Identification of neoantigen-reactive T cells in hepatocellular carcinoma: implications in adoptive T cell therapy

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INTRODUCTION

- Adoptive cell therapy (ACT) with TILs (tumor infiltrating lymphocytes) is an interesting alternative for non-responder Hepatocellular carcinoma (HCC) patients to immune checkpoint blockade.
- ACT-TIL is a form of passive therapy in which T lymphocytes are expanded *ex vivo* from tumor biopsy. Later on, these cells are selected and expanded on large-scale to finally be re-infused into the patient.
- Antigens derived from non-synonymous somatic mutations (**neoAntigens,** neoAgs) are one of the main targets of TILs.
- TILs specific for neoAgs have been described in several cancer types with a tumor mutational burden (TMB) similar to that found in HCC. Therefore, there is a likelihood of finding neoAg-specific TILs in HCC and of translating the potential of neoAg targeting into effective ACT therapies.

AIM

To determine if patients with HCC harbor T lymphocytes that can specifically recognize proteins encoded by somatic mutations uniquely expressed by autologous tumor cells.

METHOD

Patients with different HCC etiology and BCLC classification were included in the study (**Table 1**). Whole exome sequencing (WES; Agilent SureSelect Human All Exon V6 enrichment kit, Illumina Novaseq,150bp paire-end; on target 100x) of tumor and normal liver was used to identify non-synonymous somatic mutations, (**Fig. 1A**). For mutation identifications, four different variant callers (Mutect2, Varscan2, Somaticsniper and Strelka) were used. Only single Nucleotide Variants (SNVs) present in at least two different variant callers were considered as "true SNVs". Only Indel identified by either Varscan2 or Strelka were taken into account. For immunological screening, mutations were prioritized and selected using filters that accounted for expression of the variant transcript and the presence of the variant in RNA-seq. Algorithms for predicting MHC binding were not used for filtering variants. Peptides of 25 amino acids in length were synthesized containing the mutated sequences and mixed equally to form peptide pools **(Fig. 1B)**. TILs were expanded from tumors in the presence of IL-2 (**Fig. 1C**). Expanded TILs were then co-cultured with peptide-pulsed autologous antigen presenting cells (CD40-activated B cells) and evaluated for neoAg recognition by assessing IFNγ production (ELISPOT) and expression of activation markers, such as CD137, by flow cytometry (**Fig. 1D**).

RESULTS

- We generated multiple TIL cultures mostly from tumor fragments but also from digested tumors (**Fig. 2**). The numbers of expanded TILs varied among patients and tumor fragments.
- WES and RNAseq were performed from the same tumor specimens on which TILs were expanded. Approximately 94% of the mutations identified were SNVs with a median of 66 mutations per patients (**Fig. 3A**). The median of indels was 4 per patients. The value and range of tumor mutation burden (TMB) (**Fig. 3B**) was similar to those reported previously (***Alexandrov LB et al., 2013***).
- So far, we have identified neoAgs reactive TILs in 2 of 5 patients tested (**Table** 2). Flow cytometric analysis of CD137 upregulation revealed this reactivity was mediated by CD8 T cells (patient 6) and CD4 (patient 7). Screening of the remaining 8 patients is currently underway.
- As one example of the identification of neoAg reactive T cells, we show the case of patient 6 (**Fig. 4**). Three of eleven TIL cultures (F1, F3 and F4) demonstrated reactivity against peptide pool 1 (**Table 2**). To identify the specific neoAg being recognized, TILs were co-cultured with autologous B cells pulsed with each of the 16 individual peptides in peptide pool 1. IFNγ secretion indicated that F3-derived T cells recognized peptide 5, encompassing mutant HNRNPA3 (Heterogeneous Nuclear Ribonucleoprotein A3) (**Fig. 4A**). Finally, recognition of mutant peptides was compared to their wild type counterparts showing that the recognition was truly neoAg specific (**Fig. 4B**).

Table 1. *Etiology and previous treatment of the HCC patients included in this study. BCLC (Barcelona Clinic Liver Cancer classi<<fication). RE (Radioembolization); AH (Atypical hepatectomy); SR (Surgical resection); A (Ablation); R (Resection); Soraf (Sorafenib); Nivo (Nivolumab).*

ID HEPA	gender	Etiology	BCLC	previous treatment
Hepa 4	male	alcoholic	A	RE
Hepa 5	male	alcoholic and HCV	A	none
Hepa 6	male	Autoimmune hepatitis	A	AH
Hepa 7	male	metabolic	A	RE
Hepa 8	male	HCV	A	SR
Hepa 11	male	HCV	C	RE, A, R, Soraf, Nivo
Hepa 12	male	alcoholic and metabolic	A	none
Hepa 13	male	alcoholic	A	none
Hepa 14	male	alcoholic	0	none
Hepa 19	male	alcoholic	A	A
Hepa 20	male	alcoholic and metabolic	A	none
Hepa 21	male	HBV	A	A
Hepa 26	male	unknown etiology	A	none

