ShRNA-mediated Ku80 Gene Silencing Inhibits Cell Proliferation and Sensitizes to \(\gamma\)-radiation and Mitomycin C-induced Apoptosis in Esophageal Squamous Cell Carcinoma Lines

Qing-Shan YANG, Jin-Long GU, Li-Qing DU, Li-Li JIA, Li-Li QIN, Yong WANG and Fei-Yue FAN*

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To investigate the effects of Ku80 depletion on cell growth and sensitization to \(\gamma\)-radiation and MMC-induced apoptosis in esophageal squamous cell carcinoma lines. Six human carcinoma cell lines (LNcaP, K562, MDA-MB-231, MCF-7, EC9706, and K150) and normal HEK293 cell line were examined for basal levels of Ku80 protein by western blotting analysis. The suppression of Ku80 expression was performed using vector-based shRNA in EC9706 cells. Cell proliferation was determined with MTT assay and colony formation assay and tumorigenicity in a xenograft model in vitro and in vivo. Sensitivity of EC9706 cells treated with shRNA vector to \(\gamma\)-radiation and MMC was determined with colony formation assay and MTT assay. The cell cycle distribution was determined by Flow cytometry. Apoptosis induced by \(\gamma\)-radiation and MMC was analyzed using GENMED-TUNEL FACS kit. Ku80 showed higher basal levels in six carcinoma cell lines than in HEK293. The suppression of Ku80 expression decreased cellular proliferation, colony formation and inhibited tumorigenicity in a xenograft model. Furthermore, it sensitized apoptosis of the cancer cells induced by \(\gamma\)-radiation and MMC. Ku80 plays an important role not only in tumorigenesis but also in radiation resistance and chemotherapy resistance in esophageal cancer cells. Hence Ku80 may serve as a promising therapeutic target, particularly for recurrent esophageal tumors.

INTRODUCTION

Esophageal cancer is one of the most common tumors and its incidence is increasing steadily worldwide. Despite improved treatments with surgery and radiotherapy, esophageal cancer is still the leading cause of cancer death in many counties, especially in China.\(^1\) Although our understanding of the molecular biology of esophageal cancer has increased in recent years, a detailed knowledge of the molecular mechanisms underlying its formation and progression remains elusive. Furthermore, no specific molecular targets for clinical practice have yet been identified. It will be desirable in the future to develop such potent molecular targets for novel clinical treatments based on the etiology of esophageal cancer formation.

Ku80 is one component of a protein complex, the Ku80/ Ku70 heterodimer, which was originally identified as a major target of autoantibody from Japanese patients with scleroderma-polymyositis overlap syndromes two decades ago.\(^2\) Ku80 is well known for its crucial role in DNA repair. The double-strand break (DSB) is the major DNA lesion leading to chromosomal aberrations. Unrepaired DSB is likely to result in cell death. It will be likely to cause mutations or carcinogenesis under misrepaired DSB. DSB are repaired by homologous recombination (HR) and by non-homologous end-joining (NHEJ). NHEJ is the predominant mechanism in higher eukaryotes, whereas single cell organisms (such as yeast) rely more heavily on HR. Here, we are particularly concerned with NHEJ pathway. It has been shown that Ku80 can maintain the genome stability by repairing DSB through NHEJ. This process requires several factors: Firstly, Ku heterodimer is capable of both sequence – independent and sequence specific DNA binding and bridging. Then, Ku complex recruits DNA-PKcs to the DSB and activates its kinase function. Lastly, this complex stimulates DNA repair and transduces the damage/stress signal response, which may affect apoptosis and cell proliferation.\(^3,4\) These results were identified through studies of ionizing radiation-sensitive Chinese hamster cell lines and cells from severe combined immune-deficient (SCID) mice.\(^5,6\) Besides its important role in DNA repair, many reports have implicated that Ku80 is also involved in other cellular processes, such as telomere maintenance, antigen
receptor gene arrangement, regulation of specific gene transcription, apoptosis, tumor suppression, as well as regulation of the G2 and M phases of the cell cycle, suggesting that Ku80 is multifunctional housekeepers in cells.

