Recovery of Immunoglobulin VJ Recombinations from Pancreatic Cancer Exome Files Strongly Correlates with Reduced Survival

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Abstract

We assessed pancreatic cancer, lymphocyte infiltrates with a computational genomics approach. We took advantage of tumor-specimen exome files available from the cancer genome atlas to mine T- and B-cell immune receptor recombinations, using highly efficient, scripted algorithms established in several previous reports. Surprisingly, the results indicated that pancreatic cancer exomes represent one of the highest level yields for immune receptor recombinations, significantly higher than two comparison cancers used in this study, head and neck and bladder cancer. In particular, pancreatic cancer exomes have very large numbers of immunoglobulin light chain recombinations, both with regard to number of samples characterized by recovery of such recombinations and with regard to numbers of recombination reads per sample. These results were consistent with B-cell biomarkers, which emphasized the Th2 nature of the pancreatic lymphocyte infiltrate. The tumor specimen exomes with B-cell immune receptor recombination reads represented a dramatically poor outcome, a result not detected with either the head and neck or bladder cancer datasets. The results presented here support the potential value of immunotherapies designed to engineer a Th2 to Th1 shift in treating certain forms of pancreatic cancer.

Keywords Pancreatic cancer exomes · Immunoglobulin gene recombinations · B-cell infiltrates · Survival rates · The cancer genome atlas

Introduction

Genomics approaches to cancer care are rapidly entering the clinic, and in some cases represent standard of care. For

Key Points

- A very high level of lymphocyte infiltrate in pancreatic cancer, as evidenced by an immunogenomics approach.
- A clear Th2 character to the lymphocyte infiltrate.
- A significantly worse survival rate for pancreatic cancer patients with demonstrable immunoglobulin light chain recombinations detected in the microenvironment.

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example, the cancer specimen exome, representing all possible mutations, can identify mutations such as the V600E mutation in melanoma and justify Vemurafenib treatments [1]. Other approaches are extensively used in clinical trials and in big-data projects, such as the cancer genome atlas (TCGA) [2–4], for example, RNASeq (transcriptome) profiling of surgically excised tumor specimen; and genome-wide copy number variation and DNA methylation assessments.

Likewise, immunotherapies have developed extensively over the last five years, with multiple FDA approved approaches, most notably anti-immune checkpoint therapies, in melanoma and bladder cancer [5–8]. However, pancreatic cancer has not benefited from these advances, and in general, has not been studied as an immunologically active cancer. One recent study has exemplified the need for increased consideration of the possibility that immune modulation would be of benefit in pancreatic cancer, with a report of a very high level immune infiltrate in pancreatic cancers [9]. And, there are at least two clinical trials underway that could lead to increased anti-cancer immune activity in the pancreatic cancer setting (<https://clinicaltrials.gov/ct2/show/NCT02562898?term=>

<https://clinicaltrials.gov/ct2/show/NCT02575300?term=ibrutinib&cond=Pancreatic+Cancer&rank=1>, <https://clinicaltrials.gov/ct2/show/NCT02436668?term=ibrutinib&cond=Pancreatic+Cancer&rank=2>, <https://clinicaltrials.gov/ct2/show/NCT02436668?term=ibrutinib&cond=Pancreatic+Cancer&rank=3>).

Although genomic approaches are widespread in the study of cancer, there is only one approach that is common and approaching standard of care status: the tumor-specimen exome, for reasons noted above. Clearly, it will not be possible in the near future to apply all possible genomic approaches to each patient to develop personalized therapy. And, if even if such were possible in certain settings, the cost of essentially open-ended genomics applications to design personalized care is likely to be cost-prohibitive in other settings. Thus, there is an imperative to recover as much information as possible from exomes, and indeed, recent work has emphasized the value of recovery of immune receptor recombination reads from tumor specimen exome files [10–12]. These reads are thought to represent the recombinations of tumor infiltrating lymphocytes. And, while other approaches, such as RNASeq [13–17] or direct polymerase chain reaction detection of immune receptor recombinations may represent more robust recoveries, recovery of immune receptor recombination reads from tumor exome files likely represents the recovery of dominant T- and B-cell clones. In any event, past work has indicated that recovery of the recombination reads has medical and scientific value. For example, recovery of TcR- β VDJ recombination reads from bladder cancer exomes correlates with a better outcome [18]; and co-detection of TcR- α/β recombination reads in melanoma exomes correlates with PD-1 expression [19]. In this study, we have recovered T-cell and B-cell recombination reads from pancreatic cancer exomes, with results indicating that recovery of IGL and IGK recombination reads correlate with a poor outcome, among other results, all of which are particularly relevant to the ongoing clinical trials, as discussed further below.

