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Immune checkpoint blockade as a potential therapeutic strategy for undifferentiated malignancies☆

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Summary Undifferentiated malignancies (UMs) encompass a diverse set of aggressive tumors that pose not only a diagnostic challenge but also a challenge for clinical management. Most tumors in this category are currently treated empirically with nonspecific chemotherapeutic agents that yield extremely poor clinical response. Given that UMs are inherently genetically unstable neoplasms with the potential for immune dysregulation and increased neoantigen production, they are likely to be particularly amenable to immune checkpoint inhibitors, which target programmed cell death protein 1 (PD-1) or its ligands, PD-L1 and PD-L2, to promote T-cell antitumor activity. Aberrant expression of PD-L1 and, more recently, chromosomal 9p24.1/CD274 (PD-L1) and PDCD1LG2 (PD-L2) alterations can be used as biomarkers to predict responsiveness to checkpoint inhibitors. Here we evaluated 93 cases previously diagnosed as an “undifferentiated” malignancy and found that 56% (52/93) of UMs moderately to strongly express PD-L1 by immunohistochemistry (IHC). Concurrent CD274 (PD-L1) and PDCD1LG2 (PD-L2) fluorescence in situ hybridization (FISH) was performed on 24 of these cases and demonstrates a genetic gain at both loci in 62.5% of UMs. Genetic alterations at the CD274 (PD-L1) and PDCD1LG2 (PD-L2) loci were found to be completely concordant by FISH. Overall, we found that a significant proportion of UMs express PD-L1 and provide molecular support for using checkpoint inhibitors as a treatment approach for this class of tumors. © 2018 Elsevier Inc. All rights reserved.

1. Introduction

Undifferentiated malignancies (UMs), which include cancers of unknown primary, are a heterogeneous group of tumors with little to no evidence of cell lineage differentiation.

Conventional cancer therapies have relied on cell lineage of the neoplasm to direct treatment and enrollment in clinical trials. Therefore, the diagnostic workup of UMs often involves exhaustive immunohistochemical or gene expression analysis in an attempt to determine the cell lineage and/or tissue of origin. Without a clear cell lineage to guide therapy, UMs are largely treated empirically with nonspecific cytotoxic drugs (e.g. platinum-based chemotherapies, taxane, gemcitabine) that invariably result in poor outcome [1]. In the era of precision medicine, initial studies have proposed a shift away from the costly and time-consuming search for a site or cell-of-origin and a movement toward understanding the underlying specific biologic drivers. Recent, comprehensive genomic profiling of

COVID-19 and its impact on the healthcare system.
UMs has shown potential in identifying targetable genomic alterations to help individualize treatment [2-4]. While identifying genetic biomarkers and “actionable mutations” have the potential to direct therapy selection, the biologic context of genomic alterations is often still of importance and dependent on cell lineage in some instances. For example, BRAF inhibitors show very different response rates in BRAF-mutated melanoma versus colon cancer. Given this potential limitation, additional types of treatment strategies are still urgently needed to treat this aggressive class of tumors.

Immunotherapy-based targeting of programmed cell death 1 (PD-1) or its ligands (PD-L1 and PD-L2) to promote antitumor T-cell activity has emerged as a promising method to effectively treat a diverse set of cancers, irrespective of cell lineage [5,6]. Immune checkpoint inhibitors, which rely on the patient’s own T-cells to destroy tumors, have been highly effective in PD-L1 expressing tumors with high genomic instability and tumor mutational burden (TMB) [7-9]. In addition, recent studies have shown that chromosomal alterations, specifically gain of 9p24.1, which encodes CD274 (PD-L1) and PDCD1LG2 (PD-L2), may also help predict response to immunotherapy [6]. Importantly, features inherent to UMs, such as genomic instability and increased tumor mutational burden, which have previously made these tumors aggressive and challenging to treat, may now promote checkpoint inhibitor efficacy and provide a way forward. In fact, several recent studies have demonstrated increased aberrant PD-L1 expression in subsets of high-grade or poorly-differentiated tumors of identifiable cell lineage [10-14]. Even more promising, a case report of a cancer of unknown primary demonstrating both PD-L1 expression and CD274 (PD-L1) amplification showed a rapid response to the anti-PD-1 inhibitor pembrolizumab and sustained near complete remission 14 months after treatment [10]. To explore the potential utility of checkpoint inhibitors as a novel treatment approach for UMs, we evaluated PD-L1 protein expression and 9p24.1/CD274 (PD-L1)/PDCD1LG2 (PD-L2) genetic status by FISH in undifferentiated tumors.

