Epstein-Barr Virus-infected Cell Line TCC36B Derived from B Lymphocytes Infiltrating Renal Pelvis Urothelial Carcinoma

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Abstract. *This study reports an initial analysis of an EBV-infected B cell line (TCC36B), established from an urothelial carcinoma (UC) lesion of the renal pelvis. Materials and Methods: Cytofluorometric and G-banding analyses were performed for phenotyping and cytogenetics. PCR was used to detect EBV DNA, and sequence analysis to investigate mutations and deletions of the latent membrane protein (LMP)-1 gene of EBV. Results: TCC36B cells proliferated in vitro and showed positivity for surface CD19, CD20, HLA-DR and IgG(λ), indicating that they belong to B-cells. Cytogenetic analysis showed 46,XX with a unique clonal abnormality of dup(2)(p13p25). EBV DNA was detected in TCC36B cells. Sequence analysis revealed a 30-bp deletion and 7 point mutations on the LMP-1 gene in TCC36B cells. Conclusion: These results suggest the involvement of an EBV variant in the pathogenesis of UC. This cell line should thus facilitate further investigations on the aetiological role of EBV in urothelial cancer.*

Derangement of B-cell differentiation may occur as a result of genetic abnormalities of a B-cell clone, which are often followed by events leading to uncontrolled proliferation, immortalisation and malignant transformation (1). It is known that EBV can infect and immortalise human B cells, resulting in lymphoblastoid cell lines (2). EBV-carrying B-cell lines may evolve from cells transformed *in vitro*, or from individuals who have had a prior EBV infection (3, 4). While the mechanism by which these cells are immortalised remains unclear, it has been demonstrated that the cells contain most, if not all, of the EBV genome as multicopy episomes (5). In addition, EBV may protect human B cells from apoptosis through the expression of the full set of EBV encoded 'latent' proteins (6-8). The process of B-cell immortalisation by EBV is mediated by the establishment of an autocrine loop in which the cells produce a growth factor or factors that support their own proliferation (9, 10). This autocrine growth activity may be contributed by a number of soluble molecules, which have been identified to be interleukin (IL)-1 (11), IL-6 (12), IL-5 (13), lactic acid (14) and lymphotoxin (15).

During previous efforts to initiate a continuous culture of malignant cells from a surgically removed urothelial carcinoma (UC) tissue of the renal pelvis for immunobiological studies, we could not establish a UC cell line. Instead, there was growth of fibroblasts, on which lymphoid cells started to grow and form aggregates. These lymphoid cells tended to grow by themselves after few *in vitro* passages without the presence of fibroblasts, and were collectively termed as the TCC36B cell line. *In situ* hybridization analysis revealed the presence of EBV encoded RNA (EBNA) in the nuclei of UC tumour cells of the UC specimen of the patient from which the TCC36B cell line was established (16) and in those of 24% UC specimens tested (17). This study describes the establishment and characterisation of this B-cell line carrying the EBV variant with a 30-bp deletion and 7 point mutations in the latent membrane protein *(LMP)-1* gene, consistent with that found in nasopharyngeal carcinoma (NPC).

Materials and Methods

Patient and tumour tissue. A 65-year-old Taiwanese woman was diagnosed as having end-stage renal disease in 1978. Since then she has undergone regular haemodialysis. Painless haematuria was noted and a computer-assisted tomography scan revealed a mass at the right renal pelvic area. The patient underwent right nephroureterectomy

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and histopathology of the surgically removed UC lesion showed a T3 high-grade infiltrating urothelial carcinoma (16).

Cell lines and culture methods. Four human haematopoietic cell lines, Daudi (Burkitt's lymphoma), K562 (erythroleukaemia), Jurkat (T cell leukaemia), and B95-8 (EBV-producing marmoset B-cells) were maintained as suspension cultures in RPMI-1640 medium containing 10% heat-inactivated foetal bovine serum. Mechanically dispersed cells from the tumour tissue were used to initiate continuous cell lines.

