Outlier response to anti-PD1 in uveal melanoma reveals germline MBD4 mutations in hypermutated tumors

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Metastatic uveal melanoma is a deadly disease with no proven standard of care. Here we present a metastatic uveal melanoma patient with an exceptional high sensitivity to a PD-1 inhibitor associated with outlier CpG>TpG mutation burden, MBD4 germline deleterious mutation, and somatic MBD4 inactivation in the tumor. We identify additional tumors in the Cancer Genome Atlas (TCGA) cohorts with similar hypermutator profiles in patients carrying germline deleterious MBD4 mutations and somatic loss of heterozygosity. This MBD4-related hypermutator phenotype may explain unexpected responses to immune checkpoint inhibitors.
Uveal melanoma (UM) is an ocular neoplasia most often affecting populations of European ancestry and has one of the lowest mutation burdens among adult tumors\textsuperscript{1–3}. Inactivation of \textit{BAP1} (3p21), through both deleterious mutations and monosomy 3, is frequent in UM and is associated with a high risk of metastasis\textsuperscript{4}. Prognosis of metastatic UM is dismal with median survival <12 months and no systemic treatment improving survival\textsuperscript{3}. Programmed cell death protein 1 inhibitors (PD1inh), a class of immune checkpoint inhibitors, have been evaluated in UM with low overall response rates\textsuperscript{5–8}. Here we present three patients with hypermutated CpG>TpG tumors (two UM and one glioblastoma) associated with \textit{MBD4} germline deleterious mutations and somatic inactivation in the tumors. Furthermore, we provide evidence for sensitivity to immune checkpoint inhibitors in \textit{MBD4}-deficient tumors.

Results

An outlier metastatic UM patient responding to pembrolizumab. In our series of 42 metastatic UM patients treated with PD1inh, only one (UVM\textsubscript{IC}) achieved a tumor response (details in Methods)\textsuperscript{9}. UVM\textsubscript{IC} developed a metastatic UM with liver, lung, and bone lesions (Fig. 1a, c and Supplementary Fig. 1). Treatment with the PD1inh pembrolizumab resulted in complete response of known metastases 10 months later, while new non-life-threatening infracentimetric subcutaneous metastases appeared. Longitudinal monitoring of the \textit{GNAQ}\textsubscript{Q209L} mutation in circulating tumor DNA was consistent with imaging (Fig. 1b)\textsuperscript{10}. We observed peri- and intra-tumoral CD3\textsuperscript{+} lymphocytic infiltrates in all UVM\textsubscript{IC} samples (Supplementary Fig. 2). After 2 months of pembrolizumab, proportions of blood effector memory CCR7\textsuperscript{−}/CD45RA\textsuperscript{−}/CD4\textsuperscript{+} and CCR7\textsuperscript{−}/CD45RA\textsuperscript{+}/CD8\textsuperscript{+} T-cells increased from 14.5% to 21.8% and from 12.2% to...
48.9%, respectively. In comparison, the proportions of these populations in 11 non-responsive metastatic UM patients changed modestly (from 38% to 35.3%, and from 23.5% to 27.5%, respectively; Fig. 1d). These observations suggested that pembrolizumab stimulated a previously existing spontaneous cell-mediated immunity against the UVM_IC tumor.

**MBD4 defect is associated with hypermutated CpG>TpG pattern.** To explore this outlier response, we performed whole-exome sequencing (WES) of the primary tumor, the liver metastasis, and a pembrolizumab-resistant subcutaneous metastasis, as well as constitutional DNA. All cancer samples carried somatic *GNAQ*Q209L and *BAP1*R385X mutations as well as...
monosomy 3. We identified similar hypermutated profiles in all samples with >266 somatic single nucleotide variants (SNVs) per sample (87% non-synonymous SNVs) corresponding to ~19-fold increase of SNVs compared to an in-house series (Fig. 2a). Over 91% of mutations were CpG>TpG transitions, compared to <30% in other UMs (Fig. 2b). Because CpG>TpG transitions are secondary to the spontaneous deamination of 5-methylcytosines, we searched for alterations of either TDG or MBD4, encoding two glycosylases involved in 5-methylcytosine integrity. We identified a germline deleterious frameshift deletion of MBD4 (3q21.3: c.1441delT:p.F481Del*9) with loss of the second allele by monosomy 3 in all tumor samples (Fig. 2c, f). No other sample in our UM series carried a MBD4 or TDG mutation.

