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Original article

Identification of the B-cell tumor-specific molecular fingerprint using non-radiolabelled PCR consensus primers

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Summary

Background: The complementarity determining region 3 (CDR3) of the immunoglobulin (Ig) heavy chain variable region (V_H) is the most reliable molecular fingerprint for most if not all human B cells. The nucleotide sequence encoding for any B-cell tumor-specific V_H CDR3 is currently identified by PCR sequencing based on procedures involving the usage of either radioactive materials, patient/family-specific primers, or bacterial cloning.

Patients and methods: In six consecutive patients with follicular lymphoma we assessed the feasibility of a method that allows for identification of the tumor-specific V_H CDR3 using consensus primers while avoiding both radioactive materials and bacterial cloning procedures.

Results: The tumor-specific V_H CDR3 was successfully identified in all six patients in nearly half the time typically required by any other method currently utilized. The feasibility of the proposed method was not significantly affected either by the tumor-specific Ig isotype, or by the tumor infiltration in the original biopsy specimen. In the three patients for whom tumor specimen-derived hybridomas were available, the tumor-specific V_H CDR3 was also found in at least 8 of 10 of them.

Conclusions: The proposed method allows the ability to quickly identify the B-cell tumor-specific V_H CDR3 using consensus primers while avoiding radioactive materials and bacterial cloning procedures.

Key words: CDR3, consensus primers, follicular lymphoma, PCR, tumor-specific

Introduction

The nucleotide sequence encoding for the complementarity-determining region 3 (CDR3) of the human immunoglobulin (Ig) heavy chain variable region (V_H) is considered to be the best molecular fingerprint of any B cell capable of synthesizing an Ig [1]. Several studies and a number of applications are based on the elucidation of the V_H CDR3 nucleotide sequence, first and foremost those related to anti-idiotype (Id) vaccination for B-cell malignancies [2–4]. In fact, the identification of the tumor-specific antigen, that is the tumor-associated, Id-containing Ig, remains essential regardless of the nature of the Id vaccine under construction: be it either a soluble protein [5], Id-pulsed dendritic cells [6], or a DNA sequence [7]. In this context, the certainty of such identification relies on the sharp elucidation of the V_H CDR3 nucleotide sequence contained within the complementary DNA (cDNA) sequence peculiarly encoding for the whole tumor-specific V_H. However, most B-cell malignancy diagnostic samples typically contain a number of residual normal B cells. Each of these normal B cells may be characterized by a different synthesized Ig and, therefore, by a V_H CDR3 nucleotide sequence that is irrelevant to the tumor target and could hinder the polymerase chain reaction (PCR)-based definition of the V_H CDR3 clonally featured by the tumor cells. In particular, both genomic DNA and cDNA extracted from lymphoma specimens contain a percentage of tumor-specific, Ig-related V_H CDR3 nucleotide sequences that depend on the percentage of clonal tumor cells in the specimen.

Until now, the V_H CDR3 nucleotide sequence has been elucidated and/or monitored over time in a number of ways involving the use of either radiolabelled PCR consensus primers [8–9], cloning techniques [10–12], or patient-specific PCR primers [13–14]. Aside from the obvious need to standardize the methodology used in this process, we felt that the recent advent of the automatic sequencers, together with the availability of reliable PCR consensus primers might pave the way to developing a technique that avoids the use of both radioactive materials and procedures involving bacterial cloning.

So far, the most refined and best described strategy to elucidate the V_H CDR3 nucleotide sequence was published by Kobrin and Kwak in 1997 [8]. Again, it is based on the use of either ^{32}P- and/or ^{33}P-labelled PCR consensus primers.

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Patients and methods

Samples

Six relapsing FL patients underwent lymph node surgical biopsy as a preliminary idiotype vaccination procedure pending diagnosis confirmation. Indeed, one third of the lymph node was used for this purpose, while the remaining two thirds were mechanically transformed in a single cell suspension, aliquots of which were used for the molecular analysis of the tumor-specific V_H CDR3 nucleotide sequence.

