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Analysis of *NOTCH1* mutations in monoclonal B-cell lymphocytosis

Monoclonal B-cell lymphocytosis (MBL) represents asymptomatic monoclonal B-cell expansions characterized by a chronic lymphocytic leukemia (CLL) phenotype, but with less than 5.0×10^9/L circulating cells.[1-3] Clinical MBL (cMBL) is recognized during the diagnostic workup of an asymptomatic lymphocytosis.[4-6] Although the molecular pathogenesis of MBL is little known, the biological indolence of this condition is documented by the rare occurrence of genetic lesions predicting poor prognosis in CLL, such as *TP53* and *ATM* disruption.[4-6]

Recently, two independent investigations of the CLL coding genome have revealed that activating mutations of the *NOTCH1* proto-oncogene occur in approximately 10% CLL at diagnosis and their frequency increases in advanced disease phases, exemplified by the case of Richter syndrome.[7,8] Initial evidence suggests that *NOTCH1* alterations might predict an unfavorable clinical outcome in CLL. The prevalence of *NOTCH1* mutations in MBL is currently unknown.[7-11]

Here we investigated the occurrence of *NOTCH1* mutations in 63 consecutive cMBL presenting at our clinic for the initial evaluation of an asymptomatic lymphocytosis. The cMBL cohort was provided with prospectively collected peripheral blood mononuclear cell samples drawn at presentation, and with a prospectively maintained clinical database. All cMBL were analyzed for *NOTCH1*, *TP53* and *IGHV* mutations by DNA Sanger sequencing, and for FISH karyotype using the LSI13 and LSID13S319, CEP12, LSIp53 and LSIATM probes (Abbott, Rome, Italy).[5,7] A *NOTCH1* mutation (c.7544_7545delCT) that is known to be highly recurrent in CLL was also independently investigated by amplification refractory mutation system (ARMS) PCR. Patients provided informed consent in accordance with local IRB requirements and the Declaration of Helsinki. The study was approved by the Ethical Committee of the Ospedale Maggiore della Carità of Novara, Northern Italy, associated with the Amedeo Avogadro University of Eastern Piedmont (Protocol Code 59/CE; Study Number CE 8/11).

The clinical profile of the cMBL cohort was representative of this condition. Median age was 68 years (range 40-

