ANALYSIS OF IMMUNOGLOBULIN HEAVY VARIABLE CHAIN REARRANGEMENT IN CHRONIC LYMPHOCYTIC LEUKEMIA PATIENTS AMONG CHORNObYl CLEAN-UP WORKERS

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Background: A number of epidemiological studies have shown an elevated radiation-associated risk for chronic lymphocytic leukemia (CLL). The aim of the paper was to analyze immunoglobulin heavy variable chain (IGHV) rearrangement and IGHV usage in CLL cases associated with ionizing radiation (IR) exposure. Materials and Methods: Samples of 76 clean-up workers of Chornobyl Nuclear Power Plant accident of 1986 (the main group) and 194 non-exposed patients (the control group) were analyzed. Two groups of CLL patients were comparable by gender (all patients were male), age, and place of residence (rural or urban). Results: Some features of IR-associated CLL cases as compared to CLL cases in patients without history of IR exposure were revealed. Among unmutated IGHV sequences, IGHV1 genes were less commonly used (29.4% vs 48.6%; p = 0.018), while the frequency of IGHV6 genes was higher (23.5% vs 10%; p = 0.029). The unmutated IGHV sequences did not use IGHD3-16 gene (0% vs 7.9%, p = 0.038). Mutated IGHV sequences were less frequently expressed IGHV3 genes (44% vs 68.5%; p = 0.037) due low representation of IGHV3-21 (4% vs 11.1%) and IGHV3-23 (0% vs 11.1%) genes; did not use IGHD3-22 gene (0% vs 18.5%, p = 0.025); and have signs of positive selection in the HCDR regions (Σ = 0.5029 ± 0.155 vs 0.0539 ± 0.14; p = 0.013). Conclusions: The revealed differences in IGHV gene usage and B-cell receptor structure in the main and the control groups of CLL patients indirectly indicate a change in the spectrum of antigens associated with CLL under IR exposure. The possible antigenic drivers associated with CLL associated with IR exposure are discussed. Key Words: chronic lymphocytic leukemia, ionizing radiation, immunoglobulin variable heavy chain (IGHV) rearrangement.

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The question of the radiogenicity of B-cell chronic lymphocytic leukemia (CLL) remains largely controversial, although a number of epidemiological studies have shown an elevated radiation-associated risk for CLL [1–3]. Besides, CLL is the most common form of leukemia among clean-up workers of Chornobyl Nuclear Power Plant accident [2, 4].

There is some evidence that CLL is antigen-driven disease: gene-expression profile indicating activation of signaling pathways through the B-cell receptors (BCR) [5], expression of similar BCRs (stereotyped cases) [6], efficacy of drugs inhibiting BCR signaling [7], etc. A restricted number of self- or environmental antigens contribute to the selection and promote proliferation of malignant clone [8, 9].

It is known that in addition to the tumorigenic effect, ionizing radiation (IR) increases the risk of somatic and immune system disorders, persistent viral infections [10–14]. We assume that the indirect effect of IR (through imbalance of the immune system, the appearance of excess autoantigens, viral and microbial antigens) could influence the selection of B-cell clones for subsequent transformation into CLL. In this case, we can expect a difference in BCR’s structure in IR-exposed CLL cases compared to spontaneous ones. Therefore, the aim of the paper was to analyze immunoglobulin heavy variable chain (IGHV) gene rearrangement and IGHV usage in CLL cases developed in patients exposed to IR.

MATERIALS AND METHODS

Patients. The study included 270 CLL patients referred to the National Research Center for Radiation Medicine, Kyiv, during 2002–2017. The study was approved by the local Ethics Review Committee, and all patients gave informed consent prior to participation in the study. The diagnosis of CLL was based on clinical history, lymphocyte morphology, and immunophenotypic criteria. Stage of disease was established according to Rai [15, 16] and Binet [17].

CLL patients were divided into two groups — IR-exposed CLL patients (the main group) and the control group of non-exposed CLL patients. The criteria for inclusion in the study were the participation in the accident recovery work in 1986 (for the main group), male sex (since all observed clean-up workers were men), and productive rearrangement of IGHV genes. Exclusion criterion for patients of the control group was any contact with sources of IR (participation in recovery operations after Chornobyl Nuclear Power Plant accident, residence in the areas contaminated with radionuclides, work at nuclear enterprises, etc.).

