Immune Gene Rearrangements

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The tremendous diversity of the human immune repertoire, fundamental for the defense against highly heterogeneous pathogens, is based on the ingenious mechanism of immune gene rearrangements. Rearranged immune genes encoding the immunoglobulins and T-cell receptors and thus determining each lymphocyte's antigen specificity are very valuable molecular markers for tracing malignant or physiological lymphocytes. One of their most significant applications is tracking residual leukemic cells in patients with lymphoid malignancies. This so called 'minimal residual disease' (MRD) has been shown to be the most important prognostic factor across various leukemia subtypes and has therefore been given enormous attention.

Keywords: minimal residual disease ; IG/TR rearrangements ; real-time quantitative PCR ; next generation sequencing ; digital droplet PCR

1. Immunoglobulin and T-Cell Receptor Rearrangements

The remarkable ability of the human immune system to recognize and eradicate the enormous number of various antigens is based on the immune receptors [1]; the surface of T-lymphocytes is covered with the T-cell receptors (TR; TRαβ or TRγδ) and B-lymphocytes produce secreted or surface-bound immunoglobulins (IG). The tremendous diversity of the immune receptor variable domains is crucial for the specific molecular recognition of virtually any antigen [2]. Such a high degree of diversity is generated by combination of a limited number of gene segments. During this so-called somatic recombination, a DNA sequence that is unique for each lymphocyte is produced [3]. Hence, each lymphocyte bears many copies of the antigen receptor with a unique variable region that determines its antigen specificity.

1.1. Structure of the Immune Receptors

Both surface-bound and secreted immunoglobulins consist of two heavy chains (IGH) and two light chains (IGκ or IGλ), which are connected by a disulphide bond. The *IGH* gene complex consists of V ("variable") segments at the 5'-end, which are followed by a group of D ("diversity") segments and 6 short J ("joining") segments. The gene segments for the constant (C) part of the heavy chain are localized at the 3′-end of the gene complex and are responsible for the immunoglobulin class determination. IG light chains are either encoded by a kappa (*IGK*) or by a lambda (*IGL*) rearrangement. The structure of these complexes resembles the structure of the *IGH* rearrangements but is composed of less V and J segments and does not contain any D segments.

TR molecules are also composed of two chains, connected by a disulphide bond. The "classical" type of TR contains (TRα) and (TRβ) chains, whereas the "alternative" type contains (TRγ) and (TRδ) chains [4][5]. The variable domains of TRβ and TRδ contain all three types of gene segments (V, D, and J). TRα and TRγ chains lack D segments, similarly to immunoglobulin light chains. The gene complex for TRδ is localized within the gene complex for TRα (between the V and J segments). Any V-J rearrangement of the *TRA* gene segments therefore results in the loss of *TRD* gene segments, which means that complete *TRA* and *TRD* gene rearrangements can never be present on a single allele simultaneously (with the exception of combined *TRDV-TRAJ* rearrangements [6]).

1.2. Somatic (V-D-J) Recombination

Somatic recombination occurs in immature lymphocytes in primary lymphoid organs (bone marrow for B-lymphocytes, thymus for T lymphocytes). V, D, and J gene segments of IG and TR genes are rearranged and a DNA sequence, which is unique for each lymphocyte, is produced. V-D-J recombination of *IGH* and *TRB* genes is a two-step process starting with a D-J recombination followed by a V-D recombination. The recombination of the *TRD* locus starts with D-D recombination and continues with V-D and D-J recombinations. Genes for Igκ, Igλ, TRα, and TRγ are produced by a one-step V-J recombination. During each recombination a random number of gene segments is excised and the remaining segments are joined together. This gives rise to tens of millions of possible combinations of V, (D) and J segments.

This process occurs in parallel on both chromosomes. As soon as a productive rearrangement is formed, it is transcribed into mRNA. At that point, the recombination process on the second chromosome is stopped and therefore only one type of antigen receptor is produced by each lymphocyte (allelic exclusion) [7][8].

