Research Square

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Clonal Diversity of the B cell Receptor Repertoire in Patients with Coronary in-Stent Restenosis and Diabetes Mellitus

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Abstract

Background Coronary artery disease patients with diabetes mellitus may have a higher risk for clinical and angiographic restenosis. Evidence has suggested that B cells play a functional role in the progression of atherosclerotic lesions. The purpose of this study was to investigate the clonal diversity of the B cell receptor (BCR) repertoire in patients with coronary in-stent restenosis (ISR) and diabetes mellitus.

Methods In the present study, we had enrolled 21 patients with ISR and DM or not and collected the peripheral blood mononuclear cell. The DNA was extrated and then we performed DNA-seq analysis to character the B-cell receptor profiles (BCR) of CAD patients with or without ISR. The BCR diversity and the overlap was evaluate by the bioinformatics base on the DNA-seq data.

Result Seq-data showed that the diversity of amino acids was altered in patients with ISR. Specifically, 6 V gene segments as well as 41 V/J pairs exhibited different frequencies in patients with ISR, with the altered common amino acid sequences reaching 0.1–1.01% in ISR patients.

Conclusion The present findings suggest that B cells may play a role in the occurrence of ISR and that further analysis of BCR profiles would enhance understanding of ISR.

Background

Coronary artery disease (CAD) is caused by atherosclerosis, defined as proliferation, hypertrophy, and calcareous deposition in the arterial wall, resulting in reduced vascular elasticity, thrombosis, occlusion, stenosis, and other changes [1]. Coronary stent implantation has enabled management of the early complications associated with plain balloon angioplasty. Stent implantation is currently the main percutaneous coronary intervention (PCI) and has great advantages over balloon angioplasty [2]. Stent implantation can decrease the frequency of restenosis by preventing elastic recoil and constrictive remodeling. In particular, drug-eluting stent (DES) implantation has led to a 5–10% reduction in the occurrence of in-stent restenosis (ISR) [3]. The DES was coated with antibiotics and immune agents that inhibit intimal hyperplasia on its outer layer, thereby allowing the drug to slowly penetrate into the blood vessels and avoiding blood vessel obstruction by scar tissue formed after implantation. ISR is currently the major cause for recurrence of exertional angina pectoris or acute coronary syndromes after coronary angioplasty. Despite the lower occurrence of ISR through advances in stent design and polymers, it is very challenging to treat stenosis once it has occurred in these stents.

Studies have enhanced our understanding and awareness of various factors that can increase the risk of clinical and angiographic restenosis [4, 5]. Among these risk factors, the most important is diabetes mellitus (DM) [1, 6]. The underlying mechanism for the higher incidence of ISR in DM patients is likely to be complex, because the B cells and T cells involved in balancing of the immune state both contribute to ISR. Recent studies demonstrated that B cells have a pro-inflammatory role in inflammatory diseases like CAD [7–9]. B2 cells produce tumor necrosis factor (TNF)-α, interleukin (IL)-2, and IL-10 that act as

proatherogenic cytokines [10–12]. The cytokines produced by B cells enhanced immunomodulation during chronic inflammation [13]. Furthermore, the inflammation contributed to plaque formation and modulated the clinical outcomes for thrombotic complications of atherosclerosis [9]. Depletion of mature B2 cells by anti-BAFF antibodies resulted in proatherogenic chemokine production by macrophages [14]. B cell depletion reduced the development of atherosclerosis and was also a promising therapy for DM [8, 15, 16]. B cell-deficient mice failed to resolve experimental autoimmune encephalomyelitis dependent on strict B cell-derived IL-10 production [17]. These findings indicated that DM exaggerated B cell function in atherosclerosis. In the present study, we investigated the clonal diversity of the B cell receptor (BCR) repertoire in coronary ISR patients with DM to provide a reference for BCR sequences in future investigations.

Methods

Patients and study design

From January 2017 to December 2018, 21 patients with or without ISR and DM at the Center for Cardiovascular Diseases, Meizhou People's Hospital were enrolled in the study. All patients had been diagnosed with CAD, undergone PCI, and been examined for ISR within 1 year. Any lesions were confirmed by coronary CT angiography. The patients were divided into four groups: ISR with DM (ISR DM; $n = 6$), ISR without DM (ISR − DM, $n = 5$), DM without ISR (− ISR DM, $n = 5$), without ISR and DM (− ISR − DM, $n = 5$). The study was approved by the Ethics Committee of Meizhou People's Hospital (Huangtang Hospital), Meizhou Hospital Affiliated to Sun Yat-sen University, Guangdong, China. All participants provided written informed consent before enrollment in the study.

