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Analysis of a panel of druggable gene mutations and of ALK and PD-L1 expression in a series of thymic epithelial tumors (TETs)

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ABSTRACT

Introduction: Thymic epithelial tumors (TETs) are rare neoplasms with different prognosis lacking consistent molecular alterations possibly leading to targeted therapy. We collected a consecutive series of TETs aimed at investigating the mutational status of druggable genes (*EGFR, c-KIT, KRAS, BRAF, PDGFR-alpha* and *−beta, HER2 and c-MET*) and the expression of ALK and PD-L1.
Patients and methods: One hundred twelve consecutive cases of TETs and relative clinico-pathologic features were collected. Immunohistochemical expression of ALK (clone D5F3) and PD-L1 (clone E1L3N), molecular analysis of *EGFR* (exons 18–21), *c-KIT* (exons 9,11,13,14,17), *KRAS* (exon 2), *BRAF* (exon 15), *PDGFR-alpha* (exon 12) and *-beta* (exons 12, 14, 18), *HER-2* (exons 19 and 20) and *c-MET* (exons 14, 17, 18, 19) mutations were performed. Immuno-molecular results were then statistically matched with clinico-pathologic characteristics.
Results: Patients were male in 54% of cases, with a median age of 61 years (range 19–83) and affected mainly by thymoma (78%) in stage II (45%). At molecular analysis, there were 4 *c-KIT* mutations (occurring in exon 11 V559A, L576P, Y553N and exon 17 D820E) in thymic carcinomas (type C), but not in other tumor types ($p = 0.003$). No mutations were detected in other genes and none case was ALK positive. Twenty-nine (26%) cases were PD-L1 positive (65% of thymic carcinomas and 18% of thymomas). High PD-L1 expression was statistically associated with WHO classification stage type C ($p < 0.001$) and Masaoka stage III–IV disease ($p = 0.007$). In univariate analysis, WHO classification type C, advanced Masaoka stage and absence of myasthenia, but not PD-L1 expressions were correlated with worse survival; at multivariate analysis, only WHO type C confirmed its negative prognostic role.
Conclusion: A subset of TETs as thymic carcinomas can harbor *c-KIT* mutations and elevated PD-L1 expression that could represent targets of potential therapeutic use.
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1. Introduction

Thymic epithelial tumors (TETs) are rare neoplasms arising from the epithelial cells of the thymus with an incidence rate of 0.15 per 100.000 person-years in the United States [1]. According to the 2004 World Health Organization (WHO) classification, TETs are divided into thymomas (A, A/B, B1, B2, and B3 subtypes) and thymic carcinomas (TCs) [2]. Thymomas can be relatively indolent with early stage disease at diagnosis and 5-year survival of almost 80%, while TCs are more aggressive, with presentation in locally advanced or metastatic stage and a 5-year survival of only 40% [3].

Surgical resection is the cornerstone treatment of operable tumors and the only curative treatment for long-term survival. In not-resectable cases (locally advanced and metastatic disease), a multimodality approach with chemotherapy (platinum and

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anthracycline-based regimens), radiotherapy and/or surgery was considered [4]. In recent years, efforts have been made to identify the molecular pathways underlying the tumorigenesis of TETs in order to identify novel treatment strategies. However, actually new drugs are not available in clinical practice of the TET treatment and improved therapeutic strategies are absolutely required.

Therefore, the identification of targetable gene mutations is an emerging need in TET management. We retrospectively investigated the mutational status of druggable several oncogenes (*EGFR, c-KIT, KRAS, BRAF, PDGFR-alpha* and *−beta*, *HER2 and c-MET*) and the expression of ALK.

Moreover, considering the blockade of the immune checkpoint programmed death receptor ligand-1 (PD-L1)/PD-1 pathway has well-established clinical activity across many tumor types and that PD-L1 protein expression by immunohistochemistry is emerging as a predictive biomarker of response to these therapies [5], we retrospectively also examined PD-L1 expression. We investigated these immuno-molecular characteristics in a series of TETs and their relation to clinico-pathologic features and prognostic relevance.

2. Patients and methods

2.1. Patients

All consecutive patients with primary TETs (thymoma, thymic carcinoma and neuroendocrine tumors) were retrospectively retrieved from the archival files of the Unit of Pathologic Anatomy of the University Hospital of Modena (Italy), while 7 cases derived from consultation files of one of the authors (GR). A total of 112 cases were collected between 1991 and 2015. Samples were processed according to conventional protocol and the tissue was routinely fixed in 10% buffered formalin and paraffin-embedded. The histologic classification of the tumor was based on the review of all hematoxylin and eosin-stained sections and diagnostic immunostains, including cytokeratins (AE1/AE3, CK19, MNF116), CD5, p63, CD117, neuroendocrine markers (chromogranin A, synaptophysin, CD56), when necessary. All the slides were reviewed at a multiheaded microscope by two expert thoracic pathologists (GR, MM).

