

Mutational Analysis of Gene Fusions Predicts Novel MHC Class I-Restricted T-Cell Epitopes and Immune Signatures in a Subset of Prostate Cancer



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Abstract

Purpose: Gene fusions are frequently found in prostate cancer and may result in the formation of unique chimeric amino acid sequences (CASQ) that span the breakpoint of two fused gene products. This study evaluated the potential for fusion-derived CASQs to be a source of tumor neoepitopes, and determined their relationship to patterns of immune signatures in prostate cancer patients.

Experimental Design: A computational strategy was used to identify CASQs and their corresponding predicted MHC class I epitopes using RNA-Seq data from The Cancer Genome Atlas of prostate tumors. *In vitro* peptide-specific T-cell expansion was performed to identify CASQ-reactive T cells. A multivariate analysis was used to relate patterns of *in silico*-predicted tumor-infiltrating immune cells with prostate tumors harboring these mutational events.

Results: Eighty-seven percent of tumors contained gene fusions with a mean of 12 per tumor. In total, 41% of fusion-positive tumors were found to encode CASQs. Within these tumors, 87% gave rise to predicted MHC class I-binding epitopes. This observation was more prominent when patients were stratified into low- and intermediate/high-risk categories. One of the identified CASQ from the recurrent TMPRSS2:ERG type VI fusion contained several high-affinity HLA-restricted epitopes. These peptides bound HLA-A*02:01 *in vitro* and were recognized by CD8⁺ T cells. Finally, the presence of fusions and CASQs were associated with expression of immune cell infiltration.

Conclusions: Mutanome analysis of gene fusion-derived CASQs can give rise to patient-specific predicted neoepitopes. Moreover, these fusions predicted patterns of immune cell infiltration within a subgroup of prostate cancer patients. *Clin Cancer Res*; 23(24): 7596–607. ©2017 AACR.

Introduction

Recent successes using immunotherapy approaches have highlighted the potential of harnessing the immune system to treat late-stage cancers. There is an overarching view that clinical responses to immunotherapy are dependent on the presence and immune recognition of tumor-specific antigens. However, prostate cancer remains problematic given that far fewer patients demonstrate clinical responses to immunotherapy compared

with other settings. For instance, the use of vaccine strategies that target overexpressed tumor-associated self-antigens, such as prostatic acid phosphatase (PAP) and prostate-specific antigen (PSA), have shown only moderate clinical success (1, 2). Although encouraging, the general lack of robust antitumor immune responses after vaccination may be partially explained by the fact that self-antigens are often poorly immunogenic and T cells specific for such tumor-associated antigens may be tolerized. Moreover, prostate tumors generally lack an abundance of cytotoxic tumor-infiltrating lymphocytes (TIL) or have suppressed T-cell response due to engagement of checkpoint inhibition pathways (3, 4).

Checkpoint blockade and adoptive transfer of tumor-specific or engineered T cells are two promising approaches under investigation. Although these strategies have shown remarkable clinical responses in other cancer settings, it is believed that tumor antigen load may, in part, explain some of the differences between responders and nonresponders. For example, in highly mutated solid tumors such as melanoma and non-small cell lung carcinoma (NSCLC), the success of checkpoint blockade and adoptive cell transfer (ACT) of TILs has been largely attributed to the abundance of tumor neoantigens (5, 6). On the other hand, these strategies have shown less success in settings with classically low tumor mutation load (7, 8), although one recent interim report found durable clinical responses to anti-PD-1 in enzalutamide-resistant prostate cancer patients (9). Whether such responses in

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Translational Relevance

The identification of T-cell targets for immunotherapy remains a significant hurdle. Clinical studies have reported encouraging results targeting recurrent somatic point mutations. While their abundance is a factor that underlies some clinical responses, these mutations represent a fraction of possible neoantigens arising from the overall tumor mutational landscape. Prostate tumors exhibit a high frequency of genome rearrangements. Such an event can produce a CASQ, a unique peptide sequence spanning the junction of two fused gene products. Here, 74 of 85 tumors that were examined contained gene fusions. Of these tumors, 41% expressed CASQs and 87% of CASQs were predicted to generate HLA-restricted epitopes. In one case, T cells could recognize CASQ-derived peptides. Interestingly, gene fusions were strongly associated with distinct immune cell signatures. These data highlight gene fusion-derived CASQs as potential sources of neoantigens, particularly in tumors with a low frequency of somatic point mutations.

prostate cancer are associated with a higher frequency of mutations or the presence of a specific type of mutation remains to be determined.

At present, the vast majority of studies have focused on the identification of either recurrent or personalized somatic point mutations that generate tumor neoepitopes. However, given recent genomic efforts to define the broader tumor mutational landscape (e.g., single base substitutions, SCNA, translocations, chromothripsis, alternative splicing, epigenetic alterations, etc.; refs. 10–12), examining other types of mutations as potential immunogenic targets warrants further investigation.

Compared with somatic point mutations, structural genomic rearrangements are considered relatively rare events in epithelial cancers. However, these genome rearrangements are the most common type of genomic abnormality in prostate cancer (13, 14). Consequently, some of these structural genomic rearrangements are important for the pathogenesis of prostate cancer, resulting in amplification of oncogenic drivers, deletion of tumor suppressors, and fusion events that cause altered expression of tumor oncogenes (15, 16). For instance, recurrent fusions between androgen-regulated genes and ETS family genes occur in over 50% of all prostate cancer cases (17, 18) and in many cases lead to aberrant expression of oncogenic ETS transcription factors that may contribute to prostate tumorigenesis (13, 14, 18).

Tumor mutations can generate altered protein products that are distinct from those found in normal tissues. If the resulting altered protein is immunogenic, it presents an opportunity for specific targeting of the tumor by the immune system. Indeed, personalized multiepitope vaccines targeting nonsynonymous tumor mutations have demonstrated antitumor efficacy in multiple preclinical studies (19, 20), and this concept is being further investigated in early clinical trials in numerous cancer settings (21–25). Other reports have highlighted this proof-of-principle using adoptive transfer of expanded, mutation-specific TIL (26, 27). However, in mutation-low tumor settings such as prostate cancer, the pool of potential tumor neoantigens arising from somatic point mutations may be limited (28–30).

Similar to nonsynonymous point mutations, fusion events can generate altered protein products when the fusion occurs within a coding region. In this case, the breakpoint-spanning chimeric amino acid sequence (CASQ) of two fused genes can distinguish each gene partner from its respective wild-type counterpart. Given their specificity to the tumor, these unique CASQs have the potential to elicit tumor-specific T-cell responses. For instance, a multi-peptide vaccine against the CASQ of the BCR-ABL fusion protein gave rise to measurable peptide-specific immune responses and improved disease control in chronic myelogenous leukemia patients (31, 32). Despite the prevalence of genomic rearrangements across prostate cancer cases, strategies to exploit the reservoir of fusion-derived CASQs as tumor neoepitopes have not been systematically explored. Here, a stringent computational approach was used to identify prospective immunogenic fusion epitopes from transcriptomics data in a cohort of prostate adenocarcinoma patients from The Cancer Genome Atlas. While a large proportion (74 of 85) of the cases that were examined contained gene fusions, nearly half of the patients in this analysis were found to harbor CASQs, and among these, 87% of the CASQs gave rise to predicted MHC class I epitopes. These results were even more pronounced when patients were stratified into low- and intermediate/high-risk cohorts where low-risk patients contained fewer fusions and CASQs when compared with the intermediate/high-risk patients. Interestingly, there was a significant correlation between the presence of gene fusions and CASQs with distinct immune gene expression patterns in a subset of patient tumors. Thus, immunotherapy approaches in prostate cancer should consider gene fusions and their interactions with the host immune system.