Clinical Characteristics Of The Patients

Peripheral blood samples were obtained from the four groups of CAD patients using EDTA anti-coagulant tubes. The clinical characteristics were collected through a detailed medical history, physical examination with vital signs, and blood analyses. Exclusion criteria were autoimmune diseases or neoplasms, treatment with corticosteroids or other immunomodulatory therapy, or vaccination within 3 months before or after PCI. All parameters were measured in the Clinical Laboratory at Meizhou People's Hospital using standard protocols.

Sample Collection And Repertoire Sequencing

Whole blood samples (10 ml) were collected from the patients and the peripheral blood mononuclear cells (PBMCs) were immediately isolated with Ficoll-Paque (GE) according to the manufacturer's instructions. Genomic DNA was extracted from the PBMCs with a PureLink Pro 96 Genomic DNA Purification Kit (Invitrogen), and used as a template for multiplex PCR with a Multiplex PCR Kit (Qiagen). The details of the PCR primers are shown in Table 1. The Multiplex PCR protocol was as follows: pre-

denaturation at 95 °C for 15 min; 30 cycles at 94 °C for 30 s, 60 °C for 90 s, and 72 °C for 30 s; final extension at 72 °C for 5 min. Multiplex PCR amplification after the adapter, library testing qualified to go on machine sequencing. High-throughput sequencing of each captured library ensured that the sequencing data volumes met the requirements. The amplicons were gel-extracted and purified prior to library preparation and the samples were sequenced as 150-bp paired-end runs on a HiSeq™ Xten machine (Illumina).

Table 1
the primers for Multiplex PCR.

Primers	Sequence
GHV1-18	AGAGTCACCATGACCACAGAC
IGHV1-2/1–46	AGAGTCACCAKKACCAGGGAC
IGHV1-24	AGAGTCACCATGACCGAGGAC
IGHV1-3/1–45	AGAGTCACCATTACYAGGGAC
IGHV1-69/1-f	AGAGTCACGATWACCRCGGAC
IGHV1-8	AGAGTCACCATGACCAGGAAC
IGH2-70/26/5	ACCAGGCTCACCATYWCCAAGG
IGHV3	GGCCGATTCACCATCTCMAG
IGH4	CGAGTCACCATRTCMGTAGAC
IGHV5-51	CAGCCGACAAGTCCATCAGC
IGHV6-1	AGTCGAATAACCATCAACCCAG
IGHV7	GACGGTTTGTCTTCTCCTTG
IGHJ	CTGAGGAGACGGTGACCRKKGT

Table 2

Characteristics of study population at baseline

	ISR DM	-ISR DM	ISR -DM	-ISR -DM	P-value
Age ± SD	60.2 ± 7.28	63.8 ± 9.0	63 ± 5.41	61.4 ± 6.82	n.s.
Male/Femal	3M/3F	3M/2F	2M/3F	3M/2F	n.s.
Risk factor					
Smoking status,current(n)	2	1	2	4	n.s.
Hypertension(n)	3	3	1	0	n.s.
Diabetes mellitus(n)	5	5	0	0	NA
Medication					
β-blockers	2	3	3	4	n.s.
Aspirin	3	2	4	4	n.s.
ACE inhibitors	2	2	1	4	n.s.
Clopidogrel	3	3	1	4	n.s.
Statins	6	5	5	5	n.s.
Biochemistry variables					
Total Cholesterol (mmol/L)	4.00 ± 1.34	5.00 ± 0.47	4.54 ± 0.69	3.91 ± 0.57	□0.01
LDL (mmol/L)	2.16 ± 1.12	2.71 ± 0.76	2.21 ± 0.69	2.14 ± 0.49	n.s.
HDL (mmol/L)	1.30 ± 0.38	0.93 ± 0.10	1.68 ± 0.29	1.13 ± 0.29	□0.01
Triglycerides (mmol/L)	1.32 ± 0.41	3.74 ± 2.42	1.17 ± 0.32	1.73 ± 0.73	□0.05
Apolipoprotein A1 (g/L)	1.08 ± 0.21	0.87 ± 0.16	1.33 ± 0.12	0.95 ± 0.11	□0.01
Apolipoprotein B (g/L)	0.64 ± 0.24	0.83 ± 0.22	0.65 ± 0.13	0.70 ± 0.15	n.s.
Homocysteine (umol/L)	15.2 ± 2.20	16.14 ± 6.23	13.5 ± 2.02	17.6 ± 4.0	n.s.
Baseline glucose (mmol/L)	7.37 ± 1.51	7.64 ± 3.69	5.16 ± 0.71	4.75 ± 0.04	n.s.
HbAc1 (%)	9.1 ± 1.99	7.32 ± 1.33	6.5 ± 0.47	5.92 ± 0.46	n.s.
Hematological variables					
WBCs (10^3/μL)	7.86 ± 0.41	8.38 ± 1.28	8.96 ± 1.10	6.9 ± 0.59	□0.05
Neutrophil(10^3/μL)	69.86 ± 6.36	69.2 ± 7.10	70.36 ± 6.83	62.6 ± 8.57	n.s.