Immunostaining procedures. Cytospin slides were prepared from TCC36B cells. Cells on the slides were either subjected to Liu's staining, or to immunostaining with monoclonal antibodies (mAbs) using the avidin-biotin peroxidase complex (ABC) procedure as described previously (18). Following antigen retrieval process, sections prepared from formalin-fixed, paraffin-embedded tumour tissues were stained with mAb to CD20 (L26) (Dako A/S, Glostrup, Denmark).

Immunofluorescence/flow cytometry. TCC36B cells were tested for reactivity with mAbs using an indirect immunofluorescence method as described previously (18). Positive and negative controls were included and flow cytometric analysis was performed. Murine mAbs used were as follows: anti-HLA-A,B,C (W6/32), HLA-DR (DK22), CD3, CD4, CD8, CD10, CD19, CD20, CD25, CD43, CD44, CD54, CD56 and vimentin were obtained from Dako Japan Co., Ltd. (Kyoto, Japan); anti-cytokeratins, AE1/AE3 mAbs were obtained from Signet Laboratories, Inc. (Dedham, MA, USA); anti-MAK-6 mAb was obtained from Zymed Laboratories, Inc. (South San Francisco, CA, USA); and EGFR (Rl) was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Cytogenetic analysis. TCC36B cells and peripheral blood mononuclear cells (PBMCs) taken from the patient two months after the surgical removal of the tumour were subjected to chromosome preparation. After 72 h stimulation with phytohaemagglutinin (PHA) *in vitro*, PBMCs were made in metaphase by addition of colchicine at a final concentration of 0.1 mg/ml for 25-30 min. Hypotonic treatment was performed with 0.07 M potassium chloride, and cells were fixed in cold methanol-glacial acetic acid (3:1). The chromosomes spread on glass slides were stained with Wright Giemsa. Karyotypes were made and analysed on the basis of the ISCN nomenclature (19).

DNA extraction and signal amplification by polymerase chain reaction (PCR). High molecular-weight cellular DNA was extracted from cells. One hundred nanograms of DNA was used in a total reaction volume of 25 μl containing 0.5 U of Taq DNA polymerase (Perkins-Elmer-Cetus, Norfalk, CT, USA); reaction buffer (10 mM Tris, pH 8.4; 50 mM KCl; 1.5 mM $MgCl_2$; 0.01% gelatine); 0.2 mM of dNTPs; and 20 pmol/reaction of each primer. The PCR mixture was subjected to 30 cycles of amplification using standard procedures, denaturing DNA at 94˚C for 1 min, annealing at 52˚C for 2 min, and elongating at 72˚C for 1.2 min. The first pair of primers known as L1/L2 was complementary to EBV *Bam*HI L region; the sequences were as follows: L1, 5'-GGCTGGTGTCACCTGTGTTA-3' and L2, 5'-CCTTAGGAGGAACAAGTCCC-3'. The second pair of primers known as AA/AZ was complementary to EBV *Bam*HI W region; the sequences were as follows: AA, 5'-GGGATGTTTCAGCTCTTC CACC-3' and AZ, 5'-CACTCCAGAAAGCAGGACAATTCC-3'. The amplified products were resolved in a 2% agarose-EtBr gel.

DNA sequencing analysis. For analysis of mutation and deletion in the carboxy-terminal end of the *LMP-1* gene, PCR amplification was performed using a pair of primers (BN1: nucleotide positions 168390 to 168370, 5'-AGCGACTCTGCTGGAAATGAT-3' and BN2: nucleotide positions 168075 to 168095, 5'-TGATTAGCTAAGG CATTCCCA-3'). The following PCR conditions were used: 2 min of incubation at 94˚C before the first cycle, 40 cycles at 94˚C for 40 s, 55˚C for 1 min and 72˚C for 1.5 min with a 6 min extension after the last cycle. A 5 μl aliquot of PCR product was analysed on a 1.5% agarose gel at 150 volts for 45 min. The remaining PCR product was concentrated and purified with the use of a GenecleanR III kit (BiolOl; Vista, CA, USA) with sodium iodite and TBE modifier. Sequencing reactions were performed on 10 μl of the purified PCR product by an ABI PRISM Rhodamine terminator cycle sequencing ready reaction kit with ABI 377 automated sequencer (Perkin-Elmer Corporation, Applied Biosystems, Foster City, CA, USA).

