Cancer Letters ■■ (2015) ■■–■■

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Original Articles

Identification of peptide-specific TCR genes by *in vitro* peptide stimulation and CDR3 length polymorphism analysis

Hongwei Shao [a,b,1], Yanmei Lin [a,b,1], Teng Wang [a,c], Yusheng Ou [a,b], Han Shen [a,b], Changli Tao [a,b], Fenglin Wu [a,b], Wenfeng Zhang [a,b], Huaben Bo [a,b], Hui Wang [a,b], Shulin Huang [a,b,c,*]

[a] *School of Life Science and Biopharmaceutics, Guangdong Pharmaceutical University, Guangzhou, China*
[b] *Guangdong Province Key Laboratory for Biotechnology Drug Candidate, Guangzhou, China*
[c] *Southern Medical University, Guangzhou, China*

ARTICLE INFO

Article history:
Received 8 December 2014
Received in revised form 10 March 2015
Accepted 5 April 2015

Keywords:
TCR
Mutated peptide
In vitro stimulation
Immunoscope
Monoclonal expansion

ABSTRACT

Identification of TCR genes specific for tumor-associated antigens (TAAs) is necessary for TCR gene modification of T cells, which is applied in anti-tumor adoptive T cell therapy (ACT). The usual identification methods are based on isolating single peptide-responding T cells and cloning the TCR gene by *in vitro* expansion or by single-cell RT-PCR. However, the long and exacting *in vitro* culture period and demanding operational requirements restrict the application of these methods. Immunoscope is an effective tool that profiles a repertoire of TCRs and identifies significantly expanded clones through CDR3 length analysis. In this study, a survivin-derived mutant peptide optimized for HLA-A2 binding was selected to load DCs and activate T cells. The monoclonal expansion of TCRA and TCRB genes was separately identified by Immunoscope analysis and following sequence identification, the properly paired TCR genes were transferred into T cells. Peptide recognition and cytotoxicity assays indicated that TCR-modified PBMCs could respond to both the mutant and wild type peptides and lyse target cells. These results show that combining Immunoscope with *in vitro* peptide stimulation provides an alternative and superior method for identifying specific TCR genes, which represents a significant advance for the application of TCR gene-modified T cells.

Introduction

T cells play an important role in defending the host against tumor cells and viral pathogens. The antigen specificity of T cells is determined by the T cell receptor (TCR), which induces immune responses by binding peptide presented by MHC molecules on the surface of target cells/antigen-presenting cells. In recent years, a strategy to transfer TCR genes into autologous or allogeneic primary T cells in order to confer antigen specificity has been developed and refined in order to improve the application of anti-tumor adoptive T cell therapy (ACT) [1–3]. Consequently, identification of the TCR genes specific for tumor-associated antigens (TAAs) has become necessary for TCR modification of T cells. However, most TAAs are non-mutated self-antigens and thus, are molecules also expressed on the surface of normal cells [4]. As a result, high affinity TCR molecules for these self-antigens are deleted though thymic negative selection, which is necessary to avoid the development of autoimmunity. Moreover, the remaining low affinity TCRs are usually ineffective at initiating immune responses against tumor cells, which provides one explanation for the difficulties in developing efficient anti-tumor vaccines.

Point mutations that suitably change the spatial structure of peptides may enhance the immunogenicity of epitopes and improve the binding of TCRs to peptide–MHC (pMHC) ligands. More importantly, some T cells that are activated by mutated peptides can also respond to wild-type peptides [5]. For example, our previous study showed that cytotoxic T lymphocytes (CTLs) stimulated with mutated peptides could kill tumor cells expressing a wild-type peptide [6]. These studies indicate that some T cells have cross-reactivity; therefore, it is reasonable to screen and identify TCR molecules with cross-reactivity against wild-type TAA peptides by means of mutated peptide stimulation.

To isolate an antigen-specific TCR gene, one general method is to isolate and expand antigen-responsive monoclonal T cells *in vitro* followed by RNA extraction and TCR gene cloning; however, this process generally requires several months [7,8]. Single-cell PCR is another method with which to clone and identify TCR genes, but unfortunately is quite difficult and requires stringent operational practices [9–11]. In contrast, complementarity determining region

* Corresponding author. Tel.: +862039352330; fax: +862039352201.
E-mail address: shulhuang@sina.com (S. Huang).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.canlet.2015.04.001
0304-3835/

Table 1
HLA-A2 binding prediction of peptides.

