Memory T cells targeting oncogenic mutations detected in peripheral blood of epithelial cancer patients

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T cells targeting shared oncogenic mutations can induce durable tumor regression in epithelial cancer patients. Such T cells can be detected in tumor infiltrating lymphocytes, but whether such cells can be detected in the peripheral blood of patients with the common metastatic epithelial cancer patients is unknown. Using a highly sensitive in vitro stimulation and cell enrichment of peripheral memory T cells from six metastatic cancer patients, we identified and isolated CD4⁺, and CD8⁺ memory T cells targeting the mutated KRASG12D and KRASG12V variants, respectively, in three patients. In an additional two metastatic colon cancer patients, we detected CD8⁺ neoantigen-specific cells targeting the mutated SMAD5 and MUC4 proteins. Therefore, memory T cells targeting unique as well as shared somatic mutations can be detected in the peripheral blood of epithelial cancer patients and can potentially be used for the development of effective personalized T cell-based cancer immunotherapy across multiple patients.
Tumors express proteins harboring unique mutations that are absent from normal tissue. Some of these mutated proteins can trigger specific T-cell responses and therefore can potentially be recognized as neoantigens. Recent studies have demonstrated that tumor-infiltrating lymphocytes (TILs) are enriched with neoantigen-specific T cells and that adoptive cell therapy (ACT) using neoantigen-specific TILs sometimes lead to durable tumor regression. However, owing to tumor heterogeneity, targeted neoantigen(s) can be expressed in some, but not all, tumor cells, which may limit ACT efficacy. Therefore, targeting common oncogenic mutations that are more likely to be expressed in all tumor cells and are essential for tumor survival represents a more promising approach. We have recently shown that ACT using autologous TILs targeting the KRASG12D epitope could lead to tumor regression in a patient with metastatic colon cancer. However, T cells targeting common oncogenic mutations are rarely found in TILs and new, non-invasive, approaches for the identification and isolation of such cells or their T-cell receptors from TIL or circulating lymphocytes is needed.

Two major approaches have been used recently to enrich neoantigen-reactive cells from the peripheral blood of melanoma patients: PD-1-positive (PD-1+) enrichment of CD8+ T cells and tetramer isolation. However, isolation of neoantigen-specific cells from the blood of patients with the common metastatic epithelial cancers has been much more challenging. In general, the average number of mutations in common epithelial cancers is lower than in melanoma and may lead to a limited repertoire of neoantigen-reactive TILs. The low frequency of neoantigen-reactive T cells in the periphery requires highly sensitive isolation methods. In addition, unlike melanoma, establishing autologous cell lines from excised epithelial tumors is challenging with low success rates. The absence of autologous lines to validate tumor recognition by enriched T cells and the need to avoid raising de novo recognition against irrelevant antigens suggests that new approaches should focus on T-cell populations that are more likely to be clinically relevant. Although the naive T-cell (TN) repertoire is highly polyclonal and antigen inexperienced, the memory repertoire represents cells that have already been stimulated by their cognate antigens and more likely arose following infection or malignancy. Thus, the limited antigen-experienced repertoire of memory cells is ideal for in vitro stimulation (IVS)-based enrichment and isolation methods from circulating T cells. The cells or their receptors identified using such approaches are likely to arise from antigens that are efficiently processed and presented in the tumor microenvironment or its draining lymph nodes. Here, we developed a highly sensitive IVS approach starting with preexisting memory T cells and used our approach to detect and isolate T cells targeting unique as well as shared somatic mutations in the KRAS oncogene.

