## Comprehensive molecular characterization of a heavy chain deposition disease case

We herein report the complete characterization of the monoclonal Ig fragments produced by the plasma cell clone in a 65-year-old patient with a typical heavy chain deposition disease (HCDD).[1–3] The patient was referred for nephrotic syndrome and the main biological parameters are summarized in *Online Supplementary Table S1*. Kidney biopsy at diagnosis revealed a typical HCDD with nodular glomerulosclerosis and thickening of tubular and vascular basement membranes by a refractile ribbon-like material. HCDD was confirmed by immunofluorescence studies, showing linear γ1 heavy chain (HC) deposits along basement membranes and the absence of κ and λ light chain (LC) stainings, and later by electron microscopy with ultrastructural feature of monoclonal immunoglobulin deposition disease (MIDD) (*Online Supplementary Figure S1*). The patient was treated with vincristine, doxorubicin and oral dexamethasone. Overall, the treatment was well tolerated and laboratory tests conducted three months after chemotherapy showed a complete hematological and renal response (*Online Supplementary Table S1*). Ten years later, the patient relapsed with stage 3 multiple myeloma and died due to pulmonary infection.

We have recently published the most important series of HCDD cases,[3] unraveling some pathophysiological mechanisms of the disease. However, the molecular events leading to HC deposits are still incompletely understood. Deletion of the CH1 domain is supposed to be a prerequisite for the secretion of HC in the absence of a LC since unassembled HC is normally trapped in the endoplasmic reticulum (ER) by a non-covalent association with BiP/GRP78 until its association with a LC.[4] However, whether HC truncation in HCDD results from similar defect in LC production or other independent abnormalities remains to be determined. Our present study highlights the molecular events leading to the production of a monoclonal pathogenic HC in this HCDD patient.

Western blot analysis carried out on serum collected at diagnosis confirmed the presence of a truncated γ1 HC corresponding to the deposited HC (Figure 1A). Despite not being detected by immunofixation in serum, a λ-type Bence-Jones protein was observed in urine. Therefore, we sought for a λ LC in bone marrow protein extracts and serum by western blot. Interestingly, it revealed the presence of both a full-length λ LC and a shortened λ LC of approximatively 17 kDa (Figure 1B). Immunofluorescence studies carried out on medullary bone biopsy showed that both γHC and λLC are produced by the same plasma cell clone (Figure 1C).