Results

Immunohistochemical staining of the UC tissue. Sections of the original tumour from which the cell line was established were stained with mouse anti-CD20 mAb. Moderate to strong positive staining against CD20 was seen with small lymphoid cells either singly or in small aggregates adjacent to the UC tumour areas and some of the positively stained cells showed clearly infiltrating tumour cells (Figure 1A). Unstained lymphoid cells admixed with CD20 positive B cells were likely tumour-infiltrating T cells and other leukocytes.

Growth pattern of TCC36B cells. A portion of the tumour tissue was processed for cell culture in order to establish the UC cell line. Tumour-like epithelial cells and fibroblastic cells started to grow initially. However, the fibroblastic cells outgrew the tumour cells by the third week after the initiation of culture. Unexpectedly, a few small lymphoid cells were found closely adhering to fibroblasts (Figure 1B). As the number of these small lymphoid cells increased, they tended to form aggregates, which were eventually detached from the fibroblasts (Figure 1C). After 4 to 5 passages, these lymphoid cells were able to grow by themselves without fibroblasts. Liu-stained cytospin cells showed that while the majority of cells were small to medium in size, few large-sized cells were noted (Figure 1D), which appeared to be immunoblastic cells, with 'smudged' appearance.

Immunophenotyping. Both immunostaining on cytospin cell preparations and cytofluorometric analysis on monodispersed cells showed that TCC36B cells expressed HLA-A,B,C, HLA-DR, CD19, CD20, CD43, CD44, CD54, IgG (with λ-light chain) and EGFR (Rl) to various degrees, but did not express CD3, CD4, CD8, CD10, CD25, CD56, IgM and IgA

markers, vimentin, AE1/AE3 and MAK-6, indicating that TCC36B cells were B lymphocytes expressing surface IgG(λ). It should be noted that the expression of HLA-A, B and C on TCC36B cells was reduced considerably compared with that of the autologous PBMCs, as demonstrated by the percentage of positive cells (82.1% *vs*. 99.0%, respectively) and the mean fluorescence intensity (54.0 *vs*. 419.6 in arbitrary units, respectively) in cytofluorometric analysis.

Chromosome analysis. Ninety-eight percent (41/42) of metaphases showed the karyotype of 46,XX,dup(2)(p13p25). One such a complete karyotype (left panel), and three dup(2)(p13p25) chromosome markers and three normal chromosome 2 from PBMCs of the patient (right panel) are illustrated in Figure 2A.

Detection of EBV DNA. Both TCC36B cells and autologous PBMCs were tested for the presence of EBV DNA by PCR methods using two different pairs of primers, L1/L2 (for *Bam*HI L region of EBV) and AA/AZ (for *Bam*HI W region of EBV (results not shown). In PCR with the pair of L1/L2 primers, TCC36B cells showed two dense DNA bands at 237 bp and 239 bp on agarose gels and the autologous PBMCs showed a very faint band at 237 bp, while Daudi cells (positive control) showed a dense band at 239 bp. No prominent bands were detected in the autologous PBMCs, or in negative control cells (Jurkat T cells). With the pair of AA/AZ primers, TCC36B, autologous PBMCs and Daudi all showed a dense band at 239 bp, while the negative control Jurkat T-cells did not show any bands. Thus, there were some subtle differences between TCC36B and PBMCs in EBV DNA.