Similarly, in the first three patients, hybridomas were produced by fusing the same, both lymph node specimen-derived tumor and normal cells with a heterohybridoma fusion partner (K6H6/B5), as previously described [15]. Then, ten hybridomas per patient underwent the same V_H CDR3 nucleotide sequence molecular analysis as the lymph node specimen-derived original cells.

V_H CDR3 isolation

Total RNA was extracted from both types of cell samples above, using the RNeasy Mini Kit (Qiagen, Santa Clarita, California) according to the manufacturer instructions. First-strand cDNA was then synthesized using 1 μg of total RNA and the cDNA Synthesis Kit for RT-PCR (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's standard procedures. The synthesis was performed in a PTC 225 thermal cycler (MJ Research, Watertown, Massachusetts) at the following temperatures: 25 °C for 10 minutes, 42 °C for 60 minutes and 99 °C for 5 minutes.

All PCRs were performed using 2.5 μls of cDNA, 50 ng of each primer, 1 × Buffer II (purchased, like all the following PCR ingredients from Perkin-Elmer, Foster City, California), 1.5 mM of $MgCl_2$, 0.2 mM of each dNTP and 2 5 U of AmpliTaq Gold, in a final reaction volume of 25 μls. The denaturation step was performed at 95 °C for 12 minutes, and was followed by 35 amplification cycles, each including denaturation at 94 °C for 30 seconds, annealing at 53 °C for 30 seconds and extension at 72 °C for 30 seconds. Finally, the conclusive extension was performed at 72 °C for seven minutes.

Ten μl of the PCR final product were analyzed by electrophoresis in a 1.5% agarose gel (FMC, Rockland, Maine), with 0.5 μg/ml of ethidium bromide in 0 5 × TBE buffer (Gibco BRL, Basel, Switzerland). In a limited number of cases, the visualized band was not as sharp as desired, but the problem was promptly solved by repeating the electrophoresis step in 2.5% MetaPhor agarose gel (FMC, Rockland, Maine), with 2 μg/ml of ethidium bromide in 1 × TBE buffer. The PCR product-related band was then picked up by aspiration in a 10 μl tip cut at the end and eluted overnight in 20 μls of the 1 × Buffer II above.

Four μls of the eluted PCR product were subsequently PCR re-amplified using the same reagents above in terms of proportions, but in a final reaction volume of 100 μl. The thermal cycler conditions were also the same as above, except for the number of amplification cycles, reduced to 20, and the annealing temperature, increased up to 60 °C.

V_H CDR3 sequencing and analysis

The amplified product was purified using the Qiaquick PCR purification kit (Qiagen, Hilden, Germany), according to the manufacturer instructions. Cycle sequencing was then performed using the BigDye terminator kit (Perkin-Elmer, Foster City, California), according to the manufacturer's standard procedures, and both the primers by which the PCR product had been obtained. Automated sequencing was carried out in the 377 ABI-PRISM sequencer (Perkin-Elmer, Foster City, California).

The alignment of all the electropherograms obtained from PCR products, based on the use of different couples of primers, was performed using the Sequence Navigator software (Perkin-Elmer, Foster City, California). Sequence analyses were finally carried out utilizing the IgBLAST program at the NCBI web site (http://www.ncbi.nlm.nih.gov/igblast).

Results

Elucidation of the V_H CDR3 nucleotide sequence

In order to maintain in an unchanged manner as many of their methodological improvements as possible, while further refining, simplifying and modernizing the overall strategy, we decided to develop our system to elucidate the V_H CDR3 nucleotide sequence using the very same PCR primers currently used in Dr Kwak's labs at the National Cancer Institute (Table 1). These primers have been designed to anneal to highly-conserved regions within framework regions (FR) 2, 3 and 4, as well as to regions belonging to the heavy chain constant region (C_H) 1, consistent with the isotype of the antibody in question [16].