WBCs: While blood cells; HbAc1: Glycosylated hemoglobin; SD: Standard Deviation; n.s.: non-significant, NA: not applicable

	ISR DM	-ISR DM	ISR -DM	-ISR -DM	P-value
Neutrophil(%)	5.5 ± 0.63	5.86 ± 1.39	6.28 ± 0.75	4.34 ± 0.79	0.05
Lymphocyte(10^3/μL)	20.16 ± 4.71	24.00 ± 7.34	21.3 ± 5.95	27.8 ± 10.41	n.s.
Lymphocyte(%)	1.66 ± 0.40	1.84 ± 0.29	1.94 ± 0.65	1.92 ± 0.64	n.s.
Monocyte(10^3/μL)	6.60 ± 0.93	6.00 ± 0.57	7.4 ± 1.59	6.00 ± 1.63	n.s.
Monocyte(%)	0.50 ± 0.08	0.50 ± 0.11	0.64 ± 0.14	0.44 ± 0.13	n.s.
WBCs: While blood cells; HbAc1: Glycosylated hemoglobin; SD: Standard Deviation; n.s.: non-significant, NA: not applicable					

Bcr Sequence Analysis

The bioinformatics analysis was performed as follows: (1) quality control of raw data: Phred quality of > 30 in at least 80% and error rate of < 0.1%; (2) data filtering: Trimmatic was used to filter out adaptor and barcode sequences, and Flash was used for overlap of the reads [18]; (3) alignment blast: without mismatches and indel paired-end and single end-reads, the merged paired reads were confirmed identical by MiXCR [19] and aligned to the V, D, and J gene reference sequences in the IMGT database (http://www.imgt.org/) as previously described [20]; (4) characteristic gene-specific sequences (such as CDR3) were extracted from the aligned clone sequences. High-quality V, D, and J gene clone sequences were spliced into BCR clones for further analysis.

Statistical Analysis And Graphing

Statistical analyses were performed using SPSS 19 with GraphPad Prism software for graphing. All data were presented as mean ± SD, and comparisons between groups were performed by one-way ANOVA. The diversity of the BCR repertoire was calculated by the Simpson index, Chao 1 index, and Shannon–Wiener index. Values of $P < 0.05$ were considered statistically significant. The BCR overlap was calculated as previously described [21], based on the number of common amino acid clonotypes in two samples as follows: (number of common amino acid clonotypes in two samples × 2) / (total number of amino acid clonotypes in sample 1 + total number of amino acid clonotypes in sample 2) × 100. The average of all samples in each group was reported.

Results

Clinical characteristics of patients with ISR and DM

Patients who had undergone PCI with or without ISR and DM were enrolled in the study. Peripheral blood samples were collected from the patients and sent to the Clinical Laboratory for analysis. The clinical and

laboratory characteristics of the patients are summarized in Table 2. We collected data for risk factor, medication, biochemical, and hematological variables, and performed comparisons among the groups. Significant differences in lipid metabolism and hematological variables like white blood cells and neutrophils were observed (Table 2), indicating that ISR and DM can trigger immune state changes.

Different clonal diversity of the BCR repertoire in patients associated with ISR and DM

The study cohort comprised 21 patients with or without ISR and DM. All of the DNA libraries were sequenced and complete BCR repertoire data were successfully obtained using an Illumina HiSeq Xten machine. On average, 5,177,449 (range: 3,839,975–8,001,683) raw Illumina sequencing reads were obtained for each sample. After performing the quality control requirements and data filtering described in the Methods section, an average of 1,799,447 (range: 2,660,587–1,303,608) unique sequence numbers were filtered out for the alignment blast. Detailed descriptions of the sequence numbers are provided in **Supplementary Table 2**.

BCR repertoire diversity is a key feature of the humoral immune system, and creates the potential for recognition of the wide variety of antigens. To evaluate the BCR repertoire diversity, we first analyzed the Simpson index, Shannon–Wiener index, and Chao 1 index values for amino acid sequences, and found that the diversity of amino acid sequences was higher than in − DM patients. This meant that DM affected the BCR repertoire diversity in ISR patients (Fig. 1). However, in non-DM patients, a decrease in diversity was observed in ISR patients. These results indicated that ISR and DM can both change the diversity of amino acid sequences in patients.