Methods

Patient datasets and blood collection

RNA-Seq datasets (matched tumor and adjacent normal) from The Cancer Genome Atlas (TCGA) prostate adenocarcinoma cases were accessed from the Cancer Genomics Hub (<https://cghub.ucsc.edu/>; May, 2015). The patients were stratified into two groups: low-risk (PSA < 10 ng/mL Gleason Score = 6; $n = 35$) and intermediate-high risk (PSA > 10 ng/mL and Gleason Score ≥ 7 ; $n = 50$). The available clinical information for this cohort is summarized in Table 1. A list of the TCGA identifiers for patients in this study can be found in Supplementary Data (Supplementary Table S1). For *in vitro* studies, peripheral blood mononuclear cells (PBMC) were purified from whole blood of healthy donors using Ficoll-Paque PLUS (GE Healthcare) and cryopreserved in nitrogen vapor freezers. Healthy donors gave written informed consent to donate semiannual 200-mL blood draws under protocols approved by the University of British Columbia-British Columbia Cancer Agency's Research Ethics Board (REB# H07-00463). As such, this study was conducted in accordance with the Declaration of Helsinki.

Fusion detection by defuse

Fusions were detected from RNA-Seq datasets using deFuse (Version 0.6.2; ref. 33) based on reference genome and gene models from Ensembl GRCh37 release-75. In short, using default parameters for deFuse, candidate gene fusions were first identified by clustering spanning reads, which are defined as discordant mappings whose two mates were located within distinct genes. Spanning reads that mapped to multiple loci due to sequence

Table 1. Characteristics of the TCGA prostate adenocarcinoma patient cohort

Patient characteristics	
Age at diagnosis (median)	
Years	41-77 (57)
Gleason score (%)	
6	35 (41)
7	30 (35)
8	4 (5)
9	16 (19)
Preoperative PSA (median)	
0-10 ng/mL	35 (5.6)
10-20 ng/mL	35 (12.8)
>20 ng/mL	15 (26.3)
pT (%)	
T2a	5 (6)
T2b	1 (1)
T2c	34 (40)
T3a	28 (33)
T3b	15 (18)
T4	2 (2)
Regional lymph node involvement (%)	
Yes	11 (13)
No	50 (59)
Not available	24 (28)

homology or RNA splice variation were assigned to the most likely gene pairs based on the maximum parsimony principle. Once the set of fused genes was obtained, one-end anchored (OEA) reads whose mapped mates locate near each candidate fusion boundary were grouped together. Their unmapped mates were aligned to sequences near the fusion boundary in a dynamic programming formulation to obtain final breakpoints at nucleotide resolution. Additional confidence parameters were applied to eliminate false-positive fusion calls according to recommendations for deFuse (33).

***In silico* translation and CASQ identification**

To identify CASQs, we focused on genes that were fused with complimentary orientation and reading frame. For each predicted fusion, deFuse provides the orientation information describing how two genes are combined on the basis of the splitting reads that support the fusion. Fusion peptides were selected as follows: First, two genes in a fusion should concatenate in a way consistent to their strands and reading frames. In other words, fusion breakpoints should always be located downstream of the first gene, and upstream of the second gene. In addition, we only considered fusions that were in-frame, and chimeric transcripts that had lost the native ATG from the 5' gene partner were eliminated. Second, translations of chimeric peptides should pertain to the original reading frames from both parental genes. For each half of the chimeric transcript, we extract the corresponding protein sequences from the human proteome database (Ensembl GRCh37 release-75) and ensured that the translated sequence in the final chimeric peptide is identical to the peptide sequence from the same regions of the original proteins. Chimeric peptide sequences that did not share the native reading frame of their parental protein sequences were then discarded. As a result, each chimeric peptide is formed by concatenating two subsequences from known human proteins. These filtration steps ensured the prioritization of fusions encoding in-frame chimeric proteins.

MHC class I allele selection using HLAMiner

HLAMiner (Version 1.3) was used to determine the HLA haplotype of individuals at the HLA-A, -B, and -C loci from RNA-Seq (34). The top 2 predictions at the 4-digit resolution for each locus were nominated for each given patient provided that predictions differed at the 2-digit resolution.

IEDB peptide prediction

MHC_I Binding Tool (Version 2.13) from NetMHCpan package was accessed via the Immune Epitope Database (IEDB; ref. 35) and used to extract predicted high-affinity minimal peptides from CASQs on patient autologous HLA haplotypes. Fusion-spanning 8-11 mer peptides with an IEDB rank score of <2 were classified as high-affinity epitopes.

Peptide library

The TMPRSS2:ERG type VI fusion was identified as a recurrent CASQ yielding predicted HLA binding epitopes and was thus selected for further *in vitro* validation. TMPRSS2:ERG type VI fusion peptides were synthesized and purified commercially (Genscript), reconstituted and stored in pure DMSO. We chose to evaluate overlapping decamer peptides beginning at the translational initiation site N-terminal to the fusion breakpoint plus an additional nonamer peptide, beginning at position 2, based on the predicted affinity trends for these peptides on HLA-A*02:01.

MHC stabilization assay

T2 cells were originally purchased from the ATCC stock (CRL-1992) and obtained as a kind gift from Dr. Brad Nelson (BC Cancer Agency). The cells were not pathogen tested at the time they were used. T2 cells were maintained in Iscove's modified Dulbecco's medium supplemented with 20% FBS. T2 cells were plated with increasing concentrations of TMPRSS2:ERG peptides (0-80 μ mol/L) for 18 hours at 26°C. A known HLA-A*02:01 binding epitope from the melanocyte protein Melan-A/MART-1 (ELAGIGILTV) was used as the positive control. Cells were incubated for an additional 3 hours at 37°C in the presence of Brefeldin A (10 μ g/mL) and labeled with anti-human HLA-A2 FITC (Clone BB7.2, BD Biosciences). The mean fluorescence index was measured using a Guava EasyCyte 8HT flow cytometer (EMD Millipore) and data were analyzed with FlowJo V10 software.

Generation of fusion-specific T-cell lines

Monocyte-derived dendritic cell (DC) cultures were generated as described previously (36). Briefly, healthy donor PBMC were plated in 6-well plates (10^7 cells/well) and nonadherent cells removed after 90 and 150 minutes at 37°C. Adherent monocytes were supplemented with 800 IU/mL each GM-CSF and IL4. On day 6, DCs were matured with poly I:C (50 μ g/mL) for 2 days prior to harvesting for stimulation of T cells against fusion peptides. DCs were pulsed with TMPRSS2:ERG fusion peptides (10 μ mol/L/peptide), irradiated (32 Gy), and incubated with autologous PBMCs. T cells were restimulated with irradiated (50 Gy) peptide-pulsed PBMC and poly I:C after 11 days and expanded with 240 IU/mL IL2, 20 ng/mL IL15, and 20 ng/mL IL7 for two weeks. T-cell cultures were rested for 3 days in 10 ng/mL IL7 and screened for TMPRSS2:ERG reactivity by IFN γ ELISPOT as described previously (36). Individual reactive cultures were expanded using irradiated (50 Gy) allogeneic feeder cells (PBMC) supplemented with OKT3 (30 ng/mL) and IL2 (300 IU/mL) for 2 weeks.

