Immunotherapy Advances, 2022, **2**, 1–4
https://doi.org/10.1093/immadv/ltac009
Advance access publication 20 April 2022
Commentary

Target isoforms are an overlooked challenge and opportunity in chimeric antigen receptor cell therapy

Mike Bogetofte Barnkob[1,‡], Kristoffer Vitting-Seerup[2,‡], and Lars Rønn Olsen[2,*]

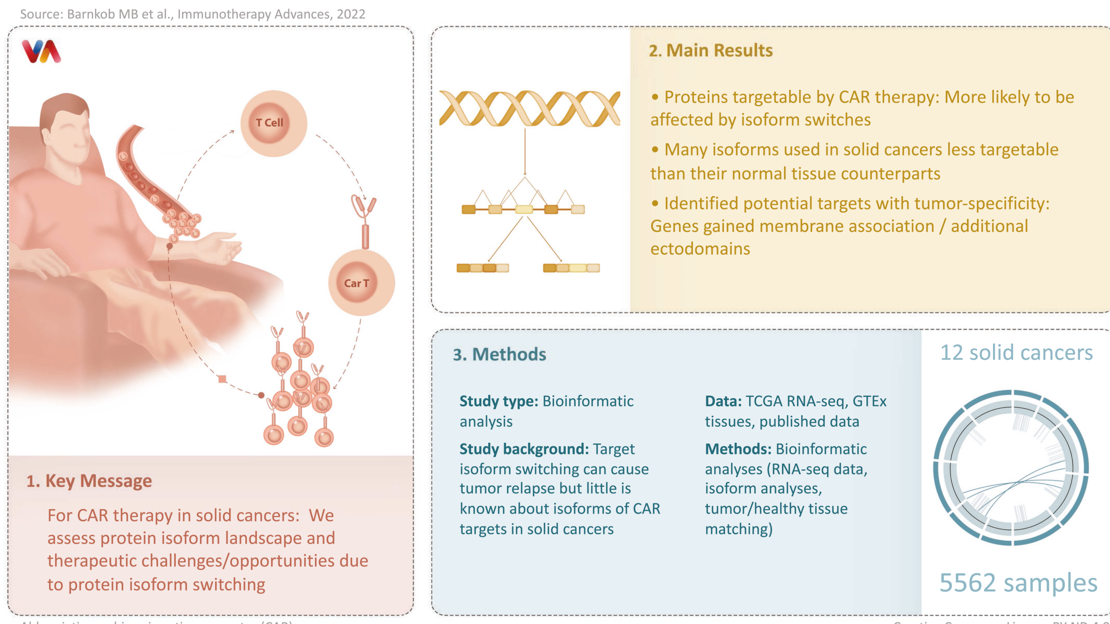
[1]Centre for Cellular Immunotherapy of Haematological Cancer Odense (CITCO), Department of Clinical Immunology, Odense University Hospital, University of Southern Denmark, Odense, Denmark and
[2]Section for Bioinformatics, Department of Health Technology, Technical University of Denmark, Lyngby, Denmark

[‡]These authors contributed equally.
*Correspondence: Lars Rønn Olsen, Kemitorvet, 204, 247, 2800 Kgs, Lyngby, Denmark. E-mail: lronn@dtu.dk

Summary

The development of novel chimeric antigen receptor (CAR) cell therapies is rapidly growing, with 299 new agents being reported and 109 new clinical trials initiated so far this year. One critical lesson from approved CD19-specific CAR therapies is that target isoform switching has been shown to cause tumour relapse, but little is known about the isoforms of CAR targets in solid cancers. Here we assess the protein isoform landscape and identify both the challenges and opportunities protein isoform switching present as CAR therapy is applied to solid cancers.

