTLA-4

CTLA-4 is a protein receptor known as CD152 and encoded by the CTLA-4 gene in humans. It is expressed in regulatory T cells but only upregulated in T cells after activation (Syn et al., 2017). When bound to CD80 or CD86 on the surface of antigen-presenting cells (APCs), CTLA-4 acts as a "shut-off" switch and transmits an inhibitory signal to T cells (Syn et al., 2017). However, the mechanism by which CTLA-4 acts in T cells remains somewhat unclear.

Antibodies to CTLA-4 may emerge additional effects when used *in vivo*, by binding and exhausting regulatory T cells (Simpson et al., 2013). Recently, CTLA-4 inhibitors have shown efficacy in overcoming immune suppression in a subset of ongoing preclinical and clinical trials of malignancies, e.g. melanoma, solid tumor, non-small cell lung cancer (NSCLC), breast cancer, prostate cancer, and many other cancers (Fig. 6) (Du et al., 2018).

Although studies about the immune landscape of PTC ongoing for an immunotherapeutic implication (Na and Choi, 2018), investigations about thyroid carcinoma with CTLA-4 protein and CTLA-4 checkpoint inhibitors are rarely reported. The role of CTLA-4 and immune response in thyroid carcinoma, especially in ATC and PDTC, remains to be elusive.

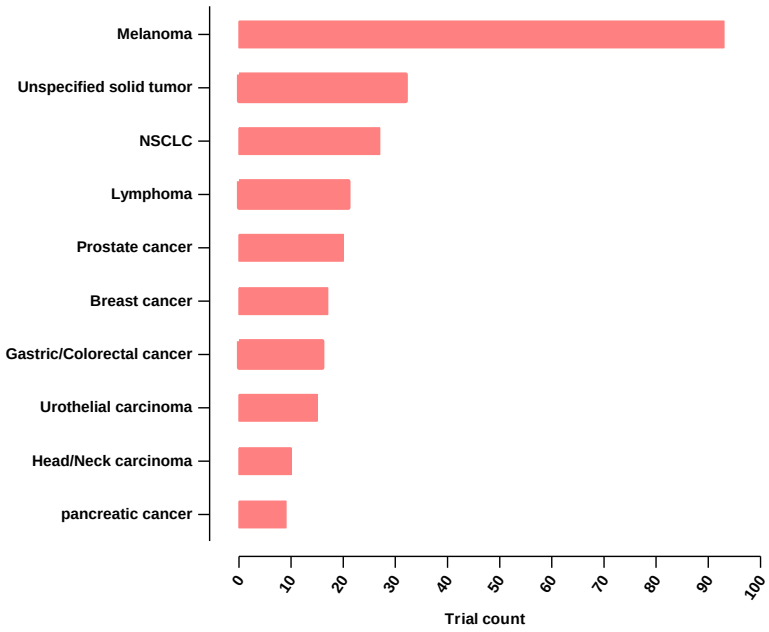

Figure 6. Top 10 cancers by ongoing CTLA-4 clinical trials. NSCLC= Non-small cell lung cancer. (Materials from https://clinicaltrials.gov/. Accessed September 20, 2018.)

1.3.2.5.3 LAG-3

LAG-3, initially discovered in an IL-2-dependent natural killer (NK) cell line in 1990 (Triebel et al., 1990), is a protein encoded by the LAG-3 gene in humans. As a cell surface molecule with multiple biologic effects on T cell function, it is expressed on both CD4 and CD8 T cells, B cells, NK cells, and plasmacytoid dendritic cells and its main ligand is MHC class II, normally it binds with ligand to keep the target cells out of being recognized and destroyed (Huard et al., 1995).

Although the precise mechanisms by which LAG-3 negatively regulates T cell function are not completely clear, LAG-3 negatively modulates cellular proliferation, activation, and homeostasis of T cells which seems to play a role in Treg suppressive function (Huang et al., 2004). Therefore, LAG-3 may be a better checkpoint inhibitor target than CTLA-4 or PD-1 in theory, because the antibodies to these two checkpoints only activate T cells function, but do not inhibit Treg activity, whereas a LAG-3 antagonist can both

downregulate the LAG-3 inhibiting signal to activate T cells and inhibit induced Treg suppressive function (Sega et al., 2014).

Recently, it has been reported that immune responses can be strongly enhanced by blockade of LAG-3 or dual blockade of LAG-3 and PD-1 (Lichtenegger et al., 2018). In 2017, it was reported that LAG-3 is expressed on TILs in NSCLC tissues, also related to a poor prognosis (He et al., 2017). Three approaches involving LAG-3 clinical development launched by the U.S. Federal Government were ongoing in these years. One is a soluble LAG-3, named IMP321, which activates dendritic cells. The other two are antibodies to LAG-3, BMS-986016 and GSK2831781, both of them take the brakes off the anti-cancer immune response (information from https://clinicaltrials.gov/. Accessed July 22, 2018). Although the combination therapies are also ongoing involving LAG-3 antibodies and CTLA-4 or PD-1 antibodies (Syn et al., 2017), observations in more patients will be required to determine the potential therapeutic mechanisms. Unfortunately, LAG-3 data in thyroid carcinoma are still missing.

1.4 Summary

In summary, thyroid carcinoma, as the most common endocrine cancer with an increasing incidence, is still challenged by the aggressiveness and poor prognosis of some subtypes: PDTC and ATC. Furthermore, the knowledges of immune checkpoint inhibitors and immunotherapy in cancer have been developed very fast and shown as a good prospective for cancer treatment. It is highlight for us to find the optimal immune checkpoint target and inhibitor for the therapies of those lethal thyroid carcinomas by detecting the expression of different immune checkpoint proteins in thyroid carcinoma tissue.

2 AIMS

2.1 Hypothesis

We hypothesize that the expression of immune checkpoint and ligand proteins, including PD-L1, CTLA-4, and LAG-3, can be detected in human thyroid malignant tissues. Furthermore, we expect differences of the immune checkpoint and ligand protein expression patterns between normal thyroid, thyroid adenoma, DTC (PTC and FTC), PDTC, and ATC tissues. The characteristics of immune checkpoint and ligand protein expression in PDTC and ATC will benefit for an improved understanding of the patient immune system and tumor microenvironment in thyroid malignancies and will better elucidate which PDTC and ATC patients can derive benefit from the promising immune checkpoint inhibitors of these target proteins.

2.2 Aims

To improve the knowledge of immune checkpoint and ligand protein characteristics in different subtypes of thyroid carcinoma to find a prospective novel treatment strategy in treating especially PDTC and ATC.

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Patients and tissues

Thyroid formalin-fixed paraffin embedded (FFPE) tissues from 105 postoperative patients were used in the study. Tonsil FFPE tissues were taken from one chronic inflammation tonsil patient. Of these, 97 thyroid samples and the tonsil tissues were obtained from the Institute of Pathology at the University Hospital Essen, Essen, Germany and eight thyroid carcinoma samples (4 PDTC and 4 ATC) were obtained from the Department of Pathology in the First Affiliated Hospital of Gannan Medical University, Jiangxi, China.

There are 17 normal thyroid, 14 thyroid follicular adenoma and 74 cases of thyroid carcinoma tissues subdivided into 19 PTC, 13 FTC, 23 PDTC, and 19 ATC. The tonsil tissues were used for PD-L1, CTLA-4 and LAG-3 positive control (Kassardjian et al., 2018; Lyford-Pike et al., 2013; Marcq et al., 2017a).

3.1.2 Reagents/Supplies

All reagents and supplies used in the study, including for H&E and immunohistochemistry (IHC) staining, are shown in Table 3. Ethanol (100%), deionized and sterile distilled water were produced in-house at the pharmacy of the University Hospital Essen, Essen, Germany.

Table 3. Reagents/Supplies.

Name	Serial No. or type	Company
Xylene	1.317.691.612	AppliChem GmbH (Darmstadt, Germany)
Neoclear	1098435000	Merck & Co (New Jersey, USA)
10 x Citrate buffer pH 6.0	ZUC028-500	Zytomed Systems GmbH (Berlin, Germany)
10 x T-EDTA buffer pH 9.0	ZUC029-500	Zytomed Systems GmbH (Berlin, Germany)
Hydrogen peroxide 35%	9683.4	Carl Roth GmbH (Karlsruhe, Germany)
Wash buffer (20 x)	ZUCO20-2500	Zytomed Systems GmbH (Berlin, Germany)
Cytochem plus (HRP) polymer bulk kit (including: Preblock, antibody dilution, postblock)	POLHRP-100	Zytomed Systems GmbH (Berlin, Germany)
DAB substrate kit (550 Tests)	DAB057	Zytomed Systems GmbH (Berlin, Germany)
Hematoxylin	1.023.101.000	Süsse Labortechnik GmbH (Gudensberg, Germany)
Eosin 0.5%	1.09844.1000	Merck & Co (New Jersey, USA)
Entellan	1.07960.0500	Merck & Co (New Jersey, USA)
Normal goat serum	G9023	Sigma-Aldrich Chemie GmbH (Munich, Germany)

Cover slips	24*60mm,24*50mm	Engelbrecht (Bremerhaven, Germany)
Slides packages	J1800AMNZ	Thermor Fisher Scientific Inc. (Waltham, USA)
Safeseal microtube	0.5, 1.0, 1.5, 2.0 ml	Sarstedt Ag & Co. Kg (Nümbrecht, Germany)
Pipette tip	10, 20, 50, 100, 200 µl	Sarstedt Ag & Co. Kg (Nümbrecht, Germany)
PaP pen	Z672548-1EA	Sigma-Aldrich Chemie GmbH (Munich, Germany)
Ethanol 100%		In-house at the pharmacy of the University Hospital Essen (Essen, Germany)
Sterile distilled water		In-house at the pharmacy of the University Hospital Essen (Essen, Germany)
Deionized water		In-house at the pharmacy of the University Hospital Essen (Essen, Germany)

T-EDTA= Trypsin-ethylene diamine tetra acetic acid, HRP= Horseradish peroxidase, DAB= 3,3′-Diaminobenzidine.

3.1.3 Antibodies and solutions

Antibodies, solutions and buffers used in the study for H&E and IHC staining of immune checkpoint proteins, are shown in Table 4 and Table 5. Solutions and buffers were prepared by experimental technicians in the research laboratory of the Department of Endocrinology, Diabetes and Metabolism or in-house in the pharmacy of the University Hospital Essen.

Table 4. Primary antibodies, secondary antibodies and cytochem plus (HRP) polymer bulk kit.

Name	Serial No.	Species	Company
PD-L1 antibody	325600	Monoclonal mouse anti-human PD-L1	Dako North America, Inc. (Carpinteria, USA)
CTLA-4 antibody	BSB2883	Monoclonal mouse anti-human CTLA-4	BioSB (Salt Lake City, USA)
LAG-3 antibody	HPA013967	Polyclonal rabbit anti-human LAG-3	Atlas Antibodies AB (Bromma, Sweden)
Cytochem plus (HRP) Polymer Bulk Kit	POLHRP-100	Poly HRP	Zytomed Systems GmbH (Berlin, Germany)
HRP antibody	A0545	Goat anti rabbit IgG-HRP	Sigma-Aldrich Chemie GmbH (Munich, Germany)

PD-L1= Programmed death-ligand 1, CTLA-4= Cytotoxic T-lymphocyte-associated protein 4, LAG-3= Lymphocyte-activation gene 3, HRP= Horseradish peroxidase.

