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A novel pipeline for V(D)J junction identification using RNA-Seq paired-end reads.

Giulia Paciello¹, Elisa Ficarra¹, Alberto Zamò², Chiara Pighi², Carmelo Foti¹, Francesco Abate¹³, Enrico Macii¹ and Andrea Acquaviva¹

¹Department of Computer Engineering, Politecnico di Torino, Torino, Italy
²Department of Pathology and Diagnostics, University of Verona, Verona, Italy
³Department of Biomedical Informatics Center for Computational Biology and Bioinformatics, College of Physicians and Surgeons, Columbia University, NY, USA
giulia.paciello, elisa.ficarra, carmelo.foti, francesco.abate, enrico.macii, andrea.acquaviva@polito.it, alberto.zamo, chiara.pighi@univr.it

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Abstract: Immunoglobulin heavy and light chains are assembled respectively from germline V, D, J and V, J segments within a process called V(D)J recombination involving the development of T and B lymphocytes. The discovery that abnormal antibodies are often related to a wide range of pathologies conducted during the last years to many studies inherent the immunoglobulin features. In particular the identification of the functional V(D)J sequence of an antibody is considered fundamental since it could allow to understand the link between a particular disease and a specific recombination in a certain tissue and to promote the engineering of therapeutic antibodies. Objective of the implemented pipeline consists in the identification of the so called ‘main clone’ that characterizes a neoplastic tissue using paired-end RNA-Sequencing (RNA-Seq) reads.

1 INTRODUCTION

The finding that abnormal antibodies are in many cases related to different pathologies, such as systemic lupus erythematosus (Fraser, 2003), multiple sclerosis (Hueber, 2002) and rheumatoid arthritis (Huang, 1998), led during the last years to an increasing interest in the study of one specific antibodies feature that is the V(D)J junction and its characterization. Thanks to the studies conducted since the mid-century it is nowadays note that the variable regions of the immunoglobulin heavy (IGH) and light (IGL) chains are assembled respectively from germline Variable (V), Diversity (D), Joining (J) and V, J segments within a process called V(D)J recombination involving the development of T and B lymphocytes (Jung, 2004), (Bassing, 2002). The afore mentioned process is capable to account for the huge variability of the immunoglobulin repertoire allowing the immune response of the organisms to a wide range of antigens. In particular, several different mechanisms are involved in the production of heavy chain variable region diversity with respect to V(D)J recombination: The introduction of nucleotides by the terminal deoxynucleotidyl transferase (TdT) (Alt, 1982) that follows the deletion at the 3’ end of the V gene, at the 5’ end of the J gene, and at both ends of the D gene which recombine, or the introduction of short inverted sequences (palindromic-regions) at the V(D)J junction, are well known examples. The identification of the functional V(D)J sequence of an antibody is becoming fundamental since it could allow to understand the link between a particular disease and a specific recombination in a certain tissue and to promote the engineering of therapeutic antibodies. The present work is inspired from two main concepts. First, the knowledge that neoplastic tumors often contain more than one type of cells called clones, since descendent from a single progenitor cell, even if they are strongly related to a specific type of cells, the so called ‘main clone’. The main clone is characterized by the remarkable amplification of a specific rearrangement of the immunoglobulin gene or T-cell receptor gene in comparison to that characterizing the other clones. Second, thanks to the evolution of High Throughput Sequencing (HTS) technology it is possible to sequence the RNA of neoplastic tissues in less than a week. Thus, in principle it is possible to identify the main clone that characterizes the tissue...
of interest by detecting the most amplified VDJ rearrangement in the samples under study in a short time, compatible with the development of focused therapies.

However, the analysis of V(D)J junction from RNA-Seq data is challenging because of two main problems: i) The recombination process makes most of the RNA reads not correctly mapping on the reference genome - This is similarly to the gene fusion detection problem (Abate, 2012), with the additional complexity that the recombinant regions are three instead of two and that they have a much smaller size with respect to fused transcripts; ii) The large variability of the junction, given by the introduction of nucleotides in the region boundaries. Current state-of-art tools, which are not based on RNA-Seq, are able to analyse a single sequence at a time to determine VDJ rearrangements. Hence, they cannot be used to identify the main clone in a tissue sample.