2. Materials and methods

2.1. Case selection

Following approval by the Institutional Review Board at Stanford University, the pathology database was searched from year 2007 to 2017 for all “undifferentiated” malignancies. Cases with the diagnosis of either “undifferentiated carcinoma” or “undifferentiated malignant neoplasm” were included in the study cohort. In total, ninety-three patients with tumors that had available formalin-fixed, paraffin-embedded (FFPE) material were identified. Tumors included biopsies and resections from a variety of anatomic sites, including the gynecologic tract, gastrointestinal tract, thorax, genitourinary, head and neck, extremities, breast, trunk, pelvis, retroperitoneum, bone and lymph node. Cases with available tissue blocks were used to prepare a tissue microarray as previously described [15]. In some cases, the exact site and/or cell of origin was not identified despite extensive radiologic and pathologic workup. Clinical information for all 93 cases in this cohort is provided in Supplementary Table 1.

2.2. Immunohistochemical staining

All immunohistochemical studies were performed on 4-μm sections prepared from formalin-fixed and paraffin-embedded tissue. An automated immunostaining platform (Leica Bond III, Leica Biosystems, Buffalo Grove, IL), and the PD-L1 rabbit monoclonal antibody (clone E1L3N, Cell Signaling Technologies, Danvers, MA) at 1:500 dilution, 30 minute incubation after pretreatment at ER2 setting for 30 minutes was used for immunohistochemical staining. Appropriate positive and negative controls were included and evaluated with the specimens tested. Based on recent literature comparing the efficacy of anti-PD-L1 clones, and our own experience with several commercial PD-L1 clones, we selected the E1L3N clone for PD-L1 immunohistochemistry. [16,17] This methodology was validated in a series of non-small cell lung carcinomas against the FDA-approved PharmDx 22C3 immunohistochemistry assay. The stained slides were reviewed by two pathologists and the percentage of immunoreactive tumor cells (0% to 100%) and the average intensity of the staining (0 - negative, 1 - weak staining above background, 2 - moderate staining, 3 - strong staining) was scored. An H-score was calculated by multiplying the percentage of immunoreactive tumor cells by the average stain intensity.

2.3. Fluorescence in situ hybridization

CD274 (PD-L1) and PDCD1LG2 (PD-L2) FISH studies were performed on 4-μm sections prepared from FFPE tissue. Twenty-four cases were analyzed, of which 7 were whole slide sections and 17 were tissue microarray sections of duplicate 0.6-mm cores. Slides were deparaffinized with CitroSolv (Fisher Scientific), digested with CytoZyme Stabilized Pepsin (SciGene), pre-treated with a sodium thiocyanate solution at 80°C (VP2000™ Pretreatment Solution; Abbott Molecular), re-fixed for 10 minutes in 10% buffered formalin, and dehydrated in an ethanol series. Dried, dehydrated slides were de-natured with a VysisHYBrite instrument at 80°C for 6 minutes and hybridized for 48 hours at 37°C with probes targeting CD274 (PD-L1) (green), PDCD1LG2 (PD-L2) (red) and the centromeric region of chromosome 9 (CEN9) (blue) (Empire Genomics, Buffalo, NY). Slides were washed with post-hybridization wash buffer (2xSSC/0.3% NP-40) at 73°C for 2 minutes and counterstained with DAPI. Cells were analyzed using an Olympus BX51 microscope with appropriate fluorescent filters and documented using CytoVision imaging software (Leica Biosystems). Fifty interphase nuclei were
analyzed for each case and the CD274 (PD-L1), PDCD1LG2 (PD-L2), CEN9 signals were quantified. In addition to the UM cases, 6 non-neoplastic controls (uterus, lung, adrenal, pancreas, stomach and tonsil tissue) were analyzed by similar methods.

Genomic alterations at the CD274 (PD-L1) and PDCD1LG2 (PD-L2) loci by were characterized by FISH. Target:control ratios, specifically that of CD274 (PD-L1):CEP9 and PDCD1LG2 (PD-L2):CEP9, were calculated for 50 cells per tumor. Tumors were scored as amplified if the target:control ratio was ≥3:1 per cell and gain if the target:control ratio was >1:1 but <3:1 per cell, per the Roemer et al. method [18]. A cutoff was generated for the “gain” category based on scored control tissue given that a subset of cells in the control tissue demonstrated a ‘gain’ target:control ratio of 2–3:1–2 likely due to plane of section artifact. Therefore, to be categorized as having ‘gain’, the percentage of tumor cells showing ‘gain’ had to be greater than that of the average control tissue percentage.