The main group consisted of 76 clean-up workers of 1986. In 8 patients, CLL developed within the first 10 post-accident years, in 34 patients — in 11–20 years after the accident and in 34 patients later than in 20 years.
after the accident. The age of patients at diagnosis ranged from 44 to 76 (median 56 years). The age of patients at the time of Chornobyl Nuclear Power Plant accident ranged from 17 to 60 (median 39 years).

Information about doses of irradiation was available from the Ukrainian State Registry of Chornobyl Catastrophe Sufferers for 32 CLL patients. The average dose was 26.76 ± 5.96 cSv (range 0.14–120 cSv, median 9.87 cSv).

The control group included 194 men aged 57.84 ± 0.67 years (median 58 years) (p = 0.986 in comparison with the main group). Urban residents predominated in both groups (88.2% in the main and 80.9% in the control group; p = 0.485).

**IGHV rearrangement analysis.** Genomic DNA for molecular analysis was extracted from peripheral blood mononuclear cells with the QIAamp Blood Mini Kit (Qiagen, UK). The IGHV gene mutational status was assessed by polymerase chain reaction amplification followed by a direct sequencing, as described above [18]. Sequences were analyzed using the IgBlast and IMGT databases. Sequences with ≥ 98% homology with the corresponding germ-line IGHV gene were considered as unmutated (UM), and cases with < 98% homology were considered to be mutated (M) [19, 20].

Analyzed IGHV sequences were presented in Genbank database under access numbers EF091900–EF091931; EF175394–EF175397; EF175401–EF175440; EF407822–EF407849; EF441744–EF441761; EF593659–EF593683; EU350410–EU350430; EU433859–EU433883; EU667594–EU667610; EU814955–EU814968; FJ694873–FJ694888; GU358656–GU358682; HM173322–HM173333; JF810256–JF810281; JQ928948–JQ928969; JX462741–JX462754; KC802095–KC802109; MN786464–MN786482.

Silent (S) and replacement (R) mutations were calculated per framework regions and complementarity-determining regions (CDRs). Absolute mutation counts were normalized by the nucleotide length of the corresponding region [21]. In M sequences, BASELINe statistics was used to determine if antigen selection occurred [22, 23]. Cases with stereotyped BCRs were identified according to A. Agathangelidis et al. [24].

Statistics were performed using the SPSS 20.0 software package (SPSS, Chicago, IL). All p values are two-sided, and p value < 0.05 was considered to be statistically significant.

**RESULTS**

**Mutational status of IGHV genes, usage of IGHV, IGHD, and IGHJ genes.** Cases with UM IGHV genes prevailed in both the main (67.1%) and the control groups (72.2%), p = 0.411. When UM sequences were subdivided into “truly unmutated” subgroup (100% identity to germ line IGHV gene), a “minimally mutated” subgroup (99% to 99.9% identity), and a “borderline mutated” subgroup (98% to 98.9% identity) according to F. Murray et al. [21], no significant differences between the groups were found (p = 0.313).

Without taking into account the mutational status of IGHV gene, a lower frequency of IGHV1 genes (p = 0.036) was detected in the main group.

Among M sequences, the frequency of IGHV3 genes was lower in the main group (44% vs 68.5% in controls; p = 0.037) (Supplement* Table1). It was mainly driven by low representation of IGHV3-21 and IGHV3-23 genes (p = 0.042). Among UM sequences, the frequency of IGHV3 genes was higher (51% vs 31.4%; p = 0.013), and the frequency of IGHV1 genes was lower (29.4% vs 48.6%; p = 0.018) in the main group than in the control. If the frequency of IGHV1-69 gene did not differ in the groups of patients (p = 0.422), the other UM IGHV1 genes were actually absent in the main group (2% vs 15% in controls; p = 0.012).

**IGHD genes** were identified in 262 cases (97%). We found an increased frequency of IGHD6 genes in the main group among UM sequences (23.5% vs 10%; p = 0.029), but not among M sequences (26.1% and 20.8%; p = 0.847).