In recent years, studies on Ku80 protein in tumor occurrence, development, treatment and prognosis are increasing extensively. Several investigations have shown abnormal expression of Ku80 protein in various cancers, including esophageal cancers. Over-expression of Ku80 made cancer acquire resistance to radiation and chemical drug. These previous findings suggested that Ku80 plays an important role in occurrence, progression and prognosis of tumor, so Ku80 protein is very likely to become a new target for radiation sensitizer. However, exact role of the Ku80 is not clear in esophageal cancer cells.

A novel molecular approach to specifically target Ku80 mRNA was used in this experiment in order to validate our obtained outcomes and clarify the occurrence of esophageal cancers. Results showed that this approach inhibited the expression of Ku80 protein. Then, the suppression of Ku80 expression decreased cellular proliferation and inhibited tumorigenicity in a xenograft model. Furthermore, it sensitized the cancer cells to γ-radiation and Mitomycin C (MMC) -induced apoptosis. All these results confirmed that Ku80 was involved in esophageal cancer development and radiation resistance, and the suppression of Ku80 might hold promise for development as a new strategy for treating esophageal cancers.

MATERIALS AND METHODS

Cells culture and reagents
Esophageal cancer cell lines (ESCC) (EC9706, K150) were obtained from the cell centre of Cancer Institute of Peking Union Medical College. LNCaP cells line, leukemia cells line K562, breast cancer cell lines (MDA-MB-231, MCF-7) and HEK293 were obtained from our laboratory. These cells were grown in RPMI 1640 medium (Invitrogen, USA) with 10% FCS (Biowhittaker, USA) at 37°C in a humidified 5% CO2 incubator. Cultured cells were rinsed twice with phosphate buffered saline (PBS) and mixed with 200 μl of lysis buffer (Beyotime Biotechnology). The cells in lysis buffer in the dish were removed using a scraper and transferred to an Eppendorf tube using a micropipette. The cells were homogenized and centrifuged 1,500 rpm for 5 minutes in a centrifuge, and the supernatant stored at −20°C. The protein concentration of the whole cell was determined using a BCA protein assay kit (Beyotime biotechnology). The protein extracts (30 μg) were incubated in sample buffer (60mmol/L Tris-Hcl, 25%Glycerol, 2%SDS, 14.4mmol/L Mercaptoethanol, 0.1%Bromophenol Blue), and boiled for 5 minutes.

Western blotting
Cultured cells were rinsed twice with phosphate buffered saline (PBS) and mixed with 200 μl of lysis buffer (Beyotime Biotechnology). The cells in lysis buffer in the dish were removed using a scraper and transferred to an Eppendorf tube using a micropipette. The cells were homogenized and centrifuged 1,500 rpm for 5 minutes in a centrifuge, and the supernatant stored at −20°C. The protein concentration of the whole cell was determined using a BCA protein assay kit (Beyotime biotechnology). The protein extracts (30 μg) were incubated in sample buffer (60mmol/L Tris-Hcl, 25%Glycerol, 2%SDS, 14.4mmol/L Mercaptoethanol, 0.1%Bromophenol Blue), and boiled for 5 minutes. These samples were electrophoresed on a 10% PAGE gel at 110v for 1.5 hours in SDS running buffer. The proteins were transferred to polyvinyl difluoride (PVDF) membrane (Bio-Rad, Hercules, U.S.A) by electrophoresis in transfer buffer at 200 mA for 1 hour. The PVDF membrane was incubated with 10 ml blocking buffer for overnight on a rocker at 4°C. The PVDF membrane was washed three times with TBS containing 0.1% Tween-20 (TBST) and then incubated with 10ml of primary Ku80 antibody after blocking. The PVDF film was washed with TBS before incubation with secondary peroxidase-labeled rabbit antibody for 1 hour at room temperature, and the protein bands were detected using an enhanced chemiluminescence kit (Boster biotechnology Co). The density of bands in the resulting film was quantified using the NIH image analysis program. The human anti-Ku80 and anti-Ku70 antibody was purchased from Cell signaling technology Co. Anti-β-actin antibody was purchased from Boster biotechnology Co.