Fig. 1 Clinical features of patients with UMs (n = 93).

<table>
<thead>
<tr>
<th>Features</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>60.5 (16-89)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>39 (42%)</td>
</tr>
<tr>
<td>Female</td>
<td>54 (58%)</td>
</tr>
<tr>
<td>Procedure</td>
<td></td>
</tr>
<tr>
<td>Biopsy</td>
<td>44 (47%)</td>
</tr>
<tr>
<td>Resection</td>
<td>49 (53%)</td>
</tr>
<tr>
<td>Primary tumor site</td>
<td></td>
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</table>

<table>
<thead>
<tr>
<th>H&amp;E</th>
<th>PD-L1 IHC</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>B</td>
</tr>
</tbody>
</table>

Fig. 2 Distribution of PD-L1 immunohistochemistry (IHC) intensity and percentage of positive tumor cells in UMs (n = 93). Representative histology by hemotoxylin and eosin (H&E) stains (A) and PD-L1 IHC for each intensity category (0 - negative, 1 - weak staining, 2 - moderate staining, 3 - strong staining, displayed from left to right) (B). Summary of the PD-L1 IHC intensity and the percentage of positive tumor cells for all UMs (individual case data provided in Supplementary Table 1) (C).

<table>
<thead>
<tr>
<th>Tumor %</th>
<th>Intensity</th>
<th>0</th>
<th>1+</th>
<th>2+</th>
<th>3+</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;10%</td>
<td>N/A</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>10–&lt;25%</td>
<td>N/A</td>
<td>4</td>
<td>5</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>25–&lt;75%</td>
<td>N/A</td>
<td>3</td>
<td>10</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>75–100%</td>
<td>N/A</td>
<td>1</td>
<td>1</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>31 (33.3%)</td>
<td>10 (10.8%)</td>
<td>17 (18.3%)</td>
<td>35 (37.6%)</td>
<td></td>
</tr>
</tbody>
</table>
2.4. Statistical analysis

Statistical analysis was performed using an unpaired Student t-test and variation among and between genetic categories (negative, gain, amplification) was calculated using ANOVA. P-values of < 0.05 were determined to be statistically significant. All statistical data was calculated with the GraphPad Prism 6 software.

3. Results

3.1. Case characteristics

The clinical features of the 93 patients with UMs included in this study are summarized in Fig. 1 and listed in Supplementary Table 1. Briefly, the UMs in our cohort were diagnosed in patients between the ages of 16 and 89 (mean age 60.5) with a male:female ratio of approximately 3:4. The cases are comprised of 47% biopsies and 53% resections. The location of the neoplasm at the time of biopsy or resection includes a wide variety of anatomic sites, such as the gynecologic tract, gastrointestinal tract, thorax, genitourinary tract, head and neck, extremities, breast, trunk, pelvis, retroperitoneum, bone and lymph node sites as shown in Fig. 1 and Supplementary Table 1.

Immunohistochemical staining for cytokeratin (pan-cytokeratin) and/or epithelial membrane antigen (EMA) was performed on 87 of the cases during their diagnostic evaluation of which 42% (36/87) displayed immunoreactivity suggestive of, but not definitive for, an epithelial origin.

3.2. Aberrant PD-L1 expression in undifferentiated malignancies

Immunohistochemical staining for PD-L1 was performed on all 93 cases using whole slide sections. Aberrant PD-L1
immunoreactivity of any degree (1 to 3+) was observed in 66.7% (62/93); however, a high proportion or 56% (52/93) of UMs demonstrated moderate to strong (2 to 3+) PD-L1 immunoreactivity. Of these cases with moderate to strong PD-L1 expression, 46% (24/52) showed diffuse immunoreactivity in greater than 75% or more of the tumor cells analyzed. The distribution of PD-L1 immunohistochemical stain intensity and percentage of positive tumor cells is summarized in Fig. 2.

3.3. Genetic analysis of the CD274(PD-L1) and PDCD1LG2(PD-L2) loci by FISH in UMs

FISH was used to assess chromosomal alterations in the 9p24.1 region containing the CD274(PD-L1) and PDCD1LG2(PD-L2) genes. A total of twenty-four cases with available material for additional analysis underwent FISH using three probes specific for CD274(PD-L1), PDCD1LG2 (PD-L2) and the centromeric region of chromosome 9 (CEP9). Copy number gain in CD274(PD-L1) and PDCD1LG2(PD-L2) was observed in 62.5% of UMs. Representative tumor histomorphology, PD-L1 immunohistochemistry and CD274(PD-L1) and PDCD1LG2(PD-L2) FISH is displayed in Fig. 3. Co-amplification or co-gain was observed in a high proportion or 62.5% (15/24) of the UMs.

