Deciphering the biology of thymic epithelial tumors

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Abstract: Thymic cancers arise from epithelial cells of the thymus and have a predilection for intrathoracic spread. Clinical behavior varies from relatively indolent to highly aggressive with a capacity to metastasize widely and adversely affect survival. Paraneoplastic autoimmune disorders are frequently observed in association with thymoma and have a significant impact on quality of life. Underlying immune deficits associated with thymic epithelial tumors (TETs) increase the risk for development of opportunistic infections and emergence of extrathymic malignancies. Advances in the molecular characterization of thymic tumors have revealed the lowest tumor mutation burden among all adult cancers and the occurrence of distinct molecular subtypes of these diseases. Mutations in general transcription factor IIi (*GTF2I*) are unique to TETs and are rarely observed in other malignancies. The infrequency of actionable mutations has created obstacles for the development of biologic therapies and has spurred research to uncover druggable genomic targets. Persistence of autoreactive T cells due to altered thymic function increases the risk for development of severe immune-related toxicity and limits opportunities for use of immune-based therapies, especially in patients with thymoma. In this paper we review emerging data on the molecular characterization and immunobiology of thymic tumors and highlight clinical implications of these discoveries.

Keywords: Thymoma; thymic carcinoma; somatic mutations; autoimmune regulator (AIRE); immunobiology

Introduction

Thymic epithelial tumors (TETs) are comprised of a family of anterior mediastinal malignancies that include thymomas, thymic carcinomas and thymic neuroendocrine cancers (1). There is considerable variation in the molecular characteristics and clinical behavior of TETs. All TETs have the capacity to metastasize widely and limit survival. Due to underlying immune deficits and persistence of autoreactive T-cells, patients with thymoma are at high risk for developing paraneoplastic autoimmune disorders (2). T-cell dysfunction also predisposes TET patients towards developing opportunistic infections and second cancers (3,4).

Histological classification of TETs is based on the World Health Organization (WHO) system, which categorizes tumors based on characteristics of the malignant epithelial cell and the degree of thymocyte infiltration (5). Staging of thymic cancers has historically been based on a surgical system developed by Masaoka and colleagues (6). However, a newly developed TNM classification is gradually replacing the Masaoka system for staging TETs (7). Early studies of genomic changes in TETs focused on a limited number of oncogenes and tumor suppressor genes, chromosomal copy number alterations and messenger RNA (mRNA) expression profiling (8,9).

The gold standard for treatment of TETs is surgical resection and it is often the only treatment modality required for patients with early-stage disease (10). Locally-advanced TETs require multimodality treatment that

includes chemotherapy and radiation therapy in addition to surgery. Patients with unresectable or metastatic disease are treated with platinum-based chemotherapy (10). Few treatment options are available for relapsed or platinum-refractory TETs. Single-agent chemotherapy is associated with modest clinical activity and limited survival. An expansion of knowledge of the biology of thymic tumors has resulted in the emergence of a limited number of targeted biologic therapies and immunotherapy (11-13).

In this paper we review the molecular characteristics of TETs with a focus on the genomic, proteomic and microRNA (miRNA) profiles of these tumors. We also describe the role of the thymus in T-cell development and the immune deficits observed in TET patients. Clinical ramifications of recent insights into TET biology are outlined briefly.

Genomic landscape of TETs

Early studies to evaluate genomic aberrations in TETs analyzed tumor samples from patients with different stages of disease and various histological subgroups using a single or limited number of molecular platforms (9,14). The overall frequency of genomic alternations was low in TETs, with a relatively higher frequency observed in clinically aggressive WHO subtype B3 thymomas and thymic carcinomas (14).

