Comprehensive analysis of cancers of unknown primary for the biomarkers of response to immune checkpoint blockade therapy

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Abstract  Background: Cancer of unknown primary (CUP) accounts for approximately 3% of all malignancies. Avoiding immune destruction is a major cancer characteristic and therapies aimed at immune checkpoint blockade are in use for several specific cancer types. A comprehensive survey of predictive biomarkers to immune checkpoint blockade in CUP were explored in this study.

Methods: About 389 cases of CUP were analysed for mutations in 592 genes and 52 gene fusions using a massively parallel DNA sequencing platform (next-generation sequencing [NGS]). Total mutational load (TML) and microsatellite instability (MSI) were calculated from NGS data. PD-L1 expression was explored using immunohistochemistry (with 5% cutoff value).

Results: High TML was seen in 11.8% (46/389) of tumours. MSI-high (MSI-H) was detected in 7/384 (1.8%) of tumours. Tumour PD-L1 expression was detected in 80/362 CUP (22%). A small proportion of CUP cases harboured genetic alterations of negative predictive biomarkers to immune checkpoint inhibitors (predictors to hyperprogression) including \textit{MDM2} gene amplification (2%) and loss of function \textit{JAK2} gene mutations (1%). Amplifications of \textit{CD274} (PD-L1) and \textit{PDCD1LG2} (PD-L2) genes were also rare (1.4% and 0.8%, respectively). The most frequently mutated genes were \textit{TP53} (54%), \textit{KRAS} (22%), \textit{ARID1A} (13%), \textit{PIK3CA} (9%), \textit{CDKN2A} (8%), \textit{SMARCA4} (7%) and \textit{PBRM1}, \textit{STK11}, \textit{APC}, \textit{RB1} (5%, respectively).

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1. Introduction

Cancer of unknown primary (CUP) are a heterogeneous group comprising approximately 3–5% of all malignancies and are associated with poor prognosis [1–3]. Usually, extensive tumour sample investigations are performed to identify the presumed tissue of origin [3–5], but in true CUP, by definition, the diagnosis of the primary cancer cannot be verified. Recently, we [6] and others [7–10] have identified numerous genetic alterations in common cancer pathways [11] in CUP, providing an opportunity to administer pathway-specific (targeted) therapies in CUP. All these studies identified at least one clinically targetable genetic alteration in CUP. In contrast to the previous studies, we utilised an extended next-generation sequencing (NGS) panel composed of 592 genes and used Archer Panel to explore the gene fusions.

In the last couple of years, a dramatic improvement in advanced cancer therapy has been achieved with immune checkpoint blockade. To date, five immune checkpoint inhibitors (pembrolizumab, nivolumab, atezolizumab, avelumab and durvalumab) targeting either programmed death 1 (PD-1) or its ligand (PD-L1) have received the US Food and Drug Administration (FDA) approval (https://www.fda.gov/) and caused a paradigm shift in treatment of various cancer types including melanoma, non–small cell lung carcinoma, renal cell carcinoma, advanced bladder carcinoma, Merkel cell carcinoma, gastroesophageal junction adenocarcinoma and classical Hodgkin lymphoma [12–23]. Several predictive biomarkers for immune checkpoint inhibitors have been proposed (PD-L1 status in tumour and inflammatory cells, tumour mutational load and microsatellite instability [MSI] status) and some have achieved companion diagnostics status (e.g. PD-L1 immunohistochemistry in certain cancer lineages and MSI status in all tumours regardless of a lineage). In addition, recent breakthrough studies revealed several predictors of hyperprogression after the therapy with the immune checkpoint inhibitors (e.g. JAK1/2, MDM2 and EGFR) [24–26]. A comprehensive molecular profiling (biomarkers) of CUP with regard to immune checkpoint inhibitors has not been conducted so far. Therefore, we decided to explore a comprehensive survey of predictive biomarkers to immune checkpoint inhibitors in a large cohort of CUP profiled at a single institution.

2. Results

2.1. Patients and histopathologic characteristics

Three hundred eighty-nine patients (53% female and 47% male) were included in the study cohort. The average patient’s age was 62.7 years. No clinically recognised primary tumour site was identified in any of the patients tested (Table 1) [3].