TMPRSS2:ERG peptide-activated T cells were enriched by FACS on CD8⁺CD137⁺ followed by reexpansion.

Immunologic correlates of CASQ

We explored correlation between total gene fusions, predicted MHC class I binding CASQ epitopes, and immunologic parameters using recently published estimates that predicted immune cell infiltrates using RNA-sequencing data (37). For immune cell infiltrates, we used published infiltration estimates for the patients included in this cohort generated, estimated via a single sample gene set enrichment analysis (ssGSEA) approach performed across multiple cancers (37). These immune cell predictions were tested for associations with predicted fusions using redundancy analysis (RDA). This is a constrained extension of principal components analysis (PCA) that employs linear modeling to associate predictor variables (here the presence/absence of CASQs and total number of fusions (log +1 transformed)) and a multivariate outcome (predicted immune cell infiltrates). Model significance was assessed via permutation tests ($n = 1,000$ permutations), all implemented in the *vegan* package of R v3.3.1 (38).

Results

Computational approach and strategy for RNA-Seq data analysis

An immunogenomic strategy was used to predict tumor-specific antigens contained within chimeric proteins using RNA-Seq datasets (Fig. 1). This approach combined three existing computational tools: deFuse, HLAMiner, and IEDB MHC class I epitope prediction software (refs. 33–35; www.iedb.org). First, deFuse identified a list of high-confidence genomic fusions from

RNA-Seq reads using a mapping-based approach. The resulting fusion transcripts encoding predicted CASQs were translated *in silico*. Second, using HLAMiner, patient-specific HLA haplotypes were extracted from matched transcriptomics data. Next, IEDB was used to interrogate CASQs against patient-relevant HLAs to generate a candidate list of high-affinity MHC class I epitopes specific to the fusion-spanning regions. Stringent criteria as defined in the Materials and Methods were used at each step to uncover potential mutations with the highest probability of generating neoepitopes for *in vitro* validation by peptide-specific expansion of CASQ-reactive T cells from the peripheral blood of healthy donors.

A proportion of tumor-specific gene fusions yield CASQ with predicted MHC class I epitopes

The frequency and recurrence of genomic fusion events were evaluated within a cohort of prostate adenocarcinoma cases from TCGA ($n = 85$). Patient inclusion was based upon clinical presentation of low-risk ($n = 35$) defined by a Gleason score of 6 and preoperative PSA < 10 ng/mL and intermediate/high-risk ($n = 50$), defined by a Gleason score ≥ 7 and preoperative PSA ≥ 10 ng/mL (Table 1). Using the approaches described above, analysis of tumor RNA-Seq datasets revealed a unique set of fusion events from each patient (range 1–65; median 8, mean 12; Fig. 2A; black bars). A complete list of gene fusions can be found in the Supplementary Data (Supplementary Table S2). The prevalence of fusion events in our cohort is consistent with previously reported transcriptomic studies in similar patient groups (39). Greater than half of the tumors (57%) in this cohort carried 10 or fewer fusions, while the top 10% of fusion-positive tumors contained greater than 25 genomic fusions. Although the mean number of putative CASQs generated by individual fusion events was low, 41% of fusion-positive tumors contained a minimum of

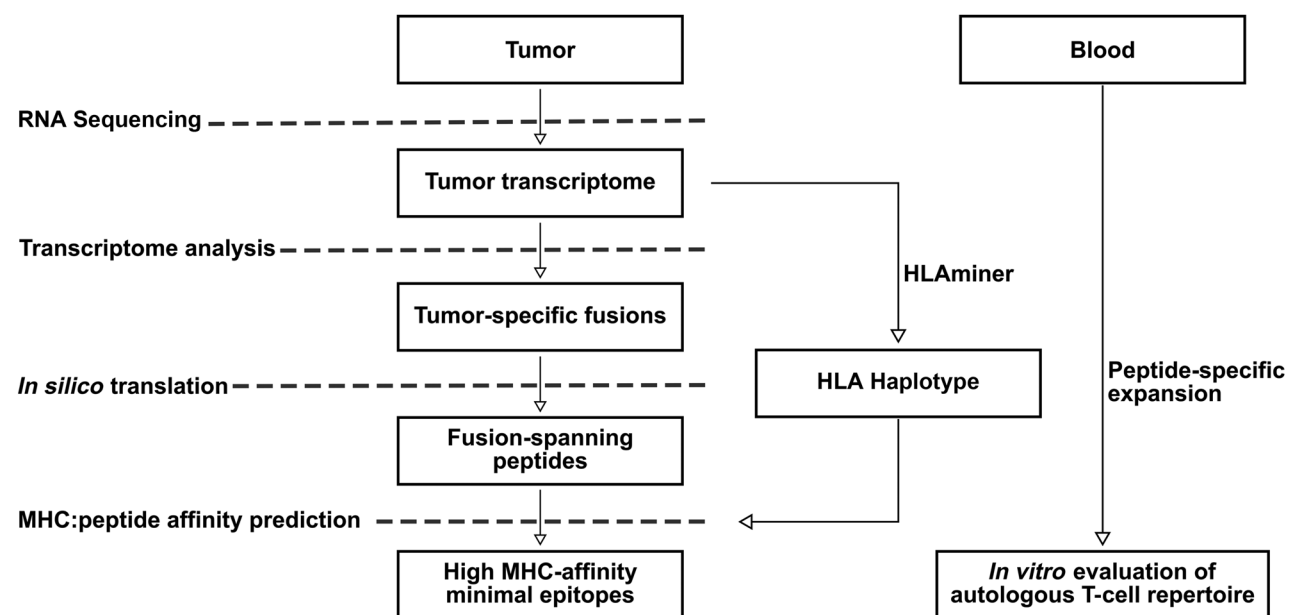
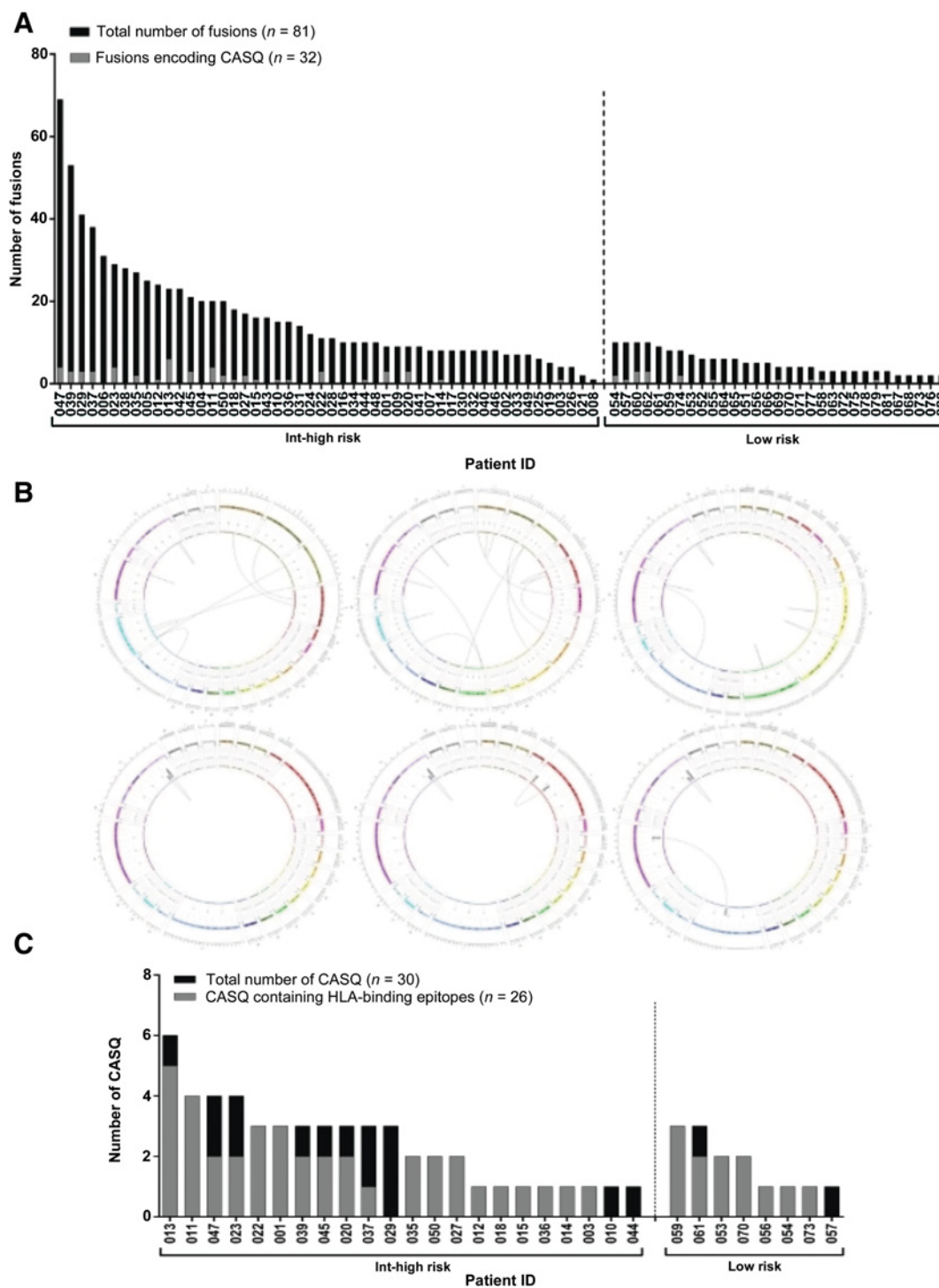