Methods

Immune Receptor Recombination Reads

To obtain a list of the barcodes (samples) and associated whole exome sequence (WXS) files needed for pancreatic adenocarcinoma (PAAD), head and neck squamous carcinoma (HNSC), and bladder cancer (BLCA) T- and B-cell immune receptors, the Genome Data Commons (GDC) web portal (<https://portal.gdc.cancer.gov/>) was queried for the TCGA provisional study data. A manifest file was then obtained for each of the three cancers (Supporting online material (SOM), Table S1). The manifests were used to download WXS BAM

(binary alignment map) files for each barcode of the three cancers using the GDC data transfer tool. To determine whether the WXS files contained productive TcR or IG recombinations, the files were searched using a collection of scripts similar to previous publications [10, 11, 18, 19], with some modifications to be described in detail in an upcoming publication. The scripts used a list of sequences to define the V and J regions of the immune receptors. The sequence matches by the scripts accounts for N-region diversity. As part of the processing, the computer scripts compiled a table in a TSV file of candidate reads when a nucleotide sequence match was found for both the V and J regions within a single read. The ImmuneGeneTics (IMGT) web tool [20] was then used to determine whether the candidate reads in the TSV files were productive or unproductive recombinations. IMGT was again referenced to determine the quantity of consecutive nucleotides that were an exact match to the V and J regions recognized by IMGT. To ensure the fidelity of the reads used in this study, only reads having a V and J match of greater than 20 nucleotides were used. For each of the three cancer datasets, the final output was then placed into separate Excel Microsoft Office Open XML Format Spreadsheets files (XLSX) for each of the following receptors: IGK, IGL, TRA, TRB, and TRD. (TRG and IGH were not studied in this report.) The exact script used for this report is in the SOM (Table S2). Selected output files for the indicated immune receptors are also present in the SOM (Tables S3).

IGK/IGL Survival Data

To compare survival differences between the IGK/IGL productive recombination barcode-subset, and the all remaining barcode-subsets (used in this study), survival data for PAAD barcodes were obtained from the GDC web portal (<https://portal.gdc.cancer.gov/>) and imported into a Microsoft Excel file. The survival data were labeled in a new column as “yes” if an IGK/IGL productive rearrangement had been found in a specific barcode or “no” if the barcode was a member of the all remaining barcodes subset. These data were then transferred to IBM statistical package for the social science (SPSS) software for Kaplan-Meier (KM) survival analysis.

RNASeq B-Cell Markers

To compare the level of B-cell markers found within the IGK/IGL productive recombination barcode-subset to all remaining barcodes (used in this study), RNASeq values for each of the 147 PAAD barcodes represented by this study were obtained from the GDC web portal and imported into a Microsoft Excel file. To separate the barcodes into two groups (IGK/IGL productive rearrangement and all remaining barcodes), a “conditional formatting” function was used to

color code barcodes in the RNASeq data that were also present in the group of barcodes identified as having a IGK/IGL productive recombination. The RNASeq data and associated barcodes were then sorted based on these grouping criteria, and statistical analysis was performed accordingly to data described in [Results](#). To analyze RNASeq value differences in the IGK/IGL productive recombination subsets for PAAD, HNSC, and BLCA, RNASeq data were imported into an Microsoft Excel file for each cancer. A “conditional formatting” function to identify duplicates was used to determine whether barcodes were present in the IGK/IGL productive recombination subset. Each barcode was then grouped into a IGK/IGL productive recombination subset or an all remaining barcode subset and analyzed further as indicated in [Results](#). The same approach was used for analyzing T-cell RNASeq markers to determine the character of T-cell infiltrate within the TRB productive recombination subset of barcodes, compared to the all remaining barcodes subset in PAAD, HNSC, and BLCA, respectively.

Supporting Online Material

Supporting online material is noted above and below; additional supporting material is available via email to the corresponding author.