Table 5. Solutions and buffers.

Name of solution	Volume	Compose
1 x Wash buffer	1000 ml	50 ml Concentrated wash buffer (20x) 950 ml Deionized water
3% H_2O_2 solution	70 ml	6 ml 35% H_2O_2 64 ml Deionized water
1 x Citrate buffer	1000 ml	100 ml Concentrated citrate buffer (10x) pH 6.0 900 ml Deionized water
1 x T-EDTA buffer	1000 ml	100 ml Concentrated T-EDTA buffer (10x) pH 9.0 900 ml Deionized water
Ethanol 96%	1000 ml	960 ml 100% Ethanol 40 ml Deionized water
Ethanol 70%	1000 ml	700 ml 100% Ethanol 300 ml Deionized water
ZytoDAB solution	1.25 ml	1.25 ml Buffer + 1 drop DAB (toxic) prepared immediately before use
Hematoxylin	70 ml	14 ml Hematoxylin (concentrated) 56 ml Deionized water
Eosin 0.3%	70 ml	42ml Eosin 0.5% 28 ml Deionized water
10% Normal goat serum	1000 µl	100 µl Normal goat serum 900 µl Wash buffer
Goat anti rabbit HRP	1000 µl	5 µl Goat anti rabbit HRP 995 µl Wash buffer

T-EDTA= Trypsin-ethylene diamine tetra acetic acid, DAB= 3,3′-Diaminobenzidine, HRP= Horseradish peroxidase.

3.1.4 Equipment and softwares

The instruments used in the experiments including the equipments used in H&E and IHC staining, the microscope, the digital scanscope system and the results analysis software are shown in Table 6.

Table 6. Equipment and softwares.

Instrument	Type	Company
Slides manual stainer	Manual stainer 12 bowls	DIAPATH (Martinengo, Italy)
Rotary microtome	Sigma 151	Sigma-Aldrich Chemie GmbH (Munich, Germany)
Tissue section float baths	GFL tissue float bath 1052	GFL (Hanover, Germany)
Drying and heating chambers	M053-230V[1]	BINDER GmbH (Tuttlingen, Germany)
Water bath	Hera cell 240	Thermor Fisher Scientific Inc. (Waltham, USA)
Pipette	0.5-10, 2-20, 10-100, 20-200, 100-1000 μL	Sigma-Aldrich Chemie GmbH (Munich, Germany)
Timer	Oregon scientific timer 73x66x18	Oregon Scientific (Oregon, USA)
Centrifuges	MAGAFUGE 16R	Thermor Fisher Scientific Inc. (Waltham, USA)
IKA shakers	Vortex 4 basic	IKA Works GmbH & Co. (Staufen, Germany)
Analytical balance	SI-114A	Denver Instrument (Denver, USA)

Microscope	Olympus BX51	Olympus Europe GmbH (Hamburg, Germany)
Aperio scanscope system	Aperio AT2	Leica Biosystems GmbH (Nussloch, Germany)
Pathology analysis software	QuPath (0.1.2)	Oracle Corporation (Redwood Shores, USA)
Statistic analysis software	GraphPad Prism 6.0	GraphPad Prism (San Diego, USA)

3.2 Methods

All investigations and experiments were conducted with the approval of the local ethics committee at the University Hospital Essen, Medicine Faculty, Essen, Germany, and the Medical Ethics Committee of Gannan Medical University, Ganzhou, P. R. China. Informed consent (The copy of certification in appendix 1: Medical ethics committee approval 1, Nr.12-5133-BO and appendix 2: Medical ethics committee approval 2).

3.2.1 H&E staining

3.2.1.1 H&E staining procedures

At least one slide from each FFPE sample was H&E stained for clinicopathological diagnosis. The procedure for H&E staining includes five steps: deparaffinization and rehydration, hematoxylin staining, eosin staining, dehydration, and embedding with entellan. The exact steps are shown in Table 7.

Table 7. H&E staining procedures.

Procedures	
• **Deparaffinization and rehydration**	
Neoclear	3 x 5 min
100% Ethanol	2 x 3 min
96% Ethanol	2 x 3 min
70% Ethanol	1 x 3 min
Rince with deionized water	once
• **Hematoxylin staining**	
Hematoxylin	5 min
Rince with deionized water	twice
Warm running tap water	10 min
• **Eosin staining**	
Eosin 0.3%	45 sec
Rince with deionized water	twice
• **Dehydration**	
70% Ethanol	Rince 2 sec
96% Ethanol	2 x 3 min
100% Ethanol	2 x 3 min
Neoclear	3 x 5 min
• **Embed with entellan**	
Slides were embedded with 2-3 drops of entellan, covered with a coverslip and dried.	

3.2.1.2 H&E staining results analysis

All samples were diagnosed and classified into different groups by certified pathologists according to the WHO classification (Hedinger et al., 1989). The diagnosis of thyroid

carcinoma was confirmed by one independent certified pathologist of the institute of Pathology at the University Hospital Essen, Essen, Germany. All H&E staining slides were scanned by the Aperio ScanScope AT2 system (Leica Biosystems GmbH, Wetzlar, Germany) and analyzed by pathology analysis software Qupath (0.1.2) system (Oracle Corporation, USA). TILs were evaluated on H&E stained sections.

3.2.2 Immunohistochemistry

3.2.2.1 Immunohistochemistry procedures

FFPE tissue sections were cut to generate 4-μm slides. Each slide was IHC stained using the following antibodies: anti PD-L1, anti CTLA-4 and anti LAG-3. Negative and positive controls were included in the experimental set-up. Each batch of IHC staining utilized a FFPE section of benign human tonsillar tissue as a positive control (Kassardjian et al., 2018; Lyford-Pike et al., 2013; Marcq et al., 2017a). As negative control, the same type of thyroid sample was treated in each batch as described in Table 8, except that the primary antibody was replaced by a solution of 1x Wash Buffer.

The procedures for IHC staining include seven steps: deparaffinization and rehydration, antigen retrieval, inactivation of endogenous peroxidase, immunostaining, hematoxylin staining, dehydration, and embedding with entellan. Citrate buffer retriever (1x) was used for subject tissues to heat epitope retrieval in three antibody groups. A 95 °C water bath was used for epitope retrieval heating method. The step of inactivation of endogenous peroxidase is blocking samples with 3 % H_2O_2 solution in a cuvette for 15 minutes.

The step of immunostaining includes primary antibody reaction, secondary antibody reaction, and biotin detection. Cytochem Plus (HRP) Polymer Bulk Kit (No. POLHRP-100, Zytomed Systems GmbH, Berlin, Germany), including preblock, antibody dilution, poly HRP, and postblock, for primary and secondary antibody reaction steps. DAB Substrate Kit (No. DAB057, Zytomed Systems GmbH, Berlin, Germany) was used in biotin detection step.

Table 8. Immunohistochemistry procedures.

Procedures	PD-L1	CTLA-4	LAG-3
• **Deparaffinization and rehydration**			
Xylol	3 x 5 min	3 x 5 min	3 x 5 min
100% Ethanol	2 x 3 min	2 x 3 min	2 x 3 min
96% Ethanol	2 x 3 min	2 x 3 min	2 x 3 min
70% Ethanol	1 x 3 min	1 x 3 min	1 x 3 min
Rince with deionized water	once	once	once
• **Antigen retrieval**			
95 °C Water bath	45 min	45 min	45 min
	1 x Citrate buffer	1 x Citrate buffer	1 x Citrate buffer
Cooling down at RT	30 min	30 min	30 min
Encircle area of tissue	PapPen	PapPen	PapPen
1 x Wash buffer	2 x 2 min	2 x 2 min	2 x 2 min
• **Inactivation of endogenous peroxidase**			
Block with 3 % H_2O_2 solution	15 min	15 min	15 min
1 x Wash buffer	3 x 5 min	3 x 5 min	3 x 5 min
• **Immunostaining**			
Add 50 µl Pre Block	10 min RT	10 min RT	10 min RT
1 x Wash buffer	1 x 2 min	1 x 2 min	1 x 2 min
Add 50 µl antibody	PD-L1 dilution 1:30	CTLA-4 dilution 1:25	LAG-3 dilution 1:200
	2 hours RT	overnight 4°C	overnight 4°C
1 x Wash buffer	3 x 5 min	3 x 5 min	3 x 5 min
Add 50 µl post block	20 min RT	15 min RT	15 min RT

1 x Wash buffer	3 x 5 min	3 x 5 min	3 x 5 min
Add 50 µl poly HRP	30 min RT	30 min RT	30 min RT
1 x Wash buffer	3 x 5 min	3 x 5 min	3 x 5 min
Add 50 µl ZytoDAB	10 min RT	10 min RT	10 min RT
1 x Wash buffer	3 x 5 min	3 x 5 min	3 x 5 min
Rince with deionized water	twice	twice	twice
• **Hematoxylin staining**			
Hematoxylin	2 min	2 min	2 min
Warm running tap water	4 min	4 min	4 min
Rince with deionized water	once	once	once
• **Dehydration**			
70% Ethanol	1 x 1 min	1 x 1 min	1 x 1 min
96% Ethanol	2 x 1 min	2 x 1min	2 x 1min
100% Ethanol	2 x 1 min	2 x 1 min	2 x 1 min
Xylol	3 x 5 min	3 x 5 min	3 x 5 min
• **Embed with entellan**			

PD-L1= Programmed death-ligand 1, CTLA-4= Cytotoxic T-lymphocyte-associated protein, LAG-3= Lymphocyte-activation gene 3, T-EDTA= Trypsin-ethylene diamine tetra acetic acid, RT= Room temperature, HRP= Horseradish peroxidase, DAB= 3,3′-Diaminobenzidine.

The last three steps in each antibody group were hematoxylin staining, dehydration, and embedding with entellan. Every rinse procedure was gently finished with wash buffer in a wash bottle and incubation step was placing the slides at RT or 4°C in a humidity chamber in horizonal level. The whole IHC procedures for the three antibodies are shown in Table 8.

3.2.2.2 Immunohistochemistry results analysis

All IHC stained sections were examined microscopically and scored independently by one pathologist. PD-L1 staining on the plasma membrane of tumor cells without or with cytoplasmic staining was account for positive staining. CTLA-4 and LAG-3 staining on the plasma membrane or cytoplasm of lymphocytes was account for positive staining (Du et al., 2018; Marcq et al., 2017b). Histopathologic analysis of PD-L1 positive staining on tumor cells or CTLA-4 and LAG-3 positive staining on TILs in tumoral or stromal compartment was performed on full-face IHC stained sections according to Salgado et al. (Salgado et al., 2015). Patterns with 5% or greater positive cells were considered positive, and those with less than 5% positive cells were considered negative for PD-L1, CTLA-4 and LAG-3 (Zwaenepoel et al., 2017).

All IHC staining slides were scanned by the Aperio ScanScope AT2 system (Leica Biosystems GmbH, Wetzlar, Germany) and the digital data for each slide were analysed by using QuPath software (Bankhead et al., 2017). Number of CTLA-4+ and LAG-3+ lymphocytes was also accounted to get the total number of positive lymphocytes counted across five low-power fields in tumor or stromal background sections in each slide. Finally, the positive expression particles for each duplicate were used for statistical analysis.