1.3. Junctional Diversity

The joining of V, D, and J gene segments is a very imprecise procedure: the ends of germline segments that are being joined are cleaved randomly [3]. Moreover, the enzyme terminal deoxynucleotidyl transferase randomly adds so called “non-templated nucleotides” (N-bases) at the junctions between the gene segments that are joined together.

These sequence-altering processes further and vastly increase the diversity of antigen receptors and their ability to recognize virtually all possible antigens.

1.4. Affinity Maturation

Naïve B-cells express unmutated IG genes, but after the recognition of antigen by B-lymphocytes in secondary lymphoid organs (lymph nodes), an enzyme called activation-induced cytidine deaminase introduces somatic hypermutations around the productively rearranged V(D)J junction and cells with higher affinity for the antigen are favoured [9][10][11]. This event, called affinity maturation, enables the production of immune receptors with very high affinity to the certain antigen [12].

1.5. Rearrangement Process during Lymphocyte Development

The regulation of the V(D)J recombination in B- and T-cells is accomplished by different accessibility of IG and TR gene due to cell-type specific chromatin structures [13][14]. The IGH D-J joining starts in the ‘common lymphoid progenitor’ stage and *IGH* V-D-J, *IGK* and *IGL* rearrangements are initiated in the pro-B cell compartment. Rearrangement of the *IGK* locus either leads to IgH/κ expression or is followed by *IGK* deletion and *IGL* rearrangement, potentially leading to IgH/λ expression. The successful assembly of IG genes plays a crucial role in guiding B-cell development: B-cells lacking the capacity to rearrange their IG genes are arrested in pro-B cell stage, but the introduction of a rearranged *IgH* transgene allows the cells to progress to a pre-B cell stage [15][16][17][18][19][20][21].

TR loci rearrange in a highly ordered way. D-D and V-D rearrangements of the *TRD* locus begin in a pro-T (DN1) stage, followed by D-J rearrangements of the *TRD* and V-J rearrangements of the *TRG* locus in pre/pro-T (DN2) stage [22][23]. This is either followed by TRγδ expression or by *TRB* rearrangement in the pre-T (DN3) stage (D-J rearrangement). The *TRB* rearrangement is completed in a pre-T (DN4) stage (V-D-J rearrangements) [24]. Lastly, *TRA* locus rearranges in a double-positive stage [23][25], which is potentially followed by TRαβ expression. Because the *TRD* locus is nested in the *TRA* locus (between *TRAV* and *TRAJ* gene segments), rearrangement of the *TRA* locus leads to the deletion of *TRD* genes. This mechanism ensures that a single cell can either express a TRαβ or TRγδ, but not both.

2. IG/TR Rearrangements in Leukemia

2.1. Leukemic Clones & Oligoclonality

The entire set of antigen receptors with different antigen specificities in one individual is called the immune repertoire. In humans it is believed to be 1011–1012 or higher [1][26]. Thanks to the above-described recombination process, each newly developed B- or T-lymphocyte carries a uniquely rearranged junctional region sequence coding its antigen receptors.

Lymphoid malignancies are clonal diseases. It is therefore commonly believed that all their cells are descendants of a single malignantly transformed B- or T-lymphocyte and that the entire malignant clone carries identical IG/TR V(D)J rearrangement(s). Consequently, the junctional region is considered as a ‘DNA fingerprint’ of each particular clone [27].

Despite the generally accepted monoclonal origin of acute lymphoblastic leukemia (ALL), already early studies using PCR and Southern blotting reported that up to 40% of the B-cell precursor ALL cases are oligoclonal at diagnosis with up to 9 leukemic rearrangements per patient [28][29][30][31][32]. More recently, highly sensitive modern techniques employing next-generation IG/TR sequencing provided evidence that the percentage of patients with oligoclonal IG/TR profiles and also the degree of oligoclonality might be considerably higher [33][34]. Interestingly, oligoclonality at diagnosis is present in 27% of T-ALL patients harboring a cross-lineage *IGH* rearrangement [35], but TR oligoclonality in T-ALL is rather rare [27].