More recently, comprehensive genomic analyses of TETs have been conducted using multiple molecular platforms. The Cancer Genome Atlas network (TCGA) analyzed 117 TET samples (107 thymomas, 10 thymic carcinomas) derived from previously untreated patients, most of whom had early-stage disease and used the following platforms: somatic copy number variations, mRNA, miRNA, DNA methylation, and reverse phase protein array (15). TETs were found to have the lowest average tumor mutation burden among adult cancers. Recurrent somatic mutations were observed in general transcription factor IIi (GTF2I), HRAS, NRAS and TP53. Integrated analyses identified four distinct molecular subtypes of TETs that showed clinicopathologic similarities to WHO subtypes B, thymic carcinoma, type AB and a mix of type A and AB (15). Mutations in the GTF2I oncogene were found predominantly in type A and AB thymoma, which is consistent with previously published data (16). A higher prevalence of aneuploidy was observed in samples derived from patients with thymoma-associated myasthenia gravis (15).

An independent effort at developing a molecular classification of TETs was conducted by Lee and colleagues by using information on DNA mutations, mRNA expression and somatic copy number alterations from the TCGA data set (17). Two independent cohorts from the NCBI Gene Expression Omnibus were used for validation of results. Four molecular subgroups were identified with the following molecular characteristics: tumors with GTF2I mutations, GTF2I-wild type tumors with expression of genes associated with T-cell signaling, and tumors with chromosomal stability and instability. These molecular subgroups corresponded with WHO subtypes A or AB, B1 or B2, B2, and B2, B3 or C, respectively.

Genomic profile in pre-treated patients with advanced-stage disease

In contrast to TCGA analysis, which was conducted on tumor tissue obtained from patients without exposure to chemotherapy, Wang and colleagues sequenced 197 cancer-associated genes in tumors derived from 78 patients (31 thymomas, 47 thymic carcinomas) with advanced-stage TETs who had received chemotherapy previously (18). Somatic mutations were detected in 39 genes in 29 (62%) thymic carcinomas and 4 (13%) thymomas. Recurrent mutations were observed in 15 genes including BAP1, BRCA2, CDKN2A, CYLD, DNMT3A, HRAS, KIT, SETD2, SMARCA4, TET2, and TP53. Nine (23%) of 39 mutated genes are known to encode for epigenetic regulatory proteins that are responsible for chromatin remodeling, histone modification, and DNA methylation. Recurrent mutations in 7 of 9 epigenetic regulatory genes, including BAP1, ASXL1, SETD2, SMARCA4, DNMT3A, TET2, and WT1, were detected in 16 (34%) cases of thymic carcinoma but were absent in thymoma samples.

Proteome profile of TETs

Ongoing attempts to define the TET proteome are focused on identifying biomarkers to distinguish between histological subtypes of the disease. Characterization of the TET proteome also has the potential to offer insights into pathogenesis and identify targets for biologic therapy. Wang and colleagues conducted one of the first studies to characterize the proteome of all subtypes of thymoma (19). Thirty-six tumor samples representing WHO subtypes A, AB, B1, B2 and B3 thymoma were evaluated by liquid

chromatography tandem mass spectrometry (LC-MS/MS) and compared with normal thymic tissue. Sixty-one proteins were found to be differentially expressed in neoplastic tissue compared with normal thymus and formed two distinct clusters consisting of thymoma subtypes AB, B1 and B2 versus subtypes A and B3. Seven of 61 proteins belonged to collagen family and were downregulated in thymomas. Dysregulation of collagen metabolism in the tumor microenvironment has been previously shown to have variable effects on tumor growth at various stages of cancer development (20). The oncoprotein, stathmin, was found to be upregulated in thymoma subtypes AB, B1 and B2. Stathmin is associated with microtubule biology and is shown to be associated with high cell proliferation, poor prognosis and resistance to microtubule-targeting drugs such as taxanes, which are used for systemic therapy of TETs (21,22). Desmoyokin, encoded by the *AHNAK* (neuroblast differentiation-associated) gene was significantly downregulated in type B thymomas but exhibited no significant differences in expression in subtypes A and AB when compared with normal thymic tissue. Hence, desmoyokin could serve as a marker to differentiate between various subtypes of thymoma. Ninety proteins were found to be differentially expressed between type A and type B3 thymoma. Biological processes associated with these proteins include apoptosis inhibition and cell cycle progression.