Among M sequences IGHD3-22 gene was absent in the main group, but was the most frequent in the control (10 cases; 18.5%), p = 0.025. Among UM sequences IGHD3-16 gene was absent in the main group, but was the most frequent in the control (11 cases; 7.9%), p = 0.038.

No significant differences in the frequency of IGHJ genes usage between groups of CLL patients were found (p = 0.303).

**Mutational pattern of M IGHV gene.** The frequency of mutation was assessed for HCDR1, HCDR2, HFR2, and HFR3 regions. Since the primers used for amplification were located in the HFR1, this region was not analyzed. In total, 316 mutations in the main group and 790 mutations in the control group were found. The largest number of mutations was in the HFR regions (63.3% and 64.2%; p = 0.835). However, the normalized mutation rate was higher in the HCDR regions compared to the HFR regions (0.191 ± 0.015 vs 0.087 ± 0.011; p < 0.0001 in the main group, and 0.221 ± 0.015 vs 0.105 ± 0.008; p < 0.0001 in the control group).

Overall, 77.8% of mutations in the main group, and 70.7% in the control group induced an amino acid change. The R/S ratio in the HCDR regions was higher in the main group (3.51 ± 0.303). However, the normalized mutation rate was higher in the HCDR regions compared to the HFR regions (0.191 ± 0.015 vs 0.087 ± 0.011; p < 0.0001 in the main group, and 0.221 ± 0.015 vs 0.105 ± 0.008; p < 0.0001 in the control group).

In the control group, there were no signs of selection in HCDR regions (Σ = 0.054 ± 0.14; p > 0.1), whereas the HFR regions were under negative selection pressure also (Σ = −0.594 ± 0.126; p < 0.005). Differences in selection strengths in the HCDR regions between group of patients were significant (p = 0.013) (Figure).
A clustering of R amino acids in HCDR1 and HCDR2 was found in 72.9% of M cases in the main group (23.7% among all cases) and in 44.4% of M cases in the control group (12.7% among all cases) \((p = 0.022)\).

Prevalence of transitions over transversions within the M sequences was observed in both groups (in the main group: \(7.16 \pm 0.69\) vs \(6.0 \pm 0.58\); \(p < 0.0001\), and in the control group \(8.92 \pm 0.59\) vs \(6.41 \pm 0.57\); \(p < 0.0001\)). The differences between groups of patients in the number of transitions \((p = 0.118)\) and transversions \((p = 0.664)\) were not significant.

When mutations were distributed along the gene, the following differences between the main and control groups of patients were revealed:

1. In patients of the main group fewer mutations were present in positions from 109 to 114 in the HCDR1 compared to the control: six in 25 sequences (four R and two S) vs 32 in 54 sequences (23 R and 9 S); \(p = 0.007\) in comparison of total number of mutations; \(p = 0.039\) in comparison of R mutations. This resulted in a lower frequency of amino acid substitutions in the last codons of HCDR1 in the main group (7.5% and 25% among total number of amino acid changes, correspondingly; \(p = 0.040\)). At the same time, the nature of amino acid changes in the HCDR1 (very dissimilar, dissimilar, similar, very similar, according IMGT) as well as in the HCDR2, HFR2, HFR3 regions did not differ between groups of CLL patients (data not presented).

2. Positions from 133 to 135 and from 163 to 165 in HFR2 were less susceptible to the mutation process in CLL patients of the main group. In the first case, only one R mutation was identified in 25 sequences from the main group compared to 14 mutations (11 R and 3 S) in the control group \((p = 0.045)\). In the second case, five mutations (3 R and 2 S) were revealed in the main group and 26 (18 R and 8 S) in the control group \((p = 0.032)\).

3. A low mutation frequency in HFR3 region was detected at positions 259–261 in the main group (1 R and 1 S) compared to the control (10 R and 13 S), \(p = 0.004\).

All M IGHV3 genes were checked for amino acid changed involved in superantigen recognition according to Silverman and Goodyear [25, 26]. In the main group, five of 11 sequences had single (4) or two (1) amino acid changes. In the control group, 10 of 37 sequences had single (8) or two (2) amino acid changes. Differences between groups were non-significant \((p = 0.247)\).