Table 1 Summary of TCR and IG productive barcodes and productive reads from PAAD, HNSC, and BLCA WXS files. (See also [Table S3](#))

Gene	Total barcodes analyzed	Barcodes with productive match	Percent barcodes with productive match	Productive reads	Productive reads per barcode, for barcodes with at least one productive read
PAAD					
IGK	147	31	21.09	117	3.65
IGL	147	49	33.33	197	3.86
IGK/IGL	147	60	40.82	314	5.23
TRA	147	98	66.67	789	8.05
TRB	147	96	65.31	799	8.32
TRD	147	29	19.72	59	2.03
HNSC					
IGK	494	62	12.55	67	1.08
IGL	494	58	11.74	76	1.31
IGK/IGL	494	100	20.24	143	1.43
TRA	494	294	59.51	670	2.28
TRB	494	291	58.91	674	2.31
TRD	494	40	8.10	50	1.25
BLCA					
IGK	389	35	9.00	45	1.29
IGL	389	38	9.77	57	1.5
IGK/IGL	389	65	16.71	102	1.57
TRA	389	150	38.56	367	2.45
TRB	389	134	34.45	296	2.21
TRD	389	18	4.63	19	1.06

Results

TRA, TRB, TRD, IGK and IGL Recombination Reads Were Recovered at Higher Rates from WXS Files for PAAD, Compared to HNSC and BLCA

To determine the profile of TcR and immunoglobulin (IG) recombinations detectable in tumor specimens for PAAD, HNSC, and BLCA, WXS files were searched for TcR and IG V(D)J recombination reads. The results of the WXS file searches revealed that the percentages of barcodes (samples) with productive reads for TRA, TRB, TRD, IGK, and IGL were significantly higher in PAAD when compared with the two control cancers ([Tables 1](#) and [2](#)). This robust recovery of recombination reads from PAAD WXS files, when compared to controls, was further illustrated by a significantly higher level of productive reads per PAAD barcode ([Tables 1](#) and [2](#); [Tables S3, S4](#)).

Recovery of IGK/IGL Recombination Reads Correlated with Significantly Worse Disease-Free Survival in PAAD

To determine the clinical relevance of the above findings ([Tables 1](#) and [2](#)), Kaplan-Meier (KM) survival regression

Table 2 *p*-values for TCR and IG productive read results from searching PAAD v. HNSC & BLCA WXS file (**95% CI**) NS, not significant. (See also Table S4)

TRA		
Comparison	Productive barcodes with p -value (N-1 proportion test)	Productive reads per barcode p -value (t-test)
PAAD vs HNSC	NS	0.0001
PAAD vs BLCA	0.0001	0.0001
TRB		
Comparison	Productive barcodes with p -value	Productive reads per barcode p -value
PAAD vs HNSC	NS	0.0001
PAAD vs BLCA	0.0001	0.0001
TRD		
Comparison	Productive barcodes with p -value	Productive reads per barcode p -value
PAAD vs HNSC	0.0011	0.0001
PAAD vs BLCA	0.0001	0.0001
IGL		
Comparison	Productive barcodes with p -value	Productive reads per barcode p -value
PAAD vs HNSC	0.0001	0.0002
PAAD vs BLCA	0.0001	0.0006
IGK		
Comparison	Productive barcodes with p -value	Productive reads per barcode p -value
PAAD vs HNSC	0.0217	0.0008
PAAD vs BLCA	0.0011	0.0017

analyses were generated to compare the survival rates represented by the barcodes with a productive recombination to all remaining barcodes. Barcodes representing a productive IGK and IGL recombination had decreased disease-free survival (Fig. 1; Table S5). This “worse-survival group” represented barcodes that were productive for either IGK, IGL, or both (herein referred to as IGK/IGL) (Fig. 1a, b). However, no such reduced survival distinctions were observed for the IGK/IGL groups for either HNSC or for BLCA (Fig. 1c-f).

RNASeq Analysis Confirms Increased B-Cell Markers in Productive IGL/IGK Barcodes Compared to all Remaining Barcodes

To verify the B-cell character of the IGK/IGL recombination group, we obtained the RNASeq values for a set of B-cell markers for the IGK/IGL group, and all remaining barcodes, using the cBioPortal.org web tool (Fig. 2). A comparison of the RNASeq values for these B-cell markers clearly indicates that the IGK/IGL group for PAAD consistently demonstrated significantly increased levels of RNASeq values for B-cell markers, when compared to all remaining barcodes (Fig. 2).