To learn more about the diversity, we created five sections based on the frequency of the BCR nucleotide sequences. The results showed that the number of productive unique BCR nucleotide sequences was highest in the 1–0.1% section, but had the lowest frequency in − ISR DM patients compared with the other groups (Fig. 2A and 2B). Similar results were found in − ISR DM patients for significantly higher percentages of high-frequency nucleotide sequences, regardless of being in the top 200, top 500, or top 1,000 BCR sequences (Fig. 2D and Supplementary Fig. 1). Regarding productive unique BCR nucleotide sequences, −ISR DM patients had a lower percentage compared with ISR DM or ISR − DM patients (Fig. 2C). These findings suggest that in DM patients, ISR can decrease the low frequency of BCR nucleotide sequence diversity, but increase the number of productive unique BCR nucleotide sequences. These data provide further evidence that ISR and DM can both affect the diversity of amino acid sequences in patients.

Differential V and J gene usage within groups compared with between groups

IgH genes are assembled from a large pool of variable (V), diversity (D), and joining (J) gene segments, and different V(D)J recombinations result in diversity of the BCR repertoire. In the present study, we did not find any significant differences in V gene or J gene segment usages within groups compared with between groups (Supplementary Figs. 2 and 3), with only some subdivisions of V gene segments showing differential usages (Fig. 3). In DM patients, we found that four V subdivision genes exhibited differences with and without ISR, namely IGHV1-18, IGHV1-3, IGHV2-70, and IGHV3-21 (Fig. 3A). Meanwhile, IGHV3-30 was lower in ISR − DM patients than in ISR DM patients (Fig. 3C), and a similar result was found in comparison with − ISR − DM patients (Fig. 3B). Therefore, IGHV3-30 appears to be affected by both ISR and DM, but whether it is regulated by both by ISR and DM in CAD patients requires further data.

The BCR repertoire is assembled by various numbers of V(D)J recombinations that affect the diversity. In total, 23 V/J paired gene usages differed between ISR DM and ISR − DM patients, comprising six down-regulated genes and 17 up-regulated genes (Fig. 3E). In non-DM patients, 12 V/J paired gene usages differed significantly in comparisons of patients with and without ISR (Fig. 3F). Compared with the ISR DM group, the − ISR DM and ISR − DM groups both had significantly lower gene usages in V subdivision genes and V/J paired genes. These findings reveal that ISR and DM can produce changes in V/J paired gene usages, and may have synergistic effects in CAD patients.

Receptor Sharing Between Isr And Dm Patients

Next, we investigated the BCR sequences for shared sequences and determined whether sharing occurred between samples within groups. As shown in Fig. 4A, there was no significant difference in the unique clonotype overlap rates in individual groups, but an increasing trend in overlaps was observed. The − ISR − DM group had the highest clonotype overlap rate, with an average overlap rate of 0.53% detected between any two − ISR − DM patient samples, while ISR DM patients had the lowest overlap rate. Based on the section divisions described above, we analyzed the overlap rates based on the frequencies of the amino acid sequences. The results suggested that ISR and DM can change the shared amino acid sequences. We did not observe any differences in the section groups, except for the amino acid frequency between 0.1% and 0.01%. In the 0.1–0.01% section, ISR − DM patients had the highest rate among the four groups and differed significantly from the ISR DM and − ISR − DM groups (Fig. 4B). Thus, it seems that ISR can increase the shared sequence rates, while DM can reverse this effect.

We further analyzed the clonotype overlap rates among the four groups. ISR DM and ISR − DM patients shared 0.50% clonotype overlaps (range: 0.001–2.31%). A similar result was found in the other parallels (Fig. 4C). An interesting class polarization that can be observed in Fig. 4C and 4D drew our attention. We collected the higher overlap rate classes, and found differences for ISR DM versus ISR − DM and ISR DM versus − ISR DM. We also performed an analysis on the section groups described above, but found no significant difference in high or low frequencies of amino acid sequences (Fig. 4E). Nevertheless, ISR and

DM can alter the diversity by changing the shared sequences, and further investigations regarding BCR specificity and activation state are warranted.

Discussion

The development of coronary stents had led the field of interventional cardiology toward a new horizon. Although the introduction of balloon angioplasty and bare metal stent (BMS) implantation has improved interventional cardiology outcomes, there are approximately 20% overall with reported rates. The widespread use of coronary stents (BMS and DES) has enhanced our understanding and awareness of risk factors that may increase the incidence of restenosis, especially in DM patients for whom the risk of ISR can increase up to 30% [1, 22, 23]. The advent of DES implantation has further decreased the incidence of ISR. Despite the lower recurrence of ISR through advances in stent design and polymers, once restenosis has occurred in these stents, it was very challenging to treat and confers great suffering and heavy financial burdens on patients. In CAD patients, DM can accelerate atherosclerosis through infiltration of inflammatory cells (macrophages and T lymphocytes), larger necrotic core size, and more diffuse atherosclerosis [24].