When impact of somatic hypermutation (SHM) on N-glycosylation sites was analyzed, no differences were found between groups of CLL patients. Germline-encoded Asn-His-Ser motif at codons HCDR2 57–59 of IGHV4-34 was abrogated by SHM in three of 10 sequences in the control group and in one of 4 sequences in the main group. New N-glycosylation sites generated by SHM were identified in three cases of the control group (in HFR3 region of IGHV4-34, g270>t, K90>N; HCDR1 region of IGHV3-15, g113>c, W38>S; and HCDR2 region of IGKV3-23, g187>a, G63>S), and in one case of the main group (in HFR3 region of IGHV4-34, c205>t, P69>S).

**Structure of HCDR3 region.** There were no differences in the HCDR3 length between the main and the control group \((p = 0.542)\) on the whole; \(p = 0.338\) in M sequences, and \(p = 0.491\) in UM sequences. The HCDR3 length was longer in UM cases compared with M cases \((p = 0.001)\) in both groups, but the differences between the subgroups “truly unmutated”, “minimally mutated” and “borderline mutated” UM sequences were not significant \((p = 0.257)\) in the main group and \(p = 0.253\) in controls.

A statistically significant negative correlation between the HCDR3 length and the number of mutations was revealed \((r = -0.449, p = 0.001)\) in the main group, and \(r = -0.446, p = 0.001\) in controls). In UM sequences, the HCDR3 length was longer in IGKV1-69 cases compared with the HCDR3 length in other IGKV1 genes (21.4 ± 0.43 a.a. vs 18.23 ± 1.03 a.a., \(p = 0.001\) in controls; 13 a.a. vs 20.85 ± 0.73 a.a., \(p = 0.016\) in the main group).

Cluster analysis of the HCDR3 region allowed us to recognize 14 of 76 cases (18.4%) with stereotyped BCRs in the main group and 44 of 194 cases (22.7%) in the control group (Supplement Table 3). Differences between groups were non-significant \((p = 0.539)\). The frequency of stereotyped cases in the groups of patients also did not differ among M sequences \((20\% \) in the main group and 15.7% in controls; \(p = 0.638\)) and among UM sequences \((16.6\% \) and 25.0%, correspondingly; \(p = 0.333\)). The most frequent in the control group were subset № 1, 2, and 6 (each subset was represented by four cases). In the main group, all subsets were represented in one case, with the exception of subset № 9 (two cases).

The most represented IGHV genes in stereotyped cases were: IGHV4-34 (3 cases), IGKV1-69 (3 cases), IGKV3-21 (2 cases), and IGKV3-48 (2 cases) — in the main group; IGKV1-69 (17 cases), IGKV1-2 (5 cases), IGKV3-21 (4 cases), and IGHV4-34 (3 cases) — in the control group. Differences between groups were non-significant \((p = 0.399)\). The same clusters were identified for both the main and control groups, namely, subset № 1 (7.1% and 9.1%, correspondingly), № 2 (7.1% and 9.1%), № 3 (7.1% and 4.5%), № 4 (7.1% and 2.3%), № 16 (7.1% and 2.3%), № 202 (7.1% and 2.3%), and № 64B (7.1% and 2.3%). The subset № 6 was absent in the main group, but was present in the control (four cases, 9.1%). Opposite, the subset № 9 was present in the main group (14.3%), but was absent in the control. Two new subsets were identified in the control group. One case (GU358667) have 80% homology of HCDR3 with our

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**Figure.** Selection strengths in the HCDR and the HFR regions in groups of observed CLL patients.
sequences 1-2*02_3-22*01_4*02_UM_FJ694875 (female CLL patient, not included in this study) and 73.3% homology of HCDR3 with public 5-51*01_3-22*01_4*02_UM_EU099233. Three cases with IGHV1-69/IGHD3-3/IGHJ6 rearrangement and 76.1% of HCDRs homology formed additional new subset.