Figure 1.

Computational approach to identify predicted immunogenic CASQs. Data are generated from RNA-sequencing of tumor tissue. Transcriptomic analysis reveals tumor-specific gene fusions and *in silico* translation was performed to identify CASQs. Patient HLA haplotype is determined via HLAMiner to generate a candidate list of MHC:peptide affinity prediction scores. T cells from the peripheral blood are interrogated against fusion-encoding predicted epitopes to assess existing immunoreactivity to patient-specific CASQs.

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**Figure 2.**

Gene fusions across the cohort of TCGA prostate adenocarcinoma patients. **A**, The number of tumor-specific gene fusions per patient dataset by deFuse analysis using a stringent systematic filtering approach to eliminate false positives. Fusions identified within the TCGA prostate adenocarcinoma cohort range from as many as 69 to as few as 1 (black bars). Gene fusions from 22 intermediate/high-risk (left) and 8 low-risk (right) patient tumors are predicted to encode at least one CASQ. **B**, Representative Circos plots display the total number of identified fusions from 3 representative patient tumors (patients 022, 011, 020, respectively, top). Circos plots display CASQs arising from those patient fusions (bottom). **C**, The total number of CASQs identified in 19 intermediate/high-risk (left) and 7 low-risk (right) patients (black bars) with a neopeptide predicted to bind to patient's autologous HLA (gray bars). As many as 6 CASQs were identified within an individual patient tumor. The majority of CASQs encode a fusion spanning epitope predicted to bind to patients' autologous HLA alleles. CASQs of Patients 029, 044, 010, and 057 yielded no predicted HLA-binding epitopes.

Table 2. Gene pairs of CASQs identified across the prostate cancer patient cohort

Patient	Fusions		
001	PMEPA1:ETV4	TMPRSS2:MORC3	
003	ABCD3:DPYD		
010	MT-CYB:MT-ND3		
011	GPBP1:MTRR	TMPRSS2:ERG	
012	LEO1:FBN1		
013	KLK2:KLK3	MAPK9:DGKB	SAMD5:SASH1
	SMYD3:TRIM58	SNAP91:BCKDHB	TMEM56-RWDD3:SNX7
014	PRCP:RAB30		
015	KDM6A:ARHGAP6		
018	CAMKK2:KDM2B		
020	TCF12:NPAS1	TMPRSS2:ERG	
022	TMPRSS2:ERG		
023	CAMKK2:KDM2B	GSK3B:ATP11B	PTPRK:ECHDC1
027	ALDH3A2:PITPNM2	SLC25A39:EFCAB13	
029	SLC45A3:EPB41L4B		
035	DLG1:CRYBG3	UBR2:XPO5	
036	TMPRSS2:TMEFF2		
037	EYA2:SYS1	HOMER2:HDGFRP3	PRUNE2:GNA14
039	FAU:SRRM1	PTBP1:UBE3C	TBC1D25:HSPA9
044	TTC39A:MRPL37		
045	POLR2J:LGALS4	VPS13B:AKAP7	
047	KANSL3:TSGA10	RPTOR:IQCH	
	SLC7A1:HRSP12	TMC6:UACA	
050	RBICC1:PTPN3		
053	HNRNPUL1:ATG10	KLK2:KLK3	
054	KLK2:KLK3		
056	KLK2:KLK3		
057	LIG3:PHF12		
059	CHD8:API51	ZMIZ1:ZCCHC24	NXPE2:EP300
061	PTEN:HECTD2	KLK2:KLK3	TORIA:COG4
070	DYM:KATNAL2	TMPRSS2:ERG	
073	MOV10:ZNHIT6		

NOTE: CASQs arising from gene fusions were found in 22 intermediate/high-risk patients (44%) and 8 low-risk patients (33%). The maximum number of CASQs identified within a single tumor was 6. The majority of CASQs involved gene partners unique to each individual; however, two recurrent fusions were observed (bold). CAMKK2:KDM2B generated CASQs in patients 018 and 023; TMPRSS2:ERG generated CASQs in patients 011, 020, 022, and 070.

one CASQ, with a maximum of six CASQs identified in a single case (Patient 013; Fig. 2A; gray bars). A complete list of the predicted CASQs for each patient is provided in the Supplementary Data (Supplementary Table S2). When the patient cohort was analyzed on the basis of risk, there was a significant stratification in the presence of fusions and CASQs. Of particular note, all of the tumors with no identifiable fusions belonged to the low-risk group of patients. In contrast, all 50 patient tumors in the intermediate/high-risk patients contained fusions. In the intermediate/high-risk group 44% of fusions were found to harbor at least 1 CASQ (Fig. 2A, left). In contrast, only 69% of low-risk patient tumors harbored fusions, and if these, 33% harbored at least one CASQ (Fig. 2A, right).