Comparison of the B-Cell Markers for the IGK/IGL Barcode Groups for PAAD, HNSC, and BLCA

The above data indicated that the IGK/IGL barcode group has a clear and dramatic, reduced survival rate, in comparison to all remaining barcodes. However, no such survival distinctions were noted for the IGK/IGL barcode groups for HNSC and BLCA (Fig. 1). Thus, we considered the possibility that the B-cell character of the IGK/IGL barcode group for PAAD was more intense than in the case of HNSC and BLCA. This possibility is supported by the fact that there are more recombination reads per barcode, for IGK and IGL, than in the case of either HNSC or BLCA (Tables 1 and 2). To further address this possibility, we obtained the RNASeq values for a variety of B-cell, Th1, and Th2 markers for the IGK/IGL groups for all three cancer datasets (Table 3; Table S6). Overall, these RNASeq values indicate a trend towards a stronger B-cell character, and a reduced Th1 character, for the PAAD IGK/IGL group. However, the RNASeq data do not reflect consistency that would allow indisputable support for the idea that the survival decrease, for the PAAD IGK/IGL barcode group in comparison to the IGK/IGL barcode groups of HNSC and BLCA, is due to the presence of B-cells or to a reduced Th1 infiltrate.

Fig. 1 **a** Kaplan-Meier (KM) disease-free survival curve for pancreatic adenocarcinoma (PAAD) barcodes with IGK/IGL productive recombinations (arrow) compared to the disease-free survival for the remaining PAAD barcodes. (SOM Table S5) Mean disease-free survival for IGK/IGL productive barcodes, 16.0 months; mean disease free survival for all remaining barcodes, 36.4 months. Log rank comparison p -value, 0.013. **b** KM overall survival curve for PAAD barcodes with IGL/IGK productive recombinations (arrow) compared to the overall survival for the remaining PAAD barcodes. (SOM Table S5) Mean overall survival for IGK/IGL productive barcodes = 22.9 months; mean overall survival for all remaining barcodes, 41.6 months. Log rank p -value, 0.07. **c** KM disease-free survival curve for HNSC barcodes with IGK/IGL productive rearrangements compared to the disease-free survival for the remaining HNSC barcodes. (SOM Table S5) Mean disease-free survival for IGK/IGL productive barcodes, 64.1 months; mean disease-free survival for all remaining barcodes, 99.6 months.

Log rank p -value, 0.815. **d** KM overall survival curve for HNSC barcodes with IGK/IGL productive rearrangements compared to the overall survival for the remaining HNSC barcodes. (SOM Table S5) Mean overall survival for IGK/IGL productive barcodes, 55.2 months; mean overall survival for all remaining barcodes = 75.8 months; Log rank p -value, 0.366. **e** KM disease-free survival curve for BLCA barcodes with IGK/IGL productive recombinations compared to the disease-free survival for the remaining BLCA barcodes. (SOM Table S5) Mean disease free survival for IGK/IGL productive barcodes, 87.7 months; mean disease-free survival for all remaining barcodes, 63.731 months. Log rank p -value, 0.761. **f** KM overall survival curve for BLCA barcodes with IGK/IGL productive rearrangements compared to the overall survival for the remaining BLCA barcodes. (SOM Table S5) Mean disease-free survival for IGK/IGL productive barcodes, 42.9 months; mean disease-free survival for all remaining barcodes, 57.6 months. Log rank p -value, 0.074