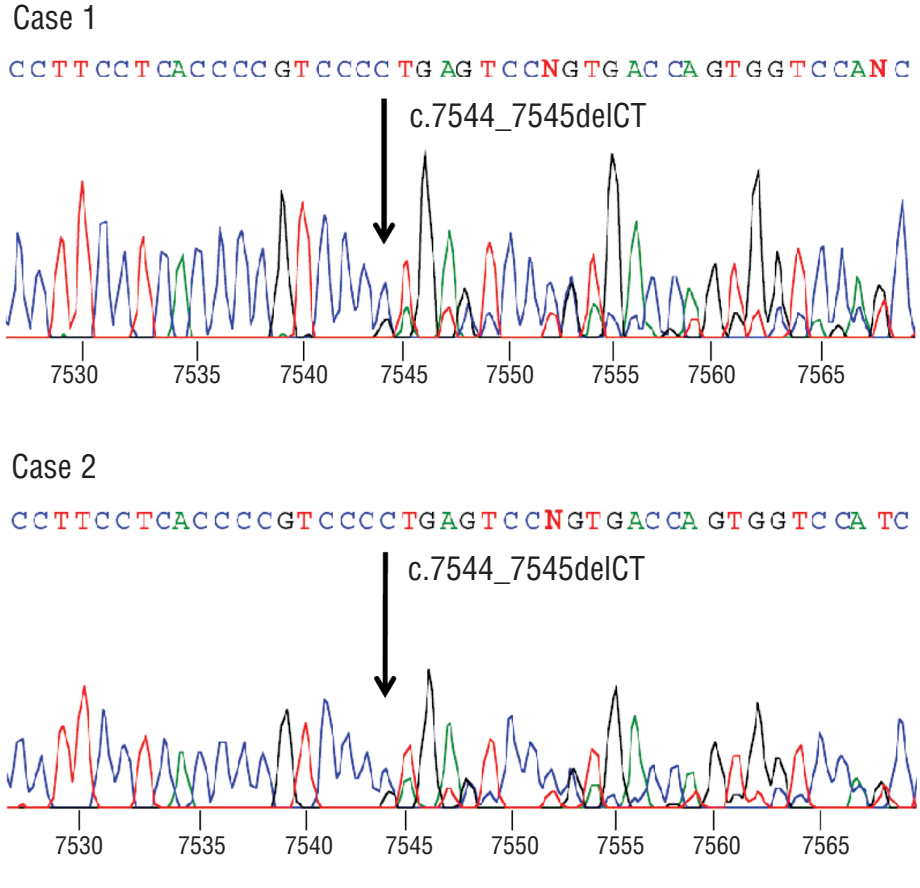

Figure 1. ***NOTCH1*** **mutations in clinical monoclonal B-cell lymphocytosis. Sequencing traces of the two clinical monoclonal B-cell lymphocytosis tumor samples (case 1 and case 2) harboring the *NOTCH1* c.7544_7545delCT mutation (RefSeq NM_017617.2); arrows point to the position of the nucleotide change.**

88 years). Male:female ratio was 33:30. Median absolute lymphocyte count was 5.0×10⁹/L (range 1.7-7.6×10⁹/L), median B-cell count was 2.9×10⁹/L (range 0.2-4.9×10⁹/L), and median CLL-phenotype lymphocyte count was 2.8×10⁹/L (range 0.2-4.9×10⁹/L). Median Hb value was 14.1 g/dL (range 11.0-16.9 g/dL), and median platelet count was 220×10⁹/L (range 148-420). The biological profile of cMBL was consistent with the indolent nature of this condition. CD38 and ZAP70 expression occurred in 8 of 60 (13.3%) and in 9 of 53 (17.0%) cases, respectively. An *IGHV* identity of 98% or over was present in 9 of 59 (15.3%) cMBLs, and stereotyped VH CDR3 in 9 of 59 (15.3%). Deletion of 13q14 was observed in 29 of 63 (46.0%) cases, trisomy 12 in none of 63, and 11q22-q23 deletion in 2 of 63 (3.2%). One of 63 (1.5%) cMBLs harbored biallelic *TP53* disruption by deletion of one allele and mutation of the second allele.

To assess the prevalence of *NOTCH1* mutations in cMBL, the *NOTCH1* mutational hotspots identified in CLL (exons 26, 27 and 34; RefSeq NM_017617.2) were initially analyzed by Sanger sequencing of tumor DNA obtained at cMBL presentation.[7,8] By this approach, *NOTCH1* mutations occurred in only 2 of 63 (3.2%) cMBL, with a prevalence that was significantly lower than that observed in a large CLL dataset (70 of 603, 11.6%) (P=0.050). In both cases, mutations were represented by a two bp frameshift deletion (c.7544_7545delCT) that represents the most recurrent (~80%) type of *NOTCH1* mutation detectable in CLL (Figure 1).[7,8] Case 1 presented with an absolute lymphocyte count of 6.3×10⁹/L, a B-cell count of 2.1×10⁹/L, and a CLL-phenotype cell count of 2.0×10⁹/L; was CD38 and ZAP70 negative; showed 3-74/2-21/4 IGHV/D/J gene rearrangement; expressed mutated *IGHV* genes (*IGHV* identity 92.01%); lacked a seterotyped HCDR3; showed a normal FISH karyotype and lacked *TP53* mutations. Case 2 presented with an absolute lymphocyte count of 6.5×10⁹/L, a B-cell count of 4.1×10⁹/L, and a CLL-phenotype cell count of 4.1×10⁹/L; was CD38 and ZAP70 negative; had 1-18/6-13/4 IGHV/D/J gene rearrangement; expressed mutated *IGHV* genes (*IGHV* identity 86.11%); lacked a seterotyped HCDR3; harbored a monoallelic 13q14 deletion and lacked *TP53* mutations.

The sensitivity of DNA Sanger sequencing does not allow the identification of a mutation whose allelic representation is less than 10%. Because *NOTCH1* mutations in CLL may be subclonal in a fraction of cases, and considering that the representation of peripheral blood monoclonal B cells in MBL is lower than in CLL, we reasoned that a mutation detection assay with a sensitivity higher than Sanger sequencing might be useful to define the true occurrence of *NOTCH1* mutations in MBL. To this purpose, we specifically designed a high sensitivity ARMS PCR assay[12] for the *NOTCH1* c.7544_7545delCT mutation, which accounts for the overwhelming majority of *NOTCH1* mutations in CLL. Despite the high sensitivity (1%) of this assay, ARMS did not identify additional c.7544_7545delCT mutations in cMBL.

The low prevalence of *NOTCH1* mutations in cMBL is consistent with the low frequency in this condition of genetic lesions that are otherwise associated with high-risk CLL, namely *TP53* or *ATM* disruption, and confirms that cMBL is characterized by an indolent biological phenotype.

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