Ku80 shRNA vector constructs
Plasmid pGCsi-U6-Neo-GFP-shRNA Expression Vector (GeneChem) was used, which contained a human U6 promoter, a GFP reporter gene and a neomycin resistance gene to enable antibiotic selection in mammalian cells. We designed two pairs of complementary oligonucleotide sequences (shRNA-H and shRNA-K) according to the mRNA sequence of Ku80 (GenBank Accession Number: NM_021141). Each anti-Ku80 target sequence corresponds to nt 619–639, 1480–1500 of Ku80 cDNA sequences. They were analyzed BLAST research to ensure that they haven’t significant sequence homology with other genes. The scrambled control plasmid (shRNA-SC) was a circular plasmid encoding a shRNA which had the sequence not present in the mouse, human, or rat genome databases. Target sequence Ku80 for shRNA-H, K and shRNA-SC are shown below:

ShRNA-H: 5’-ccaaatcctcgatttcagattcaagagatctgaaatcgaggat-tg-3’
ShRNA-K: 5’-ggaattcagatccagatctgaattcgaggat-tg-3’
ShRNA-SC: 5’-agctgacacgttcggagaattcagatctgaattcgaggat-tg-3’

ShRNA vector stable transfection
Twenty thousand esophageal cancer cells (EC9706) in a total volume of 500 μl RPMI 1640 medium were seeded in 24-well plates and incubated for 24 hours. The RPMI 1640 medium was aspirated and cells were rinsed with PBS. The cells were replenished with 100 μl fresh opti-MEM and incubated for 1 hours at 37°C in a humidified 5% CO2 incubator. Cells were treated with shRNA-H, K or shRNA-SC as negative control according to the manufacturer’s instructions. Briefly, shRNA plasmid and Oligofectamine 2000 (Invitrogen, USA) were mixed separately with opti-MEM.
and incubated for 5 minutes at room temperature. These reagents were combined and incubated for another 15 minutes before adding to the cells in opti-MEM without penicillin and streptomycin. The effectiveness of transfection was visualized by fluorescence microscopy at 24 hours after transfection. After 48 hours incubation, 600 μg/ml of hygromycin (G418) was added to the medium for selection individual colonies which were isolated after addition 10-days cultures. Selected single colony was cultured ample in 300 μg/ml G418.

**Western blot analysis**

The total amount cellular Ku80 and Ku70 protein was quantified using western blotting and NIH image analysis. As described above, the extracted proteins were subjected to 10% SDS-PAGE and then transferred into a PVDF membrane. Detection was performed by using an ECL Kit.

**Semi-Quantitative Reverse Transcription-PCR (RT-PCR)**

Total RNA was extracted from cells with TRIzol reagent (Invitrogen, USA) and quantified by UV absorbance spectroscopy. The reverse transcription reaction was performed using TaKaRa RNA PCR Kit (TaKaRa Biotechnology Co, Ltd); the newly synthesized cDNA was amplified by PCR. The reaction mixture contained 10 μl of cDNA template, 1 μl of Ku80 primer (F:5′-ggggtacatccttcctccatacatcagac-3′, R:5′-gtacrrgcgctcagcggag-3′), β-actin (F:5′-acgtagagaagagctacga-3′,R:5′-gtacrrgcgctcagcggag-3′) was used as an internal control. Amplification cycles were 94°C for 5 min, the 35 cycles at 94°C for 30 sec, 60°C for 5 min, 72°C for 2 min, followed by 72°C for 5 min. Aliquots of PCR were electrophoresed on 1% agarose gels.

**Cell proliferation assay**

Cell viability was measured by the MTT assay. Cells treated with shRNA-SC, H2, K3 and untreated cells were cultured for 1, 3, 5 and 7 days in 96-well plates at a concentration of 1000 cells/well. Each well was filled with fresh medium. At the particular times, each well was incubated with MTT for 4 hours. The liquid was removed, and dimethylsulfoxide (DMSO) was added to dissolve the solid residue. The optical density of each well at 570 nm was determined using a microplate reader (Molecular Devices, San Diego, CA).

**Colony formation assay**

Cells treated with shRNA-SC, H2 and K3 and untreated cells (1000 cells /well) were suspended in the complete medium and seeded in 100 mm culture plate. After 14 days. The cells were fixed by methanol and stained with 0.5% crystal violet. The colonies was manually counted and then photographed. Viable colonies were scored when a minimum of 50 cells were present.

**Tumorigenicity in nude mice**

The experimental protocol was approved by the China Institutional Ethics Review Committee for Animal Experimentation. Cells (1 × 10⁷ /mouse) suspended in 0.2 ml RPMI 1640 medium were injected subcutaneously into the 6-week-old male or female BALB/c nude mice at the right forward flanks. The animals were sacrificed on the 28 days after injection and the tumors were dissected and weighed.