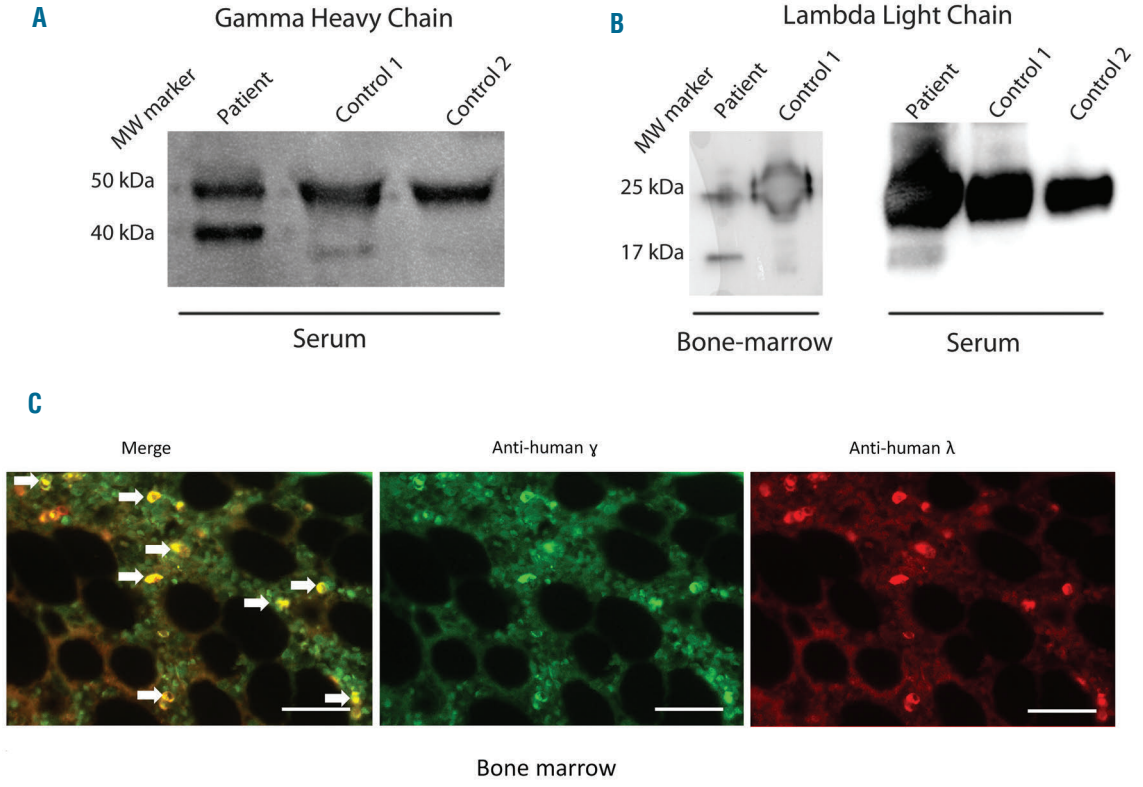


**Figure 1. Detection of truncated γ HC and λ LC in bone marrow and serum.** Western blot analysis of γ HC (A) and λ LC (B) in the serum and/or bone marrow (protein extracts) from the patient and controls. (A) The upper band (50 kDa) corresponds to the full-length γ HC, the lower band (40 kDa) corresponds to the truncated γ HC. (B) The upper band (25 kDa) corresponds to the full-length λ LC and the lower band (around 17 kDa) corresponds to the truncated LC. The truncated LC chain is detectable in both bone marrow and serum. (C) Immunofluorescence studies of a dewaxed paraffin embedded sample of medullary bone biopsy co-stained with anti-γ HC (middle) and anti-λ LC (right), the merge (left) indicating that PCs are double positive for γ and λ (white arrows). Original magnification x200, Bar = 100μM).