The cDNA obtained from the primary, diagnostic lymph node specimen of six consecutive patients with follicular lymphoma (FL) was used as a PCR template. According to the designated consensus primer set (Figure 1), and depending on the known isotype of the tumor-associated Ig, a number of primer combinations ranging from 4 to 10 per patient was used in the PCRs. In particular, since typically not all primer combinations yield a PCR product suitable for sequencing, the number of combinations successfully used (Table 2), was aimed at obtaining identical, confirmatory DNA sequences from at least two fully-independent primer combinations. Moreover, as a strict requirement, the

Table 1. Primers used for V_H fragment amplification.

FR2S1	5'-GATGAATTCGTCCTGCAGGC(CT)(CT)CCGG(AG)AA(AG)(AG)GTCTGGAGTGG-3'
FR2S2	5'-GATGAATTCTGG(AG)TCCG(AC)CAG(GC)C(CT)(CT)C(ATGC)GG-3'
FR3S1	5'-GATGAATTCACAC(AG)GC(CT)(ACG)(CT)(AG)TATTA(CT)TGT-3'
FR3S2	5'-GATGAATTCACACGGC(CT)(GC)TGTATTATTGT-3'
FR3S3	5'-GATGAATTCACACGGC(CT)(GC)TGTATTACTGT-3'
FR4AS1	5'-GACG(AG)TG(AG)CC(AG)(GT)GGT(AC)CCTTGGCCCCA-3'
CRM	5'-CCAAGCTTAGACGACGGGGAAAAGGGTT-3'
CRG	5'-CCAAGCTTAGGG(CT)GCCAGGGGGAAGAC-3'
CRA	5'-GATAAGCTTGAGGCTCAGCGGGAAGACCTT-3'

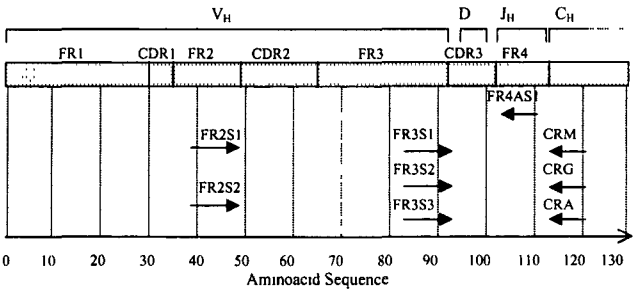

Figure 1 Schematic representation of PCR sense and anti-sense consensus primers. CRM – μ chain constant region anti-sense primer; CRG – γ chain constant region anti-sense primer; CRA – alpha chain constant region anti-sense primer.

four individual primers constituting these two primer combinations had to span different regions of the whole tumor-specific heavy chain rearranged nucleotide sequence. These regions (Figure 1) are represented by the FR2 and FR3 for the sense (S) primers (FR2S1, FR2S2, FR3S1, FR3S2 and FR3S3 in Table 1) and by the FR4 and the C_H1 for the antisense (AS) primers (FR4AS1, CRM, CRG and CRA in Table 1).

All patients' sequences were aligned using the Sequence Navigator software (Figure 2) and both their full analysis and the identification of the matching V_H CDR3 nucleotide sequences were performed using the IgBLAST program at the NCBI web site. Of course, the length of all six patients' V_H CDR3 nucleotide sequences fell within the known range of 2 to 26 aminoacids, which is 6 to 78 nucleotides [8] (Table 2). In addition, the number of non-clear-cut (N) nucleotides was remarkably low in all quality sequences (Figure 3), including those obtained from short-length PCR products, which classically tend to be mostly affected by reading difficulties within the first 20–40 nucleotides when using dye terminator cycle sequencing [17].

Concordance between tumor and tumor-derived hybridoma V_H CDR3

As further, indirect evidence for the tumor specificity of each V_H CDR3 nucleotide sequence identified by our new method, we compared those elucidated, starting from the primary diagnostic specimen, with those obtained from the corresponding hybridomas, available for the first three patients. The percentage of hybridomas that originate from tumor cells rather than from residual normal cells is always strictly dependent on the tumor cell infiltration within the original biopsy specimen. Therefore, the fact that at least 8 of 10 (Table 2) independent, stochastically-fused hybridomas shared the expected V_H CDR3 nucleotide sequence represents a further demonstration of the accuracy of the new strategy. The ability to identify the tumor-associated V_H CDR3 nucleotide sequence is mantained, even as the tumor cell infiltration within the diagnostic specimen falls as low as 50% (Table 2).