**Radiation and MMC Treatment**

The cells were irradiated at room temperature in air using a Shepherd Mark I ¹³⁷Cs irradiator. The cells were incubated with different concentrations of MMC for 48 hours at 37°C in a humified 5% CO₂ incubator.

**Anticancer drug sensitivity assay**

Cells were treated with different concentrations of MMC. Cell viability was determined indirectly by MTT assay. The cells (2 × 10⁶ cells/well) were seeded onto a 96-well culture plate. The cells were treated by 0.5 μg/ml, 1 μg/ml, 1.5 μg/ml and 2 μg/ml MMC. After cultured for 48 hours, MTT assay was performed.

**Irradiation sensitivity assay**

The 1000 cells in 100mm culture dishes were irradiated at room temperature using a Shepherd Mark I ¹³⁷Cs irradiator. Radiation doses were 1Gy, 2Gy and 4Gy (0.8Gy/min) respectively. Once all treatments were completed, the cells were processed with the colony formation assay.

**Flow cytometry**

The measurement of cell cycle distributions was described previously. Briefly, cells, either transfected with shRNA-SC, H2, K3 or untransfected, were treated with 10 μg/ml MMC or irradiated with ¹³⁷Cs (8Gy), and then further incubated for 24 hours. Cells were harvested by trypsinization and washed with PBS for three times, then fixed in ice-cold 75% ethanol. The fixed cells were treated with staining solution containing 0.2 mg/ml DNase-free RNase A and 50 μg/ml propidium iodide (PI) for 30 min at 4°C. Cell cycle phase distribution was determined by a FACS cytometry (Becton Dickinson, San Jose, CA, USA) using the cell Quest program and data were analyzed with ModFit software. The apoptotic cells were measured using GENMED-TUNEL FACS kit (GenMed, Ltd).

**DAPI staining**

Cells were irradiated with ¹³⁷Cs (8Gy) and then further incubated for 24 hours. Cells were fixed with methanol and stained with 0.1 μg/ml DAPI. Nuclei were visualized under a fluorescence microscopy. Ten different fields were randomly selected for counting 300 cells. The percentage of cells with fragmented nuclei was calculated.
**Statistical Analysis**

All of the experiments were replicated three times. The mean standard deviation, mean square errors, two-factor ANOVA, correlation and interaction of main effects were calculated using the GraphPad Prism 4.0. Appropriate comparisons were made using the Dennett’s method for multiple comparisons. A value of $P < 0.05$ was considered statistically significant.

**RESULTS**

**Over-expression of Ku80 in ESCC**

To investigate whether Ku80 abnormalities are linked to human ESCC, we first analyzed expression of Ku80 protein in different tumor cell lines and normal HEK293 cell line. Totally, at the particular time, different cells were collected. As shown in Fig. 1, over expression of Ku80 was found in tumors cells lines (EC9706 and K150, LNCaP, MDA-MB-231 and MCF-7, leukemia K562.) compared with normal HEK293 cell line. The results indicated there was higher over-expression of Ku80 in ESCC. We selected EC9706 cells line in latter experiment.

**Efficient knockdown of Ku80 using vector-based shRNA in EC9706**

RNAi technology which was utilized to knockdown Ku80 expression in EC9706 cells confirms whether endogenous Ku80 expression was essential for growth of ESCC. The vector-based shRNA plasmids (shRNA-SC, H, K) were transfected into the cells. To confirm the feasibility of our system, we observed the efficiency of transient transfection at 24hours after transfected through fluorescence microscope. The results showed that the efficiency of transient transfection was about 35% (data not shown). Then the cells were selected with G418; resistant clones were subjected to western blotting and RT-PCR to analyze the Ku80 expression. Compared with shRNA-SC cells and parental EC9706 cells, the proteins of Ku80 were reduced by 63% and 50% in shRNA-H2 and shRNA-K3 stable transfectants clones, respectively (Fig. 2A). Ku80 transcripts were reduced by about 58% and 84% in the K3 and H2 clones (Fig. 2B). However, the rest of transfectants showed no apparent changes of Ku80 expression. As to Ku70 protein, no difference was observed in transfectants clones compare with shRNA-SC cells and parental EC9706 cells (Fig. 2A).