Proteogenomic heterogeneity in thymic carcinoma

Mutational evolution and heterogeneity at metastatic sites have been characterized for various tumor types but scant data are available in the context of TETs (23,24). As part of a rapid autopsy protocol for thoracic malignancies, our group has evaluated genomic and proteomic heterogeneity between metastases from seven distinct anatomic sites in a patient with metastatic squamous cell thymic carcinoma (25). An average of 14 non-truncal driver mutations were detected and extreme mutational heterogeneity was observed between metastatic sites. The cytosine deaminase activity of apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like (APOBEC) correlated strongly with mutational heterogeneity. Transcriptomic and proteomic heterogeneity in immune signatures resulted in variations in the immune microenvironment at metastatic sites. These observations provide an explanation for variations in the degree of response to systemic therapy at different sites of disease in an individual patient and merit further evaluation.

miRNA profile of TETs

miRNAs are double-stranded, non-protein-coding RNAs involved in post-transcriptional gene expression (26). These small RNAs play important roles in thymic development and involution (27). The role of miRNAs as oncogenes or tumor suppressor genes is increasingly coming into focus and aberrant miRNA expression is present in various cancers (28,29). miRNA expression profiling of TETs has the potential to yield information on tumorigenesis, help differentiate between histologic subtypes of these tumors and uncover potential therapeutic targets.

A study of 54 TET samples derived from surgical resections in patients not exposed to chemotherapy or radiation therapy revealed differential expression of 87 miRNAs compared with normal thymic tissue (30). Differences in miRNA expression were also observed between thymoma and thymic carcinoma and between subtypes of thymoma. Amongst the most significant changes was upregulation of miR-21-5p, which promotes cell proliferation, and downregulation of miR-145-5p, which functions as a tumor suppressor (31,32).

Radovich and colleagues analyzed 13 TET samples and found overexpression of a large miRNA cluster on chromosome 19q13.42 (C19MC) in type A and AB thymomas resulting in activation of the PI3K/AKT pathway (33). In contrast, C19MC expression was absent in type B thymomas. These results provide a rationale for evaluating PI3K pathway inhibitors in TETs.

Enkner and colleagues also observed overexpression of C19MC in type A thymomas and absence of expression in thymic carcinomas (34). In addition, they found differential expression of a miRNA cluster on chromosome 14q32 (C14MC) between the two histologies with significant down regulation in thymic carcinomas. C14MC appears to have tumor suppressor properties in gastrointestinal stromal tumor and glioma (35,36). Hence, it is conceivable that downregulation of C14MC can contribute to the pathogenesis of thymic carcinoma.

Among non-clustered miRNA, miR21, miR-9-3 and miR-375 are strongly expressed in thymic carcinoma with low expression in type A thymoma whereas, miR-34b, miR-34c, miR-130a, and miR-195 are overexpressed in type A thymoma but infrequently expressed in thymic carcinoma (34). Potential biologic functions of these miRNAs include tumors suppression (miR-34b, miR-34c, miR-130a, and miR-195), oncogenesis (miR21), and immune evasion (miRNA-34), all of which might contribute

to the tumorigenicity of thymic carcinoma (34,37-39).

Evaluation of circulating miRNAs can be developed as a non-invasive tool to evaluate the effect of treatment. In a small study of 5 patients with early-stage, type B thymoma undergoing surgical resection, miR-21-5p and miR-148a-3p levels were found to be elevated in plasma at baseline and showed a significant reduction in the post-operative period (40). If validated in larger studies, these results demonstrate the potential utility of using circulating miRNAs as prognostic biomarkers in patients with TETs.

Immunobiology of TETs

Role of the thymus in T-cell development

Through a series of complex steps, the thymus gland plays a crucial role in the development of central T-cell tolerance, which is required to prevent the emergence of autoimmune diseases (41,42). This process is influenced by the autoimmune regulator (AIRE) gene and the transcription factor forebrain-expressed zinc finger 2 (Fezf2), which promote the expression of tissue-specific antigens to developing T-cells in the thymic medulla resulting in the deletion of self-reactive T-cells by negative selection (42-44). Since the process of negative selection occurs in the thymic medulla, thymic architecture has an impact on maturation of thymocytes. AIRE also promotes the development of a population of self-reactive, immunosuppressive regulatory T cells (Tregs) by positive selection, which hinder the development of autoimmune disease (45,46).