Results

Neoantigen specific TCR sequences are restricted to peripheral memory T cells. Recently, Gros et al. and others have shown that sorting PD-1+ TILs and peripheral blood enriches for tumor-reactive CD8+ T cells in patients with melanoma. To determine whether the neoantigen-reactive T cells are present in the memory compartment of epithelial cancer patients, we initially tested the phenotype of PD-1+ T cells in the peripheral blood of four metastatic gastrointestinal cancer patients (patients 4217, 4254, 4257, and 4252). Analysis of all four samples revealed that the majority of CD3+PD-1+ T cells were central memory T cells (TCM) or effector memory T cells (TEM) (Fig. 1a, b), whereas no TN cells were shown to be PD-1-positive. Interestingly, the majority of the terminally differentiated effector memory (TEMRA) cells did not express PD-1. To further address whether neoantigen-specific T cells were enriched in the memory T-cell subset, we retrospectively sorted and performed TCR-Vβ deep-sequencing of TN, TEM, and TEMRA cells from peripheral blood lymphocytes (PBL) of six epithelial cancer patients with known neoantigen-reactive TCRs that were previously found in their TILs (ref. 14, and data not published). Comparison of the TCR-Vβ sequences obtained from TILs with the sequences of the matched PBL subsets shows that neoantigen-reactive TCRs were detected only in memory cells (TCM, TEM, and TEMRA) but not in TN cells (Fig. 1c, and Supplementary Figure 1A). The frequency of the neoantigen-reactive T cells in the blood was low, ranging from 0.02 to 0.0007% of each T-cell subpopulation. Further analysis of the productive clonality based on TCR-Vβ sequences revealed a clear hierarchy of clonality among the different T-cell populations (Fig. 1d), thus demonstrating that TEM and TEMRA memory populations are significantly more clonal than TN cells.

Isolation of T cells targeting unique mutations. To test the feasibility of using memory T cells to isolate neoantigen-reactive T cells, we developed a novel IVS method (Supplementary Figure 2) and retrospectively tested our approach using PBLs from two metastatic colon cancer patients (patient 4213 and 4217, Supplementary Figure 3 and Supplementary data 1). Patient 4213 was screened in our laboratory for the presence of neoantigen-specific TILs for potential ACT, as described earlier. In brief, using whole-exome and RNA sequencing we identified somatic mutations that were present in two metastatic tumors derived from patient 4213. Two neoantigens were identified in the initial screen, SMAD5P268SnpKH and DDX1S281F (Supplementary Figure 4, Supplementary data 2). Here, we sorted memory (TCM, TEM, and TEMRA), TN and bulk PBL cells from peripheral blood mononuclear cell (PBMC) of Pt. 4213 (Supplementary Figure 1A) and co-cultured them with dendritic cells (DCs) loaded with RNA encoding tandem minigenes (TMGs) for 14 days in the presence of IL-21, IL-7, and IL-15. Each TMG comprises a string of RNA minigenes encoding identified mutations flanked on each side by 12 wild-type amino acids from the parent protein. After 14 days, memory and TN cells were re-stimulated with DCs loaded with all TMGs and sorted based on CD8+ T cells, CD4+ T cells, and expression of the T-cell activation marker 41BB to enrich for neoantigen-reactive T cells (Fig. 2a). The number of activated CD8+ T cells sorted was low, 962 from memory, and 1100 from TN (Fig. 2a), possibly owing to the low frequency of neoantigen-specific cells, as shown in Fig. 1c. Cells were then expanded and screened against all 13 TMGs to test for neoantigen recognition. Memory and naive CD8+ T cells were reactive against TMG-8 and TMG-6, respectively, whereas bulk PBL did not recognize any TMGs (Fig. 2b). To identify the specific neoantigens in TMG-8 and TMG-6, we co-cultured the enriched memory and TN cells with autologous DCs that were individually pulsed with the mutated peptides encoded by TMG-8 or TMG-6 (Supplementary Table 1). TMG-8-reactive CD8+ memory T cells recognized the SMAD5P268SnpKH mutation (Fig. 2c), whereas the TMG-6-reactive CD8+ TN cells did not recognize any single peptide from TMG-6 (Fig. 2d). The SMAD5P268SnpKH reactive memory CD8+ cells were further tested for the recognition of WT and mutated long peptides, the predicted minimal epitope and a full-length SMAD5 RNA corresponding to the mutated and WT protein sequences. As shown in Fig. 2e the SMAD5-reactive cells recognized the mutated and not the WT LP, minimal epitope and full-length SMAD5 RNA. We fluorescence-activated cell sorting (FACS)-purified the SMAD5P268SnpKH-reactive cells from Pt. 4213 TIL and memory cells and performed single-cell RT-PCR (scPCR).
to identify their TCR-Vβ and TCR-Vα sequences. Analysis of the TCR sequences revealed that the SMAD5P268inPKH-reactive TIL- and IVS-derived memory cells share matching TCR-Vβ and TCR-Vα sequences (Supplementary Tables 2 and 3). Genetically engineered autologous peripheral blood T cells expressing the SMAD5P268inPKH-reactive TCR (Supplementary Table 3) confirmed that the selective reactivity of the TCR against the mutated SMAD5P268inPKH peptide is restricted by HLA*B08:01 (Fig. 2f, g). Thus, using IVS of memory T cells can lead to the identification and enrichment of neoantigen-reactive T cells.