**Knockdown of Ku80 in EC9706 cells retard cell growth in vitro and in vivo**

For characterizing the role of Ku80 on the growth of the EC9706 cells, we measured the cell proliferation rate and the growth ability of cells in vitro. By MTT assay, up to the 3rd day a dramatic decrease in the cell growth rate (30–40%) was observed in the shRNA-H2 and K3 cells line compared with the shRNA-SC transfectants and control cells (Fig. 3B). In addition, the colony formation rate of the two cells lines was reduced (Fig. 3C). The number of colonies formed by the shRNA-H2 and K3 cells was about 50% of that of the control cells. However, no change was seen in the shRNA-SC cells (Fig. 3D).

We further observed the effects of Ku80 knockdown on the tumorigenicity in a xenograft model in vivo. At 28 days after cells were inoculated in nude mice, a remarkable reduction of tumor weight of the shRNA-H2 groups was observed compared with that of the shRNA-SC and control groups (Fig. 4A). The average tumor weight (n = 5) of the shRNA-H2 groups was about 66%, compared with the control groups and the shRNA-SC groups (Fig. 4B). The suppression of Ku80 retarded tumor growth (Fig. 4C). These results indicated knockdown of Ku80 protein in cancer cells reduced their tumorigenesis potential.

**Knockdown of Ku80 enhances EC9706 response to γ radiation and Mitomycin C**

In order to determine the correlation between Ku80 expression and radiation response, we have exposed the
Howevet, shRNA-SC did not show any significant radiation sensitization. Thus, the radiation response paralleled the change in Ku80 expression.

Another extensively study that the sensitivity of EC9706 cells transfected with shRNA-SC and shRNA-H2 to MMC were analyzed by MTT assay. We measured the MMC dose-response curves for EC9706 cell transfectants. The shRNA-
H2 cells were much more sensitive to MMC than the shRNA-SC and control cells (Fig. 5B).

Effects of Ku80 depletion on cell cycle distributions following irradiation

Irradiation sensitivity of ShRNA-H2 and K3 cells were analyzed by evaluating their ability to survive and progress through the cell cycle. Exposure to 8 Gy γ-radiation significantly increased the proportion of cells in G2/M phase \((p < 0.01)\) and decreased the proportion of cells in G1 phase \((p < 0.01)\) in shRNA-H2 and K3 cells, compared with shRNA-SC and control cells. Degree of G2/M phase arrest is more apparent in radiation state than in non-radiation state. Results indicated that knockdown of Ku80 easily escaped from G1 phase and arrested G2/M phase in the cell cycle (Fig. 6A, B).

Knockdown of Ku80 aggravated γ-radiation and MMC-induced apoptosis

We explored the sensitivity of the shRNA-H2 cells in response to radiation and MMC-induced cell apoptosis. The apoptotic fraction in the shRNA-H2 cells was increased by about 35% compared with the shRNA-SC cells and control cells under the 8Gy γ-radiation treatment \((p < 0.01)\) (Fig. 7A). The similar results were obtained while cells were treated with 10 μg/ml MMC \((p < 0.05)\) (Fig. 7A). The DAPI assay by detecting nuclear fragmentation further confirmed the γ-radiation-induced apoptosis in the shRNA-H2 cells. (Fig. 7B).

Fig. 5. Knockdown of Ku80 enhances EC9706 response to γ-radiation and Mitomycin C. A: Effect of γ-radiation on the colony survival of EC9706 cells. B: Effect of MMC on the cell survival of EC9706 cells.

Fig. 6. Cells cycle was analyzed by FACS, suppression of Ku80 expression showed G2/M phase arrest. A: cell cycle distributions were analyzed by FACS at 24 hours after cells were non-irradiated or irradiated. B: the proportions of cells in each phase of cell cycle were shown in non-radiation state and 8Gy radiation state.
DISCUSSION

Ku80 is an important DNA repair protein in the NHEJ pathway, but is also involved in other cellular processes, such as telomere maintenance, regulation of apoptosis, tumor suppression and gene regulation. Recently several investigations reported abnormal expression of Ku80 protein in various cancers. To further study the role of Ku80 in esophageal cancer, we adopted the vector-based shRNA expression systems to elucidate the functions of Ku80 in esophageal cancer cells, overcoming the limitations of transient and non-renewable nature of small interference RNA (siRNA). In this report, we have successfully constructed shRNA-Ku80 vector and selected an efficient target sequence to suppress the endogenous Ku80 expression.