During the progression of atherosclerosis, inflammatory pathways play important roles in formation of atherosclerotic plaques. A previous study suggested that inflammation can increase the occurrence of ISR, and that inflammatory biomarkers (IL-6, matrix metalloproteinases, C-reactive protein) have prognostic value in predicting the risk of ISR [25]. Among the infiltrating inflammatory cells, T lymphocytes have been extensively studied in atherosclerosis. Similar to Th1 cells that can secrete interferon-γ, T lymphocytes can activate macrophages and produce some molecules involved in plaque formation, such as Toll-like receptors (TLRs). Meanwhile, Th2 and Treg cells can secrete anti-inflammatory cytokines, such as IL-10 or transforming growth factor-β, that delay the occurrence of inflammation [26, 27]. In our previous work, we found some differences of T cell receptor repertoires in CAD patients [28]. However, it has remained unclear how B cells contribute to plaque formation. In unpublished data, we found that B cells were involved in regulation of the immune state in CAD patients. Other reports revealed that B cells acted in a pro-inflammatory manner in CAD patients [7–9]. Cytokines produced by B cells can enhance immunomodulation during chronic inflammation; for example, TNF-α, IL-2, and IL-10 produced by B2 cells promoted atherosclerosis [10–12].

In DIO mice, B cells infiltrated the adipose tissue as an early response to DIO stimulation. However, the mechanism for the B cell function was not determined. Depletion of mature B2 cells by anti-BAFF antibodies resulted in proatherogenic chemokine production by macrophages [14]. B cell depletion was also a promising therapy for DM [8, 15, 16]. DM is a chronic inflammatory disease, in which elevation of pro-inflammatory molecules can induce cell surface TLRs and retinoic acid-inducible gene I (RIG-I)-like receptors, resulting in inflammation [29–31]. Furthermore, the signaling pathway transduction by TLRs and RIG-I was crucial for innate immunity, and involved in both metabolic and cardiovascular diseases [32]. By throughput sequencing, we were able to monitor the immune state in DM patients. In a previous study, Seay et al. found that B cell receptors had a distinct tissue distribution and comparable diversity in

DM patients. In the present data, the B-cell clone diversity was changed by ISR and DM. Furthermore, DM affected the immune state mediated by B cells.

Conclusions

In summary, we have performed a comprehensive characterization of the immune BCR profiles in CAD patients with ISR and DM. Based on the obtained sequence data, we found the ISR and DM can both change the diversity and clonal distribution of BCR repertoires in CAD patients. The V/J genes had different usages between ISR and DM patients. Furthermore, a polarization of shared sequences was found between groups and ISR – DM patients had the highest clonotype overlap rate among the groups. We also investigated the disease-associated clonotypes for sharing of common or distinct amino acid characteristics, and our determination of antigenic triggers responsible for these observations provides the potential for development of targeted therapies for ISR and DM patients.

Declarations

Ethics approval and consent to participate

This study was conducted in accordance with the declaration of Helsinki. This study was conducted with approval from the Ethics Committee of the Meizhou People's Hospital (Huangtang Hospital), Meizhou Academy of Medical Sciences, Meizhou Hospital Affiliated to Sun Yat-sen University, Meizhou 514031, P. R. China.

Availability of data and material

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

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Conflict of interest

The authors declare that they have no competing interests.

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Figures

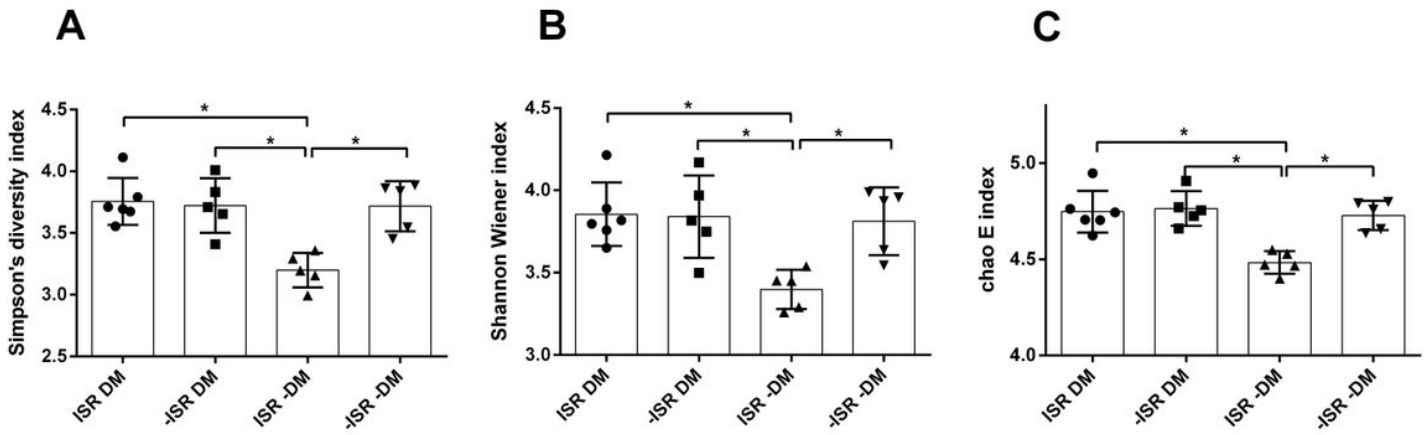

Figure 1

Diversity index of amino acid sequences. (A–C) Calculated diversity index values for amino acid sequences in the four groups. (A) Simpson index values. (B) Shannon–Wiener index values. (C) Chao 1 index values. Each dot represents information for one patient. *P<0.05, significant difference by one-way ANOVA.