Histologically, CUP were classified as adenocarcinomas (n = 175, 45%), carcinomas not otherwise specified (n = 120, 31%), squamous cell carcinomas (n = 30, 8%) or other subtypes (n = 64, 16%) (Table 1). Referring laboratories’ immunohistochemical analyses for markers of tissue of origin (e.g. wide-spectrum cytokeratins [AE1/AE3, Cam5.2], CK7, CK20, PSA, oestrogen receptor, progesterone receptor, CDX2, TTF1, napsin-A, thyroglobulin, calcitonin, neuroendocrine markers: NSE, chromogranin, synaptophysin) were non-conclusive in all analysed cases (i.e. more than one possible site of origin was considered) [3]. Board-certified pathologists reviewed all cases and selected appropriate slides for molecular profiling.

2.2. Predictive biomarkers to immune checkpoint inhibitors

Fig. 1 (Venn diagram) summarises total mutational load (TML), PD-L1 status and MSI status for the subgroup of CUP tumours that had PD-L1, MSI and TML information available (n = 362).

In the complete cohort of 389 tumour analysed, TML-high was seen in 11.8% (46/389) of CUPs, similar to the rate observed in common cancers profiled at Caris (Non-small cell lung cancer (NSCLC), bladder carcinoma, Fig. 3). In contrast to other common cancers, MSI-high (MSI-H) rate was detected in 7/389 (1.8%) of CUP cases (Fig. 3). Subsequent immunohistochemistry (IHC) analysis of MSI-H cases showed combined loss of expression of MSH2 and MSH6 or MLH1 and PMS2 mismatch repair proteins in five cases and isolated PMS2 loss in one case, while one case was not evaluable (Table 2). In addition, 12 microsatellite stable cases by NGS were also confirmed by IHC as mismatch repair proficient (no loss of expression of mismatch repair proteins).

Expression of PD-L1 (on ≥5% cancer cells) was seen in 22.5% (82/365) of tumours, while the presence of PD-L1...
expressing tumour infiltrating lymphocytes was seen in 58.7% (37/63) of cases.

2.3. Individual gene alterations detected by NGS

Fig. 2 and Supplemental Table 1 illustrate detected gene alteration frequencies in the entire cohort.

A total of 70 different genes were found with pathogenic and presumed pathogenic mutations ranging in incidence from 0.3% to 54%; the most frequently mutated gene was \textit{TP53} (54%), followed by \textit{KRAS} (22%), \textit{ARID1A} (13%), \textit{PIK3CA} (9%), \textit{CDKN2A} (8%), \textit{SMARCA4} (7%), \textit{PBRM1}, \textit{STK11}, \textit{APC}, \textit{RB1} (5%, respectively) and \textit{PTEN}, \textit{BRAF}, \textit{NF2}, \textit{BAP1} (4%, respectively). \textit{ERBB2} (HER2) was mutated in 1.5% of cases while \textit{BRCA1} and \textit{BRCA2} were each mutated in 1%.

Gene amplifications of \textit{CCND1} (5%), \textit{FGF3}, \textit{FGF4}, \textit{FGF19} (3%, respectively; all located on chromosome 11q13.3 near \textit{CCND1}), \textit{ERBB2}, \textit{MYC} (3%, respectively) were most frequent, while \textit{AKT2}, \textit{MCL1}, \textit{KRAS}, \textit{CCNE1} and \textit{MDM2} were each amplified in ~2% of the cases. Of note, amplifications of \textit{CD274} (PD-L1), \textit{PDCD1LG2} (PD-L2) and \textit{JAK2} (all located at chromosome 9p24.1) were rare (1.4, 0.8 and 1.1%, respectively).

Targetable gene fusions were identified in five cases including two \textit{FGFR2} fusions, two \textit{RET} fusions and one \textit{RAF1} fusion. Tumours in which fusions were identified as cancer driver events carried a significantly lower TML (average 6/Mb) than the complete cohort (11.0/Mb, p < 0.001).

3. Discussion

Numerous studies have identified potential predictive biomarkers to drug therapies in cancers of various, well-defined lineages [27–29]. Recent work from The Cancer Genome Atlas demonstrated that the tissue of origin of a particular cancer may be much less relevant to prognosis and response to therapy than identification of causative mutations and optimal predictive biomarkers [30,31]. Along those observations, several CUP cases that harboured activating \textit{EGFR} mutations were successfully treated with \textit{EGFR} inhibitors (e.g. gefitinib) [6,32,33]. Also, CUP cases harbouring potentially actionable \textit{ERBB2} and \textit{EGFR} gene copy alterations benefited from targeted treatments [34,35]. In our present study, we failed to detect new cases with actionable \textit{EGFR} gene alterations, so CUP remains a rare candidate for \textit{EGFR} inhibitors.