Graphical Abstract



Keywords: Chimeric antigen receptor therapy, isoform switching, epitopes, cancer

Main text

Isoforms arise when different exons are combined through RNA splicing and are translated into proteins with distinct properties. Especially in cancer cells, dysregulation and alternative splicing is thought to be involved in many hallmarks of cancer [1]. At least 75% of human protein-coding genes give rise to multiple distinct protein isoforms [2], which can severely affect the sensitivity of therapeutic targeting of specific proteins. In a recent study of 883 small molecule cancer drugs targeting 1434 different proteins, the authors found that 76% of these drugs would miss a target isoform if a switch occurred, or induce off-target effects in isoforms expressed in normal tissues [3]. Because isoforms of a protein may be differentially expressed in individual cancer cells

Received: September 29, 2021; Accepted: March 31, 2022
© The Author(s) 2022. Published by Oxford University Press on behalf of the British Society for Immunology.
This is an Open Access article distributed under the terms of the Creative Commons Attribution-NonCommercial License (https://creativecommons.org/licenses/by-nc/4.0/), which permits non-commercial re-use, distribution, and reproduction in any medium, provided the original work is properly cited. For commercial re-use, please contact journals.permissions@oup.com

[4], they may produce a pool from which escape variants can arise when selective pressure is exerted by targeted therapy. For example, exon 16 deleted splice variants in the receptor tyrosine-protein kinase erbB-2 gene (HER2; *ERBB2*) is known to decrease sensitivity to the monoclonal antibody trastuzumab targeting HER2 [5]. Such adaptive resistance to targets is increasingly recognized following chimeric antigen receptor (CAR) treatments, for example in CARs that target EGFRvIII in glioblastoma multiforme [6] and CD19 in B-cell acute lymphoblastic leukemia [7–11].

To assess the level of isoform switching in tumours, we analyzed TCGA RNA-seq data from 5562 tumour samples spanning 12 solid cancer types. For each cancer type, we analyzed the average differences between tumour and matched healthy tissue (see Supplementary Methods). As previously shown [12], protein isoform switches are very frequent in cancers (Fig. 1A). Such isoform switches greatly impact the sequences of the expressed proteins (Fig. 1B), which can negatively affect the targetable epitopes of tumour-associated antigens. In fact, the challenge is even more pertinent for antibody-based therapies, such as CAR cell therapy: our analyses showed that isoform switches in cell membrane proteins are significantly enriched in 8 of the 12 cancers investigated (Fig. 1C), with an average of 141 cell membrane proteins affected per cancer type. This means that the proteins targetable by CAR therapy are more likely to be affected by isoform switches than intracellular proteins.

The enrichment of isoform switches in cell membrane proteins compared to intracellular proteins prompted us to more thoroughly analyze both the opportunities and risks that isoform switches present. We first examined changes in signaling peptides of membrane-associated proteins as these play an important role in determining the subcellular localization and secretion status of the protein [13]. Across all 12 solid cancers, 242 membrane proteins lost signaling peptides (Fig. 1D), indicating that these isoforms would potentially no longer be secreted and thus be more amenable for targeting. However, our analysis also showed that 180 proteins not only lost their membrane association (Fig. 1E) but also 710 of those that remained membrane-bound, lost portions of their ectodomains (Fig. 1F). Adding to this, we also found that membrane proteins are overrepresented amongst genes downregulated in cancers (Supplementary Fig. S1). Jointly this shows that many isoforms used in solid cancers are less targetable than their normal tissue counterparts. Interestingly, we identified a small, but a consistent number of genes in cancer cells that either gained membrane association or additional ectodomain amino acids (Fig. 1E–F), indicating that isoform switches also lead to changes that could be potential tumour-specific targets for CAR therapy.

To better understand how isoforms might affect the success of new CAR therapies, we next examined the isoform status of the top five membrane proteins currently being tested in clinical CAR trials against solid tumours. These are the carcinoembryonic antigen-related cell adhesion molecule 5 (CEA; *CEACAM5*), Mucin-1 (*MUC1*), Glypican-3 (*GPC3*), Mesothelin (*MSLN*), and *ERBB2*.