In Fig. 2B, Circos plots from the analysis of three representative patients show the proportion and intra- and inter-chromosomal locations of tumor-specific fusion events leading to CASQs. Given the presence of CASQs in this cohort, patient-specific HLA haplotypes were identified using HLAMiner to predict a list of candidate MHC class I–restricted CASQ-derived epitopes. When the full patient cohort was considered, 41% ($n = 30$) of the tumors contained a CASQ (Fig. 2C, black bars). Of these, 86% ($n = 26$) of these patients had at least one predicted high-affinity MHC class I binding epitope (Fig. 2C; gray bars; rank score ≤ 2). Regardless of patient risk, there was a high likelihood that tumors containing a CASQ had a predicted HLA-binding epitope (Fig. 2C, left vs. right). A complete list of each patient's CASQ-derived epitopes is provided in Supplementary Data (Supplementary Table S3).

The TMPRSS2:ERG CASQ contains patient-specific HLA class I–restricted epitopes

In total, 50 gene pairs were recurrently fused throughout the cohort (Table 2). Approximately 70% of recurrent gene fusions identified within this cohort appeared to be a result of inter-chromosomal translocations based on sequence mapping across chromosomes (Supplementary Data; Supplementary Table S2). Fusions between *TMPRSS2* and *ERG* have been well annotated in prostate cancer (17). Consistent with this finding, the most prevalent recurrent gene fusion in our cohort was between *TMPRSS2* and *ERG*, with 13% of tumors ($n = 4$) expressing this fusion. One of the tumors in the low-risk and 3 of the tumors in the intermediate/high-risk cohort contained the *TMPRSS2* and *ERG* fusion. However, only 2 of the recurrent fusions encoded CASQs: CAMKK2:KDM2B and the TMPRSS2:ERG type VI fusion (exon 2:4 fusion; Table 2), both having arisen from an intrachromosomal fusion event. Moreover, TMPRSS2:ERG was the only recurrent CASQ that generated HLA-binding epitopes in multiple patients ($n = 4$). While many variants of the TMPRSS2:ERG fusion have been reported, the majority of these generate ERG frameshifts or initiate transcription downstream from the fusion breakpoint, resulting in truncated ERG gene products. The TMPRSS2:ERG fusion joining exons 2 of *TMPRSS2* and 4 of *ERG* (type VI) is the only variant in this family that encodes a CASQ that retains the native reading frame of ERG (Fig. 3). Furthermore, TMPRSS2:ERG type VI is common among these fusion variants and has

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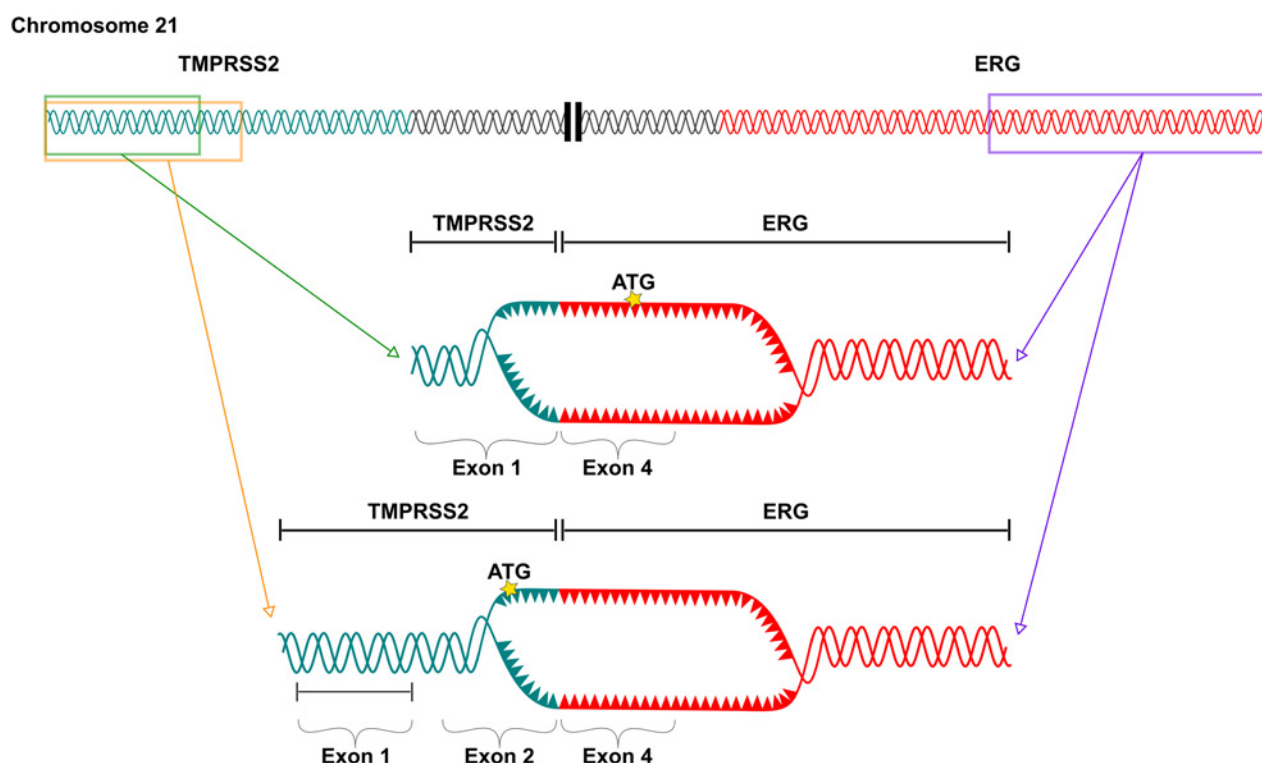


Figure 3. The recurrent fusion TMPRSS2:ERG, present in the TCGA intermediate and high risk prostate cancer patient cohort. Schematic representation of two frequently recurring TMPRSS2:ERG fusions. The type VI (2:4; bottom) fusion variant encodes an in-frame CASQ.

been reported to occur in up to 25% of cases where ERG alterations are present (40).

Of the 14 tumors that contained a TMPRSS2:ERG fusion, 23% were positive for the type VI variant ($n = 4$; Table 2). In each of these type VI cases, *in silico* analysis using IEDB predicted patient-specific HLA-binding epitopes. *In silico* epitope predictions on common HLAs revealed fusion epitopes that were predicted to strongly bind the HLA-A*02:01 allele (rank <1 ; Fig. 4A and B). These predictions were validated for HLA-A*02:01 binding *in vitro* using TAP-deficient T2 cells. Three of the TMPRSS2:ERG minimal peptides displayed no stabilization of surface MHC. In contrast, the top three ranked minimal peptides, MALNSEALSV, ALNSEALSVV, and ALNSEALSV stabilized HLA-A*02:01 above threshold in this assay (Fig. 4C). One of the fusion peptides, ALNSEALSVV, bound to MHC with similar affinity to the known HLA-A*02:01-restricted epitope modified from Melan-A/MART-1 (ELAGIGILTV; ref. 41). The results of the binding assay for the top peptides were consistent with the *in silico* epitope predictions, as these three TMPRSS2:ERG fusion peptides were predicted to bind HLA-A*02:01 with the top rank scores (4.5, 0.9, and 1, respectively).