Peptides	Type	Sequences	SYFPEITHI score	BIMAS	BepiPred affinity (nM)
$Survivin_{79-87}$	Wild	KHSSGCAFL	15	0.286	18,882.64
$Survivin_{79-87}$(H80M)	Mutated	KMSSGCAFL	23	296.997	18.77
$HIVpol_{476-484}$	Wild	ILKEPVHGV	30	39.025	72

3 (CDR3) length polymorphism analysis (Immunoscope) is a rapid and effective tool to profile the clonal repertoire of T cells and identify significantly expanded clones through detection of length differences in the CDR3 of different TCR subfamilies [12–15]. For example, in a model of heart allograft rejection in adult congenic rats, Douillard et al. analyzed the TCR β chain (TCRB) repertoire of T cells infiltrating rejected allografts and found that oligoclonal/monoclonal expansion of these T cells is reflected by an altered distribution of the TCRB CDR3 lengths [16]. Further, by combining CDR3 length analysis with sequence analysis, they identified expanded T cell clones bearing a 'public' TCRB (identical TCRB shared by multiple individuals in responding to a same antigenic epitope). Inspired by their work, in this manuscript we show the simultaneous identification of CDR3 length polymorphisms of the TCR α and β chains (TCRA and TCRB), and subsequently their sequences, in expanded T cell clones responding to a specific antigen.

Survivin, a member of the inhibitor of apoptosis family, is expressed during normal development and aberrantly in many human cancers while it is barely detectable in normal, differentiated adult tissues. Thus, a survivin-derived mutant peptide optimized for binding to HLA-A2 was selected to load DCs and activate T cells, which were then subjected to Immunoscope analysis of both TCRA and TCRB, and the monoclonal expansions of each were separately identified. Following sequence analysis, the paired full-length TCRA and TCRB genes were cloned into an adenoviral vector and subjected to adenovirus packaging and subsequent transfer into T lymphocytes via adenoviral infection. We found that TCR gene-modified peripheral blood mononuclear cells (PBMCs) could be effectively activated by mutant peptide-loaded T2 cells (used as antigen-presenting cells to avoid endogenous antigen presentation) indicating that the TCR genes identified by Immunoscope could respond to the peptides used in the T cell activation. Moreover, a cytotoxicity assay demonstrated that TCR-modified PBMCs could effectively kill cancer cells expressing the wild-type survivin peptide, which suggests a substantial cross-reactivity of the TCR.

Materials and methods

PBMCs and cell lines

PBMCs were isolated from the whole blood of healthy volunteers (HLA-$A2^+$) with Ficoll-Paque™ PLUS (GE Healthcare, Sweden) according to the manufacturer's protocol. All volunteers signed an informed consent document. The protocols used for human studies were approved by the local ethics committee.

The HEK-293 and T2 (HLA-$A2^+$) cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). HepG2 (HLA-$A2^+$/$Survivin^+$), MCF-7 (HLA-$A2^+$/$Survivin^+$), BEL-7402 (HLA-$A2^-$/$Survivin^+$), NCI-H1299 (HLA-$A2^-$/$Survivin^-$), and Jurkat E6-1 T cell lines were maintained in our laboratory. T2 cells were cultured in AIM-V medium supplemented with 100 IU/ml penicillin G and 50 μg/ml streptomycin (TBD, Tianjin, China) at 37 °C under 5% CO_2. The other cells lines were cultured in DMEM (for HEK-293) or RPMI 1640 medium (for Jurkat, HepG2, MCF-7, BEL-7402 and NCI-H1299) supplemented with 10% fetal bovine serum (FBS) (Life Technologies, Grand Island, USA) and antibiotics.

Synthesis of the Survivin HLA-A2 Binding Peptides

SYFPEITHI (http://www.syfpeithi.de/), BIMAS (http://www-bimas.cit.nih.gov/molbio/hla_bind/) and NetMHC (http://www.cbs.dtu.dk/services/NetMHC/) were used to predict peptides binding to HLA-A2 [17–19]. A wild-type survivin epitope with relatively low algorithm scores and its mutated epitope with high algorithm scores were selected for synthesis, and an HIV-derived peptide was selected for negative control (Table 1). The peptides were provided from Sinoasis Pharmaceuticals Inc. (Guangzhou, China), and the purity (90%) was verified by high-performance liquid chromatography (HPLC) and mass spectrometry (MS) analysis. The peptide-binding assay was performed by detecting the expression of HLA-A2 using flow cytometry (FCM) on the T2 cell surface after peptide loading.