In this paper we present an algorithm that addresses these issues, enabling the use of RNA-Seq to determine the main clone recombinant alleles occurring in a given tissue sample. The algorithm is based on two steps, for VJ and D alleles identification respectively. In the result section we report the details of the analysis conducted on two samples of MCL (Mantel Cell Lymphoma), highlighting the supporting reads for the most amplified clones. Validation is performed by comparing the V(D)J recombinations obtained by the proposed pipeline against the V(D)J regions obtained using state-of-art approaches applied to the sequence of the main clone (known a priori) obtained in laboratory via Polymerase Chain Reaction (PCR).

2 STATE OF THE ART

Numerous tools have been developed with the purpose of finding the best match between a rearranged sequence and the V, D and J germlines, but all of them are characterized by a different approach to the problem for what is concerning the starting point of the analysis. They try indeed to assign a specific V, D and J alleles to a unique sequence, extracted in laboratory via PCR or via High-throughput sequencing (HTS) experiments (Prabakaran, 2011), (Jackson, 2012), rather than identify, using a set of reads, the main clone recombinant alleles.

IMGT/V-QUEST (Giudicelli, 2004) is the first automatic tool developed to align both Immunoglobulin (IG) and T-cell receptor (TCR) sequences belonging to different species with the germline IG and TCR gene and allele sequences of the IMGT reference directory. Being based on Blast algorithm (Altschul, 1990) the tool results satisfying in aligning sequences in the Variable Heavy (VH) and Joining Heavy (JH) regions where large areas of sequence similarity can be found, but not in the shorter D region due to the role of the enzymatic processes in introducing or mutating bases.

JOINSOLVER (Souto-Carneriro, 2004) tries to go over this problem using a different scoring system to match D segments that gives a higher score for longer matches, being based upon consecutive nucleotide matches, whereas searches for two relatively conserved motifs ‘TAT TAC TGT’ and ‘G TGG GG’ to find the extreme points of the Third Complementary Region (CDR3).

Also IMGT/JunctionAnalysis (Monod, 2004) tries to overcome the problems related to the identification of the D allele and the nucleotides mutated or introduced by the specific processes proper of the V(D)J recombination. The junction is here defined as the region starting at the second conserved cysteine (CYS) of the V-region at position 104 and ending with the conserved tryptophan (J-TRP for IGH chains) or the conserved phenylalanine (J-PHE for the IG light chains) at position 118. IMGT/JunctionAnalysis searches the constitutive regions of the junction by comparing the user sequence with the IMGT reference directory, but since the nucleotide sequences of 3’-V region and 5’ J-region are too short to be identified by the tool, V and J allele names have to be given.

SoDA (Volpe, 2006) is another tool developed for deciphering IG and TCR gene segments composition. Initially the set of possible V, D and J segments is chosen thanks to independent unconditional pairwise alignments between the target gene and each candidate gene segments, in particular for what is concerning D segments each candidate is evaluated by alignment against the part of the target sequence between the V conserved cysteine and the J conserved tryptophan or phenylalanine. In the second phase of the pipe all the segments are at the same time aligned against the previous identified sets.

Programs such as VDJSolver (Ohm-Laursen, 2006) and iHMMune-align (Gaeta, 2007) apply instead statistical models to obtain the best fitting of the given sequence on the V, D and J alleles: Even if these methods represent an alternative way to identify the rearrangement the good performances of the tools are strictly linked to the quality and diversity of training datasets.
3 METHODS

The main clone identification is conducted in two phases within the proposed workflow.

The first, that we will call in the following VJ alleles individuation shown in Figure 1 aims to detect the V and J gene segments from which the variable regions of the different clones are arranged and to score each VJ rearrangement on the basis of the number of reads supporting it.