To examine if targeting a specific protein isoform could potentially lead to on-target/off-tumour effects in normal tissue we compared the isoforms expressed in the targeted cancer types to expression patterns in all human GTEx tissues. The number of isoforms ranged from 4 to 23 for the five genes (Fig. 2A; Supplementary Fig. S2A, S3A, S4A, and S5A), all with different expression levels between normal tissue and cancer (Fig. 2B–D; Supplementary Fig. S2B, S3B–D, S4B, and S5B). As seen in Fig. 2B–D, none of the expressed isoforms are tumour-specific in either of the three examined cancers. GPC3 was unique in having isoforms that were all associated with the cell membrane, while the other four targets had isoforms that were either secreted, associated with intracellular

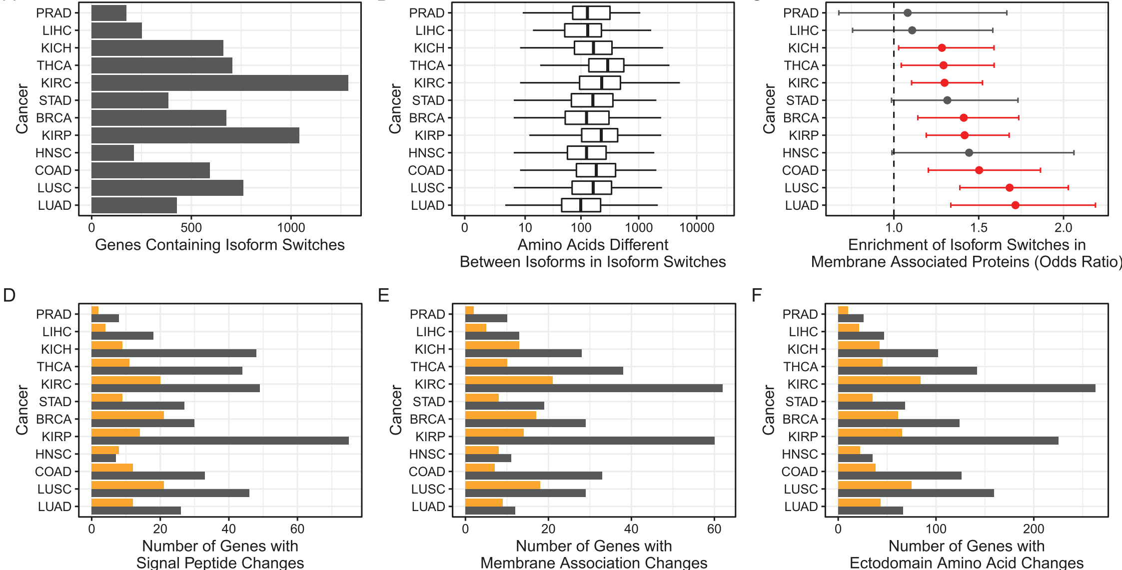


Figure 1. Protein isoform switching frequently occurs in tumour tissue. A: The number of genes containing at least one isoform switch in 12 solid cancer types. B: Boxplot showing the number of amino acids being different between protein isoforms in each isoform switch. Outliers are not shown. C: The enrichment of isoform switches in cell membrane proteins. Enrichment is given as odds-ratios (dot) along with 95% confidence interval (error bar). Color denotes false discovery rate (FDR) corrected *P*-values < 0.05. D: Number of genes where a signal peptide is gained or lost (as denoted by color) due to isoform switches. E: Number of genes where an isoform gain or loss (as denoted by color) membrane association. F: Number of genes where amino acids in the ectodomain are gained or lost (as denoted by color) due to isoform switches.