Recent advances in cancer treatment with immune checkpoint inhibitors significantly improved outcomes in several different cancer lineages (e.g. NSCLC, melanoma, urothelial carcinoma). Very limited data are available regarding the treatment of CUP patients with
immune checkpoint inhibitors, presumably due to the lack of routine testing for predictive immuno-oncology biomarkers. Recently, Groschel et al. [8] reported success with pembrolizumab, a checkpoint inhibitor (anti-PD1 drug), in a patient with CUP exhibiting focal high-level amplification of chromosome 9p including the PD-L1 gene [CD274]. Similarly, Kato et al. [9] reported a successful response to combined treatment with nivolumab and trametinib in a CUP patient whose cancer was MSI-H due to an MLH1 mutation. In our cohort, we identified 7 CUP cases harbouring MSI-H status, but the clinical response data were not available. We believe that the wider implementation of the FDA approval of immune therapy for all MSI-H cancers will lead to increased utilisation of the therapy and subsequently evaluation if its efficacy in this cancer type.

Several predictive biomarkers have recently emerged for checkpoint inhibitors and include immunohistochemical PD-L1 status and DNA MSI status. Tumour mutational load (burden) has been recently associated with response to immune checkpoint inhibitors in CUP with four other major cancer types (melanoma, NSCLC, bladder and kidney carcinomas) that have the FDA-approved immune checkpoint treatment modalities. Although no optimal predictive biomarker to assign patients for therapy with immune checkpoint inhibitors has been identified, expression of PD-L1 by immunohistochemistry is most commonly used for that purpose. Several different antibodies and thresholds are in use for associating protein expression with specific drugs in specific tumours [15,16,40]. No uniform threshold is applied in the literature [41], but for the SP142 antibody, a frequently cited threshold is 5% positivity in cancer cells, which we used in our study. With this approach, we identified 22.5% positivity for PD-L1 in CUP. This represents one of the most frequent detection rates of PD-L1 in a cancer cohort [42]. When presence of any one of the three biomarkers was taken into account, 28% of CUP cases were potentially eligible for treatment with immune checkpoint inhibitors. These findings, along with the two recently described successful CUP cases treated with immune checkpoint blockade [8,9], clearly indicate a potential for this novel treatment approach with CUP patients.

In addition to the aforementioned biomarkers, our study also revealed a small proportion of CUP cases harbouring the presence of negative predictive biomarkers (MDM2 amplification and loss of function JAK2 mutations) to immune checkpoint inhibitors (predictors to hyperprogression). These biomarkers along with JAK1 are associated with cancer progression following anti-PD-1/PD-L1 therapy [24,25].

Our study had limitations; the lack of clinical (follow-up) data did not allow us to explore the clinical relevance of the observed findings. However, we believe that our study as well as recently recognised predictive value in determination of MSI and TML status using NGS will lead to immune checkpoint inhibitors therapy in the selected patients with CUP.

In conclusion, our study showed that a substantial proportion of CUP patients are potential candidates for immunotherapy with checkpoint inhibitors, particularly with pembrolizumab.

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Table 2
Molecular profile of the 7 CUP cases with MSI-H status.