Thymomas and paraneoplastic autoimmune disorders

Changes in thymic architecture and deficient expression of AIRE and major histocompatibility (MHC) class II in thymoma results in a breakdown of central immune tolerance and a predisposition towards autoimmunity. A wide spectrum of autoimmune disorders has been described in association with thymoma (2). Myasthenia gravis is the most common paraneoplastic disorder and is observed most often in patients with WHO subtype B tumors. Thymomas with associated myasthenia gravis have a higher prevalence of aneuploidy and neoplastic thymic epithelial cells overexpress antigens that share sequence similarities with autoimmune targets (15). These findings suggest that defective immune tolerance is unlikely to be the only mechanism underlying the emergence of paraneoplastic autoimmunity.

Thymomas and immunodeficiency

Immune defects associated with thymoma have implications beyond the predisposition towards development of autoimmune disorders. Hypogammaglobulinemia and acquired T-cell immunodeficiency can increase the risk for development of opportunistic infections (47). An increase in secondary malignancies is also observed in TET patients, possibly because of defective immune surveillance (48).

Good's syndrome consists of defects in humoral and cellular immunity and is seen in approximately 5% of patients with thymoma (49,50). The classical immunological deficit associated with Good's syndrome is hypogammaglobulinemia. It is accompanied by B-cell lymphopenia, CD4 cell lymphopenia and impaired T-cell activation (51). Acquired immunodeficiency frequently coexists with autoimmunity in thymoma patients and increases susceptibility to opportunistic infections (47,52).

T-cell abnormalities can occur in thymoma patients even in the absence of Good's syndrome. Changes include an increase in the number of circulating naïve T-cells and a reduction in Treg numbers, which can be accompanied by functional impairment of Tregs and impaired mitogen responses (51,53-55).

The pathogenesis of immunodeficiency in thymoma is poorly understood. An acquired defect in CD247 in naïve T-cells has been described in patients with lymphocyte-rich thymomas, which causes T-cell receptor hyporesponsiveness and cutaneous anergy (56). B-cell lymphopenia in Good's syndrome is postulated to be caused by elimination of B-cells in the bone marrow by autoreactive CD8 T cells and T-cell mediated suppression of B-cell growth (57,58). Defects in cellular immunity in thymoma patients are also caused by anti-cytokine antibodies targeting interferons, interleukin-17 (IL-17) and IL-22 (43,59,60). Acquired immunodeficiency caused by anti-cytokine antibodies can increase the risk for development of opportunistic infections (43,61,62).

Clinical implications of TET biology

Impact of TET genomic profile on drug development

A low tumor mutation burden coupled with a low frequency of actionable mutations presents obstacles for the development of targeted biologic therapies for TETs. Apart from a few exceptions such as sunitinib and everolimus, clinical trials of targeted therapies have yielded disappointing results in patients with advanced TETs (*Table 1*).

Table 1 Selected trials of targeted therapy in advanced thymic epithelial tumors

Intervention	Target	N	Responses [%]	TTP/PFS (months)	OS (months)	Reference
Gefitinib	EGFR	26	1 [4]	4	Not reported	(63)
Imatinib	KIT	15	0	3	Not reached	(64)
Belinostat	HDAC	40	2 [5]	5.8	19.2	(65)
Cixutumumab	IGF-1R	49	5 [10]	8.2	16.2	(66)
Saracatinib	SRC	21	0	5.3	23.1	(67)
Buparlisib*	PI3K	14	1 [7]	11.1	22.5	(68)
Everolimus	mTOR	51	6 [12]	10.1	25.7	(12)
Sunitinib**	KIT, VEGF, PDGFR	23	6 [26]	7.2	Not reached	(11)

*, thymoma only; **, thymic carcinoma only. N, number of evaluable subjects; TTP, time-to-progression; PFS, progression-free survival; OS, overall survival.

This has resulted in the search for new targets to facilitate drug development. Mesothelin and exportin1 (XPO1) are examples of biologic targets expressed in TETs that can be utilized for development of tumor-directed therapy (69,70).