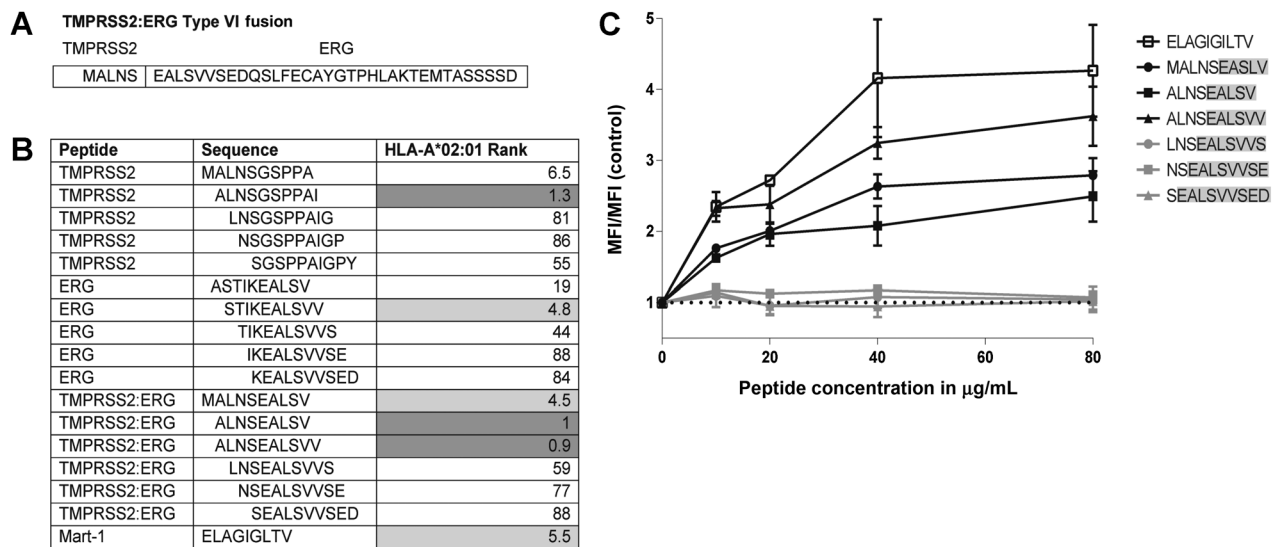
T cells recognize minimal peptides specific for the TMPRSS2:ERG type VI CASQ

Next, TMPRSS2:ERG minimal peptides were assessed for their ability to stimulate and expand T cells from peripheral blood of an HLA-A*02:01⁺ healthy donor. After two rounds of *in vitro* stimulation with peptide-pulsed dendritic cells, T-cell cultures were

monitored for antigen reactivity by IFN γ ELISPOT. Three individual peptide-reactive T-cell lines were identified by this method (Fig. 5A). Each T-cell line recognized the 10-mer peptide MALNSEALSV, as well as the 9-mer, ALNSEALSV. In addition, one T-cell line, 9E6, recognized the 10-mer ALNSEALSVV. All three T-cell lines were primarily CD8⁺ and upregulated CD137 in response to peptide stimulation (Fig. 5B). These responses are specific to the predicted epitope derived from the CASQ, as none of the T-cell lines were cross-reactive to the corresponding native TMPRSS2 or ERG peptide sequences (Fig. 5C).

Predicted immune infiltrates are significantly associated with gene fusions

Within our cohort of low- and intermediate/high-risk patients, there was a strong stratification of risk groups according to the number of fusions (Fig. 6A; Welch t test on log-transformed fusions; $P < 10^{-9}$). The likelihood of a tumor harboring at least one CASQ also trended toward an increase in the intermediate/high-risk patients (Fisher exact test; $P = 0.096$). On the basis of the CASQs and neoepitopes predicted above, one might expect that their presence or absence could impact the immune state of the tumor. This possibility was assessed with a multivariate approach to associate predicted immune cell infiltrations in these tumors with predicted fusions and CASQs using redundancy analysis. This model revealed that fusions and CASQs could predict patterns of immune cell infiltration, and together explained approximately 10% of the variation of the included immune markers (Fig. 6B–D; RDA permutation test, $P < 0.001$). However, the

**Figure 4.**

TMPRSS2:ERG peptides bind HLA-A*02:01. **A**, The TMPRSS2:ERG CASQ. **B**, Rank scores from IEDB MHC epitope predictions for each of the TMPRSS2:ERG peptides. Data includes all possible fusion-spanning 10-mer, as well as the sole 9-mer with a high-affinity binding score. Lower scores indicate an increased likelihood of peptide binding to HLA-A*02:01. Peptides which meet the predicted binding affinity threshold of rank ≤ 2 are highlighted in dark gray. Peptides with a predicted binding affinity of rank ≤ 6 are highlighted in light gray. **C**, TMPRSS2:ERG peptides MALNSEALSV, ALNSEALSV, and ALNSEALSVV each stabilize HLA-A*02:01. T2 cells were pulsed with increasing concentrations of each peptide for 18 hours at 26°C followed by 3 hours at 37°C in the presence of 10 µg/mL Brefeldin A. Cells were stained with anti-HLA-A*02 FITC for 30 minutes at 4°C and analyzed for MHC stabilization by flow cytometry. The mean fluorescence index relative to unpulsed T2 cells is shown. The HLA-A*02:01-restricted peptide from MART-1, ELAGIGLTV, was used as a positive control. The dotted line indicates the MFI of T2 cells in the absence of exogenous peptide.

significance of association with these immune markers was greater using fusions than CASQs in analyses with either as a single predictor (Permutation tests; $P < 0.001$ vs. $P < 0.011$ respectively). When focusing on subsets of immune cells identified as associating with fusions in the RDA, there were striking negative correlations between fusion load and cytolytic signals, such as predicted NK cells and CD8⁺ T cells (Fig. 6D; $P = 6.5 \times 10^{-9}$ and $P = 0.039$, respectively). These results were paralleled, in trends toward positive associations with markers related to antitumor immune suppression such as Th2 phenotypes and to a lesser extent T regulatory cells (Fig. 6D; $P = 0.03$ and $P = 0.081$, respectively). While further validation is necessary in a larger case series, these data suggest that these genomic aberrations can be accompanied by profound changes in the risk stratification and predicted immunologic status in a subset of prostate cancer patients.