Isolation of T cells targeting shared oncogenic mutations.

Owing to tumor heterogeneity and differential expression of non-synonymous mutations in cancer cells, targeting mutated driver mutations, that appear to be homogeneously expressed in cancer cells (Supplementary Table 4), can target cancer cells more efficiently. Therefore, we sought to determine whether T cells targeting shared oncogenic mutations can be isolated using our approach. We decided to focus on hotspot mutations in the proto-oncogene KRAS, which are shared at high frequencies across multiple cancers histologies. T cells targeting mutated human KRAS hotspot mutations were previously reported in human TILs, vaccinated mice, human PBMC, and in two clinical trials involving vaccination with mutated KRAS peptides. To test the use of this approach across multiple epithelial cancer types, we performed IVS of memory, TΝ, and bulk PBL, from a metastatic endometrial cancer patient (Pt.4148), using a mixture of KRASG12V, KRASG12C, and KRASG12D 24mer peptides, followed by 41BB enrichment. Exome and RNA analysis of Pt.4148 tumor showed expression only of the KRASG12V mutation (Supplementary data 3 and Supplementary Figure 5). No TILs reactive against the KRASG12V were identified in the initial screen performed in our laboratory (Supplementary Figure 6). To identify T cells targeting mutated KRAS, enriched memory, TΝ, and PBL cells were co-cultured with autologous DCs pulsed with the individual KRASG12 mutated long peptides. As shown in Fig. 3a, memory CD8 T cells showed selective reactivity against KRASG12V. LP, TΝ and bulk PBL were not reactive against any of the KRAS peptides (Supplementary Figure 7). As Pt.4148 expressed the HLA-A*11:01, which is predicted to bind KRASG12V 9mer (Supplementary Table 5), we presumed that staining the cells with A*11-9mer tetramers could address whether HLA-A*11:01 is the correct restriction element. Indeed, HLA-A*11-9mer tetramers bound 15.9% of the memory CD8 cells in the culture (Fig. 3b). Sorting the tetramer-positive cells and sequencing their TCR revealed single TCRα and TCRβ chains (Supplementary Table 3). To further test the TCR, we synthesized, cloned and retrovirally transduced the TRAV and TRBV into allogeneic PBLs, as previously described. To evaluate the specificity and the potency of the TCR we co-cultured the TCR-transduced PBLs with cancer cell lines harboring KRASG12V mutations with or without transfection with HLA-A*11:01 (Fig. 3c) or with autologous DCs pulsed with a serial dilution of the mutated 9mer and wild-type peptides (Fig. 3d). The results show that the isolated TCR selectively recognize the KRAS G12V mutation presented on HLA-A*11:01. Owing to the high prevalence of KRASG12V expression across cancers and HLA-A*11:01 allele frequencies in selected populations (14% in US Caucasians and 23% in Asian-Americans) this TCR can potentially be used as off-the-shelf reagent to treat thousands of relevant cancer patients per year.