Analysis of the LMP-1 gene and deduced products. For the molecular analysis of the carboxy-terminal end of the *LMP-1* gene to show the exact location of the deletion and the single-base mutations, PCR was performed on genomic DNA of TCC36B and the autologous PBMCs, using primers spanning this region of the *LMP-1* gene (Figure 2B). The nucleotide sequence no. 168077-168380 (corresponding to amino acid residues 286-387) was examined and compared with that of the B95-8 *LMP-1* lacking the 30-bp deletion (20). Direct sequence analysis of the PCR products revealed identical 30-bp deletion in both TCC36B and autologous PBMCs. Seven single-base point mutations at nucleotide positions 168357, 168355, 168320, 168317, 168308, 168295 and 168225 were detected. The only nucleotide position change (C → T) unique to TCC36B cells was at 168317, which was deduced to result in one amino acid change, from Gly to Asp; the former being present in both *LMP-1s* of the prototype B95-8 strain and the PBMCs. As a result of 1-2 nucleotide changes in four out of six remaining point mutations, there were three additional amino acid substitutions, Leu → Ser (168308), Gln → Arg (168320), and Gln → Asn (168355 and 168357); all being found in both TCC36B cells and PBMCs. The remaining two point mutations (168225 and 168295) were silent, since they did not result in any changes of amino acid sequences with reference to B95-8 cells.

Discussion

This study established a human B-cell line, TCC36B, capable of growing continuously *in vitro* in the absence of tumour-derived fibroblasts. Compared with the autologous PBMCs, the TCC36B cell line expressed reduced levels of surface HLA-A,B,C molecules. This cell line was characterised by the expression of CD20 and the clonal chromosomal abnormality, dup(2)(p13p25). The significance of the p13p25 duplication on the short arm of one of chromosome 2 in TCC36B cells is not immediately clear. However, a gene in the locus, 2p25, has been assigned to code for the enzyme soluble acid phosphatase (ACP1) (21). A constitutional duplication of chromosome 2p13-p21 in an individual with gingival fibromatosis and mental retardation was reported. Such an abnormality could also be associated with deafness (22). This abnormality of dup(2)(p13p25) detected with TCC36B cells was, however, not seen with autologous PBMCs obtained two months after surgical removal of the tumour.

LMP-1 expression has been shown to inhibit human epithelial cell differentiation by severe impairment of the cellular response to differentiation signals (23). In light of the strong expression of LMP-1 molecule by TCC36B cells as demonstrated in immunoblotting analysis (unpublished results), it will be of interest to examine details of the carboxy-terminal cytoplasmic domain of the molecule, which has been implicated in the increased tumourigenicity due to the 30 bp-deletion (168256-168285) (20). Of the two EBV DNA sequences (237 bp and 239 bp) detected by the pair of primer sequences, L1/L2, in TCC36B cells, one was not found in Daudi cells, and two were not found in the autologous PBMCs, suggesting that the EBV DNA sequences at or near the *Bam*HI L region of these two cell lines (TCC36B and Daudi) and the autologous PBMCs were somewhat different from each other. By another pair of primer sequences AA/AZ, one EBV DNA sequence at 239 bp at the *Bam*HI W region was detected in TCC36B cells, autologous PBMCs and Daudi cells. However, none of these PCR products were found in the Jurkat T-cells.

The presence of 30-bp deletion mutants within the carboxy-terminal cytoplasmic domain of the *LMP-1* (*BNLF1*) gene has been reported in EBV-associated malignancies (24-26). Included within the domain between nucleotide no. 168075 and no. 168380, are the major sites of serine/threonine phosphorylation (27, 28) and the two major effector regions (responsive elements) of *NF-κB* [*CTAR-1*(amino acid residues 194-232) and *CTAR-2* (residues 351-386) activating regions] (29). In both TCC36B and the autologous PBMCs, the 30-bp deletion and six single-base point mutations at nucleotide

