

Peripheral neuropathies in chronic lymphocytic leukemia: a single center experience on 816 patients

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SUPPLEMENTARY METHODS

Prognostic factors were evaluated on fresh sample or on purified frozen chronic lymphocytic leukemia (CLL) cells harvested in dimethyl sulfoxide (DMSO), collected before chemo-immunotherapy according to recent guidelines¹.

Fluorescent in situ hybridization

Fluorescent *in situ* hybridization (FISH) was performed on standard cytogenetic preparations from peripheral blood. The slides were hybridized with the multicolor probe sets LSI p53/LSI ATM, LSI D13S319/LSI 13q34/ CEP12 and RP11-17708 (Vysis-Abbott, Des Plaines, IL, USA) according to the manufacturer's protocol. Three hundred interphase nuclei were analyzed for each probe. According to the literature, cut-off for positive values (mean of normal control ± 3 standard deviation) was 4% for centromere 12 trisomy, and 10% for deletion of 11q22.3, 13q14.3 and 17p13.1^{2,3}.

Immune globulin heavy chain variable region mutation (IGHV)

To perform IGHV studies, RNA was extracted from 2×10^6 B cells using the RNeasy™ Total RNA kit (Qiagen) and reverse transcribed using the SuperScript™ Preamplification System for first-strand cDNA synthesis (Life Technologies, Inc.). The CLL cell VH gene family was assigned as previously described³ using a sense VH family-specific framework region (FR) primer in conjunction with the appropriate antisense CH primer. VH gene sequences were determined by amplifying 5 μ l of the original cDNA using the appropriate VH leader and CH primers. PCR products were sequenced directly after purification with Wizard PCR Preps (Promega, Madison, WI) using an automated genetic analyzer (3130 ABI Applied Biosystems, Foster City, CA, USA). Sequences were analyzed using IMGT/VQUEST and BLAST software⁴ to detect VDJ junction. Sequences homology $\leq 98\%$, from the corresponding germline gene, were considered mutated, as opposite to unmutated cases^{3,5}.

CD38 expression

Analyses of CD38 expression on CLL cells was carried out by incubating whole blood with 5 μ l of the following antibodies: anti-CD5 FITC (BD Biosciences, New-Jersey, USA), anti-CD38 PE (BD Biosciences, New-Jersey, USA), and anti-CD19 RPECy5 (BD Biosciences, New-Jersey, USA), for 20 minutes and at least 100,000 events were counted. Each sample was run with the appropriate isotype control antibody to define the negatively stained cells. The percentage of CD38⁺ cells was defined as the percentage of CD19⁺ CD5⁺ that were CD38⁺. The threshold for CD38 expression was set at 30%; values $\geq 30\%$ were defined as CD38⁺ and $< 30\%$ as CD38⁻^{3,6}.

ZAP70 expression

Cytoplasmic ZAP70 expression was determined by flow cytometry. Permeabilized cells were analyzed with the anti-ZAP70 antibody Alexa Fluor 488 (Caltag, Buckingham, UK), anti-CD3-phycoerythrin (PE), anti-CD56-PE (BD Biosciences, New-Jersey, USA), anti-CD19-peridinin chlorophyll protein-cytochrome 5,5 (Caltag, New-Jersey, USA) and anti-CD5 APC (BD Biosciences, New-Jersey, USA). After appropriate lymphocyte gating, cytoplasmic ZAP70 expression was determined in CD19⁺ CD5⁺ CLL cells. The threshold level for ZAP70 was set at 20%^{3,7}.

Statistical analysis

Categorical variables were compared by Chi-square (Stage and FISH) and Fisher exact test (all the other variables), when indicated, while continuous variables were compared by Mann-Whitney test. Time to peripheral neuropathy (PN) (TTPN) and overall survival (OS)

were calculated from the date of CLL diagnosis to PN or death (event), respectively, or last available follow-up (censored). Survival analyses were performed by Kaplan-Meier method and Log-rank test was used to compare OS curves between groups. Statistical analysis was performed with R (an open source statistical package downloadable from <http://www.r-project.org>).

Neurological and Neurophysiological evaluation

Neurologic assessment included strength, sensory, gate and cranial nerve evaluation; neurophysiological evaluation included electromyography, motor and sensory nerve conduction velocity, and short-latency somatosensory-evoked potentials.

Electrophysiological investigation included motor and sensory nerve conduction studies (NCS) and needle electromyography (EMG).

Compound motor action potential (CMAP) amplitude, motor conduction velocities (MCV) and distal motor latency of median, ulnar, deep fibular and tibial nerves were performed. Presence of conduction blocks were ascertained at and outside the compression sites.

Sensory nerve action potential (SNAP) amplitude and sensory conduction velocities (SCV) of median, ulnar, radial, sural and dorsal sural nerves (the most distal branch of sural nerve) were performed bilaterally.

EMG was performed using a concentric electrode: spontaneous activity, motor unit potential configuration, and recruitment were scored semi-quantitatively. The skin temperature was maintained at $\geq 32^{\circ}\text{C}$ throughout the study.

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