### Fig. 1 Identification of neoantigen-specific T cells in PBMC of epithelial cancer patients.

**a** A representative data from one of four patients showing the gating strategy used for the phenotypic analysis of PD-1-positive (PD-1+) peripheral T cells. **b** PBMC isolated from four epithelial cancer patients were thawed, rested overnight in a cytokine-free T-cell medium and stained with antibodies against CD3, CD45RO, CD62L, and PD-1. PD-1+ and PD-1-negative (PD-1−) cells were analyzed for the surface expression of the T-cell memory markers, CD45RO and CD62L. Error bars indicate the mean standard deviation between all four patients samples. **c** PBMC isolated from six epithelial cancer patients were thawed, rested overnight in a cytokine-free T-cell medium and stained with antibodies against CD3, CD45RO, CD62L, CCR7, and CD45RA. Cells were sorted based on their memory subset, washed once with PBS, frozen at −80 °C and send for TCR-Vβ sequencing. Numbers represent the percent of specific TCR-Vβ sequence in each T-cell population. A minus sign (−) was used if no sequence was identified. **d** Analysis of the productive clonality of TCR-Vβ sequences of each T-cell population isolated in **c**. (Paired t-test, ***P < 0.001, *P < 0.05) dashed line, mean
Next, we sought to apply our approach to identify and isolate TCRs targeting additional mutated KRAS neoepitopes. To that end, we stimulated T-cell subsets from PBL of a metastatic rectal cancer patient (Pt.4171). Exome and RNA analysis of Pt.4171 tumor showed expression only of the KRAS G12D mutation (Supplementary data 4). The PBL were stimulated using KRAS G12D full-length RNA or 25mer peptide following ten days of IVS, the T-cell subsets were re-stimulated, and FACS sorted based on T-cell activation markers, and further expanded, as described above. Enriched cells were further tested for recognition of KRAS G12D. Both memory and bulk sorted, CD4 cells showed specific recognition of KRAS G12D 25mer peptide-pulsed on autologous DCs (Fig. 4a). To isolate the reactive TCR, we performed an 18 h co-culture of these subsets with autologous DCs pulsed with the mutated peptide and sorted T cells that upregulated activation markers (OX40+, 41BB+, or OX40+41BB+ double positive) into a 96-well PCR plate for scPCR15. The subsets shared one TCR (TCR1), however, in the memory subset present in both sorted subsets, showed specific reactivity against the G12D peptide (Fig. 4c). Next, we evaluated the specificity and avidity of the TCR against DCs pulsed with a serial dilution of mutated and wild-type KRAS peptides (Fig. 4d) and determined that TCR recognition is restricted by HLA-DRB1*08:01 (Fig. 4e). In summary, we employed our IVS approach on T-cell PBL subsets from six metastatic cancer patients that harbored KRAS
mutation in their tumor. In three we were able to isolate TCRs targeting KRAS mutation from their memory cells (Pt.4148, Pt. 4171, Pt.4238 presented in Fig. 3, Fig. 4, Supplementary Figure 8 and Supplementary data 5, respectively). In Pt.4171 PBLs we isolated the reactive TCR from bulk CD4 as well; however, none of the reactive TCRs were detected in the naive subset (Supplementary Table 6).

**Discussion**

Employing this novel approach enabled us to identify and isolate neoantigen-reactive T cells that were present at very low frequencies in the circulation of metastatic cancer patients. Although all current IVS methods are performed on bulk or naive PBLs, our method enables the isolation of T cells or TCRs from the limited repertoire of memory precursors, which is of high relevance for personalized treatments. We identified a total of two reactivities against neoantigens, both of them also detected in TIL from these patients, and three against KRAS from a total of six patients screened using our IVS method, we were able to isolate the reactive TCR from bulk CD4 as well; however, none of the reactive TCRs were detected in the naive subset (Supplementary Table 6).

**Methods**

**IVS of naive and memory T cells.** Apheresis samples were thawed, washed, set to 5–10x6 cells/ml with AIM-V media (Life Technologies) and 1.75×10⁸ viable