Fig. 2 a Box and whisker plots for RNASeq values of PAAD barcodes with IGL/IGK productive rearrangements (left boxes) compared to RNASeq values of remaining PAAD barcodes (right boxes). For CD19, y-axis range from -50 to 400 RNASeq values, with intervals of 50 RNASeq values, and a p -value of 0.0016 (95%CI). For CD20, y-axis range from -200 to 1600 RNASeq values, with intervals of 200 RNASeq values, and a p -value of 0.0060 (95%CI). For CD22, y-axis range from -200 to 1200 RNASeq values, with intervals of 200 RNASeq values, a p -value of 0.0008 (95%CI). For CD79A, y-axis range from -200 to 1600 RNASeq values, with intervals of 200 RNASeq values, and a p -value of 0.0005 (95%CI). For CD79B, y-axis range from -100 to 900 RNASeq values, with intervals of 100 RNASeq values, and a p -value of 0.0002 (95%CI). For CD267, y-axis range from -10 to 90 RNASeq values, with intervals of 10 RNASeq values, and a p -value of .0066 (95%CI). For CD268, y-axis range from -10 to 70 RNASeq values, with intervals of 10 RNASeq values, and a p -value of .0049 (95%CI). For CD269, y-axis range from -10 to 80 RNASeq values, with intervals of 10 RNASeq values, and a p -value of 0.0003 (95%CI). For BTK, y-axis range from -100 to 600 RNASeq values, with intervals of 100 RNASeq values, and a p -value of < .0001 (95%CI). All p -values are t-test p -values

PAAD Barcodes Representing Recovery of Productive TRB Recombination Reads Demonstrate a Strong B-Cell Presence, and Reduced Th1 Character, in Comparison to the HNSC and BLCA Datasets

As previously noted, the percentage of barcodes with TRB productive VDJ recombination reads was significantly increased in PAAD, when compared to control cancers HNSC and BLCA. To determine whether there was a B-cell/Th2 character to the lymphocyte infiltrate for the overall TRB productive group for PAAD, we obtained RNASeq values for a variety of T- and B-cell markers within that group, for comparison with the equivalent (TRB productive) groups for HNSC and BLCA (Table 4; Table S7). CD8A and CD8B RNASeq values were significantly decreased in PAAD compared to both control cancers (Table 4). IFNG (interferon- γ) RNASeq values were significantly lower in PAAD when compared to both control cancers (Table 4). The Th1 specific transcription factor TBX21 (TBET) also had significantly decreased RNASeq values for the PAAD, TRB productive group when compared to the HNSC cancer dataset (Table 4). PTGDR2 (CRTH2), an important Th2 marker [21], showed significant increases in RNASeq values in the PAAD TRB productive group, in comparison to both HNSC and BLCA (Table 4). The Th2 cytokine IL4 also represented a trend of increased RNASeq values in the PAAD TRB productive group, though this trend was statistically insignificant (Table 4).

Discussion

The above analyses of the immune receptor recombination reads from WXS files representing pancreatic cancer indicate three important points: (i) pancreatic cancer has a very high level of lymphocyte infiltrate, consistent with recent report representing a variety of approaches for the assessment of pancreatic cancer, lymphocyte infiltrates [9]; (ii) the

Table 3 RNASeq values for T- and B-cell markers for the IGL/IGL productive barcode groups for PAAD, HNSC, and BLCA datasets. Notable distinctions are indicated by right side p -values. (See also Table S6)

RNASeq marker	PAAD average	HNSC average	BLCA average	PAAD v. HNSC p -value	PAAD v. BLCA p -value
CD4	1630.79	1299.18	1214.36	0.037	0.012
CD8A	346.58	481.15	390.68	NS	NS
CD8B	95.14	123.66	160.58	NS	NS
CD19	254.92	116.78	205.30	NS	NS
CD20	735.17	354.80	639.03	NS	NS
IFNG	2.64	24.51	32.19	0.001	0.002
BTK	311.69	159.81	203.25	0.001	NS
PIK3CG	155.35	137.41	77.57	NS	0.001

NS, not significant

Table 4 Average RNASeq values for barcodes with productive TRB recombination reads for the PAAD, HNSC, and BLCA datasets. Notable distinctions are indicated by right side *p*-values. (See also Table S7)

Cell type	Marker	PAAD average	HNSC average	BLCA average	PAAD v. HNSC <i>p</i> -value	PAAD v. BLCA <i>p</i> -value
CD4	CD4	1587.51	1231.34	1315.29	0.0014	0.0857
CD8	CD8A	307.65	440.50	481.78	0.0013	0.0309
CD8	CD8B	85.48	100.65	139.43	0.0085	0.0234
TH1	IFNG	2.21	24.68	39.95	0.0001	0.0001
TH1	TBX21	27.81	35.46	47.37	0.0302	NS
TH2	PTGDR2 (CARTH2)	41.32	3.95	21.53	0.0001	0.0454
TH2	IL4	1.41	0.07	0.13	NS	NS
TH17	IL17F	0.09	0.92	0.51	0.0001	0.0027
TH17	IL17A	0.10	3.06	1.42	0.0001	0.0283

NS not significant

lymphocyte infiltrate for pancreatic cancer has a strong Th2/B-cell character; and (iii) recovery of B-cell recombination reads from a subset of pancreatic cancer specimens identified those specimens as representing a reduced overall and disease-free survival rate.