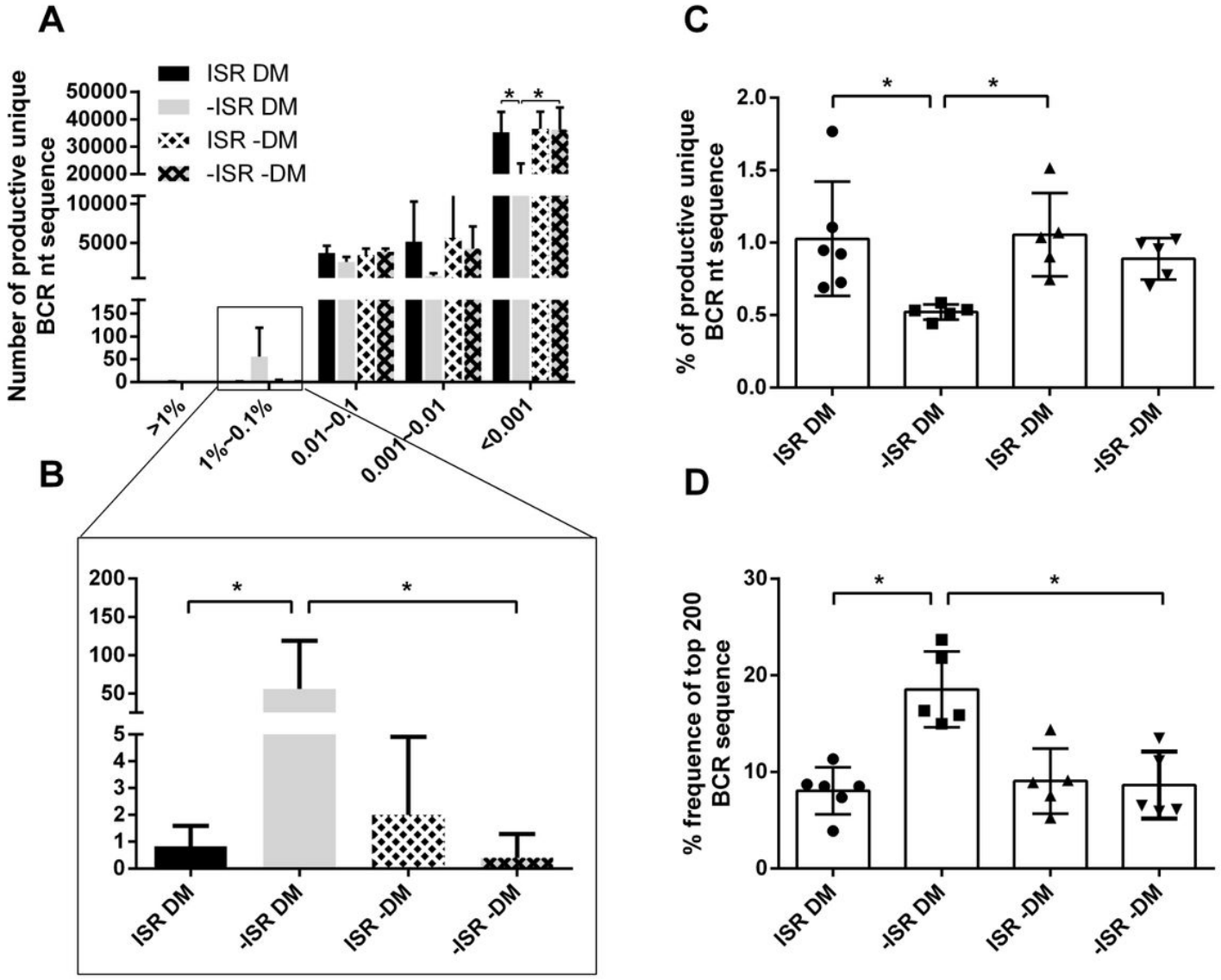

Figure 2

Clonal distribution of BCR repertoires. (A–D) Evaluation of the diversity of BCR repertoires among patients. (A, B) Mean numbers of productive unique BCR nucleotide sequences for five sections (A) and 1%–0.1% section in detail (B). The percentages of productive unique BCR nucleotide sequences in the groups are shown. (C) Mean frequencies of productive unique BCR nucleotide sequences among the four groups. (D) Frequencies of the top 200 BCR repertoire nucleotide sequences in the five groups. Data represent the mean distribution ± SD in each group. Each dot represents information for one patient. *P<0.05, significant difference between groups by a t-test. nt: nucleotide.