M sequences with stereotyped HCDR3 had weak signs of positive selection in the HCDR regions (\( \Sigma = 0.3337 \pm 0.206 \) in the main group and \( \Sigma = 0.1221 \pm 0.570 \) in controls; \( p = 0.783 \)). In contrast, non-homologous M sequences in the main group revealed positive selection in the HCDR regions (\( \Sigma = 0.5452 \pm 0.187 \)), while in the control signs of selection were absent (\( \Sigma = -0.0867 \pm 0.144 \), \( p = 0.013 \)).

Applying homology criteria described in «Patients and methods,» HCDR3 homology with various autoantibodies clones was found in eight UM cases of the control group (4.1%) and one UM cases of the main group (1.3%). Differences between groups of patients were insignificant (\( p = 0.245 \)). Homology to rheumatoid factor PH0955 was identified in three cases (KC802106 in the main group; EU350412 and EF175418 in the control group). All cases from subset № 6 (the control group) demonstrate considerable HCDR3 homology with an antiacridiolipin antibody AF460965. HCDR3s of cases HM173323 and EF175415 (the control group) were similar with HCRD3s of heterohybridoma derived from CD5− CLL B lymphocytes with rheumatoid factor activity (U86795) and natural human anti-Sm autoantibody Z46379, correspondingly.

In the main group, similarity between HCDR3s of EF091900 M case and B-cell of hepatitis C virus patient (KJ409179) was found. In the control group, HCDR3 homology with anti-viral and anti-bacterial immunoglobulin’s (Ig) clones was revealed in four cases: the JX462746 UM case was homologous to anti-tetanus toxoid (JN110652); EF407840 UM case had homology to AY944710 rotavirus-specific lgs; GU358657 UM case had homology to Y1778 herpes virus-specific lgs; and HM173331 UM case was homologous to lgs directed against respiratory syncytial virus (MG524026). None of the M CLL cases showed clear signs of homology with lgs of known specificity.

**DISCUSSION**

In this paper, we compared IGHV sequences of the leukemic cells from CLL patients among Chernobyl clean-up workers and CLL patients without history of IR exposure (control group). All examined persons were the permanent residents of Ukraine. Two groups of CLL patients were comparable by gender, age, place of residence (rural or urban). Unfortunately, data on previous diseases, lifestyle, and occupations were known only for few patients and these data were not included in the analysis.

It was revealed that CLL patients with history of IR exposure compared with non IR-exposed CLL patients of the control group have some features of the IGHV rearrangement compared with non IR-exposed CLL patients of the control group, namely:

- **UM sequences less commonly use IGHV1 genes** (except for the IGHV1-69 gene), more frequently were associated with IGHD6 genes, but did not use IGHV3-16 gene that was the most common IGD gene among UM cases in the control group of CLL patients without the history of IR exposure;
- **M sequences are less frequently expressed in IGHV3 genes and did not use IGHV3-22 gene that was the most common IGD gene among M cases in the control group of CLL patients, and have signs of positive selection in HCDR regions;**
- **There were differences in distribution of mutations in HCDR1, HFR2 and HFR3 regions.**

Earlier we studied IGHV rearrangement in IR-associated CLL patients, but the groups of examined patients were significantly smaller and more heterogeneous, as they included not only clean-up workers, but also the residents of contaminated territories, evacuees, and female patients [18]. However, we also noted a downward trend to UM IGHV1 (35.9% vs 50.5% in the control) and M IGHV3 (44.1% vs 65.9%, correspondingly) genes usage. In our opinion, identified differences suggest that antigenic determinants involved in BCR-mediated stimulation of CLL cells in patients with and without IR exposure in their history may be slightly different. The question of the nature of such antigens remains unresolved.