While the Th2 character of pancreatic cancer, overall, is well substantiated by both the recovery of IGK/IGL recombination reads (Tables 1 and 2), and the RNASeq data (Table 4), the RNASeq data is not entirely consistent with regard to Th2, as a broad category. For example, RNASeq values for the cytokines, IL5, IL10, and IL13 (data not shown) did not show the same trend as did other Th2 markers (Table 4), i.e., those values showed neither a consistent or reverse trend, with no statistical significance of association with any of the three cancer datasets above. However, IL10 RNASeq values were significantly decreased in PAAD when compared to controls (unpublished observations).

B-cell marker RNASeq values did correspond extensively and virtually perfectly with the recovery of IGK/IGL recombination reads in PAAD (Fig. 2), although the B-cell marker, RNASeq values could not be used as independent markers to establish barcode subsets with statistically significant, reduced survival associations (data not shown). Likewise, when comparing PAAD, HNSC, and BLCA IGK/IGL recombination read groups, the number of reads per barcode clearly distinguished PAAD from HNSC and BLCA, consistent with the survival distinctions among these cancers, i.e., that is the IGK/IGL group for PAAD had a clearly worse survival whereas the HNSC and BLCA IGK/IGL barcode groups did not. Interestingly, a recent report has emphasized a high level of intratumoral bacteria in PAAD, possibly related to the clearly dramatic B-cell infiltrate indicated here [22]. However, the RNASeq values representing B-markers and Th2 markers, while showing a trend and some instances of statistically significance in the distinction of the IGK/IGL groups, did not, as a group of markers, provide distinctions that were consistent

with the statistical significance of the reads recoveries (Tables 1 and 2) and survival (Fig. 1). This may be due to the fact that RNASeq markers represent complicated Th2 and B-cell subsets that cannot be grouped in a relatively simplistic manner. Or, it may be that the recovery of recombination reads provides a more consistent and reliable assessment of lymphocyte infiltrates, their basic character, and their survival impacts, at least in some cancer datasets.

It is possible that the long-term value of results presented here would be to provide more personalized prognostic information to patients who have been diagnosed with PAAD [23]. It is important to note that the 147 PAAD barcodes analyzed in this study represented a mean disease-free survival of 30.1 months and a median disease-free survival of 15.5 months (Table S5). Subdividing this group into barcodes with productive IGL/IGK recombinations and the subgroup comprised of all remaining samples provides a considerable degree of differentiation in survival. The IGK/IGL productive recombination group had a mean disease-free survival of 16.0 months compared to 36.4 months for all remaining samples (Fig. 1; Table S5). The IGK/IGL productive recombination group had a median disease-free survival of 12.8 months compared to 20.3 months for all remaining samples (Table S5).

The above results may also be of use in treatment options. Ibrutinib, a BTK inhibitor has been shown in mouse models to reduce B-cell infiltrates and exhibit antitumor effects [24]. In a separate study in mice, BTK was shown to drive pancreatic ductal adenocarcinoma through interaction of B-cells and FcRγ + tumor-associated macrophages, resulting in a predominance in Th2 type CD4⁺ cells and a decrease Th1 type CD4⁺ response [25]. As noted in the introduction, there is currently a clinical trial underway whereby Ibrutinib is used in pancreatic cancer therapy. This raises the question of whether Ibrutinib will be more successful in a subset of patients where IGK/IGL recombination reads are present at relatively high levels in the tumor specimen WXS files. In a previous report

from our group, B-cells were important for reducing the anti-apoptotic effects of IFNG [26]. And, yet another study found that inhibiting PI3K γ , a macrophage kinase, led to an enhanced CD8 T-cell response and increased survival in pancreatic ductal carcinoma [27].

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Compliance with Ethical Standards

Conflict of Interest Authors declare no conflict of interest.

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