3.2.3 Statistic analysis

One Way ANOVA test with Tukey's post hoc test was used for assessing the difference of the patients' age in different thyroid carcinoma groups, data in scatter plots and the bar graph are means ± standard deviation (SD). The Kruskal-Wallis H test with Dunn's multiple comparison test was performed for comparing the quantitative data of number of positive particles of CTLA-4 and LAG-3 between DTC, PDTC and ATC groups because the quantitative data is skewed distribution in these groups, data in box plots and the bar graph are median (interquartile range). Spearman correlation analysis was

performed for the relationships between CTLA-4+ particle number and LAG-3+ particle number in 74 thyroid carcinoma cases. For statistical analysis, GraphPad PrismTM 6.0 was used (GraphPad Software, San Diego, CA, USA). All P-values are two-sided and * $P < 0.05$ and ** $P < 0.01$ was considered statistically significant in all statistical tests.

4 RESULTS

4.1 General clinical and pathological characteristics of thyroid samples

The clinical characteristics of the 105 tissues are shown in Table 9. Among 105 tissues, 67 were female (63.8%) and 38 were male (36.2%), reflecting a female-to-male ratio greater than 1.7:1.0. Patient age ranged from 12 to 89 years, with a mean age of 52 years. There were 17 normal thyroids (16.2%), 14 thyroid adenomas (13.3%), 19 PTC (18.1%), 13 FTC (12.4%), 23 PDTC (21.9%), and 19 ATC (18.1%) tissues.

Table 9. General characteristics of patients.

Patients	**Number**	**Proportion (%)**
• **Total**	105	
• **Age range, average**	12-89 Y, 52 Y	
• **Gender**		
Male	38	36.2
Female	67	63.8
• **Histological diagnosis**		
Normal thyroid	17	16.2
Thyroid adenoma	14	13.3
PTC	19	18.1
FTC	13	12.4
PDTC	23	21.9
ATC	19	18.1

Y= Years, PTC= Papillary thyroid carcinoma, FTC= Follicular thyroid carcinoma, PDTC= Poorly differentiated thyroid carcinoma, ATC= Anaplastic thyroid carcinoma.

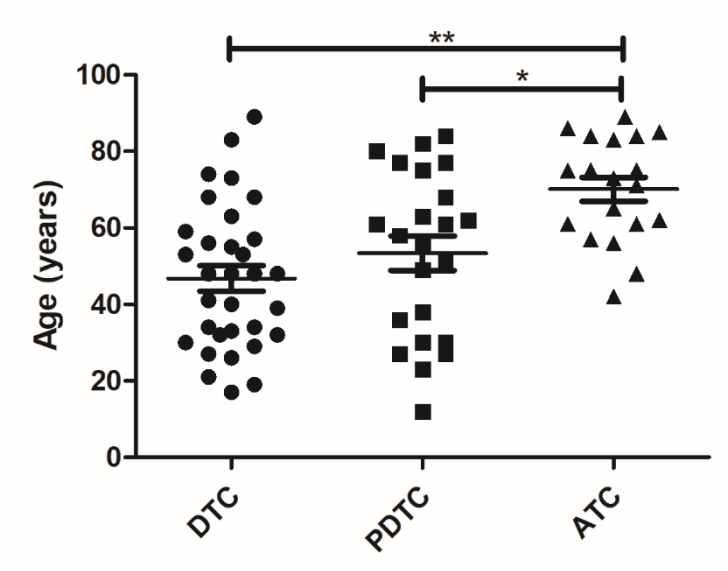

Figure 7. Age of patients in DTC (PTC + FTC), PDTC and ATC group. Data in scatter plots and the bar graph are means ± standard deviation (SD). One Way ANOVA test followed by Tukey's post hoc test was applied, * $P < 0.05$ and ** $P < 0.01$.

We compared the mean ages of the patients at diagnosis presenting with different subtypes of thyroid carcinoma. The patients presenting with ATC (mean age 70.1 ± 13.7 Y) were significantly older than those presenting with DTC (mean age 46.8 ± 18.9 Y), $P < 0.01$ or PDTC (mean age 53.4 ± 21.6 Y), $P < 0.05$. However, there was no significant difference between the patients with DTC versus those with PDTC (Fig. 7).

The three elementary features of the thyroid gland, including follicles, follicular cells, and parafollicular cells were observed in the normal thyroid tissues (Fig. 8A). Follicular adenoma is defined as a benign encapsulated tumor with follicular cell differentiation showing a uniform pattern throughout the confine nodule (Fig. 8B). Closely packed follicles, trabeculae or solid sheets were observed in the follicular adenoma samples. In addition, the surrounding thyroid tissue showed signs of compression. Some normal follicular, macrofollicular, or microfollicular patterns were also observed.

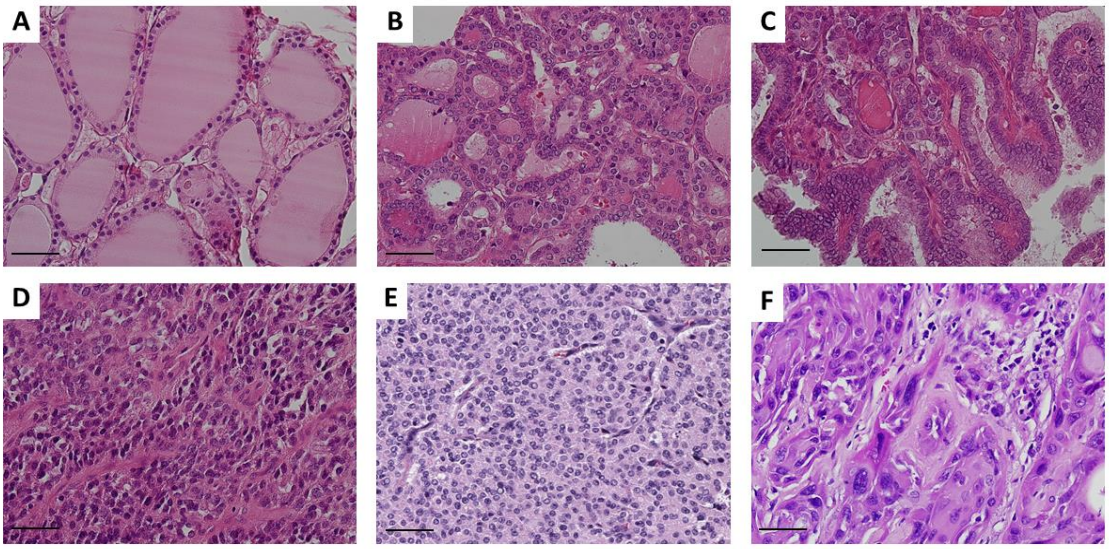

Figure 8. Representative images of H&E staining in different thyroid tissues. (A) Normal thyroid, (B) Thyroid adenoma, (C) PTC, (D) FTC, (E) PDTC, (F) ATC. (Scalebar: 50µm).

The 19 PTC cases were characterized by the formation of papillae, psammoma bodies, and a set of distinctive nuclear features. The nuclei from the PTC tissue were clear, ground glass shaped, or Orphan-Annie eyed sign. These nuclei were larger than normal follicular nuclei and overlapped with each other (Fig. 8C). FTC presented with characteristic follicular differentiation but did not show any papillary nuclear characteristics (Fig. 8D). Some FTC samples from hemorrhagic tissue showed solitary encapsulated tumors with a gray-tan-pink color. The majority of the FTC samples was minimally invasive with only a slight tumor capsular invasion. The 23 PDTC samples showed specific characteristics including a solid/trabecular/insular growth pattern and the absence of conventional nuclear features of PTC (Fig. 8E). Most of the ATC samples showed typical pathological characteristics –spindle-shaped cells, squamoid cells, and pleomorphic giant cells (Fig. 8F). Some ATC were accompanied with necrosis and hemorrhage.

4.2 Expression of PD-L1 in thyroid tissues

The PD-L1 expression patterns in the 17 normal thyroid, 14 thyroid follicular adenoma, 19 PTC, 13 FTC, 23 PDTC, and 19 ATC tissues are listed in Table 10. In the 17 normal thyroid and 14 thyroid adenoma tissues, the PD-L1 staining was negative. Among the 74 thyroid carcinomas, the 19 PTC samples were diagnosed PD-L1 negative. Two of the 13 FTC (15.4%), two of the 23 PDTC (8.7%) and seven of the 19 ATC (36.8%) samples were positive for PD-L1 expression.

Table 10. Expression of PD-L1 in thyroid tissues.

Tissue	Total (n)	Negative (n)	Positive (n, %)
Normal thyroid	17	17	0
Thyroid adenoma	14	14	0
PTC	19	19	0
FTC	13	11	2 (15.4%)
PDTC	23	21	2 (8.7%)
ATC	19	12	7 (36.8%)

PD-L1 expression on the tumor cell plasma membrane with or without cytoplasmic expression was observed in the thyroid carcinoma tissues (Fig. 9, arrow). This was similar to the expression pattern of PD-L1 on the positive control tonsillar tissue (Fig. 10A). In the two FTC tissues, PD-L1 staining was respectively observed on the plasma membrane of 20% and 40% of tumor cells. PD-L1 staining was observed on the plasma membrane in 10% and 70% of tumor cells in two PDTC tissues. In seven ATC tissues, 8% - 75% of tumor cells expressed PD-L1. In two ATC tissues, PD-L1 expression was localized on the plasma membrane of tumor cells, while the other five expressed PD-L1on the plasma membrane and in the cytoplasm of tumor cells. All negative control samples did not show PD-L1 expression in normal follicular and parafollicular cells, tumor cells, or lymphocytes (Fig. 10B).