Discussion

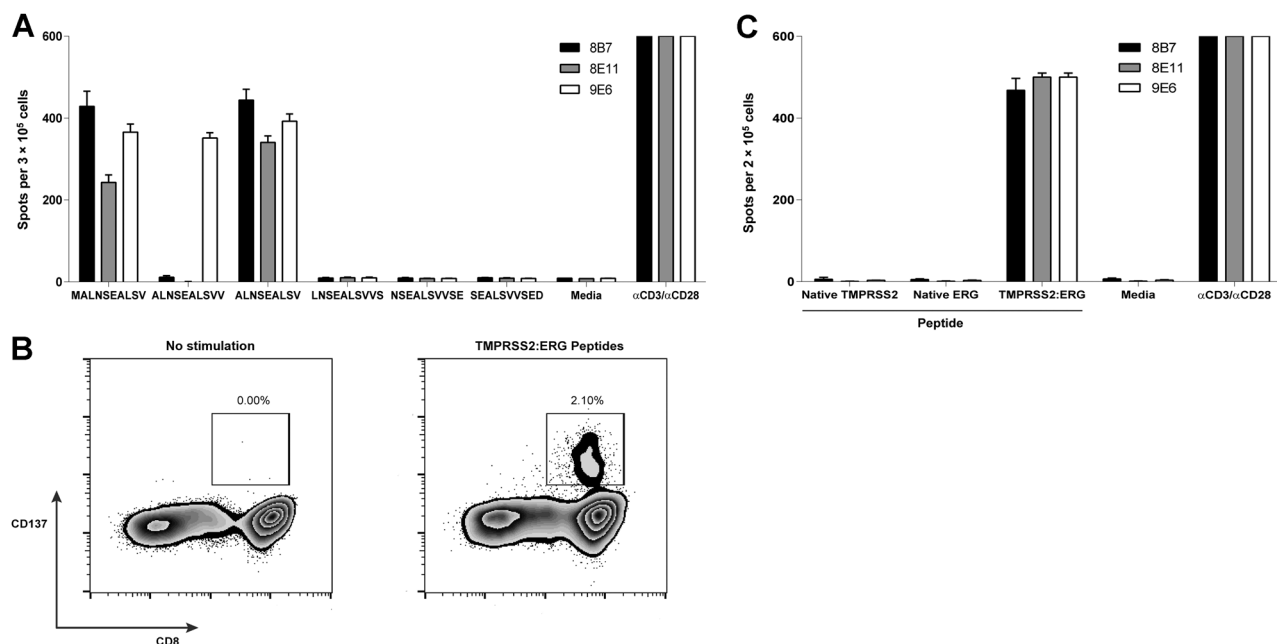
There is increasing evidence that the presence of neoantigens contribute to the success of various modalities of immunotherapy. Several correlative studies in patients treated with anti-PD-1, -PD-L1 and -CTLA-4 suggest that tumors with a high mutation burden may be more responsive to checkpoint blockade. For example, checkpoint inhibitors have been successful in melanoma and NSCLC where the average number of nonsynonymous mutations per tumor ranges from 100 to 230, with roughly 95% of these representing single-base substitutions (11). On the other hand, prostate cancers harbor fewer mutation events despite the fact that a small number of durable clinical responses to anti-PD-1

have been reported (9). However, it is not known whether these patients had a relatively higher frequency of mutations than patients who did not respond. Thus, at least for PD-1 blockade therapy the relationship between response and mutations requires further study.

Similar outcomes have been uncovered in patients who received infusions of enriched antigen-specific tumor-infiltrating lymphocyte populations and went on to show dramatic clinical responses (27, 42). However, one study in gastrointestinal cancer using similar genomic approaches described here found that out of 1,452 mutations across 10 tumors, only 18 could be recognized by CD4⁺ or CD8⁺ TILs (42). Several other studies targeting neoantigens have focused on somatic point mutations in tumor sites where this type of genomic aberration is highly abundant. Despite the abundance of point mutations observed in such tumors, it was reported that relatively few of these give rise to authentic neoantigens (43, 44). Given these data, it raises the question of whether other types of mutations may provide an alternative source of neoantigens that could be exploited for immune-based treatment strategies.

Gene fusions can introduce a variety of genetic alterations, including frameshifts, deletions, truncations, modified splicing patterns, differential inclusion of cryptic exons or introns, chimeric proteins, exchange or alteration of promoter regions, among others. In theory, the primary amino acid sequences generated from many of these events have the potential to elicit a tumor-specific immune response. Our analysis focused only on the coding region fusions that preserve the parental sequence of each gene partner across the breakpoint. While this likely results in an under-representation of the number of

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**Figure 5.**

T cells from an HLA-A*02:01⁺ healthy donor recognize the three TMPRSS2:ERG peptides with predicted affinity for HLA-A*02:01. All T cells were assessed for IFN γ secretion by ELISPOT after 20 hours of coculture with pools of overlapping minimal peptides (10 μ mol/L). **A**, T cells secrete IFN γ in response to stimulation with TMPRSS2:ERG type VI fusion peptides. **B**, TMPRSS2:ERG-specific CD8⁺ T cells upregulated CD137 upon stimulation by a pool of TMPRSS2:ERG peptides (MALNSEALSV, ALNSEALSVV, and ALNSEALSV). **C**, TMPRSS2:ERG-specific T cells do not cross-react with either the corresponding native TMPRSS2 or ERG peptides.

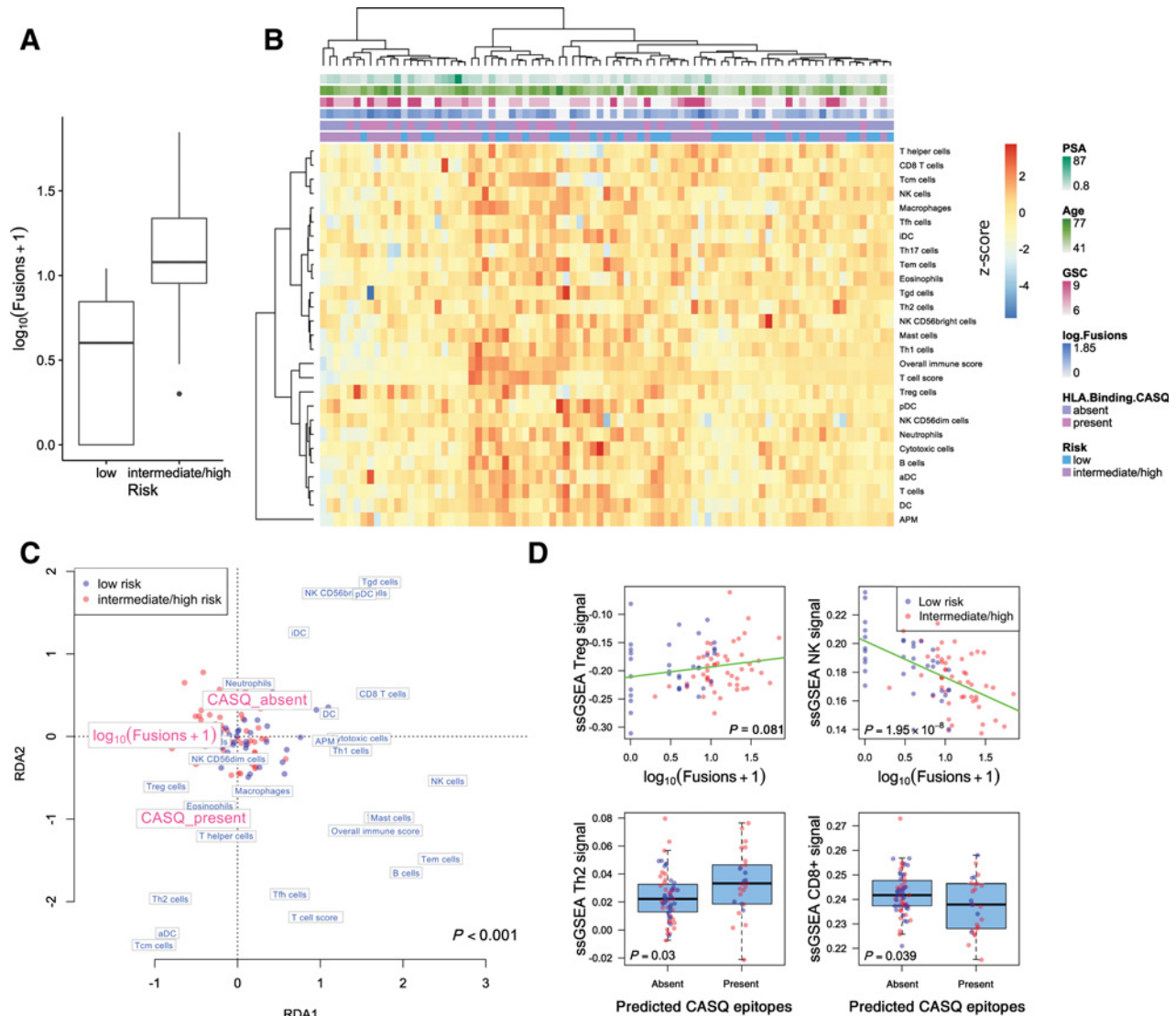
possible neoepitopes generated by this class of mutations in prostate tumors, this strategy is intended to streamline the identification of putative epitopes with the highest likelihood of biological relevance, allowing for a more focused selection of minimal peptides for empirical validation. By this rationale, intronic fusions were also excluded from the final analysis as these sequences would be either biologically nonfunctional or removed posttranscriptionally.