An oligoclonal IG/TR rearrangements profile is a consequence of continuing rearrangement and secondary rearrangement processes via the active recombinase machinery in these immature lymphoid malignancies [27]. Besides, up to a quarter of CLL patients harbor multiple dominant productive *IGH* rearrangements [36][37]. Only one third of these

cases exhibit two clonal populations with distinct immunophenotypes [38], but each productive *IGH* rearrangement corresponds to a different B-cell clone also in immunophenotypically monoclonal cases [39].

2.2. IG/TR Rearrangement Profiles in Leukemias

Leukemic transformation leads to a cell differentiation arrest, which has direct impact on the IG/TR gene rearrangement configuration. Therefore, rearrangements in leukemic cells differ from the physiological repertoire and distinct rearrangement profiles can be identified according to age at diagnosis and genetic aberrations [40][41][42].

B-cell precursor ALL results from a leukemic transformation of a lymphoid precursor at an early stage of B-cell differentiation and it is therefore not surprising that over 80% of both adult and pediatric cases carry an *IGH* rearrangement and around 40% of them carry an *IGK* rearrangement [43][44]. As might be expected following the same logic, over 90% of T-ALL cases harbor a *TRB* rearrangement [45], over 80% a *TRG* and almost 70% (adults) or 40% (children) a *TRD* rearrangement [43][44]. Exceptionally, however, there are also ALL cases with all IG/TR loci in germline configuration—those are most probably derived from very immature progenitor cells.

Although cross-lineage rearrangements have not been detected in human thymocytes and their frequency in B-cells is very low (<0.5%) [46][47], these so-called illegitimate rearrangements have been identified in leukemic cells besides the lineage-consistent rearrangements: *IGH* rearrangements in 22% of T-ALL cases [35] and TR rearrangements in 80–90% of patients with B-ALL [48][49]. Cross-lineage rearrangements in B-ALL have several special characteristics, compared to regular rearrangements: *TRB* rearrangements contain particularly the most downstream Vb gene segments and solely the Jb2 segment, *TRG* rearrangements involve Jg1 segments in 70% of cases, and 80% of *TRD* rearrangements are represented by incomplete Vd2-Dd3 or Dd2-Dd3 junctions, which are rare in T-cells [44][48][50][51][52]. *TRB* rearrangements are virtually absent in pro-B-ALL and in infants, and patients with complete *TRB* gene rearrangements show a more mature IG/TR profile (higher frequency of *IGK*, *TRG*, and Vd2–Ja rearrangements) [53]. Remarkably, the frequency of cross-lineage Vd2-Dd3 rearrangements significantly decreases with age at diagnosis, while cross-lineage *TRG* rearrangements are rarely found in patients below 2 years of age [27][41]. In T-ALL, the cross-lineage *IGH* rearrangements are rather immature, as they are characterized by a high frequency of incomplete D-J rearrangements and frequent usage of most downstream Dh6-19 and Dh7-27 and most upstream Jh1 and Jh2 gene segments [35]. Cross-lineage rearrangements are rare in mature B- and T-cell malignancies, probably due to the absence of recombinase activity [54][55][56]. This corresponds with the reported decreasing incidence of cross-lineage *TRG* rearrangements in more mature B-ALLs: pro-B (57%), common (47%), pre-B (42%), and mature-B (0%) ALL [49]. Also, in more mature T-ALLs with biallelic *TRD* deletions and completed *TRA* rearrangements the *IGH* gene rearrangements are virtually absent [35]. In contrast to CLL, a mature B-cell malignancy, high incidence of non-coding/out-of-frame rearrangements was observed in ALL, suggesting that antigen selection pressure does not play a crucial role in ALL [57].

In CLL, so called stereotyped B-cell receptors are a common phenomenon. Their complementarity-determining region 3 (CDR3) sequences are closely similar (share structural features like V-gene, length, amino acid composition) among unrelated cases, suggesting that stimulation by (auto)antigens may play a role in CLL pathogenesis [58][59]. The *IGH*/*IGK*/*IGL* repertoires in CLL are biased and differ from repertoires in normal B-cells [60][61][62][63]. Additionally, certain V-segments (*IGHV3-21* and *IGHV1-69*) are associated with poor outcome [57]. Furthermore, presence of somatic mutations in variable heavy chain genes defines two CLL subtypes associated with a different clinical course. About half of CLL cases have more than 98% identity to the closest germline V-gene (“unmutated”), which corresponds to inferior outcome compared to patients with “mutated” CLL (less than 98% identity) [64][65].