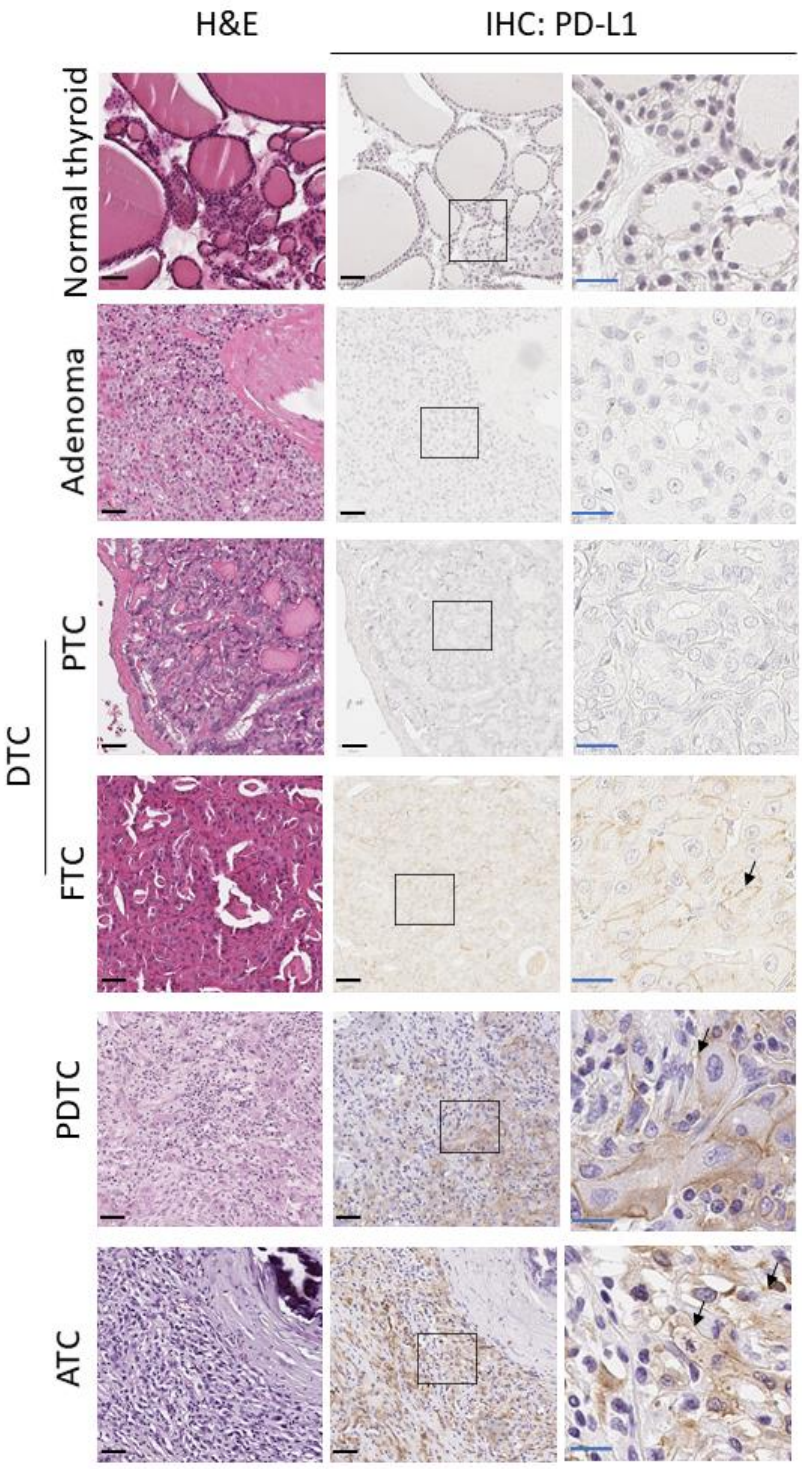

Figure 9. Expression of PD-L1 in normal thyroid, thyroid adenoma, and thyroid carcinoma tissues. H&E staining was used for clinicopathological diagnosis of FFPE human thyroid sample. Immunohistochemistry analysis in FFPE human thyroid carcinoma using a PD-L1 polyclonal antibody (Product # 325600). PD-L1 negative

staining in normal thyroid, thyroid adenoma, and PTC tissues. Representative examples of PD-L1 positive cases in FTC, PDTC, and ATC. PD-L1 expression was localized on the plasma membrane of tumor cells with or without cytoplasmatic staining (arrow). (Black Scalebar: 50 μm, Blue Scalebar: 20 μm).

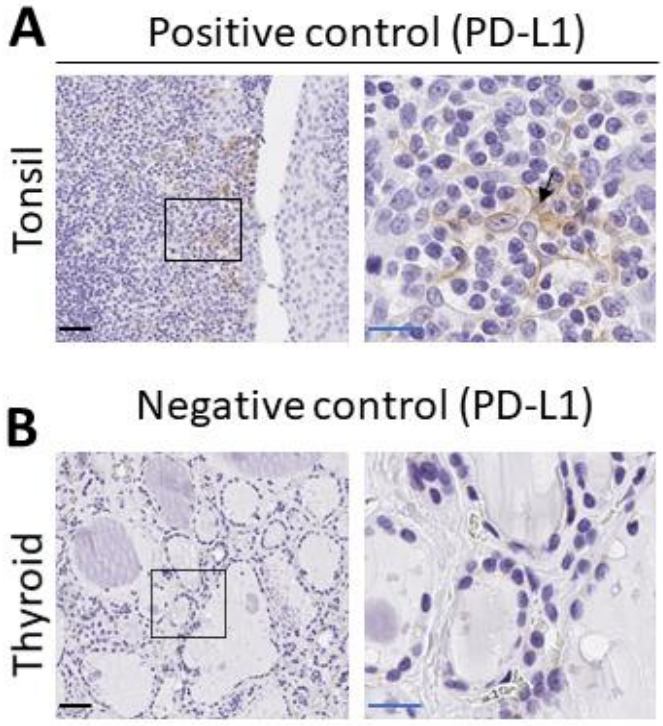

Figure 10. Positive control and negative control of PD-L1. (A) Positive control: PD-L1 staining was localized on the plasma membrane of lymphocytes with or without cytoplasmatic and nuclear staining (arrow). (B) Negative control (without primary antibody): no positive staining. (Black Scalebar: 50 μm, Blue Scalebar: 20 μm).

4.3 Expression of CTLA-4 in thyroid tissues

The CTLA-4 expression patterns in the 105 thyroid tissues are listed in Table 11. No CTLA-4 positive case was diagnosed in the 17 normal thyroids and 14 thyroid follicular adenoma tissues. CTLA-4 positive expression in the lymphocyte membrane or cytoplasm was diagnosed in 17 thyroid carcinoma tissues, including three of the 19 PTC (15.8%),

two of the 13 FTC (15.4%), five of the 23 PDTC (21.7%), and seven of the 19 ATC (36.8%) samples.

Table 11. Expression of CTLA-4 in thyroid tissues.

Tissue	Total (n)	Negative (n)	Positive (n, %)
Normal thyroid	17	17	0
Thyroid adenoma	14	14	0
PTC	19	16	3 (15.8%)
FTC	13	11	2 (15.4%)
PDTC	23	18	5 (21.7%)
ATC	19	12	7 (36.8%)

Representative images of CTLA-4 expression in the thyroid samples are shown in Fig. 11. CTLA-4 was partly expressed on the plasma membrane and cytoplasm of lymphocytes. A weak to moderate CTLA-4 expression on the plasma membrane and cytoplasm was also observed in most thyroid epithelial and tumoral cells (Fig. 11). In most positive tissues, CTLA-4 expression was observed in the lymphocytes both in the tumor compartment and in the stromal compartment. In the tonsil tissue (positive control), membranous staining with or without cytoplasmic staining in the lymphocytes was observed. In addition, a weak to moderate CTLA-4 expression was observed in most tonsil epithelial cells (Fig. 12A). All negative control samples did not show CTLA-4 expression in normal follicular and parafollicular cells, tumor cells, or lymphocytes (Fig. 12B).

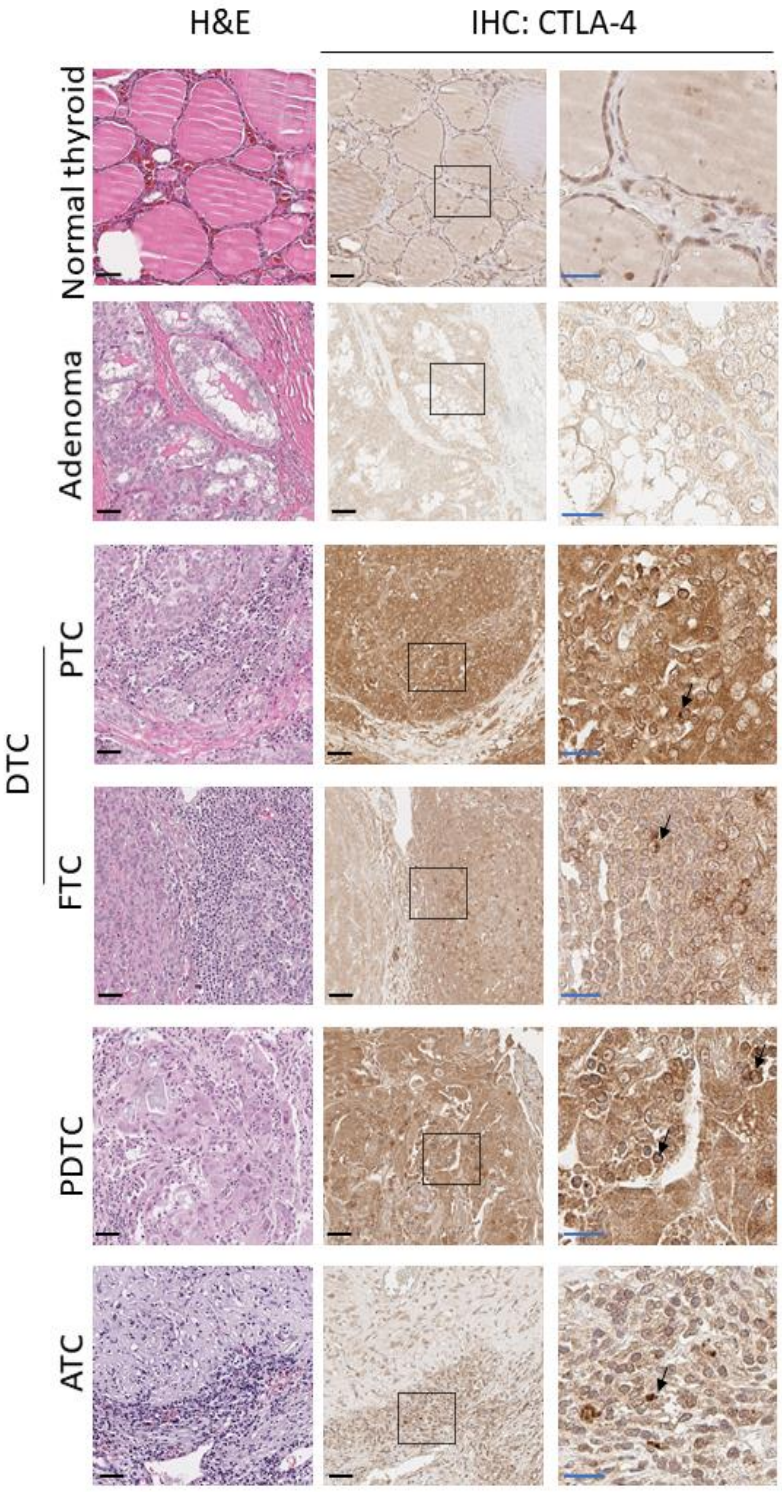

Figure 11. Expression of CTLA-4 in normal thyroid, thyroid adenoma, and thyroid carcinoma tissues. H&E staining was used for clinicopathological diagnosis of FFPE human thyroid sample. Immunohistochemistry analysis of FFPE human thyroid tissues using a CTLA-4 polyclonal antibody (Product # BSB2883). CTLA-4 negative staining in

normal thyroid and thyroid adenoma tissue. Representative examples of CTLA-4 positive staining in PTC, FTC, PDTC, and ATC. CTLA-4 was localized on the plasma membrane of the lymphocytes in the tumor compartment or in the stromal compartment with or without cytoplasmatic staining (arrow). (Black Scalebar: 50 µm, Blue Scalebar: 20 µm).

