Responsiveness to PD-1 Blockade in End-Stage Colon Cancer with Gene Locus 9p24.1 Copy-Number Gain

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Running Title: Colon Cancer 9p24.1 Copy-Number Gain and Checkpoint Blockade

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Abstract

Most patients with spread of large bowel cancer to other organs do not respond to immune therapy. We identified a rare gene mutation, termed 9p24.1 copy-number gain, in this otherwise incurable tumor that provoked an immune therapy response. We identified the chromosome 9p24.1 copy-number gain in a liver metastasis biopsy from a patient that had refractory disease to standard therapies, by gene-panel sequencing of DNA. Following immune checkpoint blockade with pembrolizumab (anti–PD-1), the patient experienced conversion of the tumor phenotype from one with epithelial features to that of an inflamed microenvironment, demonstrated by high-resolution RNA sequencing. Circulating tumor DNA disappeared over the first two weeks of therapy. As assessed by standard radiographic measurement, the patient had a partial response that was durable. This patient’s response may support the use of histology-agnostic immune checkpoint blockade in solid tumors that carry the rare 9p24.1 copy-number gain.
Introduction

The immune context of the tumor microenvironment (TME) determines the response to immune checkpoint blockade (ICB) in colorectal cancer (CRC; 1, 2). Favorable survival following ICB in metastatic disease from the minority of cases with primary CRC deficient in the DNA-mismatch repair (MMR) function is reflected by either high tumor mutational burden or positive microsatellite-instability (MSI) status (3, 4). Unlike for non-small-cell lung cancer (NSCLC), tumor-cell expression of the ligand of the programmed death receptor (PD-1), PD-L1, has not been used as a predictive biomarker of ICB response in CRC.

Despite the extensive immune-cell infiltrate recruited by the malignant Reed-Sternberg cells in classic Hodgkin’s lymphoma, the cytotoxic immune cells are anergic. This is frequently caused by Reed-Sternberg-cell copy-number gain (CNG) of chromosome 9p24.1, which includes loci for CD274, PDCD1LG2, and JAK2. This CNG leads to enhanced expression of PD-L1, the PD-1 ligand PD-L2, and Janus kinase-2 (JAK2) and thereby maintains Reed-Sternberg-cell survival signaling, unless the evasion of T-cell cytotoxicity is revoked by ICB (5-8). CNG of the 9p24.1 locus, associated with susceptibility to ICB, has been observed in rare lymphoma entities, and its prevalence in solid tumors is low, estimated to only 0.2% of CRC cases (9).

The ‘Actionable Targets in Cancer Metastasis’ (MetAction) study (ClinicalTrials NCT02142036) established expedited and safe mutation profiling of an individual patients’ progressing metastatic cancer following failure of systemic therapies, in order to offer histology-agnostic, molecularly matched medication for end-stage cancer (10). The treatment-refractory 9p24.1-CNG colon cancer case reported here illustrates the utility of this pipeline and further delineates the ICB response by diverse modalities.
Materials and Methods

Study procedures

The study was approved by the Institutional Review Board, the Regional Committee for Medical and Health Research Ethics of South-East Norway (reference number, REK 2013/2099), and the Norwegian Medicines Agency. It was performed in accordance with the Declaration of Helsinki. Written informed consent was required for participation. The study procedures and endpoints have been described previously (10) and are, together with details about the gene mutation profiling, provided in Supplementary Methods (10-12).

DNA/RNA extracted from biopsy-sampled metastatic tissue and from a whole blood sample as germline control at enrollment was sequenced using the Ion Oncomine™ Comprehensive Assay v1 (Thermo Fisher Scientific). The median sequencing depth was ~3000x, enabling calculation of the approximate mutant-allele fraction in heterogeneous tissue samples. The DNA sequence data was analyzed by the Torrent Suite™ software (Thermo Fisher Scientific); specifically, copy-number variants were detected in the copy-number detection module of the Ion Reporter™ software (Thermo Fisher Scientific). The called gene variants were filtered prior to assessment and prioritization. The Molecular Tumor Board interpreted the findings with regard to the likelihood of involved tumor signaling activity, delivering conclusions that the following Clinical Tumor Board used to recommend potential systemic tumor-directed medication.

Fluorescence in-situ hybridization analysis of the resected primary and locally recurrent tumors was performed to assess chromosome 9p copy-number, using a commercial probe pair that included a 350-kilobase 9p21.3-specific probe covering CDKN2A and CDKN2B, labeled with PlatinumBright™550, and a 440-kilobase 9q21-specific probe spanning from RH40366 to SHGC-82248, labeled with PlatinumBright™495 (both Kreatech™ probes KBI-10402).
Cell-free DNA was extracted from plasma sampled at each CT examination. The quantitative digital PCR assay, capable of discriminating between the \textit{KRAS} p.G12D and wildtype sequences, was performed on the Raindrop™ system (RainDance™ Technologies).