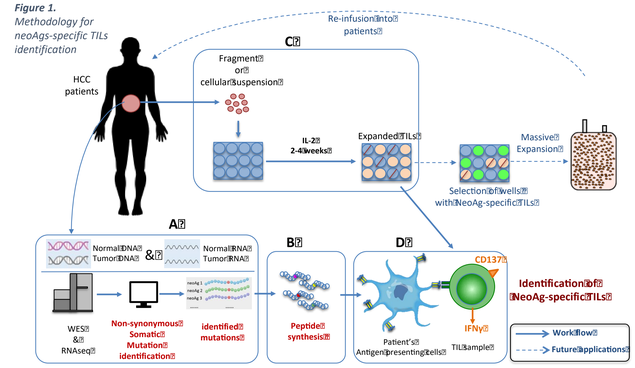


Figure 1. *Methodology for neoAgs-specific TILs identification*

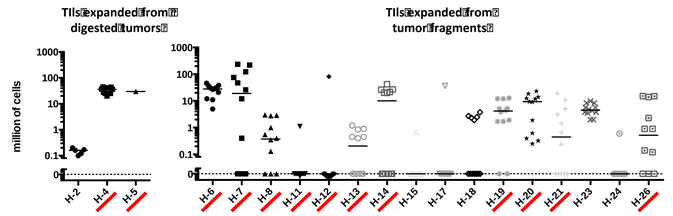


Figure 2. *TILs were expanded from digested tumors or tumor fragments from the indicated HCC patients (H) in the presence of IL-2. The dots represent the number of expanded cells of each of the mini cultures. Highlighted patients were those selected for immunological screening.*

Table 2. *Peptides containing the mutation were synthesized and mixed equally to form peptide pools (PP). Autologous activated B cells were pulsed with PP and co-cultured with expanded TILs. NeoAg recognition was assessed by IFNγ production (1 and 2) and/or CD137 expression (2). TIL cultures that were positive in the preliminary screening assays were subsequently co-cultured with B cells pulsed with individual peptides derived from the PP to identify the specific neoAgs being recognized. (F) fragment from which expanded TILs were able to recognize neoAgs. "NeoAg Recognition" column shows PP or individual pept recognized by expanded TILs. (3) Due to the low number of F2-derived TILs available, the second screening in patient 7 was done with mixtures of 2 peptides instead of individual peptides. F2-derived TILs recognize either peptide 7 or 8, and peptide 19 or 20 from PP1 and PP2 respectively.*

Ptx	# candidate NeoAgs	# pept (pool pept)	TIL well tested	# TIL well recognizing NeoAg	NeoAg Recognition (1)		TIL subset (2)
					Preliminary Screening	Secondary Screening	
					Peptide Pool	Individual pept	
Hepa-4	93	98 (5)	10	0	0	-	-
Hepa-6	68	72 (4)	11	3 (F1,F3,F4)	PP1	$HNRNPA3^{M71V}$	CD8
Hepa-7	77	83 (5)	7	1 (F2)	PP1 & PP2	PP1 (Pept 7 or 8) PP2 (Pept 19 or 20) (3)	CD4
Hepa-8	11	11 (1)	7	0	0	-	-
Hepa-12	22	17 (1)	6	0	0	0	-

Figure 3. (A) *WES was used to identify non-synonymous somatic SNVs and indels using data from tumor and non-tumor samples as described in Methods. **(B)** Tumour mutational burden (TMB) was calculated as the number of somatic mutations (SNV+indels) per 35,7 Megabase pairs (the targeted genomic regions of Agilent SureSelect Human All Exon V6 library)*

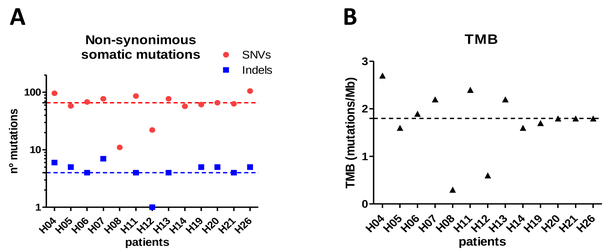
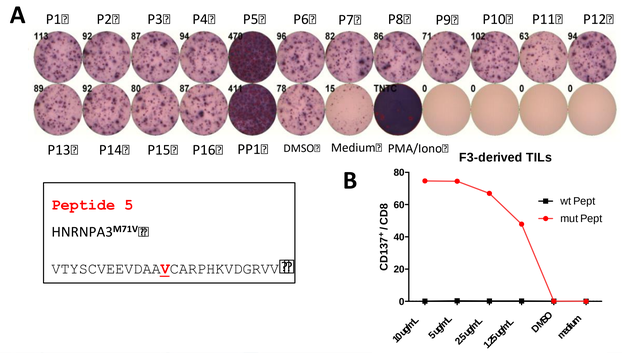


Figure 4. *F3-derived TILs from patient 6 recognized PP1 (Table 2). In the secondary screening, F3-derived TILs were cultured alone (medium), with PMA+Ionomycin or with autologous B cells pulsed with each of the 16 individual peptides contained in PP1, the mixture PP1 or vehicle (DMSO). **(A)** ELISPOT showing recognition of mutant peptide 5 ($HNRNPA3^{M71V}$) and PP1. **(B)** CD137 upregulation assay showing specific recognition of the mutant peptide but not the WT peptide.*



CONCLUSIONS

- Our observations demonstrate that HCC, generally considered to be non-immunogenic, do elicit in vivo immune response against somatic mutations uniquely expressed by autologous tumor cells
- These data provide a rationale for developing new personalized immunotherapeutic treatments for patients with HCC by targeting NeoAgs.

REFERENCES

Alexandrov LB et al. Signatures of mutational processes in human cancer. *Nature. 2013;500(7463):415-21.*

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