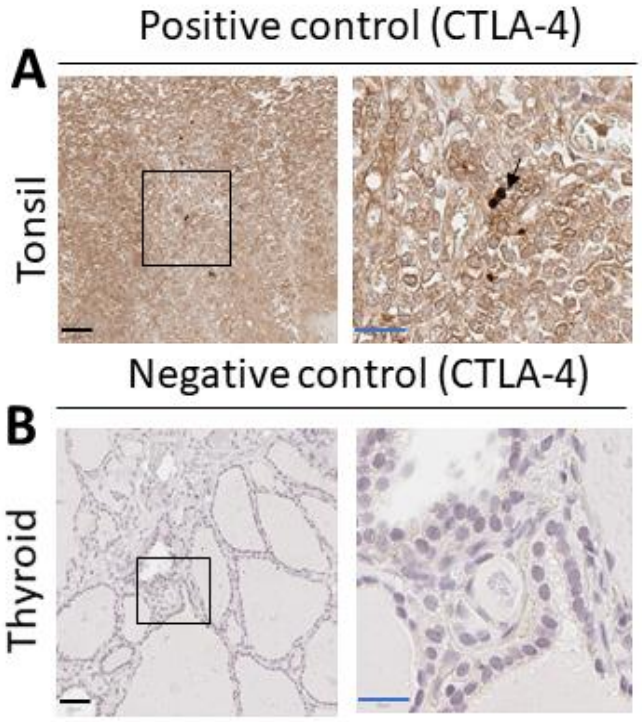

Figure 12. Positive control and negative control of CTLA-4. (A) Positive control: CTLA-4 expression was localized on the plasma membrane of lymphocytes with or without cytoplasmatic and nuclear staining (arrow). (B) Negative control (without primary antibody): no positive staining. (Black Scalebar: 50 µm, Blue Scalebar: 20 µm).

Quantification of CTLA-4 expression was performed using the digital image analysis software Qupath. Five high magnification images from different regions on each slide were analyzed in all thyroid carcinoma, and the total number of CTLA-4 positive granules per slice were compared between the carcinoma subtypes. The number of CTLA-4 positive granules was significantly higher in ATC compared to DTC, $P < 0.05$. However,

there was no significant difference in the number of CTLA-4 positive granules between ATC and PDTC and between PDTC and DTC (Fig. 13).

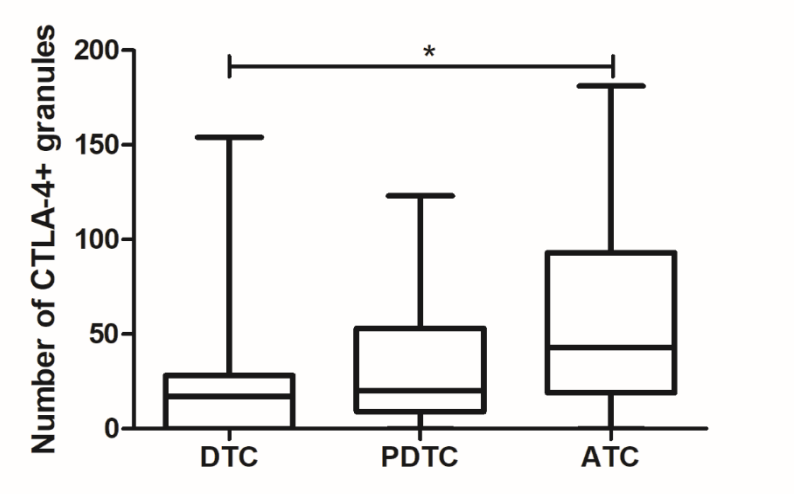

Figure 13. Number of CTLA-4 positive granules in DTC (PTC + FTC), PDTC and ATC. Quantification of CTLA-4 expression from IHC images. Data in scatter plots and the bar graph are median (interquartile range). Kruskal-Wallis H test followed by Dunn's multiple comparison test was applied, * $P < 0.05$.

4.4 Expression of LAG-3 in thyroid tissues

The expression pattern of LAG-3 in different thyroid tissues is listed in Table 12. No LAG-3 positive case was diagnosed in any of the 19 normal thyroid tissues. Only one of the 14 thyroid follicular adenomas (7.1 %) was found to be positive for LAG-3. The 74 thyroid carcinoma samples showed different levels of LAG-3 expression (Table 12). Seven of the 19 PTC (36.8 %), three of the 13 FTC (23.1%), four of the 23 PDTC (17.4%), and nine of the 19 ATC (47.4%) samples were diagnosed LAG-3 positive.

Table 12. Expression of LAG-3 in thyroid tissues.

Tissue	Total (n)	Negative (n)	Positive (n, %)
Normal thyroid	17	17	0
Thyroid adenoma	14	13	1 (7.1%)
PTC	19	12	7 (36.8%)
FTC	13	10	3 (23.1%)
PDTC	23	19	4 (17.4%)
ATC	19	10	9 (47.4%)

In our study, some normal thyroid epithelial cells, follicular adenoma cells, and tumor cells showed weak LAG-3 expression in the nuclei with or without plasma membranous and cytoplasmic expression (Fig. 14). LAG-3 expression in thyroid epithelial cells and tumor cells was heterogeneous and no specificity was observed between different groups. Some lymphocytes showed LAG-3 expression on the membrane with or without plasmatic and nuclear staining in benign and malignant thyroid tissues. A membranous expression of LAG-3 was observed in the lymphocytes located in the tumor compartment and in the stromal compartment of thyroid carcinoma (Fig. 14). LAG-3 expression on the membrane and cytoplasm of lymphocytes is considered positive LAG-3 expression regardless of positive staining in the epithelial and tumor cells. A weak LAG-3 expression was observed in most of the tonsillar epithelial cells. The tonsillar lymphocytes showed positive membranous staining with or without cytoplasmatic and nuclear staining (Fig. 15A). All negative control samples did not show LAG-3 expression in normal follicular and parafollicular cells, tumor cells, or lymphocytes (Fig. 15B).

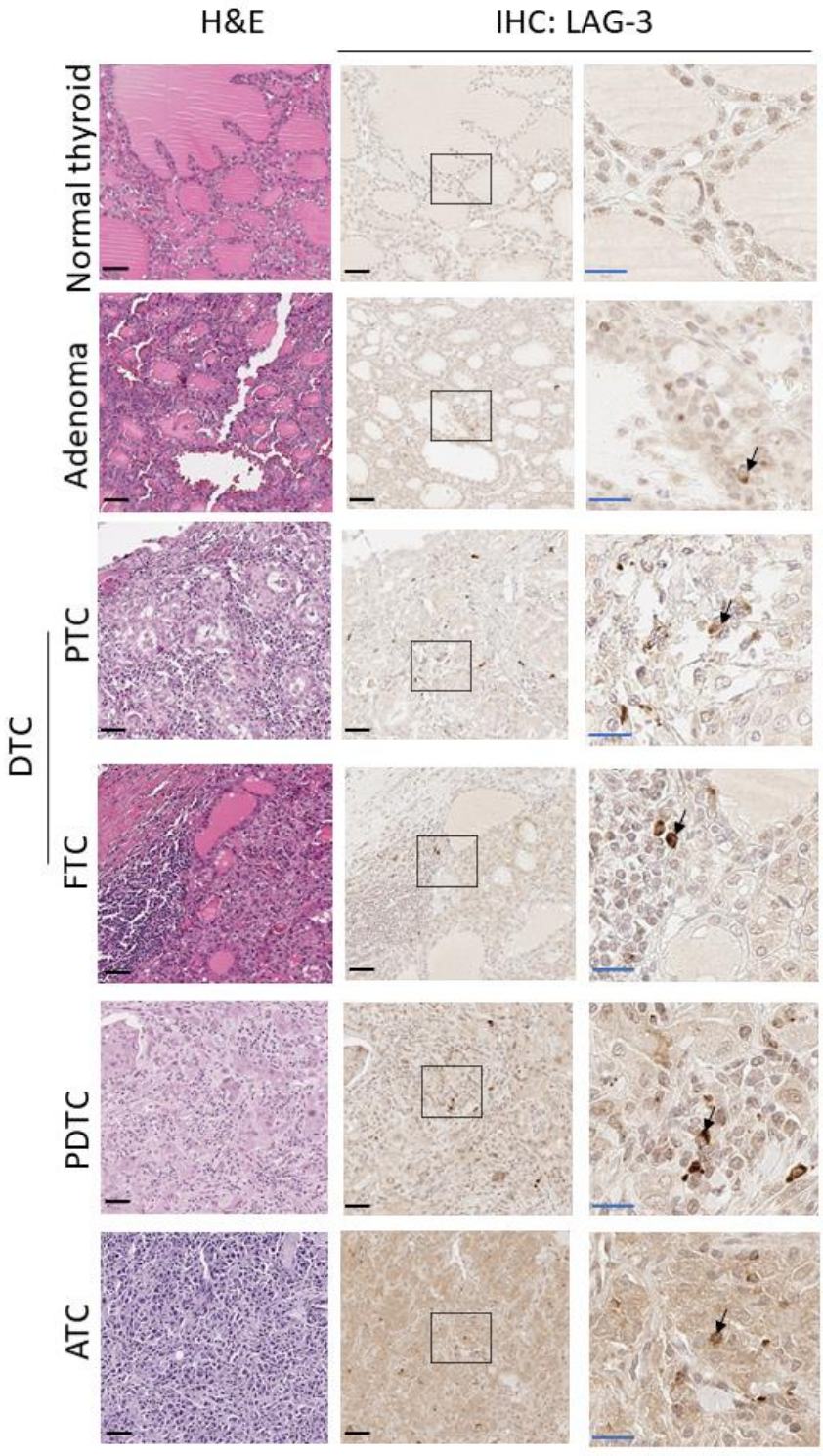

Figure 14. Expression of LAG-3 in normal thyroid, thyroid adenoma, and thyroid carcinoma tissues. H&E staining was used for clinicopathological diagnosis of FFPE human thyroid sample. Immunohistochemistry analysis of FFPE human thyroid tissues using a LAG-3 polyclonal antibody (Product # SPA13967). LAG-3 negative staining in

normal thyroid tissue. Representative examples of LAG-3 expression in thyroid adenoma, PTC, FTC, PDTC, and ATC. LAG-3 expression was localized on the plasma membrane of lymphocytes in the tumor compartment or in the stromal compartment with or without cytoplasmatic and nuclear staining (arrow). (Black Scalebar: 50 µm, Blue Scalebar: 20 µm).

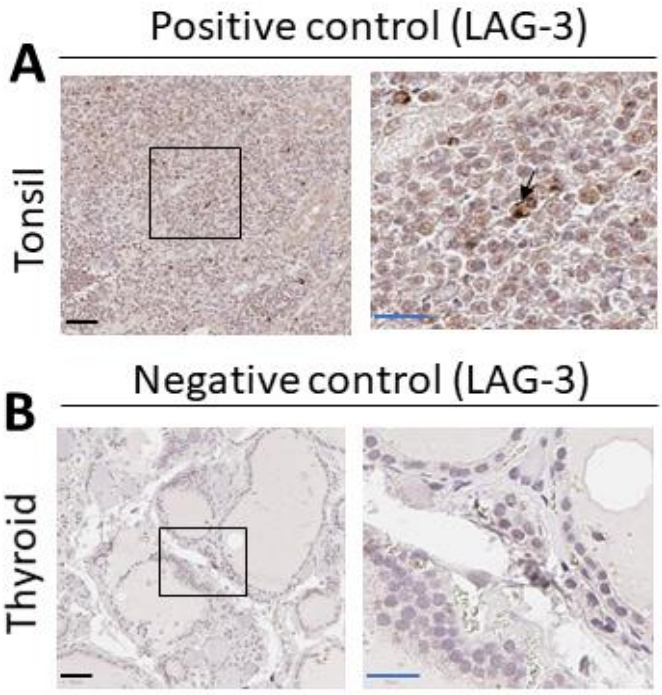

Figure 15. Positive control and negative control of LAG-3. (A) Positive control: LAG-3 expression was localized on the plasma membrane of lymphocytes with or without cytoplasmatic and nuclear staining (arrow). (B) Negative control (without primary antibody): no positive staining. (Black Scalebar: 50 µm, Blue Scalebar: 20 µm).