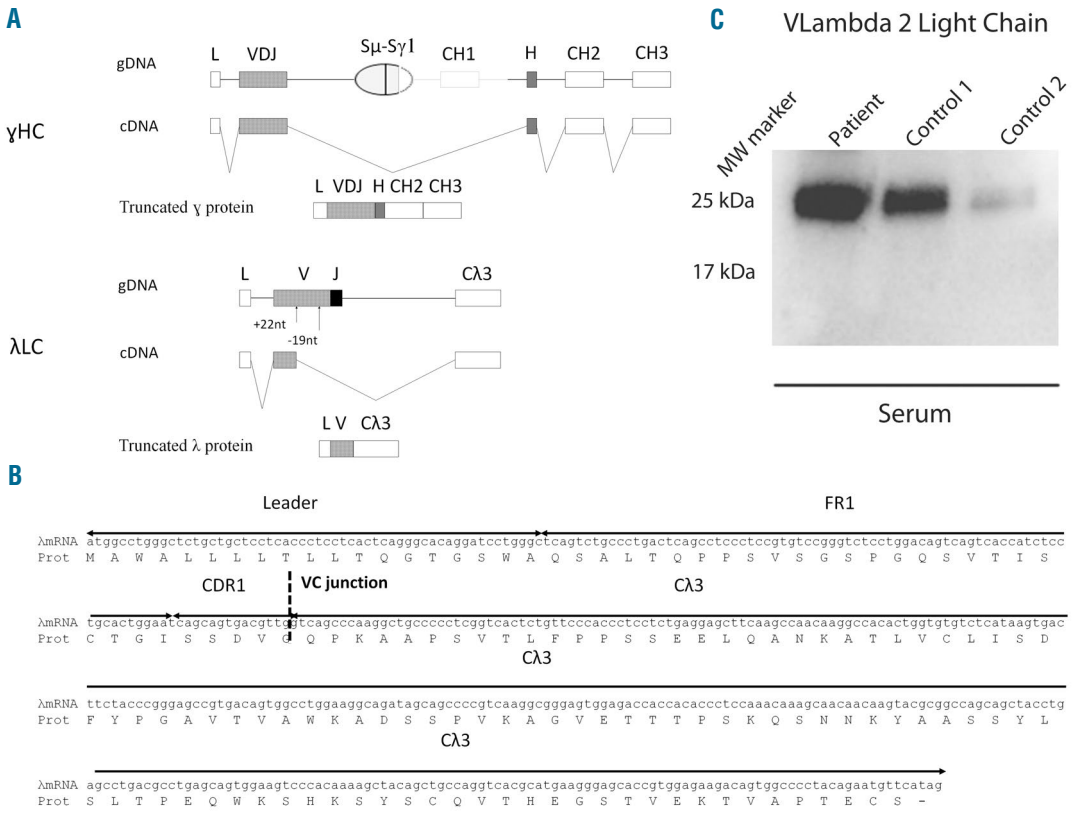


**Figure 2. Determination of LC and HC mutations.** (A) Schematic representation of the transcripts and proteins alterations observed on the γ HC and λ LC. (B) λ mRNA sequence and the deduced amino-acid sequence. (C) Western blot analysis of the circulating λ LC with an anti-Vλ2 antibody compared to the western blot carried out with an anti-λ antibody. Note the absence of the 17 kDa band, corresponding to the truncated λ LC detected with the anti-λ LC.

We determined the corresponding sequences from cDNAs derived from bone marrow extracts. We confirmed the presence of a monoclonal CH1-truncated HC sequence, composed of the rearranged VH3-30/DH2-15/JH4-derived germline genes (identity = 93.06 % for VH gene and 83.3 % for JH gene) directly spliced to the hinge/CH2/CH3 exons (*Online Supplementary Figure 2*). Further analysis of the monoclonal γ1 HC primary transcripts showed a large genomic deletion from the end of switch γ1 region to the middle of the CH1/hinge intron (Figure 2A). The PCR amplifications of λ LC cDNA confirmed the presence of a monoclonal truncated LC with a large deletion of the 3' part of the variable (V) domain, from the end of the CDR1 to the junction (J) region (Figure 2A). The remaining 5' part of the V domain, derived from the germline Vλ2-18 gene, was directly spliced onto the Cλ3 exon to form an in-frame truncated λ LC (Figure 2B and *Online Supplementary Figure S2*). We next analyzed the λ LC primary transcripts and found two abnormalities in the V domain: a 22 pb addition in FR2 and a 19 pb deletion in FR3. The 22 pb addition corresponded to a duplication of the CDR1/FR2 upstream region leading to a frameshift with the appearance of a stop codon at the beginning of the FR2 (*Online Supplementary Figure 2*). Frameshift in the Vλ exon may result from somatic hypermutations as it was previously shown for HC V domains.[5] When occurring in such HC V regions, a frameshift may lead to a complete decay of the transcript,[6] consequently resulting in free LC secretion (as seen in ~10% of plasma cell dyscrasias) since LCs are not retained in the ER in the absence of HC pairing. Alternatively, a frameshift may result in an alternative splicing leading to the production of a HC with a truncated V domain as observed in heavy chain diseases (HCD).[5] In the present case, an alternative splicing in the Vλ exon using the cryptic site TG/GTAGT in CDR1 led to the skipping of the premature stop codon and the production of truncated in-frame mRNA λ LC (*Online Supplementary Figure S2*). Since most of the Vλ domain is missing in the truncated LC, we performed a western blot using an anti-Vλ2 domain antibody[7] which proved positive for the full length polyclonal λ LC containing Vλ2 domains but failed to detect the shortened monoclonal V domain (Figure 2C).

