

Lack of Ku80 Alteration in Multiple Myeloma

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Chromosomal rearrangement involving the immunoglobulin gene locus, as a result of marked chromosomal instability, is the hallmark of human multiple myeloma (MM) cells. Since Ku80 plays a key role in the non-homologous end-joining (NHEJ) system, we investigated whether Ku80 alteration contributes to this genetic instability by examining its status in 16 MM cell lines. Our study demonstrated a lack of Ku80 alterations at the protein, mRNA and gene level in 15 out of the 16 cell lines. Only the U266 cell line carried a missense mutation of Ser335Leu in one allele of the cDNA. Six marrow samples derived from myeloma patients also did not show any aberrant Ku80 protein, in terms of size. Accordingly, Ku80 alteration is unlikely to be involved in MM, in disagreement with a previous study reporting frequent presence of a 69-kD Ku80 variant (Ku86v) with reduced DNA binding activity in MM cells.

Key words: Multiple myeloma — Ku80 — Non-homologous end-joining (NHEJ) — Missense mutation

In mammalian cells, the non-homologous end-joining (NHEJ) system is required for double-strand breaks (DSBs), which are generated during physiological V(D)J recombination of immunoglobulin (*Ig*) and T-cell receptor (*TCR*) genes in lymphoid cells and as a result of DNA damage induced by radiation or chemotherapeutic agents. The known factors involved in NHEJ include Ku70, Ku80, DNA-dependent protein kinase catalytic subunit (DNA-PKcs), XRCC4 and ligase IV. The Ku heterodimer consisting of Ku80 (also known as Ku86) and Ku70 is critical for this repair system in that it functions as an initiator of NHEJ because of its ability to bind DNA DSBs and to recruit complexes of XRCC4, DNA-PK and DNA ligase IV.^{1–3} The importance of Ku protein for DSB repair and genetic stability is also apparent from the extremely increased radiation sensitivity and the defect of specific V(D)J recombination in Ku-deficient cells,⁴ accompanied by an inadequate DSB repair system, which leads to a high frequency of chromosomal aberrations in Ku70^{–/–} and Ku80^{–/–} mice.⁵ Recently, dysfunction of both NHEJ and apoptotic pathways has been demonstrated to lead to lymphoid malignancies in Ku80^{–/–}, p53^{–/–} and XRCC4^{–/–}, p53^{–/–} mice, which develop disseminated pro-B cell lymphomas carrying chromosomal translocations involving *Ig* heavy chain gene (*IgH*) and *c-Myc* gene loci, accompanied by increased chromosomal instability.^{6,7} Therefore, Ku80 and XRCC4 have roles in suppressing chromosomal rearrangement and in maintaining the integrity of the genome.

Moreover, human multiple myeloma (MM) cells feature extreme chromosomal instability and harbor characteristic

chromosomal translocations involving switch sequences of *IgH* loci from the early stage, as represented by monoclonal gammopathy of undetermined significance (MGUS), through to late stages such as refractory MM and plasma cell leukemia.^{8,9} It thus appears to be of considerable interest to investigate how such chromosomal instability arises in normal plasmablasts, which are believed to be the origin of the MM cells. On the basis of the above-mentioned findings, we examined whether Ku80 aberration contributes to the development of human MM.

A total of 16 MM cell lines (ODA, SACHI, U266, ILKM2, ILKM3, ILKM8, SKMM-1, XG7, FR4, NOP1, KM4, KM5, KM7, NCU-MM-1, JIN3, AMO1) were used.¹⁰ For protein analysis, six marrow samples, which contained more than 40% of atypical plasma cells in total nucleated cells, derived from patients with MM were additionally used after informed consent had been obtained. Total RNA used for northern blot analysis was isolated by means of the guanidine isothiocyanate/cesium chloride ultracentrifugation method. A 10- μ g aliquot of total RNA was loaded onto each lane and capillary blotting followed by hybridization was performed as described previously.¹⁰ For reverse transcription (RT)-polymerase chain reaction (PCR) analysis, 5 μ g of total RNA was reverse-transcribed to cDNA in an overall volume of 20 μ l in the presence of MMLV-RT (GIBCO BRL, Tokyo) according to the manufacturer's instructions, and 5 μ l of the resultant reaction mixture was subjected to 35 cycles of PCR amplification in an overall volume of 50 μ l using the specific primer pairs shown in Fig. 1A. Amplified cDNA was then subcloned into pGEMT-Easy vector (Promega, Madison, WI) and at least 10 independent clones were sequenced from both sides using T7 and SP6 primers with the aid of an