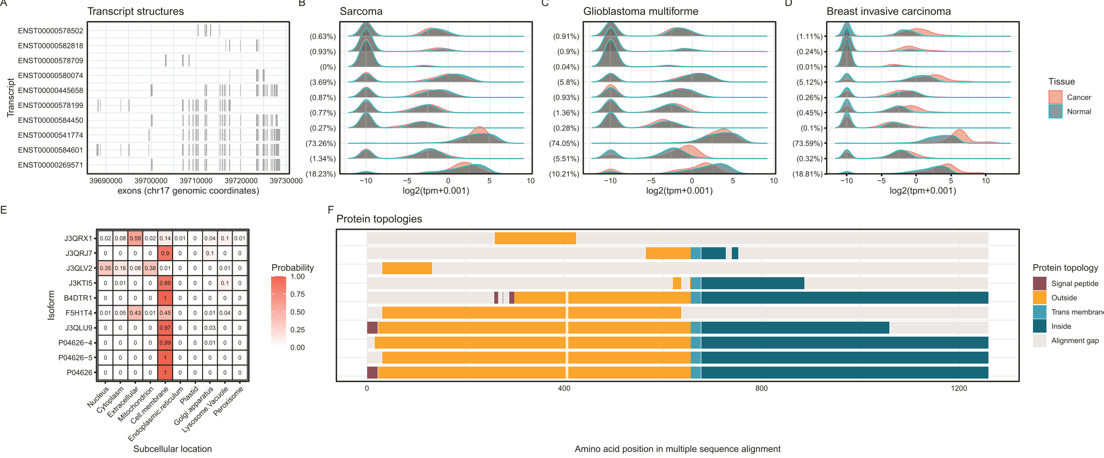


Figure 2. Isoform characteristics of ERBB2. A: Genomic coordinates of exons making up the six transcripts. B–D: Stacked density distributions of transcript expression in all healthy tissues combined vs sarcoma, glioblastoma multiforme, and breast invasive carcinoma, respectively. Average transcript frequencies in cancer are listed in y axis labels. E: Predicted subcellular locations of each of the protein isoforms shown as probabilities of each location. F: Multiple sequence alignment of the protein isoforms with topological annotation, where 'outside' and 'inside' denotes orientation to the cell membrane or an intracellular membrane. Note that Ensembl transcript IDs in A correspond to UniProt IDs in E in a row wise manner.

membranes, the cytoplasm, or the cell membrane (Fig. 2E; Supplementary Fig. S2C, S3E, S4C, and S5C). As indicated by our analysis across tumour types (Fig. 1), we found significant alignment gaps in all cell membrane-associated protein isoforms (Fig. 2F; Supplementary Fig. S2D, S3F, S4D, and S5D). These observations give rise to a number of challenges, exemplified here with HER2 (*ERBB2*), which is an important growth factor receptor across a number of cancer types (Fig. 2). For ERBB2, there were both conserved and variable regions in the ectodomain of the membrane-bound isoforms (Fig. 2F). Should the targeted CAR epitope be located in a variable region of the ectodomain it may lead to lower treatment efficacy, or in severe cases, therapy-induced target loss. Likewise, the expression of secreted epitope harboring isoforms may cause off-tumour effects if the secreted isoforms bind to other cells, or decrease efficacy by changing the pharmacokinetics between the CAR and the cellular-bound isoform(s) [14].

We also wished to explore how sensitive newly proposed CAR targets would be in terms of variable epitope expression and secretion status should an isoform switch occur. To this end, we analyzed the targets proposed in a recently published study by MacKay *et al.*, in which the authors systematically examined the expression patterns of 13,206 genes across 20 different cancers and 44 normal tissues in an effort to identify novel targets [15]. This study represents the most comprehensive of its kind but does not include analyses of the expression of different protein isoforms. In total, the authors highlight 65 potential new targets for CAR therapy. We found that 51 of these target genes express more than one protein isoform in at least one of the 20 cancers, and none of these target isoforms have completely identical ectodomains. Additionally, out of the 65 targets, 34 express isoforms with different subcellular locations, and 27 express isoforms that are potentially secreted (Supplementary Table S1).