We inferred the clonal structure and observed that the primary tumor presented multiple subclones, which is unusual in UM, while metastases were more homogeneous (Supplementary Figs. 3a and 4). We then observed that each metastasis shared more SNVs with the primary tumor than with other metastases, suggesting polyphyletic clones (Supplementary Fig. 3b, c). Furthermore, each metastasis presented 18–44 new SNVs, again dominated by CpG>TpG (>93%), compared to the predicted initial clones, while cohort analyses demonstrate that UMs usually acquire a mean of two SNVs during metastatic progression (Supplementary Fig. 4). Altogether, these data suggest an ongoing MBD4-related mutagenic process during tumor progression, as has been observed with APOBEC in other cancers.

MBD4 germline mutations in UM and glioblastoma. To investigate the frequency of MBD4 and hypermutation in an independent UM cohort, we analyzed the TCGA UM dataset (N = 80 patients). This identified an additional hypermutated UM case (patient UVM_1; Fig. 2a). This tumor was carrying a BAP1 mutation and monosomy 3 as well as 474 SNVs (305 non-synonymous SNVs) corresponding to a 36-fold increase of SNVs as compared to the overall TCGA UM series. Again, the SNVs were predominantly CpG>TpG (460/474; 97% of SNVs). This patient furthermore carried a germline c.1562G>T:p.D521Pfs*4 MBD4 splice-site variant and somatic loss of the wild-type allele due to tumor monosomy 3. Analysis of RNA-seq demonstrated that this splice-site variant was associated with exon 7 skipping and a frameshift (Fig. 2c, e, f). No other MBD4 or TDG mutation was identified in this series. We further analyzed the pan-cancer TCGA series (>10,000 tumors; Supplementary Table 1) and identified 4831 hypermutated tumors (>200 SNVs per tumor) of which 20 cases, including UVM_1, were enriched in CpG>TpG mutations (≥2.55; Fig. 2d). Of these 20 cases, patient GBM_4 presented a glioblastoma carrying 1149 SNVs (440 non-synonymous SNVs) and a germline c.335+1G>A:p.R335fs*5 MBD4 mutation with somatic loss of heterozygosity leading to the use of a cryptic splice donor site, loss of 88 bases, and a premature stop codon (Fig. 2c, e, f). The three other hypermutated CpG>TpG glioblastoma cases did not carry any identifiable deleterious MBD4 or TDG mutation. The germline MBD4 mutations identified in patients UVM_IC, UVM_1, and GBM_4 are rare in the general population with minor allele frequencies ranging from ~0.0000007 to ~0.000002. To be noticed, none of these 20 hypermutated cases carried somatic MBD4 indels together with mismatch repair deficiency (two colorectal and one endometrial adenocarcinomas); the molecular mechanism of hypermutation in the other cases remains undetermined.

Discussion

A role for MBD4 germline mutations in cancer predisposition was hypothesized 18 years ago. The identification of two UM cases with MBD4 germline mutations is intriguing, and possibly related to the frequent monosomy 3—where MBD4 is located—in this disease. Integrating our institutional cohort and the TCGA UM cohort, MBD4 germline deleterious mutations were present in 2% of UM patients (2/102). Both UVM_1 and GBM_4 tumors presented before the age of 50, earlier than median ages (60 in UM and 65 in glioblastoma). However, none of the three patients had a reported personal or familial history of invasive cancer. In this regard, Mbd4 knockout mice models are associated with increase of CpG>TpG transitions without increased tumor incidence, except Apc-deficient backgrounds. Thus, MBD4 inactivation may not be sufficient to initiate tumorigenesis but may play a significant role in tumor progression.

Because high mutation burden is predictive of response to immune checkpoint inhibitors, PD1inh have shown a high activity in hypermutated mismatch repair-deficient tumors leading to the tissue-agnostic approval of PD1inh in these tumors. Hence, while the limited activity of PD1inh in UM patients may be explained by a low mutation burden, the MBD4-related high mutation load probably contributed to the dramatic response in the UVM_IC patient. These observations open avenues for clinical trials providing tissue-agnostic access to PD1inh to treat patients with MBD4-deficient tumors.