It is known that CLL cells with UM IGHV genes recognize epitopes associated with apoptosis and oxidation [27, 28]. Imbalance of lipid metabolism, the accumulation of oxidized lipoproteins, and the appearance of apoptotic cells accompany chronic inflammatory diseases, including atherosclerosis. Oxidation-specific epitopes are dominant targets of innate natural antibodies in mice and humans [29]. In CLL, the intensity of IGHV binding with such antigens depends on the structure of HCDR3 region. According to R. Catera et al. [27], CLL sequences belonging to subsets № 1 and № 28 predominantly reacted with low-density lipoproteins modified by malondialdehyde, whereas the sequences of subset № 9 reacted better with apoptotic cells. The sequences of subset № 6 showed a weak reaction with both apoptotic cells and low-density lipoproteins modified by malondialdehyde. No reactions were observed with sequences of subset № 2 and heterogeneous M IGHV4-34 sequences. Strong binding of immunodominant oxidation-specific epitopes by UM IGHV1-69 belonged to subset № 7H was found additionally [30]. However, in our study, differences in the frequency of these subsets in the main and control groups were not significant. Only one difference was evident when comparing the two groups of CLL patients — a significant reduction in IGHV1 gene frequency, except IGHV1-69. These "other" UM IGHV1 genes (IGHV1-02, 1-08, 1-18, 1-24, 1-46, 1-58) had a short the HCDR3 region. We do not exclude that a) their antigenic reactivity differs from the reactivity of other UM IGHV genes; b) in the group of IR-exposed CLL patients, the response to such antigens is impaired or these antigens are less common drivers for the selection and proliferation of malignant clone.

The second group of antigens, presumably involved in the pathogenesis of CLL, is represented...
by superantigens. CLL cells are often compared with B1 lymphocytes [31–33]. One of the properties of B1 cells is to provide an early immune response to T-independent antigens [34]. Bomben et al. [35] described CLL cases with IGHV3-23 expression and suggested that IGHV3-23 sequences involved in superantigen binding via residues located outside the conventional antigen-binding sites. In their study, expression of IGHV3-23 gene, mostly M, was found in 134 of 1426 (9.4%) of the sequences. In study of Murray et al., IGHV3-23 was one of the most common genes among M cases (12.1%) [21]. In our study, the frequency of IGHV3-23 gene usage among M IGHV genes in the control group (11.1%) was comparable with literature data. The lack of MIGHV3-23 expression in the main group may indicate a reduced effect of antigens causing the expansion of this gene. At the same time, the preservation of conservative binding sites in the amino acid sequence coding by IGHV3 genes indicates that, in general, the response to superantigens in the main group of CLL patients was not impaired.

Of particular interest is the increase in the number of heterogeneous M sequences with signs of positive antigenic selection in the HCDR regions in CLL patients with history of IR exposure. Such sequences may reflect immune response on several viral or microbial antigens [36]. The associations of CLL with bacterial [28] and viral infections (hepatitis C virus, cytomegalovirus — CMV) [37–41] have been discussed. On the other hand, we previously showed that clean-up workers of Chornobyl Nuclear Power Plant accident had a significantly greater incidence of CMV infection and hepatitis C virus seropositivity as compared with the control group of IR-non-exposed persons [14].

However, we did not reveal a clear homology of HCDR3 regions of M CLL cases with Ig sequences directed against viral or bacterial antigens. Nevertheless, the reactivity of such sequences toward different infectious agents cannot be excluded. A possible explanation may be that immunization of healthy individuals is also accompanied by the appearance of antigen-experienced B memory subsets and accumulation of highly M Ig genes with unique HCDR3s even toward the same antigenic epitope [46]. In study of Kostareli et al. [39], the simultaneous presence of CMV and Epstein — Barr virus DNA assessed by real-time polymerase chain reaction in CLL patients was strongly associated with IGHV4-34—expressed cases belonging to the subsets N° 4 and N° 16. Nevertheless, above-mentioned CLL sequences did not reveal homology with anti-viral antibodies according to IgBlast database.

Based on all these data, we suggest that the detected CLL cases expressing M IGHV genes with signs of positive selection in HCDR regions can be associated with persistent infectious processes. The frequency of such cases is significantly higher among IR-exposed CLL patients compared with CLL patients without IR exposure in their history. However, the exact nature of viral or microbial antigens that may be CLL-drivers under IR exposure has not been established in our study.

In summary, the revealed differences in IGHV gene usage and BCR structure in the main and the control groups of CLL patients indirectly indicate a change in the spectrum of antigens associated with CLL under IR exposure.

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