2.3. Stability and Sensitivity of IG/TR Rearrangements as MRD Targets

Since IG/TR rearrangements are not directly related to the oncogenic process, they may vanish over time due to the outgrowth of subclones or ongoing and secondary rearrangements in leukemic blasts with active IG/TR recombination machinery. This might lead to an underestimation of MRD level if a rearrangement is only present in a small subclone, or even a false-negative MRD result if the rearrangement is fully lost during the disease course. It has therefore been recommended to use at least two leukemia-specific rearrangements to detect MRD in ALL to lower the risk of obtaining a false negative result [66].

Studies comparing the rearrangement profiles at diagnosis and during the disease course or at relapse are almost exclusively focusing on pediatric patients. It has been shown that oligoclonality at diagnosis is the most powerful predictor of ongoing clonal evolution in ALL: particularly in childhood BCP-ALL, significant differences in stability were observed between monoclonal and oligoclonal rearrangements: 89% of monoclonal vs. 40% of oligoclonal rearrangements are preserved at relapse [67]. In this study, roughly 85% of monoclonal *IGH* and *TRD* rearrangements remained stable

between diagnosis and relapse. Among monoclonal IGK-Kde rearrangements the percentage of stable targets is even higher (95%), probably due to their end-stage character [68]. A study comparing IG/TR profiles at diagnosis and relapse of B-ALL employing high throughput IG/TR sequencing confirmed that the overall stability of IG/TR rearrangements is rather low in (27% of clonal rearrangements were preserved), but also showed that the stability of large clones is way higher (84%) [69]. At relapse, the general characteristics of the IG/TR gene profiles are comparable to those at diagnosis but exhibit a lower degree of oligoclonality and more frequent *TRD* gene deletions, which fits with the hypothesis of ongoing clonal selection and continuing rearrangements [67]. In T-ALL, the IG/TR rearrangements profiles at diagnosis and relapse are more stable: 97% and 86% of TR rearrangements are preserved at relapse in adult and childhood T-ALL, respectively [70]. *TRD* rearrangements are the most stable ones (100% of rearrangements preserved at relapse), followed by *TRG* (89%) and *TRB* rearrangements (82%) [70].

Besides different stability during the disease course, IG/TR MRD targets also vary in sensitivity of the derived real-time quantitative polymerase chain reaction (RQ-PCR) assays. The sensitivity is primarily determined by the combinatorial and junctional diversity of the CDR3 regions. Therefore, RQ-PCR assays based on rearrangements from IG/TR loci that contain more V/D/J gene segments in their germline sequence (higher combinatorial diversity) generally have higher sensitivity. Similarly, complete rearrangements that contain D-segments (*IGH*, *TRB*, *TRD* V-D-J rearrangements) provide higher sensitivity than complete rearrangements without a D-segment (*TRG*, *IGK* rearrangements) and incomplete rearrangements. For example, complete *IGH* rearrangements represent the most sensitive group of targets, usually reaching the sensitivity of 10^{-4} [43]. Also, complete *TRB* rearrangements provide decent sensitivity thanks to their extensive junctional regions [70]. The lower combinatorial diversity in incomplete *TRB* rearrangements provides an explanation for slightly lower sensitivities in this group of targets [53]. In contrast, the sensitivity of *TRG* targets is usually considerably limited (a sensitivity of at least 10^{-4} is reached in less than half of the patients), owing to the restricted size of their junctional regions and the non-specific amplification of highly abundant polyclonal *TRG* rearrangements in normal T-cells [71]. Intriguingly, *TRG* rearrangements contain significantly higher number of inserted nucleotides and lower number of deleted nucleotides in T-ALL than in BCP-ALL, which seems to be the most important predictor for reaching good sensitivity [71].

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