Solid tumours have largely been refractory to CAR T-cell therapy [16]. Here we show that the targetability of most of the currently used and proposed antigens for CAR therapy against solid tumour targets may be negatively affected by isoform switching, as has been observed with current CD19-specific CAR therapies [7–9]. Our analyses highlight the importance of considering target expression beyond the expression of the canonical protein isoform, as isoforms can have very different characteristics in terms of expression, targetable epitopes, cellular location, and secretion status. We also find that membrane proteins in particular are often down-regulated perhaps as a consequence of cancer evolution [17] or immunoediting. Tumour cells have to detach themselves from the 'communication network' that helps maintain tissue homeostasis and corroboration not only between cells but also upregulate receptors that enable them to increase the uptake of nutrients and molecules for growth [18]. We hypothesize that as cancer cells evolve, subtle changes in membrane-receptors via isoform switching might enable cancer cells to change the communication signals they receive without compromising growth. Another explanation for these findings could also be that selective pressure via immune recognition favoring clones expressing fewer receptors.

As cancer cells are often enriched in alternative splice variants due to excessive DNA damage or chemotherapy [19, 20], it is possible that patients could be stratified before CAR therapy in case variants that lack the targeted epitope exists pre-treatment. As data from clinical trials become available, it will therefore be highly interesting to examine the distribution of isoforms in solid cancers before and after CAR therapy.

To overcome the challenge of isoform switching, bispecific CARs could be utilized, as has been explored using a HER2/MUC1 bispecific CAR [21], although it will still be important to consider all expressed isoforms of these targets. Indeed both of these targets have a high number of isoforms, as shown in our analysis above. Another approach could be to promote specific isoform expression through the use of histone deacetylase inhibitors or DNA-demethylating therapy. Here we suggest another possible solution, namely specifically targeting the small number of isoforms that are not lost, but enriched in cell-membrane proteins in cancer cells. These might provide unique opportunities for discovering truly cancer-specific targetable epitopes. To identify such potential targets, transcript- or exon-level expression analysis should

be combined with information concerning the subcellular localization of variants at the protein level. Such analyses would provide a space for potential targets but would need to be validated using proteomic measurements and preclinical testing. However, technologies such as targeted single-cell mass spectrometry [22] are maturing to enable high-throughput, single-cell analysis, and making this a reachable goal. As such, further studies into subclonal isoform expression are warranted and may lead to the discovery of the first truly tumour-specific CAR targets.

Supplementary material

Supplementary data are available at *Immunotherapy Advances* online.

Acknowledgements

The Editor-in-Chief, Tim Elliott, and handling editor, Tao Dong, would like to thank the following reviewer, Abdullah Kahraman, and an anonymous reviewer, for their contribution to the publication of this article. The authors wish to thank the reviewers for their helpful comments and suggestions.

Author contributions

All authors contributed equally to the study conceptualization. LRO and KVS performed the data analyses. All authors contributed equally to writing and editing the manuscript.

Funding

This work was funded by the Independent Research Fund Denmark (grant 8048-00078B to LRO).

Conflicts of interest

MBB has received consulting honorariums from Janssen and Kite/Gilead. The remaining authors declare no competing interests.

Data availability

The datasets were derived from sources in the public domain: The Xena Browser (https://xenabrowser.net, dataset ID 'TcgaTargetGtex_rsem_isoform_tpm').

Ethical approval

Not applicable.