RNA was extracted from liver metastasis biopsies sampled at study enrolment and 2 weeks of treatment for high-resolution sequence analysis that yielded a minimum of \(~35\) million reads per sample. Each transcript was quantified and compared for expression level in the two biopsy specimens. Differentially expressed genes were analyzed for significant biological functions and signaling pathways inherent in the tumor phenotype responses using the Ingenuity® Pathway Analysis software. Details on these procedures are provided in Supplementary Methods.

\textbf{Study treatment}

Following conclusion of the diagnostic procedure, the patient had a baseline computed tomography (CT) examination and commenced treatment with pembrolizumab (2 mg/kg every 3 weeks). Evaluation CT examinations were undertaken every 9 weeks. Routine blood tests were done within the standard patient work-up.

\textbf{Results}

\textbf{Case history}

In October 2015, a 51-year-old woman with an otherwise unremarkable medical background presented with anemia and was diagnosed with a conventional-type colon adenocarcinoma located in the hepatic flexure and extending into the right liver lobe. The tumor was positive for the \textit{KRAS} p.G12D mutation and MSI markers and devoid of the MMR protein PMS2. The patient was referred to neoadjuvant oxaliplatin-based chemotherapy [the
Nordic FLOX regimen (13)], but CT evaluation after 3 cycles revealed locally progressive
disease. The patient underwent a right-sided hemicolectomy and in-continuity resection of the
involved liver segments. Histological examination of the surgical specimen confirmed
margin-negative ypT4bN0V0-stage disease. The patient was not offered adjuvant oncologic
therapy.

Repeat CT scanning 4 months after the surgical procedure showed a locally recurrent
tumor comprising the duodenum and pancreas, and the patient proceeded directly to salvage
surgery. The histological examination noted a positive margin towards the posterior
abdominal wall and tumor infiltration in a portal vein. One month post-operatively the patient
commenced a 5-week-long chemoradiotherapy regimen (delivered to the involved area of
dissection).

A repeat CT record 3 months after completion of the adjuvant treatment revealed one
metastatic lesion in each liver lobe in addition to an enlarged portal hilum lymph node. The
patient was assessed for palliative irinotecan-based chemotherapy [the Nordic FLIRI regimen
(14)], but disease progression was noted after 4 cycles. Immune checkpoint inhibitors are not
approved by Norwegian health authorities for treatment of advanced MSI-positive CRC
within the public health services, thus, the patient was ineligible in routine clinical practice. In
February 2017, she was enrolled in the MetAction study.

**Metastasis genome profiling**

Following study enrollment, ultrasound-guided core biopsies were taken from the
metastatic lesion in the left liver lobe without procedure-induced complications. Tumor-cell
content was 30% as assessed by tissue imprint. The DNA sequencing revealed point
mutations commonly observed in CRC (APC p.R216X, KRAS p.G12D, and FBXW7
p.R465H), each with approximately 30% mutant-allele fraction, in addition to an estimated
≥4-fold relative 9p-CNG (Fig. 1A), confirmed by fluorescence in-situ hybridization (Fig. 1B). Acknowledging the approximations by applying the Oncomine™ Comprehensive Assay to estimate the tumor mutational burden, defined as the number per megabase of coding synonymous base substitutions and indel mutations, but excluding known driver mutations (15), only one of the identified mutations was eligible. This corresponded to a tumor mutational burden rate of 5, which is typical for MMR-proficient CRC (16).

**Clinical response**

Following tumor board consensus supporting treatment with a PD-1 inhibitor and patient’s commencement of pembrolizumab treatment, blood tests showed decline in tumor markers already at 2-3 weeks (Fig. 2A). The moderately elevated liver enzymes decreased over the entire treatment course; liver function markers as well as differential blood counts, electrolytes, and other renal function markers have all been within reference limits throughout therapy. The first CT evaluation after 3 treatment cycles revealed a partial response, according to the Response Evaluation Criteria in Solid Tumors v1.1, which remained consolidated at the 1-year treatment evaluation (Fig. 2B). The patient has been continuing the treatment for 20 months with ongoing partial response (by November 2018).

As shown by Supplementary Fig. S1, the patient experienced a rise in plasma free-T4 and a corresponding fall in thyroid-stimulating hormone only 2-3 weeks after commencement of therapy. Grade 1 hyperthyroidism (by Common Terminology Criteria for Adverse Events v4.0) was evident when the patient started the third treatment cycle, whereas grade 2 hypothyroidism was recorded at commencement of cycle 4. Since then, the patient has been on continuous medication with levothyroxine that has been dose-adjusted based on the continuous monitoring of plasma thyroid-stimulating hormone and free-T4. The patient has not reported other adverse events.
Circulating tumor DNA (ctDNA) response

Plasma analysis showed a decline in the KRAS mutant-allele fraction from 23% at baseline to 2.4% at 2 weeks of study treatment (Fig. 2C). A mutant-allele fraction of only 0.1% was detected in the concurrent liver metastasis biopsy. Plasma specimens sampled at each of the CT evaluations were negative for KRAS p.G12D (Fig. 2C).