Result reproducibility regardless of tumor-specific Ig isotype

A primary requirement for any method used to elucidate tumor-specific V_H CDR3 nucleotide sequences is reproducibility, regardless of the corresponding Ig isotypes. The lack of this feature would make the entire strategy unreliable and consequently not routinely applicable, particularly in the setting of both idiotype vaccination [18] and minimal residual disease monitoring [19–20]. FL cells display a clonal IgM on their surface in nearly 50% of cases, followed by IgG in most of the remaining cases and only occasionally express IgA. Rarely, the tumor cell express' no Ig on its surface [21]. Our method accurately identified the tumor-associated V_H CDR3 nucleotide sequence in all six patients; the tumor isotypes were IgM in two cases, IgG in three cases, and IgA

Table 2. CDR3 nucleotide sequence of the six patients.

UPN	Isotype	NC	CDR3 Sequence	NH	TI (%)
1	IgM	6/10	5'-GGGGAAGTGGGAGCGGAT-3'	9/10	60
2	IgM	3/6	5'-GGTATATATTTCTGTGCGATAGAGGGCGACGGTGACTCCCATTGGGGCCC GGGGACT-3'	10/10	50
3	IgA	2/4	5'-TATGACGTCCGCCGACACGGGCGTTTATTATTGTGCGACHGGTCCCGGTCG HGAATCCAATCG-3'	8/10	50
4	IgG	4/4	5'-CTAAGATCGGAAGATACGGCTCGATATTTCGTTGCGGCCCAGAATGGGGGT GGTCTTGATCCC-3'	NA	85
5	IgG	2/4	5'-CTTAATTGTAGTAGTTGTCTACGGAATATGTTTAAGGGGTCTGGCTCGTACT ATAAC-3'	NA	70
6	IgG	3/4	5'-AAATTCCTCTGGGGATCCCCGTTATTCCTATTA-3'	NA	75

Abbreviations: NC – number of primer combinations successfully utilized (see text); NH – number of hybridomas sharing the tumor-specific CDR3 sequence; NA – not available; TI (%) – percentage of tumor infiltration in the diagnostic specimen.