Importantly, none of the 24 cases studied showed discordant alterations in CD274(PD-L1) and PDCD1LG2(PD-L2). Of note, one case showed statistically significant (P < 0.05) CD274(PD-L1) and PDCD1LG2(PD-L2) discordance as well as CD274(PD-L1) and PDCD1LG2(PD-L2) target:control ratio discordance within individual cells scored; however, the overall genetic categorization of this neoplasm was entirely concordant between the two gene loci.

3.4. PD-L1 protein expression and CD274(PD-L1) genetic alterations

PD-L1 protein expression was assessed in relation to chromosomal alterations in CD274(PD-L1). Both PD-L1 expression intensity (0 to 3+) and percentage of positive tumors cells (0% to 100%) were used to generate an H-score derived from multiplying the two values together (0–300). The average and median PD-L1 H-score was the highest in the CD274(PD-L1) amplified cases; however, no statistically significant differences (P > 0.05) were observed between the H-scores in the different genetic categories. This finding reflects the fact that cases without CD274(PD-L1) copy number gain demonstrate a wide range of PD-L1 protein expression and can have robust and diffuse PD-L1 protein expression that is independent of chromosomal alteration. There is a trend towards increasing PD-L1 H-scores in the copy number amplified group which were higher than those in the FISH-negative and gain groups (P = 0.045). A graphical representation of the distribution of the PD-L1 H-scores by genetic category is shown in Fig. 4.

4. Discussion

Undifferentiated malignancies constitute a unique group of aggressive neoplasms for which the overall survival is poor with current treatment strategies, indicating an unmet need for novel, more effective therapies. Immunotherapy has recently shown promise across many tumor types, however, its potential for the treatment of UMs has not been extensively evaluated. This study was undertaken on a large cohort of UMs for biomarkers that portend response to checkpoint inhibitors and argues for their potential use as a novel treatment strategy for this class of tumors.

In our study of 93 UMs, we found that a high proportion (56%) demonstrates moderate to strong expression of PD-L1 protein in neoplastic cells, suggesting that many of these neoplasms share a common pathobiology of immune checkpoint dysregulation. Currently, assessing for PD-L1 expression by immunohistochemistry is the best characterized and clinically utilized biomarker for predicting response to immunotherapies; however, interpretation can be complicated by factors such as the antibody clone used and tumor heterogeneity, among others. Here PD-L1 immunohistochemistry was performed using the E1L3N clone, which has been shown to have comparable, if not superior, staining to that of the 22C3 and 28–8 clones, which are being used as companion diagnostics for pembrolizumab and nivolumab, respectively, making our findings clinically relevant for current ongoing trials using these therapies [16]. While over a third of tumors demonstrated no (33.3%) to only weak (10.8%) staining, an important caveat of using PD-L1 immunohistochemistry as a biomarker is the potential underestimation of dynamic changes since only a
snapshot of the tumor biology is represented at any one time. In fact, several groups have shown that tumor positivity is subject to change when tested on different specimens (eg, biopsy versus resection) and in the setting of different environmental factors (eg, radiation) [19-22]. Importantly, while PD-L1 expression correlates with a higher response rate to checkpoint inhibitor therapies, it is not entirely predictive of therapeutic efficacy, as some tumors lacking PD-L1 staining have also been shown to respond [6,23-27]. Although PD-L1 expression in immune and stromal cells was not formally characterized in this study, non-tumor cell expression was also observed in some cases and may contribute to anti-tumor immune inhibition.