On the basis of current genome sequencing data, prostate tumors harbor an average of 3,866 somatic point mutations where the majority are unique to an individual patient. Of these, nonsynonymous mutations account for only about 0.5% of the total mutation load (45). Furthermore, only a fraction of these would ultimately generate *bona fide* neoantigens, restricting the scope of potential antigens that could be targeted. Mutations arising from gene fusion events occur more frequently in prostate cancer than in other solid tumors, with reports ranging from 43 to 213 fusions per tumor (45). Despite their prevalence, the immunogenicity of gene fusions in prostate cancer has not been explored. One potential reason may be due to the difficulties encountered during the confident identification of fusion calls from RNA-Seq data, specifically during the elimination of false positives (33). While multiple sequencing methods are now able to identify and resolve the breakpoint sequences of gene fusions, using RNA-Seq can be advantageous for discovering events that have a higher probability of predicting functional relevance, as only expressed and translated fusions are of interest during the identification of T-cell-targetable epitopes. Thus, prostate cancer is an ideal setting for evaluating the potential use of

patient-specific fusion proteins as targets for T-cell-based immunotherapies.

Here, a computational strategy was used to evaluate the extent of CASQs generated by fusion events. This analysis identified fusions in 87% of the entire cohort. However, most of this was due to the intermediate/high-risk patients where all tumors contained fusions. In contrast, only 69% of the low-risk patients were found to harbor fusions. Similarly, 41% of all patients contained a CASQ, whereas 33% of the low- and 44% of the intermediate/high-risk patients were found to possess predicted CASQs. Within the group of patients whose tumors harbored CASQs ($n = 30$), 86% ($n = 26$) had at least one predicted chimeric epitope. The frequency of HLA-binding epitopes was similar between the low- and intermediate/high-risk patients. Therefore, although the proportion of fusions encoding CASQs was small, the presence of CASQs was a predictor of MHC class I binding epitopes. The majority of predicted CASQ-derived epitopes in this population were patient-specific. In fact, only two recurrent gene fusions, *CAMKK2:KDM2B* and *TMPPSS2:ERG*, yielded predicted epitopes in more than a single patient ($n = 2$ and 4, respectively; Fig. 4A), supporting the notion that, like somatic point mutations, targeting fusion mutations may be suited as a personalized approach. Whether any of the CASQs generate aberrant cell surface protein expression is not yet known, but could represent compelling targets using chimeric antigen receptor (CAR) T-cell therapy. Indeed clinical trials targeting PSMA with CAR-T cells trial are underway (46, 47).

TMPPSS2:ERG fusions were identified in 23% of tumors in our cohort and was within the range of what has been previously reported. Although several variants of this fusion have been

**Figure 6.**

Gene fusions and predicted CASQ-derived epitopes are associated with predictions of tumor-infiltrating immune cells. **A**, Predicted number of fusions differs across risk groups ($P < 10^{-9}$). **B**, Heatmap of predicted immune cell infiltrate levels and immunologic parameters for patients in this cohort, extracted from the ssGSEA-based predictions of Senbabaoglu and colleagues (2016). Annotation tracks represent the presence of predicted CASQ epitopes (CASQ), log-predicted total tumor fusions, Gleason Score, patient age at diagnosis, and PSA level. **C**, Redundancy analysis associating tumor fusions and CASQ epitopes with patterns of immune cell infiltration shows a significant multivariate effect (Permutation test, $P < 0.001$). Triplot representing patients (red and blue circles), immune predictions (blue), and explanatory variables (light red). **D**, Plots of univariate associations of predicted immune cell infiltrates with fusion and CASQ epitope predictions.

identified, the type VI (2:4) fusion is the only known variant which preserves the native translation initiation site of TMPRSS2 while ERG remains in-frame, producing functional ERG with a 5 amino acid N-terminal extension imparted by TMPRSS2 (Fig. 3; ref. 40). In our study, type VI fusions were present in 4 tumors (13%) and in each case led to predicted high-affinity fusion epitopes on autologous HLAs. Indeed, due to the prevalence of ERG alterations in prostate cancer, one study reported vaccine-induced immunity to ERG-derived HLA-A*02:01-restricted epitopes (48).

In this study, peptide-pulsed dendritic cells were used to expand TMPRSS2:ERG-specific T-cell lines from an HLA-A*02:01⁺ healthy donor. However, several criteria must still be satisfied for a given mutation to produce an authentic neoepitope; for example, the parent protein must undergo endogenous antigen processing to produce the immunogenic peptides. One limitation of our study was the lack of HLA-matched tumor specimens with confirmed TMPRSS2:ERG type VI expression to test the authenticity of this fusion against TMPRSS2:ERG-specific T-cell lines that were expanded *in vitro*.

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Finally, there was a significant negative relationship between tumor fusions and the presence of cytolytic immune signatures, and the number of fusions strongly stratified patients across low and intermediate/high risk groups. In general, cytolytic immune responses such as the expression of NK-cell and CD8⁺ T-cell markers negatively correlated with tumor fusions, and, to a lesser extent, predicted CASQs. Consistent with this reduction in cytolytic indicators, there was a positive association between Th2 genes. In addition to this, there was a trend towards positive associations between Tregs and the presence of fusions and CASQs. Although this exploratory analysis examined on a small subset of patients, our results suggest the possibility of functional interactions between genome rearrangements and host immune responses that may impact antitumor immunity. It could be speculated that the negative relationship observed between tumor fusions and cytolytic immune responses may have resulted from immune selection against these potential neoantigens during tumor evolution. A similar circumstance could also exist for these immune signatures when considering the lower frequency of both fusions and CASQ in the low-versus intermediate/high-risk patients. Indeed, such a scenario may be possible as prostate cancer risk segregates with a unique immune signature based on a specific mutation profile. However, further validation in independent cohorts is needed to confirm these findings. Nonetheless, this information could expand the potential scope of mutations, including CASQs and other gene fusions, as an alternative avenue for immune-based approaches targeting prostate cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Mutational Analysis of Gene Fusions Predicts Novel MHC Class I–Restricted T-Cell Epitopes and Immune Signatures in a Subset of Prostate Cancer

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