<table>
<thead>
<tr>
<th>Case</th>
<th>MSI-NGS</th>
<th>MMR-SEQ (mutation)</th>
<th>IHC MMRP</th>
<th>Other NGS</th>
<th>TML</th>
<th>PD-L1</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1</td>
<td>MSI-H</td>
<td>Wild type</td>
<td>No loss (MSH6 fail?)</td>
<td>BRAF V600E</td>
<td>10</td>
<td>Negative</td>
</tr>
<tr>
<td>#2</td>
<td>MSI-H</td>
<td>Wild type</td>
<td>MLH1/PMS2 loss</td>
<td>CTNNB1</td>
<td>11</td>
<td>Negative</td>
</tr>
<tr>
<td>#3</td>
<td>MSI-H</td>
<td>MSH2 (R621X), MSH6 (F1088fs)</td>
<td>MSH2/MSH6 loss</td>
<td>KRAST12D</td>
<td>16</td>
<td>Not performed</td>
</tr>
<tr>
<td>#4</td>
<td>MSI-H</td>
<td>Wild type</td>
<td>MLH1/PMS2 loss</td>
<td>BRAF V600E</td>
<td>66</td>
<td>Negative</td>
</tr>
<tr>
<td>#5</td>
<td>MSI-H</td>
<td>MSH6 (F1088fs/S616F), PMS2 mutation result unknown</td>
<td>Isolated PMS2 loss</td>
<td>KRAST12V</td>
<td>48</td>
<td>Negative</td>
</tr>
<tr>
<td>#6</td>
<td>MSI-H</td>
<td>Wild type</td>
<td>MLH1/PMS2 loss</td>
<td>KRAST12V</td>
<td>9</td>
<td>Positive (5%, 3+)</td>
</tr>
<tr>
<td>#7</td>
<td>MSI-H</td>
<td>MSH2 (D680X 49)</td>
<td>Loss of MSH2/MSH6</td>
<td>MSH2 D603V</td>
<td>31</td>
<td>Negative</td>
</tr>
</tbody>
</table>

IHC = immunohistochemistry; MMRP = mismatch repair protein; MSI-H = microsatellite instability-high; NGS = next-generation sequencing; PD-L1 = programmed death-ligand 1; TML = tumour mutational load.
immune checkpoint therapy, but to achieve maximum detection success multiplatform testing may be necessary.

4. Materials and methods

Formalin-fixed paraffin-embedded tissue samples from 389 consecutive patients with verified CUP [3] were used in the study. All the tested cases were previously characterised as CUP by the referring pathologists and oncologists (mainly from the United States) who submitted the specimens for molecular profiling over 34-month period. The haematoxylin and eosin stained slides were re-reviewed by a board-certified pathologist (Z.G.) to confirm the diagnosis of CUP. All assays were performed in CLIA/CAP/ISO15189 certified clinical laboratory (Caris Life Sciences, Phoenix, AZ).

The samples were analysed with massively parallel, NGS platform that included 592 genes (NGS, NextSeq, Illumina, San Diego, CA) [43]. TML was calculated using nonsynonymous missense mutations; common germline variants excluded. A high TML was considered ≥17 mutations/Mb. This threshold was previously validated and was based on the MSI and NGS data comparisons (more info is available here: https://www.carismolecularintelligence.com/wp-content/uploads/2016/12/TN0291-v1_Total-Mutational-Load-Immunotherapy-REVERSED-PAGES.pdf). Copy number variation was tested by NGS and was determined by comparing the depth of sequencing of genomic loci to a diploid control as well as the known performance of these genomic loci. Calculated gains ≥6 copies were considered amplified.

MSI was calculated from the NGS data by direct analysis of short tandem repeat tracts in the target regions of sequenced genes. The count only included alterations that resulted in increases or decreases in the number of repeats; MSI-H was defined as ≥46 altered
microsatellite loci (this threshold was established by comparing to the polymerase chain reaction–based MSI FA results from ~2100 cases [44,45]).

ArcherDx FusionPlex Assay (ArcherDX, Boulder, CO) was used to detect gene fusions; 52 gene targets were analysed in 156 tumours. The panel of tested gene fusions is available here: https://www.carismolecularintelligence.com/tumor-profiling-menu/mi-profile-usa-excluding-new-york/.

IHC was used to detect expression of PD-L1 (SP142 antibody) and, in some cases, presence of PD-1 expressing tumour infiltrating lymphocytes (NAT105 antibody), using an automated staining platform (Ventana Medical Systems, Inc., Tucson, AZ). Tumour cells were considered positive for PD-L1 if ≥5% of cancer cells exhibited moderate (2+) membranous positivity [6,41,46,47]. Benign tonsil samples served as a positive control for PD-L1.

In addition, IHC (Ventana) was used to assess the expression of mismatch repair proteins (MLH1, MSH2, MSH6 and PMS2) in seven MSI-H confirmed cases and 12 additional MSI-stable cases with available tissue [48].

Conflict of interest statement

Joanne Xiu, Jeff Swensen and Zoran Gatalica are all employees of Caris Life Sciences.

Semir Vranic declares no conflict of interest.

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The preliminary data from the study were presented at the ESMO 2017 Congress that was held on September 8–12, 2017 in Madrid, Spain.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ejca.2018.02.021.

References