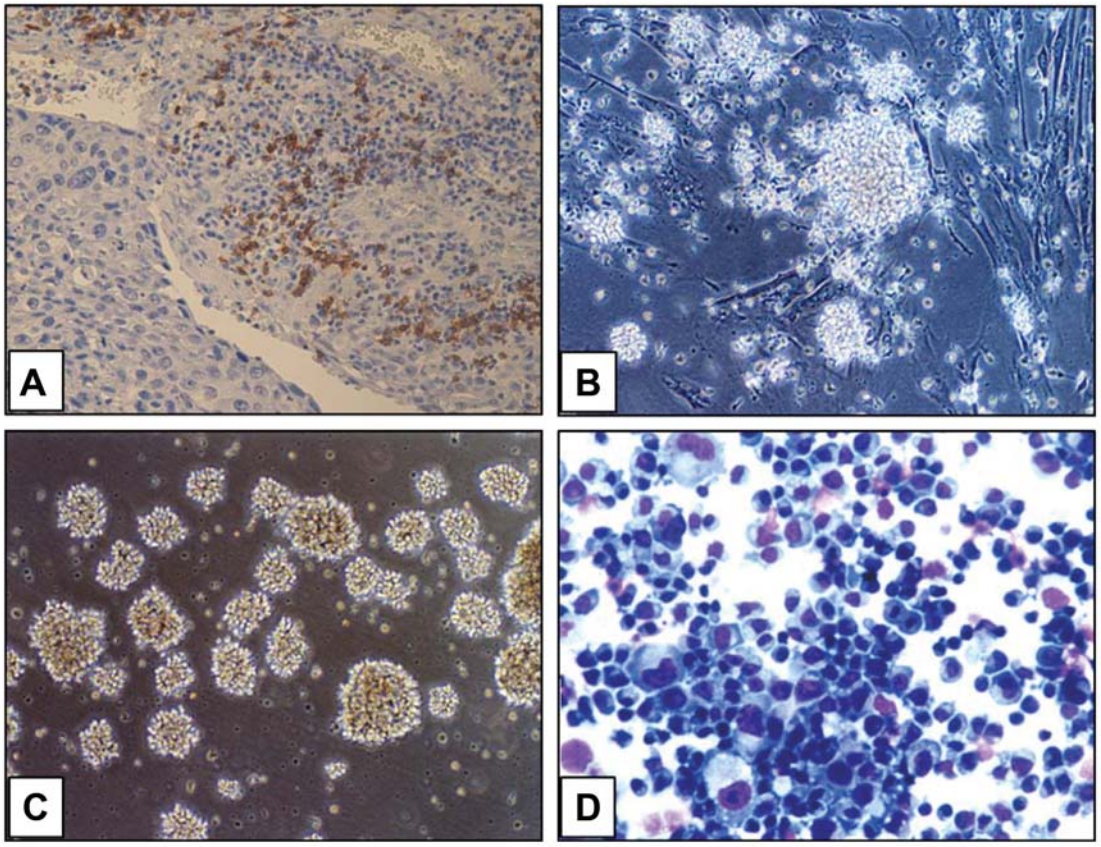

Figure 1. *Live cultured cells of TCC36B cells and immunostaining/chemical staining of tissue sections/cultured cells from a renal pelvic UC lesion from which TCC36B cell line was derived. A: Immunostaining with anti-CD20 mAb of sections from a formalin-fixed, paraffin-embedded renal pelvic UC, from which the TCC36B cell line was derived (original ×200). B: Adherence of single or aggregates of TCC36B cells to fibroblasts in the early culture is noted (original ×100). C: Many aggregates of TCC36B cells in varying sizes floating in the culture medium after 4 passages in vitro can be seen (original ×100). D: Cellular morphology of TCC36B cells on a cytospin slide after Liu's stain can be seen (original ×400).*

positions 168357, 168355, 168320, 168308, 168295, and 168225 were identified. These changes have been shown to occur frequently in most, if not all, EBV-positive normal and reactive lymphoid tissues (25) and tumour tissues of patients with NPC (24, 30), as compared with those on the prototype B95-8 EBV *LMP-1* that lacks the 30-bp deletion. One nucleotide position difference (C → T) found only in TCC36B was at the 168317 position, which was deduced to result in one amino acid difference, Asp instead of Gly. The latter amino acid was present in the *LMP-1* of both the autologous PBMCs and the prototype B95-8 cells.