Quantification of LAG-3 expression was performed using the Qupath software. Five high magnification images from different regions on each slide were analyzed in all thyroid carcinoma, and the total number of LAG-3+ granules per slice were compared between the carcinoma subtypes. The number of LAG-3+ granules was significantly higher in ATC compared to PDTC or DTC, $P < 0.05$. However, there was no significant difference in LAG-3 expression between PDTC and DTC (Fig. 16).

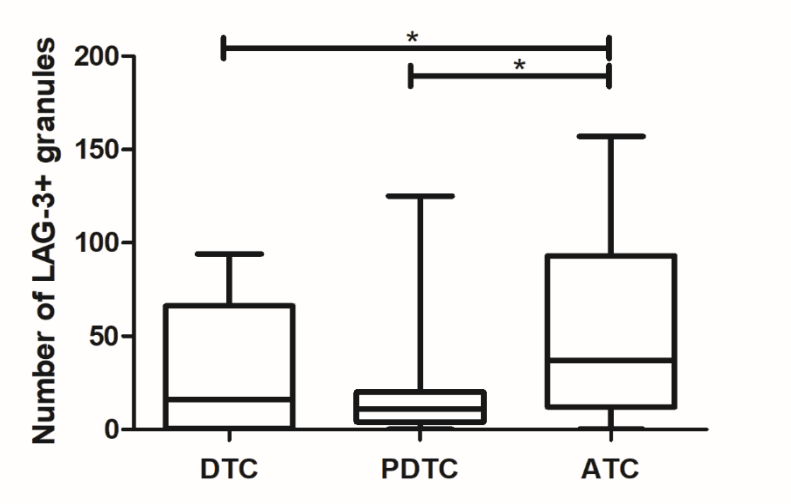

Figure 16. Number of LAG-3 positive granules in DTC (PTC + FTC), PDTC and ATC. Quantification of LAG-3 expression from IHC images. Data in scatter plots and the bar graph are median (interquartile range). Kruskal-Wallis H test followed by Dunn's multiple comparison test was applied, * $P < 0.05$.

4.5 Characteristics of PD-L1, CTLA-4, and LAG-3 expression in thyroid carcinoma

In this study, we investigated the expression of PD-L1, CTLA-4, and LAG-3 in the normal thyroid, thyroid adenoma and malignant thyroid tissues. We found that PD-L1, CTLA-4 and LAG-3 were significantly up-regulated in thyroid carcinoma compared to normal thyroid and thyroid adenoma tissues (Table 10, 11, 12). We also found differences of the expression of PD-L1, CTLA-4, and LAG-3 in 74 thyroid carcinoma tissues. Only 14.9% (11/74) of the thyroid carcinomas were diagnosed PD-L1 positive, while 23.0% (17/74) diagnosed positive CTLA-4 and 31.1% (23/74) diagnosed positive LAG-3.

The expression patterns of immune checkpoint proteins in different subtypes of thyroid carcinoma were variable. In ATC, the most aggressive thyroid carcinoma, PD-L1, CTLA-4, and LAG-3 were expressed with the highest positive rates of 36.8% (7 of 19), 36.8% (7 of 19), and 47.4% (9 of 19) respectively compared to 8.7% (2 of 23), 21.7% (5 of 23), and 17.4% (4 of 23) in PDTC, and 6.3% (2 of 32), 15.6% (5 of 32), and 31.3% (10 of 32) in DTC (Fig. 17).

Among the 74 thyroid carcinoma tissues, there were only three cases (4.1%) that expressed all three immune checkpoint proteins, PD-L1, CTLA-4, and LAG-3. Interestingly, some thyroid carcinoma tissues with positive CTLA-4 staining also had positive LAG-3 staining on lymphocytes. Among the 74 thyroid carcinoma tissues, 10 tissues (13.5%) were diagnosed CTLA-4 and LAG-3 positive, whereas 44 tissues (59.5%) were negative for both CTLA-4 and LAG-3 in their TILs (Fig. 17). We compared the number of CTLA-4 and LAG-3 positive granules in 74 thyroid carcinoma tissues and found that CTLA-4 expression was positively correlated with LAG-3 expression (R = 0.6119, $P < 0.01$) (Fig. 18).

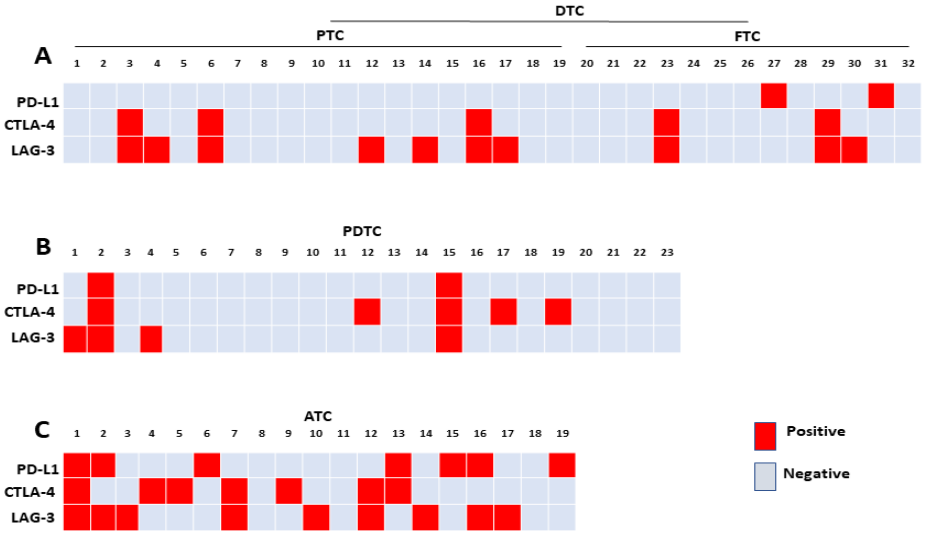

Figure 17. Distribution and prevalence of PD-L1, CTLA-4, and LAG-3 in DTC (PTC + FTC), PDTC and ATC. Expression of PD-L1, CTLA-4, and LAG-3 in (A) DTC (n= 32), (B) PDTC (n= 23), and (C) ATC (n= 19).

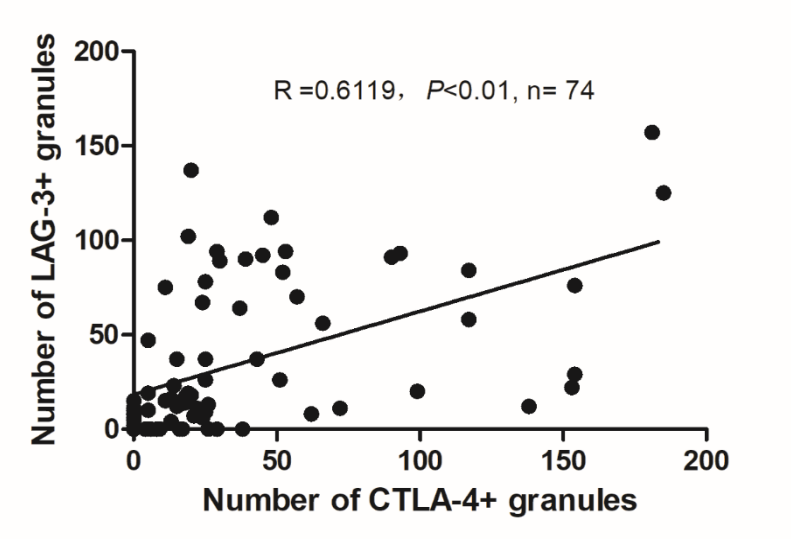

Figure 18. Spearman correlations of the number of CTLA-4 and LAG-3 positive granules in 74 thyroid carcinoma cases. Analyses showed a positive correlation (R = 0.6119) between the number of CTLA-4 and LAG-3 positive granules (Spearman's Rank coefficient analysis, $^{**}P < 0.01$, n=74).

Among our samples, we found one interesting ATC case coexisted with PDTC (Fig. 19). We analyzed the characteristics of PD-L1, CTLA-4 and LAG-3 expression in different subtype tumor epithelial compartments and necrotic regions with lymphocytes in this sample. PD-L1 was expressed in ATC tumor cells and lymphocytes in necrotic regions but was not expressed in the PDTC epithelial compartments. CTLA-4 was not expressed in the ATC and PDTC compartments and the necrotic regions. LAG-3 positive lymphocytes were detected in the ATC compartments. LAG-3 expression was also observed in lymphocytes in the necrotic regions but not in the PDTC compartments.

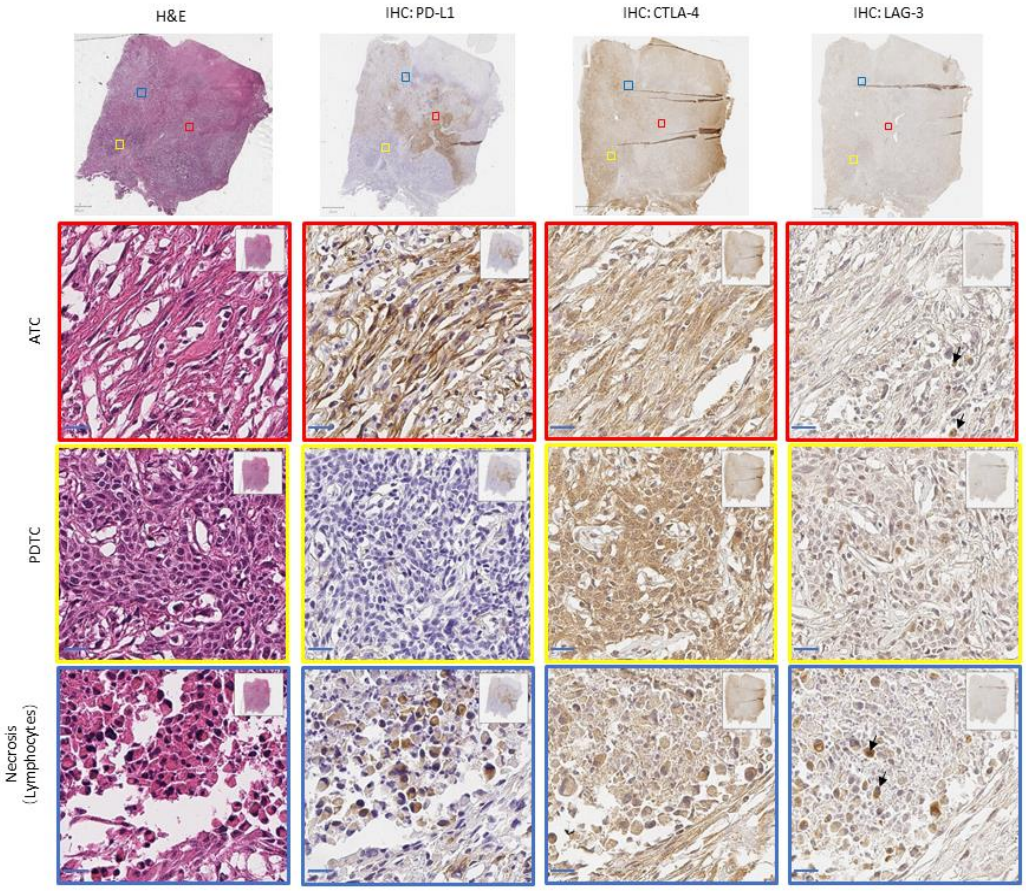

Figure 19. PD-L1, CTLA-4 and LAG-3 expression in one thyroid malignant tissue with PDTC and ATC characteristics. H&E staining was used for clinicopathological diagnosis of FFPE human thyroid sample. PD-L1 expression was observed in ATC tumor cells and lymphocytes in the necrotic regions but not in the PDTC compartments. CTLA-4 was not expressed in the lymphocytes in the ATC and PDTC compartments and the necrotic regions. LAG-3 positive lymphocytes were detected in the ATC and necrotic regions (arrow), but not in the PDTC compartments. (Blue Scalebar: 20 μm).