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**Fig. 3** Identification, isolation, and analysis of KRASG12V-reactive TCR. a 41BB-enriched CD8 memory T cells were expanded, and their reactivity was tested against autologous DCs pulsed with the indicated peptides. b 41BB-enriched CD8 memory T cells are stained with KRASG12V 9mer tetramers; unstimulated CD8 cells were used as control (left). c 41BB upregulation and ELISPOT IFNγ secretion assay of TCR-transduced allogeneic T cells. T cells co-cultured with cell lines naturally expressing G12 mutations ± HLA-A11 transduction. d IFNγ secretion of TCR-transduced cells against autologous DCs pulsed with the indicated concentrations of mutated and WT 9mers. A representative of at least three experiments.
cells were incubated in T175 (Corning Inc.) at 37°C. After 2 h, the flasks were washed 2–3 times vigorously with phosphate-buffered saline (PBS) to collect non-adherent cells for T-cell sorting. For the adherent cells, 30 ml DC media were added, comprised of RPMI containing 5% human serum, 100 U/ml penicillin and 100 µg/ml streptomycin, 2 mM L-glutamine, 800 IU/ml GM-CSF (Leukine) and 200 U/ml IL-4 (Peprotech), and cells were incubated at 37°C, 5% CO2. On day 4 or 5, cells were harvested and freshly used or frozen for further use. DCs were seeded into low-attachment 12 or six-well plates for peptide loading or TMG transfection. For peptide loading, DCs were loaded with 10^10^5, cells were harvested and freshly used or frozen for further use. DCs were seeded with autologous DCs pulsed with mutated and wild-type KRAS peptides. T cells were cultured with autologous DCs pulsed with either KRASG12D or KRASWT peptides. G12D or WT peptides. IFN γ spots per 2e4 cells were added to each well and incubated at 37 °C for 12 h. For TMG, RNA transfection lipofectamine or electroporation were used.

***For TcR, in patients 4148, 4171, and 4238, instead of IL-2 and IL-2, 5 ng/ml of IL-15 and IL-7 were used in the second feeding and 10 ng/ml in the third feeding. Immature DCs were thawed resuspended in DC medium + cytokines and transfected into low-attachment 12- or 6-well plates for peptide loading. For peptide loading: DCs were loaded with 10–15 µg/ml peptide or peptide pools for 2–12 h. For TMG, RNA transfection lipofectamine or electroporation were used and the cells were incubated for 8–12 h prior to IVS 1. DCs were harvested by vigorous washing with PBS and incubated for 5 minutes in 5 ml 0.9 mM ethylenediaminetetraacetic acid–phosphate-buffered saline. DCs were washed with DC medium and resuspended at a concentration of 5e6/ml.

Non-adherent cells were spun, resuspended in 50/50 medium comprising 1:1 mix of RPMI-1640 with L-glutamine (Lonza) and AIM-V (Gibco) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 12.5 mM HEPES, and 5% human serum, and rested overnight at 37°C, 5% CO2. After 120 min, non-adherent cells were collected, and the cell mix were transferred into individual wells of a 48-well plate. Cells were incubated for 24 h. Cells were harvested, washed with sorting buffer (PBS, 1% FCS, 0.5 mM EDTA) and stained with Anti-human CD3, Anti-human 41BB, Anti-human OX40 and Anti-human CD4.

First feeding—Check cells under the microscope and calculate the required amount of medium to give 0.5 ml per well. In all, 500 µl of warm CTL medium containing 60 ng/ml of IL-2 and 3000 IU/ml of IL-2 (referring to the final concentration in the culture medium) were added to each well of a six-well plate. Cells and medium from each individual well of the old 12-well plate were transferred to individual wells of the new six-well plate. Cells were incubated for 72 h. From this step cells can be kept in CTL medium containing 3000 IU/ml IL-2 and expanded until the second IVS and 41BB/ OX40 enrichment.

Monocyte-derived, immature dendritic cells were generated using the plastic adherence method. In brief, autologous apheresis samples were thawed, washed, set to 5–10e6 cells/ml with AIM-V media (Life Technologies) and then incubated at ~16 cells/cm² in an appropriate size tissue culture flask and incubated at 37 °C, 5% CO2. After 120 min, non-adherent cells were collected, and the flask was vigorously washed with PBS, and then adherent cells were incubated with RPMI (Life Technologies) containing 3% human serum 100 U/ml penicillin and 100 µg/ml streptomycin, 2 mM L-glutamine, 800 IU/ml GM-CSF and 800 U/ml IL-4 (Peprotech). On day 4–7, fresh DCs were collected. Fresh or freeze/thawed DCs were used in experiments on day 4–5 after initial stimulation.