Methods

Case report. A 76-year-old woman was diagnosed with a stage IIIA UM localized on the left eye choroid (UVM_IC). She had a past medical history of breast ductal carcinoma in situ diagnosed at age 74, and no familial history of cancer. Importantly, no prior mutagen exposure was identified. She underwent enucleation for her UM. Histopathological examination revealed a tumor measuring 10.6 mm in diameter and 10 mm in thickness of mixed spindle cell/epithelioid cell morphology. No sceral or optic nerve infiltration was observed. As her tumor presented with monosomy 3, the patient was deemed at high risk for metastatic disease and close surveillance was instituted. Nine months after enucleation, computed tomography detected a unique liver lesion. Resection of the lesion with clear margins revealed a UM metastasis. Four months later, the patient experienced a relapse with new liver, lung, and osseous lesions. Treatment with pembrolizumab (2 mg per kilogram of body weight every 3 weeks) was initiated. Ten months later, liver and lung metastases exhibited a complete response, while osteolytic bone lesions showed sclerotic features suggestive of a response. Longitudinal monitoring of circulating tumor DNA (gDNA) in plasma was consistent with the clinical and imaging status (Fig. 1b). The patient has now been receiving pembrolizumab for >22 months without visceral tumor progression. In order to identify the mechanisms implicated in sensitivity to pembrolizumab, immune response markers were analyzed in the primary tumor and in the removed liver metastasis collected prior to pembrolizumab therapy. Pathological examinations showed CD3+ peri- and intra-tumoral lymphocytes infiltrates in both samples (Fig. 1b); however, CD8+ infiltrates were only observed in the primary tumor.
With respect to PD-L1 expression, 30% of the primary tumor was positive for PD-L1 versus 20% in the metastasis. However, 90% of peri-tumoral stroma and immune infiltrates (IC) were positive in the metastasis expressed in UVM-IC. UVM-IC is a 41-year-old woman without past medical history of cancer, diagnosed with a stage IIIA UVM localized to the choroid. No metastatic recurrence was reported after a follow-up of 39 months. The tumor showed focal lymphocytic infiltration with low CD8+ and moderate CD27+ (which codes for PD-L1) RNA expression.

**DNA sequencing.** Samples were histologically reviewed by a pathologist before nucleic acid extraction in order to select samples with at least 30% of melanoma cells. DNAs were extracted from snap-frozen samples, except for the primary tumor UVM-IC, which was extracted from a formalin-fixed paraffin-embedded (FFPE) sample. Germline DNA was extracted from unaffected tissues (healthy liver or blood). DNAs were extracted from frozen samples using phenol (Invitrogen, Carlsbad, CA, USA) by the Centre de Ressources Biologiques (Institut Curie tumor biobank) and from FFPE using the Nucleospin Tissue Kit (Macherey-Nagel, Düren, Germany) then subsequently purified on Zymo Spin™ IC (Zymo Research, Irvine, CA, USA). DNAs were quantified by Qubit (Thermo Fisher Scientific, Waltham, MA, USA) and integrity was assessed by BioAnalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA).

WES libraries were prepared using the Agilent SureSelectXT Clinical Research Exome kit (Agilent Technologies) from 1 µg of DNA isolated from initial libraries with median size of 300 bp according to the manufacturers’ protocols. Libraries were 100 bp paired-end multiplex sequenced on the Illumina HiSeq 2000 (Illumina). WES depth was a priori settled up to sequence germline DNA at 30× and somatic DNA at 100×. The library from UVM-IC primary tumor was prepared using the Agilent SureSelectXT HS kit (Agilent Technologies) from 20 ng of DNA isolated initial libraries with median insert size of 300 bp according to the manufacturers’ protocols. Libraries were 100 bp paired-end multiplex sequenced on the Illumina HiSeq 2000 (Illumina). WES depth was a priori settled up at 100×.

**Mutation calling.** Sequencing quality was assessed by FastQC. Reads were aligned to the human genome (hg19) with Bowtie2 2.1.0.15. PCR duplicates were removed using Picard Tool MarkDuplicates v1.97. RNA-seq reads were aligned using STAR (v2.5.0a) to the human genome (hg19) with Gemma alignments (v1.1.6). RNA-seq reads were aligned using STAR (v2.5.0a) to the human genome (hg19). GSEA (v4.0.3) was used to enrich the genome-wide raw reads with the default version 4.2.3 and its packages GenomicAlignments (1.12.2) and GenomicRanges (1.28.4) from data downloaded through the GDC portal. Expression data of the tumors from UVM-IC and GBM-14 were explored through the cBioportal.

**Data availability.** Sequence data have been deposited in and are available from the European Genome-phenome Archive database under number EGAS00001002761.

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Author contributions
M.R. and L.M. conceived the study, interpreted the data, and wrote the manuscript. A.H. and A.F. performed bioinformatic analyses. S.G. and R.L.B. performed pathological analyses. T.P. performed bioinformatic analyses. V.S. performed radiological evaluations. A.R. performed and analyzed ctDNA experiments. A.M. performed and analyzed FACS experiments. S.D. performed Sanger sequencing. V.R. performed next-generation sequencing, M.G. prepared patient specimens. M.P. performed pathological analyses. S.T. and N.C. provided patients specimens and critical advice. S.R.-R. interpreted the data and provided critical advice. F.-C.B. and O.L. interpreted the data and provided critical advice. P.M. and S.P.-N. provided patients specimens and critical advice. M.-H.S. conceived and guided the study, interpreted the data, and wrote the manuscript. All authors reviewed and approved the final manuscript.

Additional information
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