5 DISCUSSION

Thyroid carcinoma is the most common cancer of the human endocrine system and its prevalence has increased over the last 20 years (Rahib et al., 2014). Although most patients present with an indolent tumor with a favorable prognosis, some aggressive thyroid carcinoma, including PDTC and ATC have a poor prognosis and can be lethal (Alonso-Gordoa et al., 2015). Therefore, new treatment strategies are urgently needed for patients with PDTC and ATC.

Investigations have shown that cancers possess different mechanisms to circumvent host immunosurveillance (Kim et al., 2007). One such mechanism is the up-regulation of immune checkpoints in immune cells and their ligands in tumor cells. Immunotherapy with immune checkpoint inhibitors has begun to revolutionize the therapy of several malignancies (Jung and Antonia, 2018). There is not enough evidence on immunotherapy in thyroid carcinoma besides some case reports (Kollipara et al., 2017; Saini et al., 2018). Examining the role of immune checkpoints in aggressive thyroid carcinomas such as PDTC and ATC, and harnessing the power of the new immune checkpoint inhibitors are necessary and important in thyroid carcinoma treatment. The aim of this study was to evaluate the expression of immune checkpoint and ligand proteins in different types of thyroid carcinoma and to analyze the possibility of immunotherapy in aggressive thyroid malignancies. In this study, we investigated the expression of PD-L1, CTLA-4, and LAG-3 in human normal thyroid, thyroid follicular adenoma, PTC, FTC, PDTC, and ATC tissues by IHC. Our results demonstrate that different subtypes of thyroid carcinoma have different immune checkpoints and ligand protein expression patterns.

5.1 PD-L1 expression in thyroid carcinoma

PD-1/PD-L1 pathway has been found to negatively regulate human immune response in malignancies (Fourcade et al., 2009). Many cancers have been shown to express PD-1 or PD-L1 and PD-1/PD-L1 inhibitors, such as Keytruda, Opdivo, and Tecentriq, were

developed and are in various stages of clinical trials (Guan et al., 2017; Romano and Romero, 2015). Furthermore, progression in PD-1/PD-L1 inhibitor therapy has opened a new era in the treatment of melanoma, NSCLC and Hodgkin's lymphoma (Chae et al., 2018; Sunshine and Taube, 2015).

Since PD-1 was first found to be expressed in PTC in 2012 (French et al., 2012), some endocrinologists and oncologists focused on the characteristics of PD-1/PD-L1 expression in thyroid carcinoma (Rosenbaum et al., 2018; Zwaenepoel et al., 2017). However, insufficient data on thyroid carcinoma patients treated with PD-1/PD-L1 inhibitors have affected the widespread use of immune checkpoint inhibitors in these patients (Antonelli et al., 2018). Moreover, there are very few large-scale studies on the expression of immune checkpoints and ligand proteins in PDTC and ATC.

IHC studies have shown that PD-L1 is expressed in the cytoplasm and plasma membrane in many tumor cells, e.g. melanoma, NSCLC and renal cell carcinoma (RCC) (Hamid et al., 2013; Mahoney et al., 2015; Topalian et al., 2012). After analyzing the expression of PD-L1 in 57 gastric adenocarcinoma patients by IHC, Schlosser et. al found that the staining pattern was mainly cytoplasmatic with some membranous staining (Schlosser et al., 2016). In this study, PD-L1 positive staining in FTC and PDTC tissue was respectively observed on the plasma membrane. In the seven PD-L1 positive ATC samples, two showed expression on the plasma membrane of tumor cells, while the other five expressed PD-L1 on the plasma membrane and in the cytoplasm of tumor cells.

In our results, 19 normal thyroid tissues and 14 thyroid adenoma tissues were negative for PD-L1 staining, and only a positive PD-L1 staining rate of 14.9% (11/74) was observed in thyroid carcinoma. It was reported that high expression of PD-L1 is correlated with invasiveness and poor disease-free survival in PTC (Chowdhury et al., 2016). Some researchers found PD-L1 expression significantly increased in an invasive encapsulated follicular variant of PTC as compared to noninvasive follicular thyroid neoplasm with papillary-like nuclear features (Fu et al., 2017). However, in our results, none of the 19 PTC samples showed PD-L1 positive expression. The explanation for this difference

could be that the PTC sample size we studied was relatively small (19 PTC samples). Ahn et al. reported that tumoral PD-L1 was expressed in 6.1% of PTC, 7.6% of FTC and 22.2% of ATC (Ahn et al., 2017). We also observed an increase in PD-L1 positivity with the increase in tumor invasiveness. Two out of 13 FTC (15.4%), two out of 23 PDTC (8.7%) and seven out of 19 ATC (36.8%) samples were PD-L1 positive. This study demonstrates the characteristics of PD-L1 expression in thyroid carcinomas varying in differentiation, including DTC, PDTC, and ATC.

Last year, PD-L1 over-expression in ATC and exceptional response with immunotherapy in one ATC patient have been reported (Kollipara et al., 2017; Zwaenepoel et al., 2017). Our data also demonstrated that aggressive thyroid carcinoma, including PDTC and ATC, appears to have a higher frequency of PD-L1 expression than the normal thyroid and thyroid adenoma tissue or low aggressive thyroid carcinoma (PTC and FTC). It suggests that immunotherapy using PD-1/PD-L1 checkpoint inhibitors are a potential option for PDTC and ATC patients. The expression of PD-L1 in many malignancies, such as melanoma, NSCLC, breast cancer, and RCC patients present an important additional risk factor and should be considered as a potential immunotherapeutic target (Choueiri et al., 2014; D'Incecco et al., 2015; Ghebeh et al., 2006). We expect that PD-1/PD-L1 inhibitor therapies may be effective in thyroid carcinoma patients with aggressive features that cannot be cured by standard therapies. In future more preclinical and clinical studies are necessary to characterize the immune response in PDTC and ATC and to investigate the potential for PD-1/PD-L1 blockade therapy.

5.2 CTLA-4 expression in thyroid carcinoma

CTLA-4 is the receptor of an immune checkpoint pathway that plays an important role in the negative regulation of T cell activation and maintaining of self-tolerance (Chikuma, 2017). It is well accepted that CTLA-4 is frequently overexpressed in a variety of malignancies, such as melanoma, NSCLC, and other solid tumors (Erfani et al., 2012; Kvistborg et al., 2014). Accordingly, it is speculated that high CTLA-4 expression could

be involved in the dysregulation of the immune response in cancer and is associated with tumor growth and progression (Seidel et al., 2018). Moreover, CTLA-4 inhibitors have shown efficacy in overcoming immune suppression in some ongoing preclinical and clinical trials (Sakamuri et al., 2018). However, there are only few studies that focused on CTLA-4 expression in thyroid carcinoma and immunotherapy using CTLA-4 inhibitors for endocrine malignancies. Recently, the overall deregulation of CTLA-4 ligands mRNA in PTC and ATC tissues and its role in ATC progression that could be targeted with the novel immune therapeutic strategies was reported (Tuccilli et al., 2018). The investigation of CTLA-4 expression in thyroid carcinoma is crucial for understanding tumor-derived immune deregulation and is necessary to develop immune therapy strategies for aggressive thyroid carcinoma.

In theory, most of the CTLA-4 may be expressed on the surface of T cells (Valk, Rudd et al. 2008). Previous studies have reported the mechanism of immune suppression by CTLA‐4 expressed in T cells (Jain et al., 2010). Some studies have reported the expression of CTLA-4 in tumor cells (Kim et al., 2016; Salvi et al., 2012). In our study, a weak to moderate heterogenous CTLA-4 staining was observed in the cytoplasm of tonsil epithelial cells in positive control groups and in the cytoplasm of most thyroid epithelial cells in all benign tissues and tumor cells on malignant thyroid tissues, we postulate, that positive epithelial staining is unspecific and therefore only used T cell positive staining for our analysis. In contrast to the positive staining on thyroid epithelial cells and tumor cells, analysis of tumor-infiltrating T cells revealed more information about the immune response in cancer growth. Only the membranous CTLA-4 staining of the lymphocytes was considered positive in this study. In the carcinoma group, the percentage of positivity was increased with the aggressiveness of the tumor, five out of the 32 DTC (15.6%), five out of the 23 PDTC (21.7%) and seven out of the 19 ATC (36.8%). The number of CTLA-4 positive granules was significantly higher in ATC compared to DTC (*, $P < 0.05$). This finding provides a first and promising indication for the prognostic role of CTLA-4 expression in thyroid carcinoma. A similar negative

prognostic impact of CTLA-4 expression on TILs has been demonstrated for gastric adenocarcinoma (Salvi et al., 2012). One study demonstrated that patients overexpressing CTLA-4 in esophageal cancer cells tend to have a poor prognosis (Zhang et al., 2016).

5.3 LAG-3 expression in thyroid carcinoma

As a cell surface immune checkpoint protein, LAG-3 binds with the MHCII molecule on APCs (Andrews et al., 2017). LAG-3 is mostly expressed on T cells, B cells, Tregs, and natural killer cells (Castelli et al., 2014). LAG-3 was also found to be expressed on dysfunctional or exhausted T cells that are developed during tumor progression (Mishra et al., 2016). In addition, LAG-3 mRNA can be found in the splenic red pulp, the thymic medulla, and the base of the cerebellum (Workman et al., 2002). However, there is no report about LAG-3 expression in thyroid carcinoma.

Hald et. al reported that LAG-3 displayed a homogenous membranous and diffuse cytoplasmic staining and that its expression was confined to the TILs (Hald et al., 2018). In our study, as some tonsil epithelial cells in positive control groups, some normal thyroid epithelial cells, follicular adenoma cells, and tumor cells showed weak LAG-3 expression in the nucleus with or without plasma membrane and cytoplasmic expression. We also only used T cell positive staining for our analysis because LAG-3 staining in thyroid epithelial cells and tumor cells was heterogeneous and no specificity was observed between different groups.

Our results demonstrated that the expression of LAG-3 was increased on lymphocytes in human thyroid carcinoma. None of the 17 normal thyroids (0%) were diagnosed LAG-3 positive and only one of the 14 thyroid follicular adenomas (7.1%) was LAG-3 positive on the lymphocytes. Twenty-three out of the 74 thyroid carcinomas (31.1%) expressed LAG-3 on the TILs, which indicate the immunosuppressive status of thyroid carcinoma. In addition, when we quantified the number of positive TILs in thyroid carcinoma subgroups, we found the number of LAG-3+ TILs were significant higher in ATC compared to PDTC or DTC ($P < 0.05$). This suggests that the presence of LAG-3 in TILs

facilitates thyroid carcinoma proliferation and resists immune system-mediated tumor cell eradication, resulting in aggressive and less differentiated thyroid carcinoma. These findings provide the rationale for a potential immunotherapeutic strategy involving LAG-3 blockade for treating ATC.