Genomic DNA and Total RNA was purified using the QIAGEN AllPrep DNA/RNA kit (cat #80204) for patients 4213, 4148, 4238, and 4171 fresh tumor (fTum) and matched normal apheresis samples following manufacturer’s suggestions. Whole-exome library construction and exon capture of ~20,000 coding genes was prepared using Agilent Technologies SureSelectXT Target Enrichment System (cat# 5190-8846) for paired-end libraries coupled with Human All Exon V6 RNA
bait (cat# 5190-8863) (Agilent Technologies, Santa Clara, CA, USA). WES libraries were subsequently sequenced on a NextSeq 500/550 desktop sequencer (Illumina, San Diego, CA, USA). The library was prepped using gDNA (3 μg) isolated from the fresh tumor tissue following the manufacturer’s protocol. Paired-end sequencing was done with an Illumina High-output flow cell kit (300 cycles) (cat# FC-408). Flanking 30 bp of either RNA with the Illumina reads and a stranded library prep kit following the manufacturer’s protocol. RNA-Seq libraries were paired-end sequenced on our NextSeq 500/550 desktop sequencer (Illumina, San Diego, CA, USA) again using the same mechanism described above to generate 25 million paired-end reads.

**Sequence alignment, processing, and variant calling.** Output from the sequenced and converted to fastq format using illumina’s bcflastq program. Reads were trimmed for quality and to remove and adapter sequence with Trimmomatic software. Once trimmed, Exome reads are aligned to hg19 genome using novoalign from novocraft to create initial bams. RNA-seq reads are aligned to hg19 using STAR two pass alignment procedure. Both RNA-seq and Exome bam files are preprocessed according to the GATK best practices protocol. Exome SNVs are called using Strelka, Somatic Sniper, Varscan2 and Mutect. Insertions and deletions (In/Dels) are called using python script. This script produces 25 mers with 12 aa for neoantigens. The regions primers by Beckmann Coulter.

**Construction of tandem minigene and in vitro transcription.** For TMGs construction, each non-synonymous variant identified a minigene was constructed encoding the mutant amino acid flanked by 12 amino acids of the wild-type sequence. TMGs were cloned into pCR2.1 using a linearization of the constructs, phenol water and extracted with ethanol. Prior to the mutation be located closer than 12 aa from the beginning or end of a transcript, the maximum number of base that can think are used. For In/Del mutations, the corresponding change is made to the cDNA sequence, and then 2 aa before the mutation (where possible) are extracted as well as all amino acids beyond the mutation up until the 1st stop codon is encountered. If no stop codon is encountered, the neotope will encompass all sequence up to the end of the cDNA transcript.

**Peptide pulsing and RNA liposomal transfection.** Peptides were made in-house or purchased from GenScript or peptides & elephants. In brief, autologous or allogeneic DCs were harvested, washed and resuspended at 5-10×10^6 cells/mL. Concentration in DC media supplemented with 890 IU/mL GM-CSF and 800 U/mL IL-4. Next, cells were incubated with peptides for 2-12 h at 37°C, 5% CO2. Prior to co-culture DCs were collected, washed twice with PBS, and resuspended in 50/50 media and then used for co-culture assays.

**Co-culture assays:** IFN-γ ELISPOT, ELISA assays and flow cytometry for activation markers CD34 and CD137 staining. When DCs were used as target cells, 3×4-1×5 cells/well were used in 96-well plates. When cells lines were used as target cells, 2×4-5×4 cells/well were used in 96-well plates. 3×4-2×4-1×2 cells/well effecter T cells were used in 96-well plates. All co-cultures were performed in 50/50 media in the absence of exogenously added cytokines. Phorbol 12-myristate 13-acetate: ionomycin mixture (eBioscience) was used as a positive control. In HLA-blocking assays target cells were incubated with 20–50 μg/mL blocking antibodies for 2 h at 37°C, 5% CO2, and then effector cells were added and incubated for 12-18 h.