There were collected 38 (34%) thymic biopsies and 74 (66%) surgical resection specimen, of which 66 (89%) cases underwent to complete resection. In according to 2004 WHO classification [2], there were 87 (78%) cases of thymomas (11 type A, 11 type B1, 26 type B2, 16 type A/B, 4 type B1/B2, 8 type B2/B3, 11 type B3), 20 (18%) cases of thymic carcinomas (type C) and 5 (4%) cases of atypical carcinoids. According to Masaoka-Koga staging, there were 27 surgically resected cases in stage I, 45 in stage II and 2 in stage III. In the 38 cases lacking surgical resection, we applied the Masaoka-Koga scheme according to the imaging studies, with 5 cases in stage II, 18 in stage III and 15 in stage IV.

Furthermore, according to Moran-Suster classification [3] there were 68 (60.7%) typical thymomas, 18 (16%) atypical thymomas, 21 (18.8%) thymic carcinomas and 5 (4.5%) neuroendocrine carcinomas (grade 2).

The relevant clinico-pathological data were collected by review of patients' charts, pathologic reports and information from referring physicians. The study was conducted in accordance with the precepts of the Helsinki Declaration and all data were handled anonymously according to the national laws.

2.2. Immunohistochemical (IHC) stains

In each case, 4-μ thick sections were obtained from a representative block. Sections were air dried overnight at 37 °C, then deparaffinized in xylene and rehydrated through a decreasing concentration of alcohol to water. Endogenous peroxidase activity was blocked by immersion for 10 min with 3% hydrogen peroxide (H2O2) in methanol. Incubation with primary antibodies was accomplished with a modified streptavidin–biotin–peroxidase technique using an automated immunostainer (Benchmark XT, Ventana Medical Systems, Tucson, AZ); 3-3diaminobenzidine was used as the chromogene and Harris's hematoxylin as the counterstain. The panel of the primary antibodies used in the study and their technical characteristics are the following: CD117 (clone A4502, Dakopatts, Glostrup, Denmark; 1:200 dilution), CD5 (clone SP19, Ventana; prediluted), p63 (clone 4A4, Ventana; prediluted), chromogranin (clone LK2H10, Ventana; prediluted), synaptophysin (polyclonal, Ventana; prediluted), pan-cytokeratins (clone AE1/AE3, Ventana; prediluted), pan-cytokeratins (clone MNF116, Ventana; prediluted), CD56 (clone XXX, Ventana; prediluted), ALK (clone D5F3, Ventana; prediluted), PD-L1 (clone E1L3N, 1:500, CellSignaling Technology, Danvers, MA).

Negative and positive controls were included in each batch. Negative controls for specificity of staining were carried out by immunostaining duplicate sections with nonimmune mouse IgG, at the same concentration as that of the corresponding primary antibody. A normal tonsil tissue was used as a positive control for CD117 (mast cells), CD5 (T lymphocytes) and p63 (squamous epithelium), while a pulmonary typical carcinoid serve as positive control for chromogranin and synaptophysin. The percentage of positive cells and the intensity of staining (0: negative; 1+: weak; 2+: moderate; 3+: strong) were recorded, and a lesion was considered positive when at least 10% of the cells reacted with a moderate or strong intensity on the relevant subcellular localization (nuclear for p63, cytoplasmic for chromogranin and synaptophysin and cytoplasmic/membranous for CD117 and CD5). For the PD-L1 expression, the score proposed by Katsuya et al. [6] was adopted in the study. ALK expression was calculated as positive or negative, as previously suggested [7], using an ALK-positive inflammatory myofibroblastic sarcoma as IHC control in each batch. In 12 randomly selected cases (including 7 thymomas and 5 thymic carcinoma) ALK FISH test was performed using a break-apart probe specific to the ALK locus (Vysis LSI ALK dual-color, break-apart rearrangement probe; Abbot Molecular, Abbot Park, IL) according to the manufacturer's instructions (see supplemental file).

2.3. DNA extraction and mutational analysis

Five-μm thick sections obtained from a representative paraffin-embedded block were deparaffinized by xylene, and tumor DNA was extracted using an identical manual microdissection method as previously described. Microdissected tumor cells were subject to proteinase K treatment in a digestion buffer (50 mM Tris [pH 8.5], 1 mM EDTA, 0.5% Tween 20) and then incubated overnight at 37 °C. PCR was performed in 20 μL reactions containing 2.0 μL DNA, 2 μL of commercial PCR buffer (Applied Biosystem), 1.0–1.5 mM MgCl2, 200 μM of each dNTP, 20 pmol of each primer, and 3 units of AmpliTaq gold polymerase (Applied Biosystem). PCR reaction was carried out on Uno II Thermoblock (Biometra, Gottingen, Germany). Initial denaturation at 94 °C for 10 min was followed by 41 cycles, and a final extension step (7 min at 72 °C). The cycles included denaturation at 95 °C for 1 min, annealing at 55–58 °C for 1 min, and extension at 72 °C for 2 min. The amplified DNA was electrophoresed on 2% agarose gel for 1 h at 110 V. The amplification products were then purified by using MinElute PCR purification Kit (Qiagen) as indicated by the manufacturer's instructions. PCR products were then sequenced in both directions with ABI Prism BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems), using the same primers as those employed for PCR. PCR products were finally purified by Centri-Sep Spin Columns (Applied Biosystem) and subsequently runned on the ABIPrism 310 automatic sequencer

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