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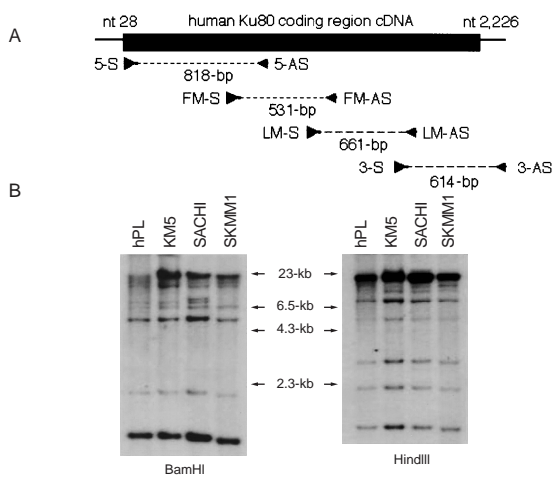


Fig. 1. A. Schematic representation of amplification of cDNA fragments by means of RT-PCR. The coding region of Ku80 cDNA is indicated by shading. The location and size of each amplified fragment are indicated below the cDNA scheme. Nucleotide numbers used in this figure are the same as those described by Mimori *et al.*¹³⁾ The primer segments used for the PCR were 5-S, 5'-GCTGTTGTGCTGTGTATG-3'; 5-AS, 5'-CATCCACAAGTGTCCAAG-3'; FM-S, 5'-CCTGCCGACTG-ACCATTG-3'; FM-AS, 5'-GGCAGCTGCACATACTACTA-3'; LM-S, 5'-CAGCAAGAGATGATGAGGC-3'; LM-AS, 5'-GAC-ACTGCCTTCAGCCAG-3'; 3-S, 5'-AGGATCAAGTGACTG-CTCAG-3'; 3-AS, 5'-AGCATCACAGCGATGGCAG-3'. B. No genomic rearrangement was detected by Southern blot analysis using the Ku80 cDNA probe in MM cell lines. An additional 8-kb band was seen in the lane of SACHI cells when digested with *Bam*HI, but this was considered as resulting from either a genetic polymorphism or a minimal deletion within an intronic sequence, since no such band was detected in *Hind*III or *Eco*RI digestion and this cell line expressed no aberrant mRNA.

ABI3100 capillary DNA sequencer (Applied Biosystems, Foster City, CA). A mixture of a 818-bp fragment of the 5' site and a 661-bp fragment of the 3' site of Ku80 cDNA was used as a probe for northern analysis. Immunoblotting analysis was performed as described previously.¹¹⁾ In brief, total cell lysate derived from 1×10^6 cells was fractionated on to 7.5% SDS acrylamide gel and transferred to a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). The primary antibodies used for western analyses consisted of an affinity-purified anti-carboxy terminal portion of Ku86/Ku80 (c-20) goat serum (Santa Cruz Biotechnology, Santa Cruz, CA), anti-amino terminal (aa 8–221) Ku86/Ku80 (S10B1) mouse monoclonal antibody (mAb) (Labvision, Fremont, CA) and anti- β -actin (I-19) goat serum (Santa Cruz Biotechnology) for quality and quantity control. After the reaction with secondary peroxidase-linked antibodies, the specific signals were detected with an ECL system (Amersham Life Science, Bucking-

hamshire, UK). The rearrangement of the *Ku80* gene was screened by employing Southern blot analysis using Ku80 cDNA probes, which were a mixture of the four RT-PCR products shown in Fig. 1A.

In order to examine the genomic status of the *Ku80* gene in a panel of 16 MM cell lines, we first performed Southern blot analysis using a Ku80 cDNA probe covering nearly the entire coding region. No major deletions or rearranged bands were detected in the DNAs digested completely with *Bam*HI, *Hind*III or *Eco*RI restriction enzyme (Fig. 1B, some data not shown). We next characterized Ku80 expression in the 16 MM cell lines at the protein and mRNA levels, as shown in Fig. 2, A and B. Neither lack of 80-kD Ku80 expression nor aberrant-sized product was observed in any of the cell lines by means of immunoblot analysis using c-20 antiserum raised against the C-terminus of the protein. Moreover, the amount of the protein was similar in all the cell lines. Recently, predominance of the 69-kD variant of the Ku80 protein (Ku86v), which results from C-terminal truncation and is characterized by reduced DNA binding activity resulting in an inability to form complexes with DNA-PKcs, was reported in 84% (12 out of 14) of fresh MM cells by Tai *et al.*¹²⁾ We next used the same mAb (S10B1), which recognizes the N-terminal portion of Ku80, as that used by Tai *et al.*¹²⁾ Contrary to their previous observation, we found that the normal 80-kD Ku80 continued to be present in all of the MM cell lines, and no Ku86v protein was detected. In order to exclude the possibility that the Ku86v protein had been lost during the establishment of the MM cell lines, we additionally analyzed six primary myeloma samples by immunoblot analysis (Fig. 3). All of the cell lysates derived from tumor samples showed the presence of the normal Ku80 protein and the absence of the 69-kD Ku86v. Consistent with this result, northern analysis showed only two normal forms of transcripts, the 3.4 and 2.6 kb size, in all of the MM cell lines, while no aberrant messages were identified. Accordingly, we have no explanation as to why the previous study by Tai *et al.* found the presence of Ku86v and the absence of normal Ku80 protein except for the possibility that some sort of specific protease truncated the c-terminal portion of Ku80 during the time-consuming process of plasma cell purification and preparation of the cell lysate that they used. As the possibility exists that inactivating mutation in the coding sequences may lead to functional loss of the Ku80 protein, we performed sequencing analysis of the RT-PCR products. Only one cell line (U266) contained a missense mutation, Ser335Leu, of the *Ku80* gene in one allele, although the other allele harbored the wild type (Fig. 2C). Coexistence of both wild-type and mutated alleles in U266 cells was confirmed, since 6 out of 15 clones (531-bp FM-S–FM-AS fragment) subcloned into pGEM-T Easy vector showed the mutated sequence while the remaining 9 were