**Antibodies**

*Patient informed consent* All patient samples were obtained in the course of a National Cancer Institute Institutional Review Board-approved clinical trial. Patients provided informed consent.
The following titrated anti-human antibodies were used for cell surface staining: CCR7–BBS15 (cat. 565869, 1:12.5 dilution) or FITC (cat. 561271, 1:12.5 dilution), CD45RO-PE-Cy7 (cat. 560608, 1:7 dilution) or APC (cat. 559865, 1:7 dilution), CD45RA-APC (cat. 561210, 1:7 dilution) or BD Horizon™ V450 (cat. 560362, 1:7 dilution), CD262L-PE (cat. 555544, 1:7 dilution), CD4-PE (cat. 566679, 1:100 dilution), FITC (cat. 56561005, 1:100 dilution), BV421 (cat. 562659, 1:100 dilution), CD3-APC (cat. 560127, 1:100 dilution) or APC-H7 (cat. 560176, 1:100 dilution), CD4–FITC, PE, PE-Cy7, APC-H7 (clone: SK3), CD8-PE-Cy7 (cat. 335787, 1:100 dilution), OX40-PE-Cy7 (cat. 563663, 1:7 dilution) or FITC (cat. 555837, 1:7 dilution), 41BB-APC (cat. 550890, 1:7 dilution). All antibodies were from BD Biosciences. For MHC blocking assays, the following antibodies were used: pan-class-I (clone: W6/32, produced from hybridoma and used at 1:20 dilution), pan-class-II (BD pharmpingen, cat. 555556, 1:20 dilution), HLA-DR (clone: HB55, produced from hybridoma and used at 1:20 dilution), HLA-DP (Leinco, cat.H127, used at 15 μg/ml), and HLA-DQ (BD pharmpingen, cat. 555862, 1:20 dilution). For cells stimulation, purified anti-CD3 was used (Miltenyi Biotech, cat.130-093-387, used at 30 ng/ml).

Reporting summary
Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.

Data availability
The exome and RNA sequencing data for all patients have been deposited in the sequence read archive at NCBI under the accession code PRJNA507557. Each sample include the patient name, number of the resected metastatic lesion that was sequenced (1Met for example) and the sequencing output (exome or RNA-seq). Normal tissue sequencing data is marked as N (for example 4217N-exome). Patient 4217 exome and RNA sequencing data have been deposit under the accession codes 4217-3Met-exome, 4217-2Met-exome, 4217-1Met-exome, 4217-3Met-RNA-seq, 4217-2Met-RNA-seq, 4217-1Met-RNA-seq, 4217N-exome. Patient 4213 exome and RNA sequencing data have been deposit under the accession codes 4213-3Met-exome, 4213-2Met-exome, 4213-1Met-exome, 4213-3Met-RNA-seq, 4213-2Met-RNA-seq, 4213N-exome, 4213-2Met-exome, and 4213-2Met-exome. Patient 4238 exome and RNA sequencing data have been deposit under the accession codes 4238Met-exome, 4238N-exome, and 238Met-RNA-seq. Patient 4148 exome and RNA sequencing data have been deposit under the accession codes 4148-2Met-RNA-seq, 4148-1Met-RNA-seq, 4148-1Met-exome, 4148N-exome, and 4148-2Met-exome. Patient 4171 exome and RNA sequencing data have been deposit under the accession codes 4171Met-RNA-seq, 4171N-exome, and 4171Met-exome.

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Author contributions
G.C. developed the IVS method and performed the experiments regarding the identification of T cells targeting unique mutations. R.Y. performed the experiments regarding the identification of T cells targeting shared oncogenes. A.P. performed the TCR single-cell sequencing. D.C.D and M.P. contributed to the development of the IVS method and for some of the experiments. Y.C.L. performed single-cell sequencing for the identification of the G12V-specific TCR. J.J.G and L.J. performed all bioinformatic analysis. S.R., L. T-N, M.J, and A.S performed experiments. T.P. performed the DNA and RNA sequencing and technical support. We thank the medical arts service at the NIH for ow cytometry support, and other members of the Surgery Branch for helpful discussions and technical support. We thank the medical arts service at the NIH for figure editing. A patent applications related to KRASG12V (U.S. application no. 62/594,244) was filed. This research was supported by the Center for Cancer Research intramural research program of the National Cancer Institute.

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