Identifying amplifications of chromosome 9p24.1 is an emerging biomarker that provides a genetic basis for aberrant PD-L1 expression and correlates with a robust response to checkpoint inhibitor therapy [9,10]. In addition to PD-L1 protein expression, we analyzed chromosomal alterations at the CD274(PD-L1) and PDCD1LG2(PD-L2) loci by FISH. We found that 62.5% of UMs show chromosomal gain at the CD274(PD-L1) and PDCD1LG2(PD-L2) loci and that co-amplification strongly correlated with aberrant PD-L1 protein expression. Given the high degree of genomic instability in many UMs, it is not surprising that several tumors showed genetic alterations of chromosome 9p24.1. Previous studies in Hodgkin lymphoma have shown that while chromosomal gain of 9p24.1 by CD274(PD-L1)/PDCD1LG2(PD-L2) is associated with advanced stage disease and shorter progression-free survival, checkpoint inhibitors are highly effective in patients with a genetic basis for PD-L1 overexpression [9,18]. Amplification of CD274(PD-L1) by FISH has been observed in a variety of cancers and has been shown to correlate with mRNA and protein expression [18,28-32]. Incorporation of 9p24.1 amplification status will likely be of prognostic utility and a complimentary biomarker to PD-L1 immunohistochemistry. To our knowledge, CD274(PD-L1) and PDCD1LG2(PD-L2) amplification status is not yet used in the clinical setting, but will likely become a more mainstream diagnostic assay in the near future.

Given that the genetic status of either CD274(PD-L1) or PDCD1LG2(PD-L2) could be useful in helping predict tumor response to immune checkpoint blockade, we examined both loci separately in this study. The genetic status of CD274(PD-L1) and PDCD1LG2(PD-L2) was concordant for all tumors tested, corroborating findings in other tumor types [18,28,29]. This high degree of concordance is accounted for by the close proximity (approximately 40 kb) of CD274(PD-L1) and PDCD1LG2(PD-L2) at the chromosome 9p24.1 locus. These results underscore that a simplified FISH assay with a single target probe to either CD274(PD-L1) or PDCD1LG2(PD-L2), in addition to a control probe to the centromeric region of chromosome 9, would be effective as a widely applicable clinical FISH assay.

Genome-wide approaches will also likely be used to assess for CD274(PD-L1) and PDCD1LG2(PD-L2) copy number variations independently, or as a complimentary method to FISH. Several groups have detected genetic alterations in CD274(PD-L1) by comparative genomic hybridization or deep sequencing in various differentiated tumor types [29,33-35]. Importantly, CD274(PD-L1) copy number gains were also shown to correlate with mRNA and protein expression [10,33-35].

While CD274(PD-L1) and PDCD1LG2(PD-L2) amplification is one potential mechanism for aberrant PD-L1 expression in UMs, there are likely multiple modalities to achieve immune dysregulation as the tumor biology is complex in these neoplasms. Despite the majority of CD274(PD-L1) amplified tumors showing strong PD-L1 protein expression, no statistical significance was found between the PD-L1 H-scores in amplified versus and non-amplified UMs. This result can be partially explained by the fact that non-amplified tumors showed a wide range in PD-L1 protein expression. Although we are underpowered to detect a true difference in PD-L1 H-scores across FISH subgroups (negative, gain, amplification), our data support the notion that there are numerous mechanisms contributing to upregulation of PD-L1 expression, of which copy number alteration is only one. This explains the heterogeneity in PD-L1 H-scores in the copy number negative group. There does appear to be a trend towards increasing PD-L1 H-Scores in the copy number amplified group, which has biologic plausibility. In larger cohorts of Hodgkin lymphoma and squamous cell carcinoma of the cervix and vulva, statistical significance between PD-L1 expression and genetic status was observed [18,28]. Interestingly, however, in other cancer types, such as triple-negative breast cancer, no significant association was observed between 9p24.1 genetic status and PD-L1 protein expression [32]. Additional complexity in the PD-L1 regulatory network will require further and extensive study and validation to fully understand anti-tumor immune dysregulation.

Lastly, a recent case report describes a 44-year-old woman with a refractory high-grade metastatic tumor of unknown primary that was successfully treated with a check-point inhibitor. Specifically, the tumor was found to have a high mutational load, focal high-level amplification of chromosome 9p, including the CD274(PD-L1) locus, as well as high mRNA and protein expression of PD-L1. After several rounds of failed multimodality therapy, pembrolizumab was initiated with near-complete response at 6 months and sustained durable remission at 14 months [10]. This case provides a strong rationale for the broader application of checkpoint inhibitors to this class of tumors.

In summary, our results show a high proportion (>50%) of UMs aberrantly express PD-L1 protein in tumor cells. While immune dysregulation in UMs is likely multifactorial, amplification of CD274(PD-L1) is seen in a subset of tumors and often coincides with robust aberrant PD-L1 protein expression. Our data suggest that the majority of UMs may be sensitive to immune checkpoint inhibitors and provide a new treatment strategy for such aggressive tumors that currently have a devastating prognosis and poor survival rates.
Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.humpath.2018.06.034.

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