The second, we will call D alleles individuation which purpose is to recognize for the most supported VJ couple identified before, the D allele introduced during the recombinational process. The proposed pipeline was applied to 2 samples of MCL for which the quality was previously assessed. RNA was extracted using Allprep QUIAGEN Columns and then sequenced in 100 bp paired-end reads using an Illumina HiSeq1000. For these samples the VDJ rearranged sequence of the main clone was retrieved in laboratory via PCR.

3.1 VJ alleles individuation

The individuation of the V and J alleles involved in the main clone rearranged sequence is performed during this step following the activities depict in Figure 1 and detailed below.

Initial Alignment. The starting point for determining the list of the VJ rearrangements is the alignment of short RNA-Seq paired-end reads to the reference genome (hg19). The alignment is performed using Bowtie (0.12.8) (Langmead, 2009) in order to retrieve from the initial set of data only those reads that don’t map on the genome due to splicing events that potentially could involve also V, D and J alleles in the process called V(D)J recombination. We will call these reads VDJ Unmapped Reads.

VJ encompassing reads retrieving. The reduced dimension of the new dataset, allows in this step for performing a more accurate mapping of the VDJ Unmapped Reads on the V and J alleles using Blast (2.2.25) (Altschul, 1990) with default parameters. Objective of the this alignment is to retrieve from the VDJ Unmapped Reads only those mates mapped on the 272 V alleles or on the 16 J alleles proper of the IGH locus. We define in this step a VJ recombination if, given a read, a mate is mapped on a V allele and the other on a J allele: We call these reads Encompassing VJ Reads. It is worth noting that the remarkable polymorphism occurring among the considered genes, in addition to homology (Li, 2002), conduct the same read to define different VJ couples.

VJ couples sorted occurancy calculation. Based on the number of reads supporting the recombination, each of the identified VJ couples is scored. Because of homologies inside the same allele, the reads that present multiple matches are removed. A sorting based on the number of Encompassing VJ Reads supporting the recombinations is so performed. A list containing all the identified clones is given at the end of this phase: The most supported VJ couple is here defined as that characterizing the main clone.

3.1.1 D alleles individuation

In order to identify for the most supported VJ couple the recombining D allele, only those mates belonging to a VJ encompassing read that don’t map totally on the V or J allele are retrieved.

These mates are aligned using Shrimp aligner (2.2.2) (Stephen, 2009) on the D genes. Once again a sorting based on the occurancy of each D allele is performed and the most supported allele considered as the recombede allele for the specific VJ couple.

4 RESULTS

In Figure 2 are shown the results obtained applying our pipeline on the two samples under study. Sub-figure A and B are respectively relative to the five
Figure 2: Supporting reads for the five clones most supported by reads. Subfigure A and B report respectively for Samples 1 and 2 on x-axis the number of reads supporting the VJ recombinations detected (darker bar) and the identified D allele (lighter bar) for the recombinations indicated on y-axis. The rectangular box highlights the main clone detected by the developed pipeline.

Table 1: V(D)J recombinations identified by different tools for the sequence of the main clone obtained via PCR in laboratory. Table a and b report respectively for Samples 1 and 2 the V, D and J assignments given by different tools to the sequence obtained in laboratory via PCR for the main clone.

(a) Sample 1

<table>
<thead>
<tr>
<th>Tool</th>
<th>V(D)J recombination</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMGT/Junction</td>
<td>IGHV3-34<em>01 IGHD3-22</em>01 IGHJ4*02</td>
</tr>
<tr>
<td>JOINSOLVER</td>
<td>IGHV3-34<em>01 IGHD3-22</em>01 IGHJ4*02</td>
</tr>
<tr>
<td>VDJsolver</td>
<td>IGHV3-34<em>01 IGHD3-22</em>01 IGHJ4*02</td>
</tr>
<tr>
<td>SoDA</td>
<td>IGHV3-34<em>01 IGHD3-22</em>01 IGHJ4*02</td>
</tr>
</tbody>
</table>