Most recently, we determined EBV DNA in 66 UC specimens by real-time PCR. The tumor status of poor differentiation was found to be correlated with the high load of the EBV genome in non-muscle invasive urothelial carcinoma (31).

The relationship between the renal pelvis UC and TCC36B cells and the precise role of EBV plays in the development of UC tumour remain enigmatic at this time. However, it will be important to determine whether the EBV DNA variant and the mutant *LMP-1* detected in TCC36B cells could also be detected in the UC tumour cells of the lesion. Unfortunately, it was not possible to cultivate UC tumour cells from the same tumour lesion successfully, or to isolate the population of UC cells from this tumour in sufficient numbers for molecular analysis. More work is therefore required to determine the precise role(s) of EBV in UC pathogenesis and/or tumour progression using additional UC tumour specimens, freshly purified UC cells and tumour-associated B cells from different TCC patients. This is the first example of EBV-infected B cell lines associated with UC being reported. TCC36B cells should facilitate further investigations of the role of UC tumour-infiltrating B cells and the EBV variant with the mutant *LMP-1* in the pathogenesis of a significant proportion of urothelial malignancies (17).

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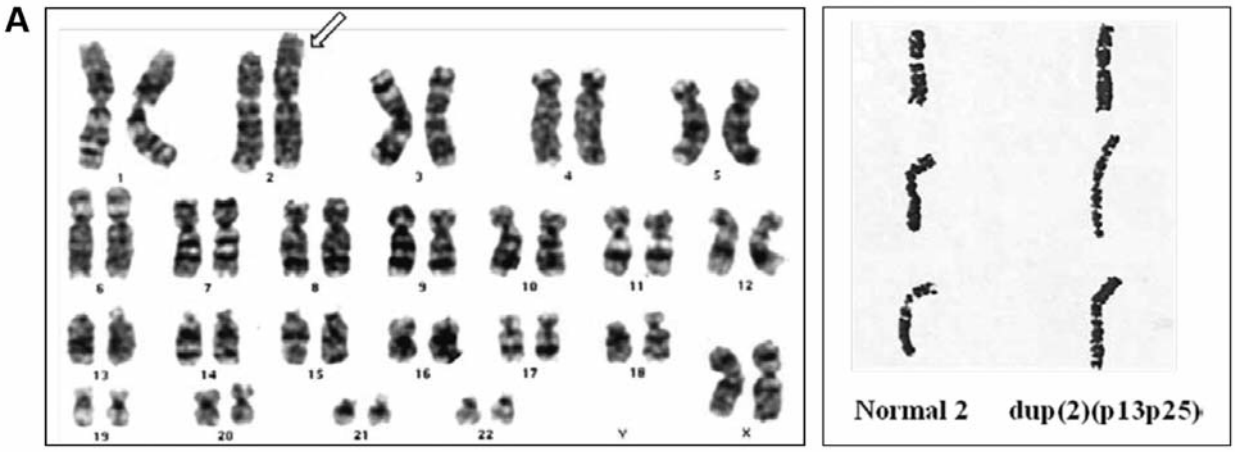

B LMP-1 Nucleotide No. 168075-168380 examined

Antisense primer BN2:5'-tgattagctaaggcattccca-3' Sense primer BN1:5'-agcgactctgctggaaatgat-3'