References

1. Creason A, Haan D, Dang K et al. A community challenge to evaluate RNA-seq, fusion detection, and isoform quantification methods for cancer discovery. *Cell Syst* 2021;12(8):827–38.e5. https://doi.org/10.1016/j.cels.2021.05.021
2. Frankish A, Diekhans M, Ferreira A-M et al. GENCODE reference annotation for the human and mouse genomes. *Nucleic Acids Res* 2019; 47(D1):D766–73.
3. Ji Y, Mishra RK, Davuluri RV. In silico analysis of alternative splicing on drug-target gene interactions. *Sci Rep* 2020; 10(1):134. https://doi.org/10.1038/s41598-019-56894-x
4. Wu S, Zhang H, Fouladdel S et al. Cellular, transcriptomic and isoform heterogeneity of breast cancer cell line revealed by full-length single-cell RNA sequencing. *Comput Struct Biotechnol J* 2020; 18:676–85. https://doi.org/10.1016/j.csbj.2020.03.005
5. Mitra D, Brumlik MJ, Okamgba SU et al. An oncogenic isoform of HER2 associated with locally disseminated breast cancer and trastuzumab resistance. *Mol Cancer Ther* 2009; 8(8):2152–62. https://doi.org/10.1158/1535-7163.MCT-09-0295
6. O'Rourke DM, Nasrallah MP, Desai A et al. A single dose of peripherally infused EGFRvIII-directed CAR T cells mediates antigen loss and induces adaptive resistance in patients with recurrent glioblastoma. *Sci Transl Med* 2017; 9(399).
7. Sotillo E, Barrett DM, Black KL et al. Convergence of acquired mutations and alternative splicing of CD19 enables resistance to CART-19 immunotherapy. *Cancer Discov* 2015; 5(12):1282–95.
8. Plaks V, Rossi JM, Chou J et al. CD19 target evasion as a mechanism of relapse in large B-cell lymphoma treated with axicabtagene ciloleucel. *Blood* 2021;138(12):1081–5. https://doi.org/10.1182/blood.2021010930
9. Fischer J, Paret C, El Malki K et al. CD19 isoforms enabling resistance to CART-19 immunotherapy are expressed in B-ALL patients at initial diagnosis. *J Immunother* 2017; 40(5):187–95.
10. Zhang Z, Chen X, Tian Y et al. Point mutation in CD19 facilitates immune escape of B cell lymphoma from CAR-T cell therapy. *J Immunother Cancer* 2020; 8(2).
11. Asnani M, Hayer KE, Naqvi AS et al. Retention of CD19 intron 2 contributes to CART-19 resistance in leukemias with subclonal frameshift mutations in CD19. *Leukemia* 2020; 34(4):1202–7.
12. Vitting-Seerup K, Sandelin A. The landscape of isoform switches in human cancers. *Mol Cancer Res* 2017; 15(9):1206–20.
13. Hegde RS, Bernstein HD. The surprising complexity of signal sequences. *Trends Biochem Sci* 2006; 31(10):563–71.
14. Attwood MM, Jonsson J, Rask-Andersen M et al. Soluble ligands as drug targets. *Nat Rev Drug Discov* 2020 19(10):695–710.
15. MacKay M, Afshinnekoo E, Rub J et al. The therapeutic landscape for cells engineered with chimeric antigen receptors. *Nat Biotechnol* 2020; 38(2):233–44.
16. Milone MC, Xu J, Chen S-J et al. Engineering enhanced CAR T-cells for improved cancer therapy. *Nat Cancer* 2021; 2(8):780–93.
17. Lin C-Y, Lee C-H, Chuang Y-H et al. Membrane protein-regulated networks across human cancers. *Nat Commun* 2019; 10(1):3131.
18. Kampen KR. Membrane proteins: the key players of a cancer cell. *J Membr Biol* 2011; 242(2):69–74.
19. Boutz PL, Bhutkar A, Sharp PA. Detained introns are a novel, widespread class of post-transcriptionally spliced introns. *Genes Dev* 2015; 29(1):63–80.
20. Urbanski LM, Leclair N, Anczuków O. Alternative-splicing defects in cancer: splicing regulators and their downstream targets, guiding the way to novel cancer therapeutics. *Wiley Interdiscip Rev RNA* 2018; 9(4):e1476.
21. Wilkie S, van Schalkwyk MCI, Hobbs S et al. Dual targeting of ErbB2 and MUC1 in breast cancer using chimeric antigen receptors engineered to provide complementary signaling. *J Clin Immunol* 2012; 32(5):1059–70.
22. Schoof EM, Furtwängler B, Üresin N, et al. Quantitative single-cell proteomics as a tool to characterize cellular hierarchies. *Nat Commun* 2021; 12(1):3341.