(b) Sample 2

<table>
<thead>
<tr>
<th>Tool</th>
<th>V(D)J recombination</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMGT/Junction</td>
<td>IGHV1-8<em>01 IGHD1-26</em>01 IGHJ6*03</td>
</tr>
<tr>
<td>JOINSOLVER</td>
<td>IGHV1-8<em>01 IGHD1-26</em>01 IGHJ6*03</td>
</tr>
<tr>
<td>VDJsolver</td>
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</tr>
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<td>SoDA</td>
<td>IGHV1-8<em>01 IGHD1-26</em>01 IGHJ6*03</td>
</tr>
</tbody>
</table>

clones most supported by reads detected in Sample 1 and in Sample 2. On the x-axis is reported the number of supporting reads for the recombinations indicated on the y-axis. In particular the darker bar represents the number of mates supporting the VJ rearrangements whereas the other the number of mates supporting the D allele detected for the considered recombination.

The main clone for each of the sample has been correctly identified as the one with the maximum number of supporting reads (see the rectangular boxes in Figure 2 A and B). Furthermore it can be noticed that for each of the analysed sample, the reported recombinations involve alleles belonging to the same V, D and J immunoglobulin subgroup. The observed feature allows to affirm that all the recombinations pointed out in Sample 1 and 2 describe in reality the same clone: The noticed behaviour, expected since the first alignment performed, can be indeed explained considering polymorphisms and homologies occurring in IGH genes (Li, 2002). Both polymorphisms and homologies introduce a bias in the alignment leading the same mate to be mapped on different genes.

In Samples 1 and 2 (see Figure 2 A and B) the J allele involved in the five most supported recombinations belongs respectively to IGHJ4 and IGHJ6 subgroup: In particular two specific members of these families can be distinguished that are IGHJ4*02 for Sample 1 and IGHJ6*03 for Sample 2. IGHV4 and IGHV1 subgroup are instead the reported alignments for the two analysed Samples. The D allele reported
in each of the presented graphic is that characterized by the highest score for the specific recombination after Shrimp alignment: The other D alignments were indeed supported by a not considerable number of reads. In both the Samples the D specific allele and not only a D subgroup is maintained along all the recombinations detected: IGHD3-22*01 for Sample 1 and IGHD1-26*01 for Sample 2.

In order to validate our identified main clone sequences, highlighted with a rectangular box in Figure 2 A and B, we verify if our V, D and J rearranged alleles were the same as those obtained by inserting the main clone PCR sequence in different online tools. We perfomed this comparison against four free available tools: IMGT/JunctionAnalysis (Monod, 2004), JOINSOLVER (Souto-Carneriro, 2004), VDJ-solver (Ohm-Laursen, 2006) and SoDA (Volpe, 2006).

As it is possible to note from Table 1, despite methods and algorithms implemented by the different tools are different, all of them agree about the V, D and J allele assignments of the sequence obtained in laboratory via PCR. The main clone detected by the above mentioned tools is for both the Samples the same we identified applying our pipeline.

5 CONCLUSIONS

The arising interest in understanding the correlation between a specific pathology and the detection of abnormal antibodies with the main purpose of promote the engineering of therapeutic antibodies conducted us to develop a new approach to the analysis of the V(D)J junction of mature B cells. Differently from the other available tools our pipeline aims at identify the recombinant V, D and J alleles by starting from a set of RNA-Seq paired-end reads rather than from a single sequence. The results obtained on two Samples of MCL, confirmed by several available tools on the main clone PCR validated sequence, conducted us to affirm that the implemented pipeline is capable to manage the typical sequence features characterizing V, D and J alleles in other words homologies and polymorphisms.

Future works will aim at validate the developed flow on other neoplastic datasets and than at identify the specific main clone sequence by considering all the enzymatic processes above mentioned acting during the VDJ recombination. We also intend to optimize the proposed algorithm in order to identify and characterize subclones or divergent clones in a neoplastic population and follow them up over time since it is worth noting that during lymphoma development the B cell repertoire can evolve.

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