It is well known that limitless cell growth is one of important alteration in cancer cell phenotypes. We firstly observed the effects of Ku80 depletion on cell growth in esophageal cancer cells. In this study, we demonstrated that knockdown of Ku80 affects not only tumor cell growth but also tumor-igenicity phenotypes in esophageal cancer cells by cell proliferation assay, colony formation assay and tumorigenicity in nude mice in vitro and in vivo. These results are consistent with previous studies, most of which have shown that combination of Ku80 depletion and radiation inhibited tumor growth in H1299 cells, and suppression of Ku80 expression inhibited the proliferation of HeLa cells. But our observations disagree with those results of Uegaki, which indicated Ku80 did not affect cell growth and genomic integrity. Here, the probably mechanisms of inhibition of cell proliferation by Ku80 depletion may include: 1. Ku80 is closely related to the maintenance of telomere structure. Previous studies showed that there was direct relation between Ku80 and telomerase. Suppression of Ku80 expression decreased telomerase activity, made chromosome ends gradually shorten, promoted cell senescence and apoptosis; 2. Ku80 may act as gene regulator, it can affect HER-2, NF-kB, EGFR, p50 gene transcription, and these genes can promote cell proliferation.

One of the current major issues in esophageal cancer treatment is resistance to radiation and chemotherapy. Several investigations showed that over-expressions of Ku80 have a positive-relation to radiation resistance. Using gene knockdown technology and antisense RNA technology, Ku80 depletion can significantly enhance the tumor cells sensitivity to radiation. In this study, we have shown that suppression of Ku80 expression sensitized esophageal cancer cells to apoptosis induced by γ-radiation and MMC. Our results also indicated that cells of Ku80 depletion easily escaped from G1 phase and arrested in G2/M phase, and then cells may develop into multinucleated giant cells with characteristic aneuploid that results in apoptosis. The probably reason that Ku80 depletion escape from G1 phase is that Ku80 mainly occurred in G0, G1 or early S phase of cell cycle which is involved in repairing DSB via NHEJ. In general, content of cellular Ku80 increased during G1 phase and reached maximum in the latter part of S phase while cells were irradiated. However, Keng et al reported that cells of Ku80 mainly lie in the late G1 population were slightly less radiosensitive than cells of Ku80 mainly lie in other cell cycle stages. Our results showed that Ku80 mainly enriched in S phase in EC9706 cells in the non-radiation state, but it mainly lie in G1 phase in γ-radiation state. While expression of Ku80 protein was inhibited by shRNA-H2 vector, G1 phase was apparently reduced. Then cells were arrested in G2/M phase in γ-radiation state. Because the suppression of Ku80 expression decreased Ku80 end-binding activity and repair ability, cell can escape from G1, S phase into G2 phase. Meanwhile, the rest of repair pathways increased such as HR, which made cells arrest in G2/M phase. But our results disagree with the results of Myint, which indicated that the cell cycle distribution for the parental and Ku80 deficient cell lines does not alter after cells were treated with Cisplatin. Our results also indicated that Ku80 depletion enhanced radiation sensitivity; there was significantly synergistic effect between Ku80 depletion and radiation. So Ku80 depletion may become radiation sensitizer, which would be an appealing therapeutic strategy. Hence, many recurrent esophageal cancers are refractory to radiation therapy and chemotherapeutic agents.

In summary, we have shown in this report that targeted Fig. 7. Effects of Ku80 depletion on apoptosis induced by radiation and MMC. A: Percentage of sub-G1 phase was analyzed at 24 hours after cells were irradiated by 8Gy 137Cs and treated with 10 μg/ml MMC. B: Morphologic changes in cell nuclei visualized by DAPI staining. Arrows indicate examples of cells with fragmented nuclei.
Ku80 knockdown by vector-based shRNA prevents cell proliferation and sensitized cancer cell to radiation and MMC-induced apoptosis in esophageal cancer cells. Nevertheless, the underlying molecular mechanisms of this action of Ku80 remain unknown and require further research...

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