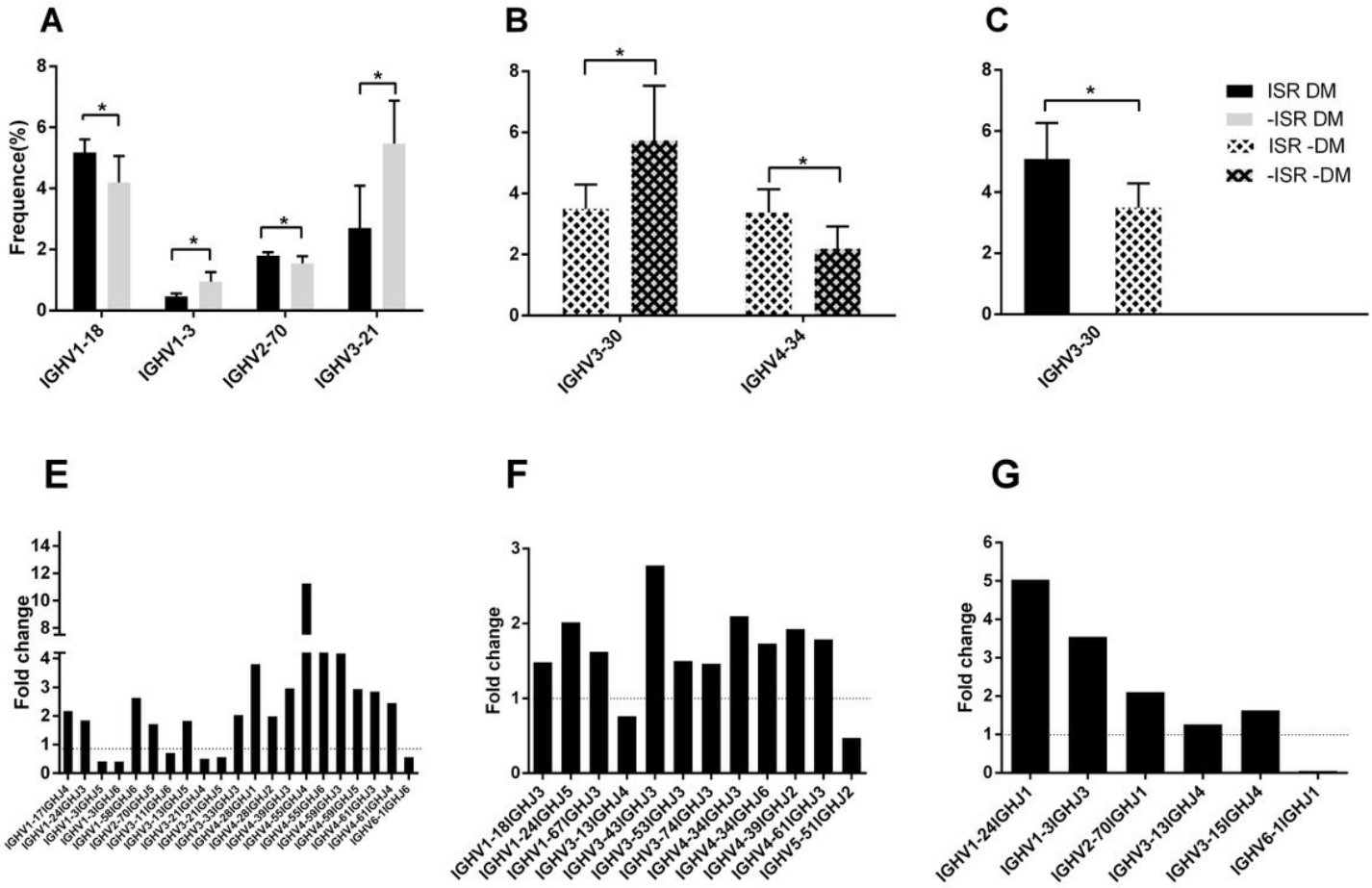

Figure 3

Significant difference of V/J gene usage (A-G) The mean frequency of V subgroup gene usage were shown each group and the significant difference of V subgroup gene usage for (A) ISR DM compared to -ISR DM, (B) ISR -DM compared to -ISR -DM and (C) ISR DM compared to -ISR -DM. Data are represented as mean±SD of the group. Comparison of V/J paired gene usage between groups. The fold change of significant difference usage in V/J paired gene compared between groups for (E) ISR DM compared to -ISR DM, (F) ISR -DM compared to -ISR -DM and (G) ISR DM compared to ISR -DM were shown. All P-values were less than 0.05 by one-way ANOVA. Fold change= the mean frequency in one group divided by the mean frequency in other group.* indicate that the difference is significant (*P□0.05).