Given our findings, we decided to study the capacity of this truncated LC to associate with a HC in an *in vitro* model. For this purpose, we stably transfected the murine hybridoma/myeloma cell line SP2/0 with the truncated λ LC and the patient's truncated HC or full-length HC obtained by the addition of the CH1 domain. A control full-length λ LC was also transfected with the full-length or the truncated HC. We confirmed that despite its abnormalities, the truncated λ LC can be secreted, as observed in the patient (Figure 3A). Then, to detect HC/LC association, we performed a hybrid ELISA using anti-γ HC antibodies for coating and anti-λ LC for revealing. Our results showed that the truncated LC do not associate with the full-length HC (Figure 3A). Finally, the intracellular/secreted ratio for the full-length HC in cell culture was nearly ten times higher compared to the

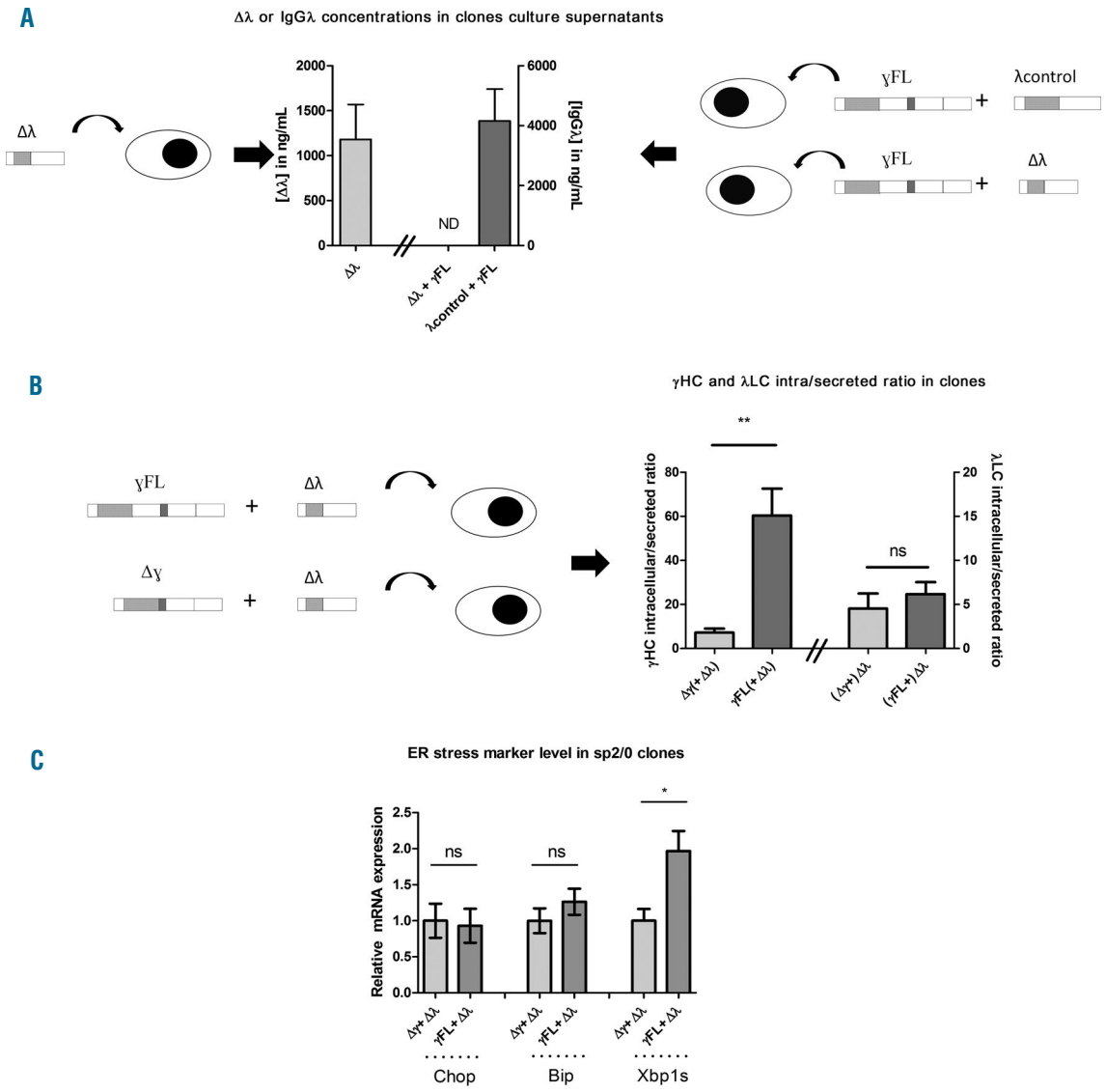


**Figure 3. *In vitro* study of the truncated LC/HC mispairing and resulting higher ER stress in cells expressing a solitary complete γ HC in a SP2/0 cell line. model.** (A, left) ELISA analysis of supernatants from SP2/0 cells transfected with the truncated λ LC (Δλ) or (right) transfected with the full-length HC (γFL) and the truncated λ LC (Δλ) or a control λ LC (λ control). Note the absence of association between the truncated λ LC and the normal reconstituted HC (ND=non detectable). (B) Determination of the intracellular/secreted ratio of SP2/0 clones expressing the full-length (γFL) or truncated HC (Δγ) co-transfected with the truncated λ LC. The high ratio indicates that products were retained intracellularly. Note that the intracellular/secreted ratio of the truncated λ LC was similar in both cases, demonstrating that the full-length HC retention is not due to a general secretory defect of the cells. (C) Quantitative expression of ER stress marker *Chop*, *Bip* and *Xbp1s* in a SP2/0 cell model containing a full-length HC (γFL) or a truncated HC (Δγ) associated with the truncated λ LC. Note the two-fold increased rate of *Xbp1s* in cells expressing the γ FL HC. Data are shown as mean ± SEM from 6 clones obtained per condition which express the highest levels of HC and/or LC. ** =*P*<0.01; *=*P*<0.05; ns: non-significant.

truncated HC (Figure 3B). These results confirmed that in absence of a pairing LC, a complete HC is retained intracellulary and that CH1 domain deletion facilitates HC secretion. This result prompted us to determine if the deletion of the HC CH1 domain could benefit plasma cell fitness. We analyzed the transcriptional expression of endoplasmic reticulum stress markers *Chop, Bip* and *Xbp1s* on SP2/0 clones expressing complete or truncated HC. We found that clones expressing