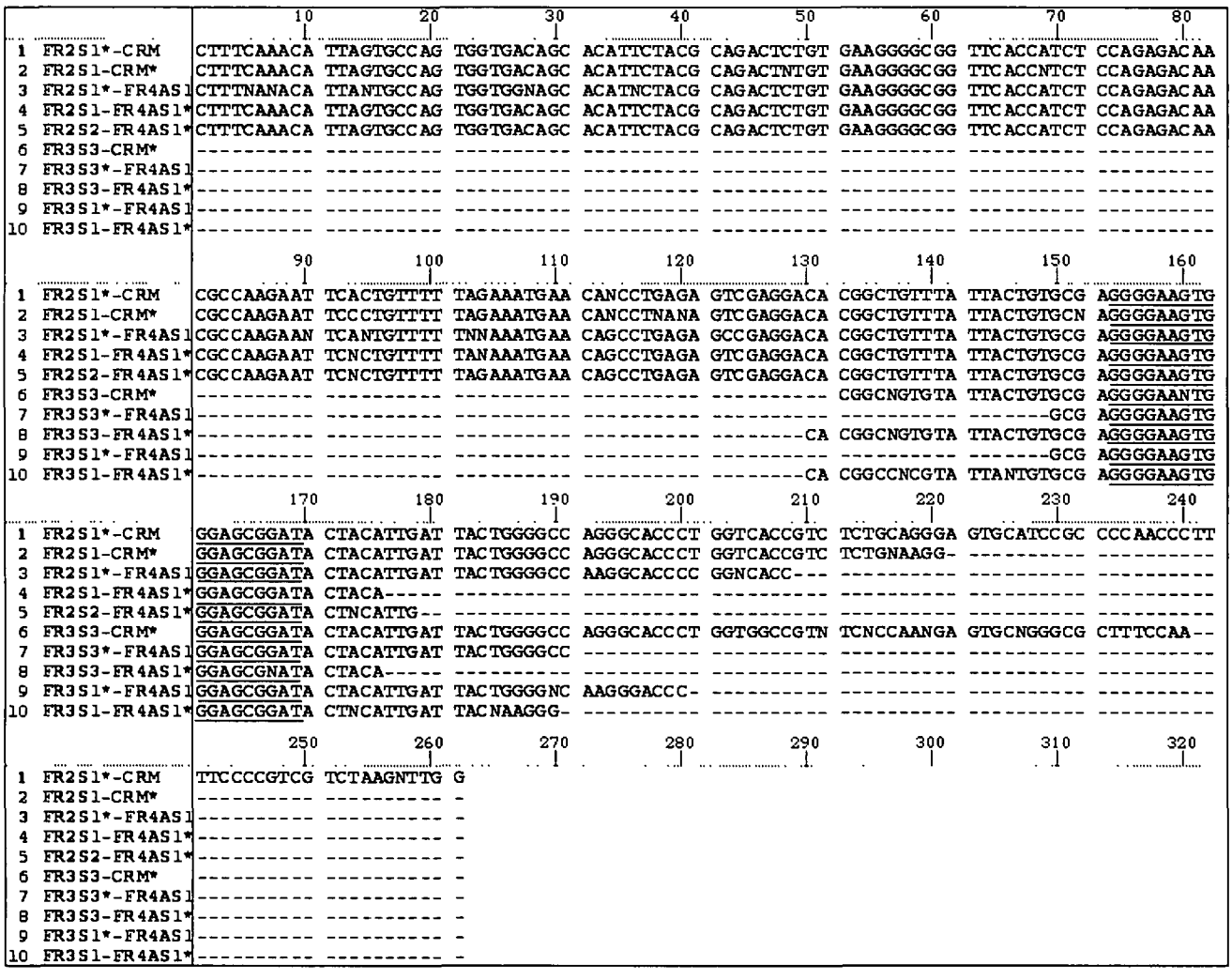

Figure 2. Alignment of IgBLAST program-analyzed nucleotide sequences all referring to a single, representative patient (patient no 1). All CDR3s are underlined for better magnification. The asterisks indicate the primers used for sequencing.

in one case (Table 2). We can therefore surmise that the C_H consensus primers used in Dr Kwak's labs in a radiolabelled-primer setting are similarly useful when used in our procedure.

Discussion

From a conceptual standpoint, the difference between elucidating the tumor-specific V_H CDR3 nucleotide sequence from an agarose- [4, 9] rather than from a polyacrylamide- [8] extracted PCR product band might appear small. Moreover, the former will contain a greater number of lg V_H CDR3 nucleotide sequences derived from the whole diagnostic specimen, regardless of their length, while the latter will only contain those sharing the unique length corresponding to that of the tumor-specific V_H CDR3 nucleotide sequence [8]. In other words, the former will be characterized by a definitely higher degree of contamination due to irrelevant V_H CDR3 nucleotide sequences. In fact, due to the higher resolution power characterizing the run of a PCR sample on a polyacrylamide gel, the PCR product band extracted and purified from it contains only all V_H CDR3 nucleotide sequences sharing the length of the tumor-specific V_H CDR3 [8]. On the contrary, most if not all PCR product bands extracted and purified from various types of agarose gel will contain at least some V_H CDR3 nucleotide sequences characterized by a different length. However, the method we have described here, based on an agarose-extraction and purification of the PCR product band, has yielded in a limited number of unselected patients an optimal rate of tumor-specific V_H CDR3 nucleotide sequence identifications in an easy and reproducible way, while avoiding the use of radioactive materials, patient specific primers, or bacterial cloning procedures. This relatively new method appears to make B-cell malignancy molecular fingerprinting less labor-intensive and time-consuming (Table 3) regardless of its applications, which include the monitoring of minimal residual disease. Finally, in terms of procedure impact on therapeutic strategies such as idiotype vaccination, this method makes it easier to identify the tumor-specific Ig, regardless of the subsequent use of