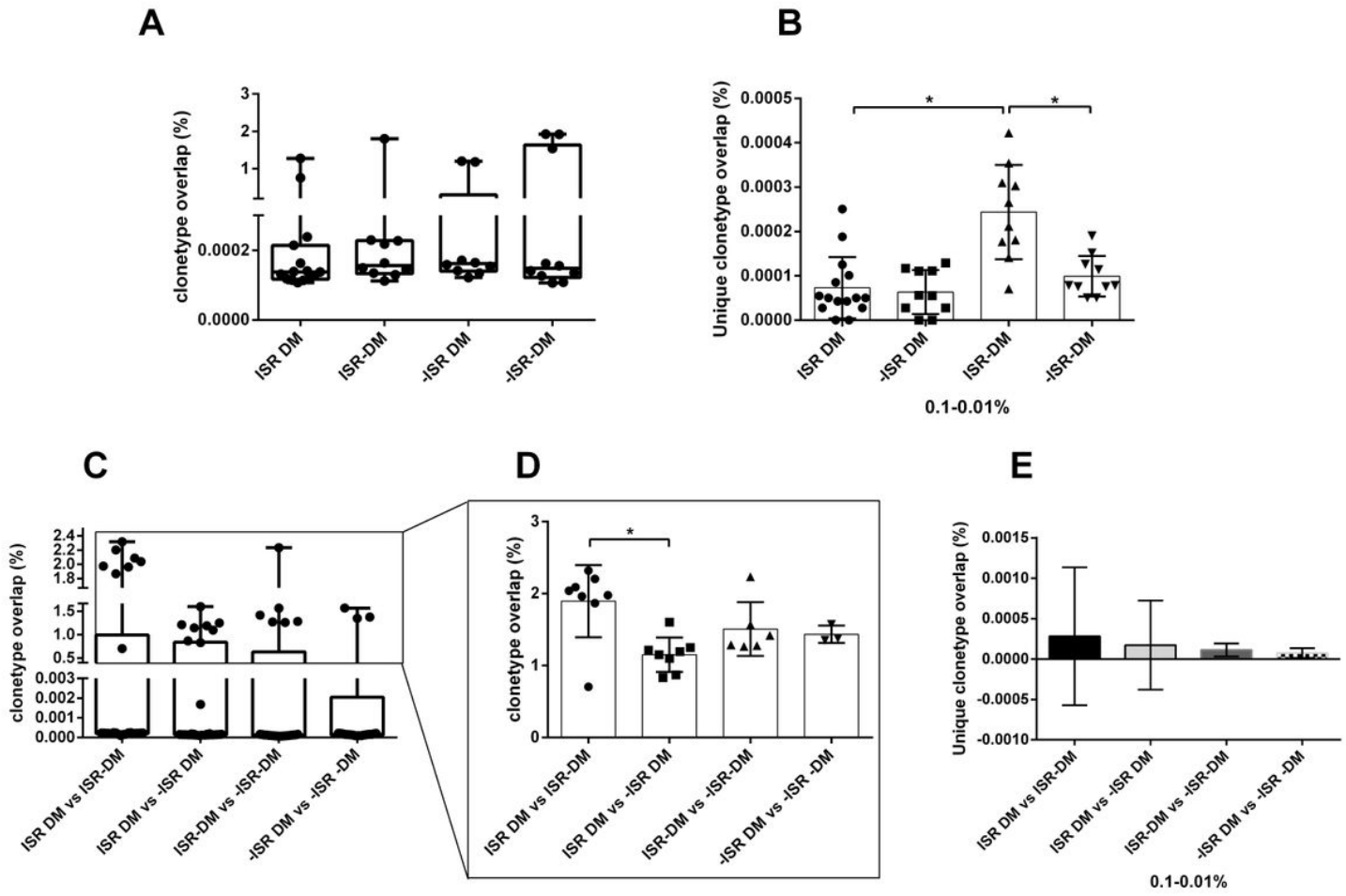

Figure 4

Unique clonetype overlap rate in each and between groups. (A) The data show the unique clonotype overlap of clonetype in ISR DM, ISR -DM, -ISR DM, -ISR -DM group. (B) The data showed the unique clonotype overlap between 0.1% and 0.01% in each group. (C) The data show the unique clonetype overlap rate between groups: ISR DM vs ISR-DM ISR DM vs -ISR DM ISR-DM vs -ISR-DM -ISR DM vs -ISR -DM. (D) The data showed the unique clonotype overlap >0.1% between groups. (E) The data showed the unique clonotype overlap base of the sequence frequency between 0.1% and 0.01% within groups.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementfiles.docx