a solitary full-length HC present a two-fold increased rate of *Xbp1s* (*P*=0.01) compared to clones expressing the truncated one (Figure 3C). We also observed a slight but non-significant increase of *Bip* (*P*=0.3) while *Chop* was unchanged. As previously mentioned, in the absence of LC, the HC is retained in the ER by BiP binding to CH1 domain,[4] a situation which was shown to lead to few CH1-truncated HC in a LC-deficient mouse model.[8] Consequently, we suggest that in the present case, the defect of the LC could have been the first event promoting the deletion of the CH1 domain of the HC in order to avoid HC intracellular retention and alleviate reticulum stress. Recently, several studies have shown that excessive ER stress induced apoptosis of PCs,[9] that inhibition of LC production by siRNA triggered apoptosis due to unpaired HC accumulation in ER and unfolded protein response[10] and that truncated LC selectively inhibited plasma cell differentiation and survival.[11] Consequently, we hypothesize that the CH1 deletion could act as a prosurvival event

since the intracellular retention of the full-length HC would likely have caused the loss of the PC clone due to ER stress. Accordingly, the very rare occurrence of true nonsecretory plasma cell disorders likely accounts for the toxicity of solitary HCs, the loss of LC or H/L pairing being lethal for the plasma cell except in case of HC truncation.

Truncated LC were not observed in other HCDD cases with LC characterization[3] but shortened LC transcripts resulting from internal deletions of the VJ exon and/or of splicing aberrations were also found in cases of Burkitt's lymphoma[12] or in non-secreting myeloma[13] and it cannot be excluded that point mutations without effects on the LC length could also impede H/L association. Our study gives an example of the relevance to monitor free LC in HCDD or other HC-related diseases since the same clone is producing both HC and LC. The intrinsic toxicity of isolated full-length HC in plasma cells also underpins the relevance of therapeutics aimed at inhibiting or altering LCs, for instance by siRNA[10] or antisense oligonucleotide (AON)-induced exon skipping.[11]

To conclude, we hypothesize that in this patient the onset of HCDD started with the alteration of the LC, followed by the deletion of the CH1 domain of the HC. This eventually relieved ER stress and thus improved the fitness and survival of the abnormal plasma cells. Further investigations must be conducted on other cases, in order to appreciate on a larger series how often H/L mispairing occurs in HCDD and can thus be considered as the initial alterations affecting these malignant B-cell clones.

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