5.4 Expression of immune checkpoint and ligand proteins in thyroid carcinoma

In this study, we investigated the expression of PD-L1, CTLA-4, and LAG-3 in different thyroid tissues. We found a moderate increase in the expression of CTLA-4 and LAG-3 on lymphocytes in thyroid carcinoma tissues compared to the normal thyroid and thyroid adenoma tissues. We also found a lower expression of PD-L1 (11/74, 14.5%) compared to CTLA-4 (17/74, 23.0%) and LAG-3 (23/74, 31.1%) in our samples. This suggests that CTLA-4 and LAG-3 may have a higher potential as immune checkpoint markers than PD-L1 in thyroid carcinomas and may have a potential impact in immunotherapy. Interestingly, we found a significant correlation between the number of CTLA-4+ and LAG-3+ TILs in 74 thyroid carcinoma cases (r=0.6119, **, $P < 0.01$), suggesting that the combination of CTLA-4 and LAG-3 immune checkpoint inhibitors might be a potential treatment strategy in dual positive patients.

Immunotherapy by using immune checkpoint inhibitors was considered as potential treatment option for aggressive thyroid carcinoma (Naoum et al., 2018). We detected PD-L1, CTLA-4 and LAG-3 expression in different types of thyroid carcinoma and found that the expression of immune checkpoint and ligand proteins was increased in the aggressive tumor entities PDTC and ATC. ATC, the most lethal thyroid carcinoma, showed the highest expression of PD-L1, CTLA-4 and LAG-3. Even in one ATC tissue coexisting with PDTC, PD-L1 and LAG-3 positive granules were detected in the ATC and necrotic compartment but not in the PDTC compartment, CTLA-4 was negative in this tumor sample.

Our data demonstrate that some immune checkpoints and ligands might promote tumor progression by inhibiting the anti-tumor T cell immunity, inducing tumor-specific T cell

apoptosis and T cell-mediated cytotoxicity associated with a shorter survival in ATC. Our results demonstrate the differential expression of various immune checkpoint and ligand proteins in thyroid carcinoma and provide a rationale for the use of checkpoint inhibitors for treating thyroid carcinomas with different aggressiveness and prognosis.

One limitation of this study is that clinical data of our patients like treatment strategies and survival times are missing. So, a study of associated clinicopathologic features is needed to validate the potential of immunotherapy for PDTC and ATC in future.

In conclusion, this work has several potential implications. First, the confirmation of elevated PD-L1, CTLA-4 and LAG-3 expression in some thyroid carcinomas support the continued development of immune checkpoint inhibitors and immunotherapy in thyroid malignancy. Second, the expression levels of PD-L1, CTLA-4 and LAG-3 in ATC were higher than in DTC and PDTC. This information may be used to develop a novel therapy for ATC. Third, our results demonstrated a significant correlation between the number of CTLA-4+ and LAG-3+ particles in thyroid carcinomas suggesting that a combination of CTLA-4 and LAG-3 immune checkpoint inhibitors might be a potential treatment strategy for some dual positive thyroid malignancies.

6 SUMMARY

Successful therapy of thyroid carcinoma is still challenged by the aggressiveness and poor prognosis of some types: poorly differentiated thyroid carcinoma and undifferentiated thyroid carcinoma. We investigated the expression of immune checkpoint and ligand proteins in thyroid carcinoma and got some knowledge about the immune microenvironment in thyroid carcinoma. Our research found that programmed death-ligand 1, cytotoxic T-lymphocyte-associated protein 4 and lymphocyte-activation gene 3 proteins were overexpressed in thyroid carcinoma. There was a significant correlation between cytotoxic T-lymphocyte-associated protein 4+ and lymphocyte-activation gene 3+ particles in 74 thyroid carcinoma cases. In addition, the expression rate of programmed death-ligand 1, cytotoxic T-lymphocyte-associated protein 4 and lymphocyte-activation gene 3 in undifferentiated thyroid carcinoma was higher than in differentiated thyroid carcinoma and poorly differentiated thyroid carcinoma.

Our results show that the investigated immune checkpoint and ligand proteins are potential targets for aggressive thyroid malignant tumor immunotherapy. Moreover, our data suggest that combination of cytotoxic T-lymphocyte-associated protein 4 and lymphocyte-activation gene 3 immune checkpoint inhibitors might be an attractive treatment strategy for thyroid carcinoma patients with dual positive expression of these proteins. It is important to continue our research on thyroid tumor immunotherapy to validate the optimal immune checkpoint target and thus to find out suitable inhibitors for thyroid carcinoma therapy.

Zusammenfassung (In Deutsch)

Die erfolgreiche Therapie des Schilddrüsenkarzinoms wird durch die Aggressivität und die schlechte Prognose einiger Entitäten, wie des schlecht differenzierten und undifferenzierten Schilddrüsenkarzinoms herausgefordert. Wir untersuchten die Expression von Immun-Checkpoint- und Ligand-Proteinen in Schilddrüsenkarzinomen und erhielten Erkenntnisse über die immunologische Mikroumgebung in Schilddrüsenkarzinomen. Unsere Forschungen zeigten, dass die Proteine programmed death-ligand 1, cytotoxic T-lymphocyte-associated protein 4 und lymphocyte-activation gene 3 in Schilddrüsenkarzinomen überexprimiert werden. Bei 74 Schilddrüsenkarzinomen gab es einen signifikanten Zusammenhang zwischen der cytotoxic T-lymphocyte-associated protein 4+ und lymphocyte-activation gene 3+ Expression. Darüber hinaus zeigten programmed death-ligand 1, cytotoxic T-lymphocyte-associated protein 4 und lymphocyte-activation gene 3 im undifferenzierten Schilddrüsenkarzinom signifikant höhere Expressionsniveaus im Vergleich zum differenziertes Schilddrüsenkarzinom und schlecht differenziertes Schilddrüsenkarzinom.

Unsere Ergebnisse zeigen, dass die untersuchten Immun-Checkpoint und Ligand-Proteine potenzielle Angriffspunkte für eine Immuntherapie aggressiver Schilddrüsenkarzinome darstellen. Darüber hinaus deuten unsere Ergebnisse darauf hin, dass die Kombination von cytotoxic T-lymphocyte-associated protein 4 und lymphocyte-activation gene 3 Immun-Checkpoint-Inhibitoren für Schilddrüsenkarzinompatienten mit dualer Expression dieser Proteine ein attraktiver Behandlungsansatz sein könnte. Es ist wichtig, unsere Forschung zur Immuntherapie von Schilddrüsentumoren fortzuführen, um das optimale Immun-Checkpoint-Ziel zu validieren und somit geeignete Inhibitoren für die Therapie von aggressiven Schilddrüsenkarzinomen zu finden.

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8 APPENDIXES

8.1 List of tables

8.2 List of figures

8.3 Abbreviations

ACT	Adoptive cell transfer
AJCC	American Joint Committee on Cancer
ALL	Acute lymphoblastic leukemia
APCs	Antigen-presenting cells
ATA	American Thyroid Association
ATC	Anaplastic thyroid carcinoma
B7-H1	B7 Homolog 1
BRAF	proto-oncogene v-Raf murine sarcoma viral oncogene homolog B
cHL	Classical Hodgkin lymphoma
CI	Cancer immunotherapy
CT	Computed Tomography
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
DAB	3,3′-Diaminobenzidine
DLBCL	Diffuse large B-cell lymphoma
DTC	Differentiated thyroid carcinoma
EANM	European Association of Nuclear Medicine
EBRT	External beam radiation therapy
ETA	European Thyroid Association
FDA	Food and Drug Administration
FFPE	Formalin-fixed paraffin embedded tissue
FNA	Fine needle aspiration
FTC	Follicular thyroid carcinoma
GAL9	Galectin-9

GM-CSF	Granulocyte-macrophage colony-stimulating factor
H&E	Hematoxylin and eosin
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HNSCC	Head and neck squamous cell carcinoma
HPV	Human papilloma virus
HRP	Horseradish peroxidase
IFN-α	Interferon-α
IFN-γ	Interferon-γ
IHC	Immunohistochemistry
IL-2	Interleukin-2
LAG-3	Lymphocyte-activation gene 3
MAbs	Monoclonal antibodies
MAPK	Mitogen-activated protein kinase
MCC	Merkel cell carcinoma
MHC	Major histocompatibility complex
MSI-H	Microsatellite instability-high
MTC	Medullary thyroid carcinoma
NGS	Next generation sequencing
NK	Natural killer cells
NSCLC	Non-small cell lung cancer
OS	Overall survival
PD-1	Programmed cell death receptor 1
PD-L1	Programmed death-ligand 1
PD-L2	Programmed death-ligand 2
PDTC	Poorly differentiated thyroid carcinoma

PET-CT	Positron Emission Tomography–Computed Tomography
PIK3CA	Phosphatidylinositol-4, 5-bisphosphate 3-kinase, catalytic subunit alpha gene
PMBCL	Primary mediastinal large B-cell lymphoma
PTC	Papillary thyroid carcinoma
PTH	Parathyroid hormone
RAI	Radioactive iodine
RCC	Renal cell carcinoma
RT	Room temperature
T3	Triiodothyronine
T4	Thyroxine
TCR	T-cell receptor
T-EDTA	Trypsin-ethylene diamine tetra acetic acid
TERT	Telomerase reverse transcriptase
TH	Thyroid hormone
TILs	Tumor-infiltrating lymphocytes
TIM-3	T-cell immunoglobulin and mucin-domain containing-3
TP53	Tumor protein p53
WHO	World Health Organization

9 ACKNOWLEDGEMENTS

"*Now that I have set foot on the road, so, nothing should prevent me to go along this road" -Immanuel Kant*

I would like to express my most sincere thanks from my heart bottom to my mentor and advisor, Prof. Dr. Dr. med. Dagmar Führer-Sakel. She has given a shining example of the intensity, creativity and kindness it takes to succeed as a female scientist. Her guidance and support give me an opportunity to realize my dream for my medicine doctor degree, which should have been finished for me 10 years ago.

I also thank my respectable, assiduous and decent endocrine surgery supervisor, Prof. Dr. med. Dr. h. c. mult. Henning Dralle. He let me open my eyes not only for science and medicine but also for great Deutsche culture. I should thank PD Dr. med. Frank Weber, my vice supervisor and clinical knowledge mentor for his encouragement, guidance and continuous support ever since I came to Essen two years ago. I express my sincere gratitude to Prof. Dr. Lars Möller, Dr. Denise Zwanziger, Dr. Holger Jäschke and Dr. Sören Latteyer for their kindness and selfless help. Their remarkable ability to explain things clearly, paying attention to details, and their in-depth knowledge, have benefitted me greatly during my research project and have motivated me a lot.

Then, I would like to express my gratitude to Prof. Dr. Kurt Werner Schmid and Dr. Sarah Theurer, for teaching me the knowledge of pathology and providing suggestions for my research project. I am very grateful to Julius Göbel, Stefanie Rehn, and all members of my lab for their kindness and support. At last, I thank my wife, my dear Mrs. Xiulian Liao, not only for the goodness to me but also for taking good care of my parents and our daughter and son when I went abroad for my study in Germany.

10 CURRICULUM VITAE

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