Generation of the DCs

Freshly-isolated PBMCs were cultured at 37 °C under 5% CO_2 for 4 hours. The nonadherent cells were collected and stored in liquid nitrogen until use. The adherent cells were cultured with 1000 units/ml IL-4 (R&D Systems, Minneapolis, MN) and 1000 units/ml recombinant granulocyte-macrophage colony stimulating factor (GM-CSF) (R&D Systems, Minneapolis, MN) in AIM-V medium. Half of the medium was changed every 3 days. On day 7, tumor necrosis factor-α (TNF-α) was added to the culture at a concentration of 10 ng/ml. After an additional 3 days of incubation, the mature DCs were harvested.

Primary CTL induction cultures

CTL induction was performed as described previously [20,21], with some modification. Briefly, mature DCs were pulsed with 10 μg/ml of the Survivin epitope peptide in the presence of 2 μg/ml $β_2$-microglobulin for 4 hours. The peptide-pulsed DCs were harvested, washed with PBS, and fixed with 2% paraformaldehyde. The fixed peptide-pulsed DCs were washed three times with PBS and mixed at a 1:20 ratio with autologous lymphocytes (the nonadherent cells obtained above) in AIM-V medium with 100 units/ml IL-2. Half of the medium was changed, and additional aliquots of peptide-pulsed DCs were added to the cultures every 3 days. On day 10, the lymphocytes were harvested and the total RNA was extracted with TRIzol (Life Technologies, Carlsbad, USA) and stored at –20 °C until use.

CDR3 length polymorphism analysis

With primers specific for the constant region of TCRB (TCRBC: 5′-CTCAAACACAGCGACCTC) and TCRA (TCRAC: AGTCTCTCAGCTGGTACACG), first-strand cDNA synthesis was performed using the PrimeScript II 1st Strand cDNA Synthesis Kit (Takara Clontech, Kyoto, Japan). The CDR3 length polymorphism analysis of TCRB was performed as described previously [22,23]. The CDR3 length polymorphism of TCRA was similarly analyzed with a set of primers designed based on previous reports (Table S1) [24]. Multiplex PCR was performed with mixed primers (universal primers: specific primers = 60:1 mole ratio) for each group. After cleaning, the amplified products were subjected to capillary electrophoresis. A dominant CDR3 peak is defined as monoclonal when the area under a single peak is 50% or more of the total area under the curve [25]. An oligoclonal expansion is defined as occurring when there is a dramatic reduction of other CDR3 signals except for a few (usually 2–3) peaks whose distribution is not Gaussian.

Cloning of TCR genes

To obtain the full-length TCR genes, primers with a restriction site were designed based on the TCRBV and TCRAV subfamilies to be cloned (Table S2). The amplified products were ligated into the T-vector (Promega, Madison, USA) and subsequently transferred into *Escherichia coli*. Twenty transformants were selected and subjected to sequence analysis for each transformation reaction.

Construction and packaging of recombinant TCR adenovirus

The TCRB gene was first inserted into the BamH I/Sal I site of the shuttle plasmid pDC315 (Microbix Biosystems, Toronto, Canada) to produce the recombinant plasmid pDC315-TRB. The TCRA gene was inserted into the EcoR I/Sal I site of the bicistronic plasmid pIRES2-EGFP to produce the recombinant plasmid pIRES-TRA-EGFP. Then, the TCRA gene and IRES element were cut from pIRES-TRA-EGFP with EcoR I and BstX I restriction enzymes and inserted into the corresponding site of pDC315-TRB to produce the recombinant plasmid pDC315-TRA-IRES-TRB.

Recombinant adenovirus packaging was performed as described previously [26,27]. Briefly, the shuttle plasmid, pDC315-TRA-IRES-TRB, and the modified backbone plasmid, pAd5/F35L, were co-transfected into HEK-293 cells. Ten to fourteen days after the transfection, plaques could be observed. The cells were collected when approximately 80% cytopathic effect (CPE) was observed, and the virus was released by four cycles of freezing and thawing. After the TCR gene identification and

Please cite this article in press as: Hongwei Shao, et al., Identification of peptide-specific TCR genes by *in vitro* peptide stimulation and CDR3 length polymorphism analysis, Cancer Letters (2015), doi: 10.1016/j.canlet.2015.04.001