Metastasis phenotype response

The RNA transcript profile of the left liver lobe metastasis (the individual RNA sequence counts are provided in Supplementary Table S1) changed over the first 2 weeks of PD-1 blockade (Supplementary Table S2). Although expression of CD274 (PD-L1) and JAK2 diminished, PDCD1LG2 (PD-L2) expression increased in the on-treatment biopsy (Supplementary Table S3). Among the most discriminating RNA profile-defined functions altered between the baseline and 2-week samples were activation of responses ascribed to immune-cell trafficking, lipid metabolism, and inflammation, whereas biological features related to epithelial neoplasia were inhibited (Fig. 3A and Supplementary Table S4).

Moreover, profile-defined signaling pathways that were induced by pembrolizumab included activation of the liver-X/retinoid-X hormone receptor transcription factors (LXR/RXR) and acute-phase reaction with the complement system (Fig. 3B and Supplementary Table S5). An 18-gene T-cell-inflamed expression profile that predicted response to pembrolizumab across multiple solid tumors has been reported (17). In our dataset, a majority of these interferon-γ-responsive genes were activated in the metastasis at 2 weeks of pembrolizumab treatment, whereas IDO1 expression was repressed (Fig. 3C).
Discussion

We report a radiographically partial and durable response of advanced colon cancer on PD-1 blockade based on 9p24.1-CNG detected in a metastatic liver lesion. A decline in circulating routine tumor markers and the mutant KRAS DNA was observed already following the first therapy cycle, mirroring the conversion of the metastasis phenotype from epithelial features to characteristics of an inflamed TME. The response measured with each of diverse modalities was consistent with the MetAction diagnostic procedure concluding that the CNG of chromosome 9p24.1 represented the driver alteration. This cause of ICB susceptibility is rare in solid tumors and particularly in CRC (9). One other case of advanced 9p24.1-CNG colon adenocarcinoma treated with a PD-1 inhibitor has been reported (18).

The therapeutic response came with a common toxicity, a transient hyperthyroidism preceding hypothyroidism that was managed by hormone substitution. Patients with advanced NSCLC have better response and outcome measures to ICB if they developed thyroid dysfunction, which was an independent predictive factor (19, 20). This suggests a mechanistic connection between the thyroid hormone metabolism and ICB efficacy. In mice, the T4 metabolite triiodothyronine stimulated tumor-antigen-presenting dendritic cells to impel interferon-γ-mediated T-cell cytotoxicity (21).

Some studies have reported on the analysis of ctDNA to assess ICB efficacy in advanced cancer. A prospective pilot study of 10 patients with detectable ctDNA at commencement of therapy, including one MSI-positive CRC case, showed that complete ctDNA loss at the first treatment assessment (8 weeks) was associated with durable radiographic tumor response as well as long survival outcomes (22). An association between ctDNA and radiographic outcomes was also reported in 24 NSCLC cases (23). Our case showed 30% KRAS mutant-allele fraction in the baseline metastasis and only 0.1% at 2 weeks of treatment. The corresponding figures for ctDNA, 23% versus 2.4%, may reflect different
methodological sensitivity or tumor load at other metastatic sites. Nevertheless, plasma KRAS p.G12D was undetectable from the first radiographic response evaluation at 9 weeks onwards.

The conversion of the liver metastasis phenotype following initiation of the PD-1 inhibitor, as portrayed by the RNA sequence data, was perhaps the most intriguing observation. The interrelated phenotypes comprising CD274 (PD-L1) and JAK2 expression and sequences ascribed to intestinal epithelial neoplasia were attenuated, consistent with a rapid and substantial tumor-cell elimination. In contrast, expression of the chromosome 9p24.1 partner PDCD1LG2 (PD-L2) was augmented in the 2-week biopsy sample. It has been proposed that PD-L2 positivity of tumor cells and TME cells predicts treatment response to PD-1 blockade through T-cell reactivity (24, 25). Hence, the heightened PD-L2 expression in the on-treatment metastasis specimen might have reflected activation of tumor-targeting immunity.