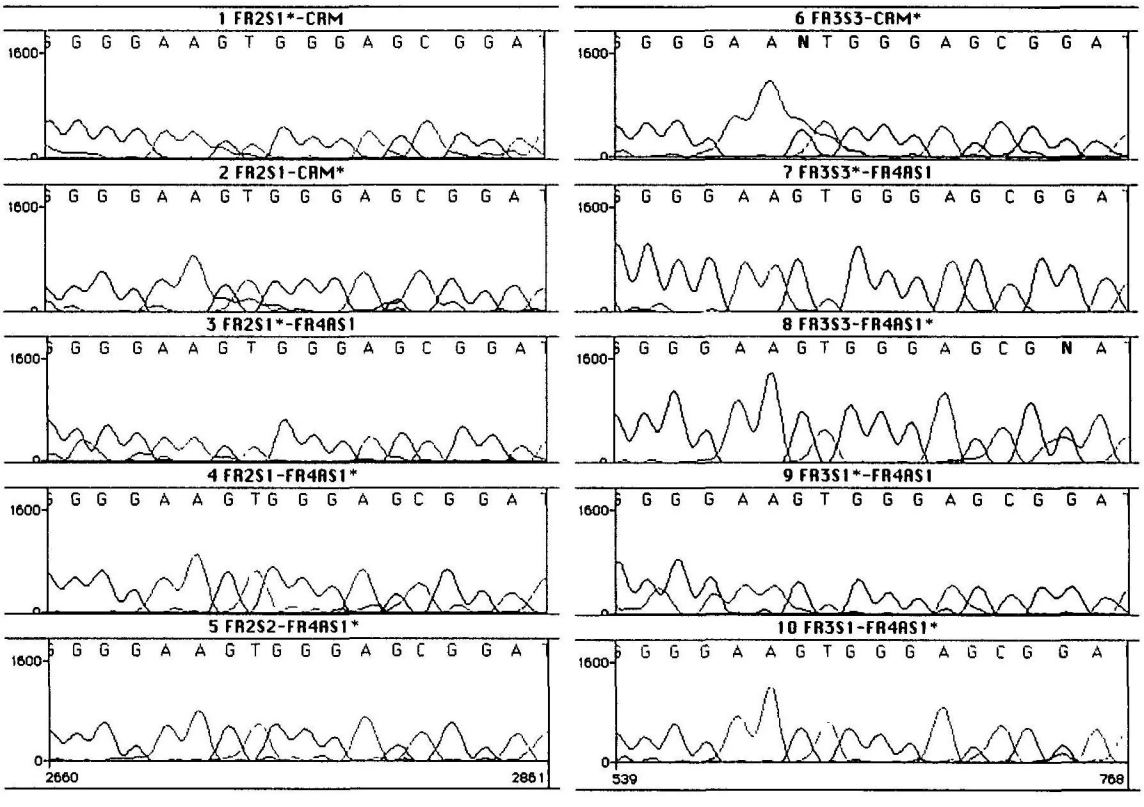

Figure 3. V_H CDR3 nucleotide electropherogram segments obtained from several primer combinations used for a single, representative patient (patient no. 1). The asterisks indicate the primers used for sequencing.

Table 3. Technical comparison between the new method and those previously published.

Method / Features	Hawkins et al[4]	Kobrin & Kwak[8]	Hsu & Levy[9]	Osterborg et al[10]	Trojan et al[12]	Davis et al[14]	New Method
ESTIMATED TIME REQUIRED	5-6 days	5-6 days	4-5 days	5-6 days	5-6 days	6-7 days	2-3 days
RADIOACTIVE MATERIALS	None	^{32}P and ^{33}P	^{35}S	None	None	^{35}S	None
BACTERIAL CLONING	Yes	No	No	Yes	Yes	No	No
PCR PRIMERS	V_H family-specific and consensus	Consensus only	V_H family-specific and consensus	V_H family-specific and consensus	V_H family-specific and consensus	V_H family-specific, consensus and patient-specific	Consensus only

this information and, therefore, of the nature of the idiotype vaccine core.

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