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Nucleotide No. 168--   223 225                                    256
                        ↓ ↓                                        ↓
     B95-8  Nucleotide  aga acc caa aag cag cgt agg aag gtg tgg atc acc gcc gcc atg gcc gga
      PBMC  Nucleotide  --t --- --- --- --- --- --- --- --- --- --- *** *** *** *** *** ***
    TCC36B  Nucleotide  --t --- --- --- --- --- --- --- --- --- --- *** *** *** *** *** ***
     B95-8  Amino acid  Ser Gly Leu Leu Leu Thr Pro Leu His Pro Asp Gly Gly Gly His Gly Ser
      PBMC  Amino acid  --- --- --- --- --- --- --- --- --- --- --- *** *** *** *** *** ***
    TCC36B  Amino acid  --- --- --- --- --- --- --- --- --- --- --- *** *** *** *** *** ***

                                285           295              308        317 320
                                  ↓             ↓                ↓          ↓   ↓
     B95-8  Nucleotide  atc atg act atg acc gcc gcc tcc gtc tgt cat caa agg cgg gcc ctg gtc
      PBMC  Nucleotide  *** *** *** *** --- --- --- a-- --- --- --- -g- --- --- --- -c- ---
    TCC36B  Nucleotide  *** *** *** *** --- --- --- a-- --- --- --- -g- --- --- -t- -c- ---
     B95-8  Amino acid  Asp His Ser His Gly Gly Gly Gly Asp Ser Met Leu Pro Pro Gly Gln Asp
      PBMC  Amino acid  *** *** *** *** --- --- --- --- --- --- --- Ser --- --- --- Arg ---
    TCC36B  Amino acid  *** *** *** *** --- --- --- --- --- --- --- Ser --- --- Asp Arg ---

                                                        355 357                370
                                                          ↓ ↓                   ↓
     B95-8  Nucleotide  acc tcc ttt gtt ttc aac ctc ttc cgt caa ttg tgg agg gcc tcc a
      PBMC  Nucleotide  --- --- --- --- --- --- --- --- --- --- a-t --- --- --- --- -
    TCC36B  Nucleotide  --- --- --- --- --- --- --- --- --- --- a-t --- --- --- --- -
     B95-8  Amino acid  Gly Gly Lys Asn Glu Val Glu Glu Thr Leu Gln Pro Pro Gly Gly
      PBMC  Amino acid  --- --- --- --- --- --- --- --- --- --- Asn --- --- --- ---
    TCC36B  Amino acid  --- --- --- --- --- --- --- --- --- --- Asn --- --- --- ---
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Figure 2. *Chromosomal G-banding and DNA sequence analyses of TCC36B cells. A: A complete (left panel) and partial karyotypes of 3 cells (right panel), exhibiting the clonal marker chromosome, dup(2)(p13p25). The marker in the left panel is pointed out by an arrow. Three apparently normal chromosome 2s are shown with the abnormal marker chromosome counterparts side by side for comparison. B: DNA sequence analysis of the carboxy-terminal end of the LMP-1 gene isolated from TCC36B cells and autologous PBMCs. The nucleotides (168075 to 168380) and amino acid sequences of the equivalent region of the prototype B95-8 LMP-1 are included for comparison. No differences in nucleotide sequences between 168075 to 168221 and 16371 to 16380 from three sources of cells were detected. The segment marked by *** *** *** *** signifies the region of sequence with 30-bp deletion, which was absent from the B95-8 LMP-1. There are two identical point mutations at the positions 168355 (T → A) and 168357 (G → T) for both TCC36B and autologous PBMCs, resulting in a substitution of one amino acid (Gln → Asn) when compared with the B95-8 LMP-1. The three additional point mutations common to both TCC36B and PBMCs are at positions 168308, 168320 and 168225, the former two positions of which resulted in two amino acid changes from Leu → Ser and Gln → Arg, respectively. The only point mutation in the TCC36B LMP-1 standing out from LMP-1 of both in B95-8 cells and PBMCs is at the position 168317 (C → T), resulting in one amino acid substitution (Gly → Asp).*

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