The computational RNA sequence analysis further revealed enrichment of pembrolizumab-induced functions and signaling pathways with designations such as immune-cell trafficking but also inflammation, acute-phase response, and complement system activity. The complement complex belongs to the rapidly responding innate immunity and exerts opposing effects within tumor-antigen presentation and T-cell-mediated cytotoxicity on the one side and TME activation of tumor-supportive systemic inflammation on the other (26). However, the data also supported pembrolizumab-induced activity of interferon-γ-responsive genes involved in tumor-eliminating T-cell effects (17). The patient has had no clinical or biochemical evidence of systemic inflammation during the treatment course. Expression of the interferon-γ-responsive IDO1, encoding the essential indoleamine 2,3-dioxygenase-1 of various TME-cell types that participate in immune tolerance to tumor antigens (27), was repressed at 2 weeks of ICB. Noting the failure or termination of clinical trials adding an
indoleamine 2,3-dioxygenase-1 inhibitor to PD-1 blockade, it may be that this particular combination is mechanistically redundant.

The RNA transcript profile also revealed a possible role of lipid metabolism and particularly LXR/RXR activity in the patient’s metastatic cancer on ICB response. In mouse models, administration of an LXR agonist reduced the numbers of systemic and tumor-infiltrating myeloid-derived suppressor-cells (an innate immune-cell population), which evoked T-cell cytotoxicity and restrained tumor growth; similar effects on peripheral blood myeloid-derived suppressor-cells and cytotoxic T-cells were observed in patients (28). It has been known for almost three decades that LXR/RXR function as co-regulators of thyroid hormone receptor-mediated transcription (29). However, in our patient’s case, it is not clear how the initial hyperthyroidism and the concurrent ICB-induced LXR/RXR activity in the metastatic cancer were mechanistically interrelated.

The patient described here, presenting to the MetAction study with metastatic colon cancer refractory to all standard therapies, has experienced durable, near-complete radiographic tumor regression following administration of pembrolizumab based on the detection of 9p24.1-CNG in a liver metastasis. Our experience may support the use of PD-1 blockade in histology-agnostic cases with 9p24.1-CNG.
Data Sharing

Request to inspect and analyze the data that underlie the results reported in this article, including the sequence datasets that are stored in the Services for Sensitive Data facility at University of Oslo, should be directed to the corresponding author, and access will be provided in accordance with the General Data Protection Regulation of the European Union.

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References


Figure 1.

Chromosome (Chr.) 9p copy-number gain. Panel A shows the genes within the 9p amplicon, as covered by the Oncomine™ Comprehensive Assay and each with its relative copy-number gain, identified in the liver metastasis specimen sampled at study enrolment. Panel B shows nuclei from the primary tumor specimen with 9p21.3 copy-number gain [9p21.3 (red signals) to 9q21 (green signals) ratio of 3.4 from the average copy numbers of 4.7 for 9p21.3 and 1.4 for 9q21], as assessed by fluorescence in-situ hybridization.
Figure 2.

Tumor response to the study treatment. Panel A shows amounts of circulating tumor markers over the treatment course. The start of a new treatment cycle corresponds to the given week numbers. Panel B shows one transverse-view image from each of portovenous-phase contrast-enhanced computed tomographic scans of the abdomen recorded before commencement of the study treatment (top panel), at the first evaluation (middle panel), and at the 1-year evaluation (bottom panel). Longest cross-sectional perpendicular measures at these points were for the right liver lobe lesion: 120 mm × 100 mm, 56 mm × 46 mm, and 25 mm × 15 mm; for the left liver lobe lesion: 125 mm × 66 mm, 79 mm × 37 mm, and 52 mm × 22 mm; for the short axis of the portal hilum lymph node (not depicted on the selected images): 26 mm, 22 mm, and 13 mm. Panel C shows the circulating amounts of the mutant KRAS relative to the wildtype variant over the treatment course. The inserted histograms show the quantification by digital PCR of the two KRAS alleles before the start (top panel) and at 2 weeks (middle panel) and 9 weeks (bottom panel) of the study treatment.
Figure 3.

Phenotypic response to the immune checkpoint blockade. Panel A shows annotated functions, as defined by the Ingenuity® Pathway Analysis software, based on differentially expressed genes \((n = 4,168)\) in the 2-week \textit{versus} baseline biopsy specimen from the left liver lobe metastasis, ranked by \textit{z}-score. The resulting functions (Supplementary Table S4) were filtered according to the following criteria: \textit{z}-score higher than 3.4 or lower than \(-1.6\), \(p < 3 \times 10^{-6}\), and removal of redundant functions. Panel B shows the software’s signaling pathway annotation of the input genes, ranked by \textit{p}-value and following removal of redundant pathways (from Supplementary Table S5); patterned bars, no \textit{z}-score. Panel C shows log\(_2\) fold-change (FC) expression of 18 interferon-\(\gamma\)-responsive genes, which define a profile predictive of response to immune checkpoint blockade (17), in the 2-week \textit{versus} baseline biopsy specimen.