Mesothelin is a cell surface glycoprotein with high levels of expression in most thymic carcinomas (71). Several drugs are in development to target mesothelin including anetumab, an antibody-drug conjugate that is undergoing evaluation in patients with advanced thymic carcinoma (NCT03102320) (69).

XPO1 is involved in nuclear export of tumor suppressor proteins, including p53 and inhibition of XPO1 in TET cells has been shown to induce anti-tumor activity *in vitro* (70). Selinexor, a small-molecule inhibitor of XPO1, is under evaluation in a phase II trial in patients with advanced TETs (NCT03193437).

Effect of genomic profile on survival and disease recurrence

Certain genomic characteristics of TETs appear to be of prognostic value. In a study of genetic alterations in patients with advanced TETs, the presence of somatic mutations was associated with shorter survival (median survival 59 versus 142 months; P<0.05) (18). Mutations in p53 were also associated with worse overall survival (OS) compared with p53-wild-type tumors (median survival 19 versus 106 months; P=0.0003) (18). A negative impact of p53 mutations on survival was also detected in 123 TET cases derived from the TCGA database. Median survival in patients with p53-mutated tumors was 25.4 months. Presence of a p53

mutation was also associated with a shorter time to disease relapse (median disease-free survival was 9.7 months versus not evaluable in p53-wild-type cases due to a low frequency of relapse) (72).

Presence of a *GTF2I* mutation and enrichment of genes involved in T-cell signaling are associated with favorable disease-free survival and OS in contrast to tumors with chromosomal instability (17).

Other prognostic markers under investigation include expression of the genes *SOX2* and *CA9*, which are reported to be associated with shorter survival in TET patients and the methylation status of the genes *KSR1*, *ELF3*, *ILRN* and *RAG1*, which have variable effects on OS (73-75).

Defective immune tolerance and risk of immunotherapy

Defects in central immune tolerance and persistence of autoreactive T-cells in patients with thymoma places them at high risk for developing toxicity related to immune activation resulting from immunotherapy. Unsurprisingly, an increased frequency of immune-related adverse events has been observed in early trials of immune checkpoint inhibitors and cancer vaccines in TET patients despite exclusion of patients with a history of paraneoplastic autoimmune diseases (13,76-78). For immunotherapy to emerge as a safe and feasible alternative for TET patients, especially for those with advanced thymoma, it is imperative to develop strategies to identify patients at risk for immune-related toxicity and establish treatment protocols for adverse events that are observed in response to treatment.

Altered T-cell function and risk of infections and secondary malignancies

Immune dysfunction places TET patients at risk for development of opportunistic infections (3,47). Early recognition and treatment of infectious complications is important in reducing morbidity and improving quality of life. Opportunistic infections observed in patients with thymoma include mucocutaneous candidiasis, pneumocystis pneumonia, cytomegalovirus and herpes virus infections, cryptococcosis and non-tubercular mycobacterial infections (3,47). Patients with hypogammaglobulinemia and recurrent infections can potentially benefit from prophylactic administration of intravenous immunoglobulin.

TET patients are also at increased risk for developing extrathymic malignancies including non-melanoma skin cancers, non-Hodgkin lymphoma, cancers of the gastrointestinal tract, thyroid cancer and lung cancer (4,79). Since second cancers can adversely affect survival, appropriate surveillance and management should be instituted per established guidelines.

Conclusions

The past decade has witnessed a rapid increase in the understanding of the biology of thymic cancers. This process has been facilitated by the availability of next-generation sequencing technologies, the ability to conduct multiplatform analyses and the establishment of thymoma and thymic carcinoma cell lines. Ongoing studies are uncovering novel biologic targets that can be harnessed to develop new systemic therapies. Greater insights into the immunobiology of TETs are resulting in improved management of autoimmune and immunodeficiency disorders and have the potential to mitigate the risks associated with immunotherapy. Efforts to address existing gaps in the knowledge of TET biology will hopefully translate into improved patient outcomes in the future.

Acknowledgments

Footnote

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