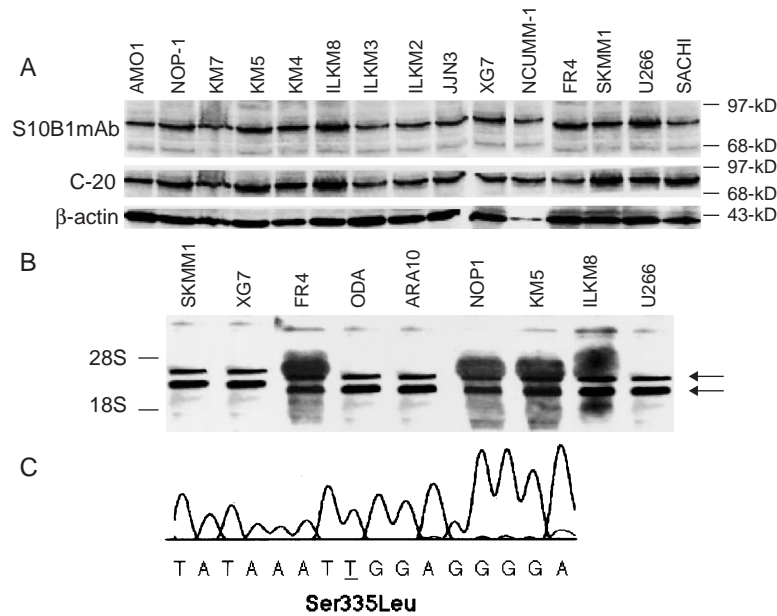


Fig. 2. A. Ku80 protein expression in MM cell lines detected by western blot analysis using polyclonal c-20 and monoclonal S10B1 antibodies. Ubiquitous expression of the Ku80 protein with no aberration in size was found in all MM cell lines. B. Ku80 mRNA expression detected by northern blot analysis. All MM cell lines show both 3.4- and 2.8-kb mRNAs without any aberrant messages. C. A missense mutation found in an allele of the U266 cells. A nucleotide exchange from C to T at codon 335 resulted in replacement of the Ser with the Leu residue.

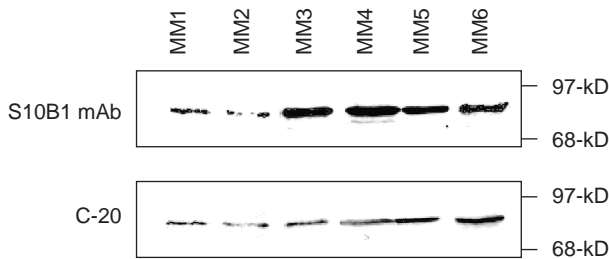


Fig. 3. Ku80 protein in primary myeloma samples detected by immunoblot analysis. All six cell lysates derived from bone marrow mononuclear cells of myeloma patients show normal 80-kD Ku80 protein when using polyclonal c-20 and monoclonal S10B1 antibodies.

consistent with the wild type. This mutation was unique and did not seem to be a polymorphism, since we could not find the same amino acid replacement in the cDNAs derived from peripheral blood mononuclear cells of 15 normal volunteers and from 50 hematopoietic cell lines other than those we used in this study (Komatsu *et al.*, unpublished data). Currently, it remains unknown if this unique mutation may have a dominant negative effect on

the wild-type Ku80 protein or have lost its normal function, leading to haplo-insufficiency in terms of the NHEJ pathway in U266 cells. In conclusion, our findings suggest that Ku80 alteration is unlikely to be involved in the chromosomal instability frequently found in human MM cells. However, further investigations of additional DSBR genes, such as *XRCC4*, *Ku70*, *DNA-ligaseIV* and *DNA-PKcs*, are needed to confirm this conclusion, since dysfunction in any of these components may cause the identical phenotype, which is likely to show chromosomal rearrangements that would contribute to the tumorigenesis in MM.

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