• ESOPHAGEAL CANCER •

Alterations in expression, proteolysis and intracellular localizations of clusterin in esophageal squamous cell carcinoma

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Abstract
AIM: To investigate biogenesis and intracellular localizations of clusterin to elucidate the potential molecular mechanisms implicated in tumorigenesis of esophageal mucosa.

METHODS: Semi-quantitative RT-PCR for multi-region alteration analysis, Western blot for different transcriptional forms and immunohistochemical staining for intracellular localizations of clusterin were carried out in both tissues and cell lines of ESCC.

RESULTS: The N-terminal deletions of the clusterin gene and the appearance of a 50-53 ku nuclear clusterin, an uncleaved, nonglycosylated, and disulfide-linked isoform, were the major alterations in cancer cells of esophagus. Naturally the 40 ku clusterin was located in the connective tissue of the lamina *propria* of epithelial mucosa and right under the basal membrane of epithelia, but it was disappeared in stromal mucosa of esophagus and the pre-matured clusterin was found positive in cancerous epithelia.

CONCLUSION: The N-terminal deletion of clusterin may be essential for its alterations of biogenesis in ESCC.

INTRODUCTION

Clusterin, a 70-80 ku heterodimeric, disulfide-linked glycoprotein is expressed in a wide variety of tissues and secreted in all human fluids[1-3]. Human clusterin is encoded by a single copy gene located on chromosome 8p12 and 8p21 with nine exons and eight introns, spanning approximately 17 kb[4,5]. Clusterin gene has a single functional promoter and a single transcript mRNA, 1.6 kb in length, containing an N-terminal hydrophobic leader sequence. There are two forms of clusterin: one set of proteins is directly for secreted into humour, and the other forms are expressed in the cytoplasm and nucleus. The secretory form of the clusterin protein is produced by translation on membrane-bound ribosomes from the first AUG codon of the full-length clusterin mRNA and is targeted to the endoplasmic reticulum (ER) by an initial leader peptide. Subsequently, this -60 ku pre-clusterin protein containing 427 amino acids has to be further glycosylated in the ER and proteolytically cleaved between R205 and S206 into a mature protein discrete α- and β-chains, held together by disulfide bonds in Golgi[1, 6]. External secretory clusterin is a 70-80 ku heterodimeric glycoprotein that appears as a -40 ku α- and β-subunits smear by sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) electrophoresis under reducing conditions[7-9]. Recent data suggest that secretory clusterin acts as a molecular chaperone to scavenge denatured proteins and cellular debris outside cells following specific stress-induced injury such as heat shock[10-14].

Clusterin has been found highly conserved and implicated in a variety of biological processes including lipid transport, epithelial cell differentiation, transformation, and regulation of apoptosis in numerous models of epithelial cells during hormone ablation[15-20]. It is induced during regression of most hormone-dependent secretory epithelial cells as one of the most potent proteins of the rat ventral prostate or mammary gland[9,20-23]. Overexpression of secretory clusterin in human cancer cells caused drug resistance and protection against certain cytotoxic agents that induce apoptosis[24-26]. In human prostate cancer cells, overexpression of clusterin provides protection against TNFα-induced cell death and oligonucleotide directed antisense inhibition enhances spontaneous cell death in untreated cultures[24]. Clusterin may have a cytoprotective role in epithelial cell death. There are significant alterations in the biogenesis of clusterin during apoptosis, which lead to the appearance of a 50-53 ku uncleaved, nonglycosylated, disulfide-linked isoform that accumulates in the nucleus of MCF-7 cells[20]. Nuclear clusterin synthesis is a product of alterative splicing, in which the exon II, containing the first AUG and encoding the ER-targeting peptide, was omitted. This "death" form of the clusterin protein was proposed to be synthesized from a second in-frame AUG codon in exon III as translation start site. The short mRNA produces the 49 ku precursor nuclear clusterin which overexpression acts as a pro-death signal, inhibiting cell growth and survival[27-29].

Clusterin mRNA and protein was recently shown to be down-regulated in esophageal squamous cell carcinoma (ESCC), the major malignant tumor occurred in epithelium of esophagus[7]. Thus, to the author's knowledge, it is unclear whether alterative splicing clusterin is also involved and the possible roles of clusterin in process of the ESCC. We additionally studied, by multi-regional RT-PCR, Western blot

and immunohistochemical staining, the levels of expression and cellular distribution of clusterin in both tissues and cell lines of human ESCC.

MATERIALS AND METHODS

Tissue sample
The esophageal specimens were obtained from patients diagnosed with ESCC by the pathologists that assisted in our previous work in Cancer Hospital of Chinese Academy of Medical Sciences and Beijing Yanjing Hospital[7]. The study was approved by the Institutional Review Board. Briefly, immediately the specimens were dissected manually into several aliquots (about $0.3\,cm^3$ in size), quickly frozen in the liquid nitrogen and, then stored at -80 ℃ until analysis. Carcinoma tissues were obtained from poorly, moderate, and well differentiated ESCC. The corresponding normal tissues were obtained from the distant edge of dissected esophagus. For immunohistochemical (IHC) staining, tissues were fixed in 700 mL/L ethanol or 40 mg/L neutral formalin and embedded in paraffin.

Cell lines
Human ESCC cell lines, EC0156 and EC0132, were generated in our laboratory from ESCC tissues.

RNA isolation and semi-quantative RT-PCR
Total RNAs were isolated from ESCC specimens and cell lines with RNeasy MinElute cleanup kit (QIAGEN, Valencia, CA) according to the manufacture's instruction. RNA quality was assessed on agarose gel electrophoresis and spectrophotometric analysis. Reverse transcription reactions were performed on 5 µg of total RNAs using SuperScript™ First-Strand synthesis for RT-PCR II kit (Invitrogen, Carlsbad, CA) at 42 ℃ for 80 min, and 0.5-1 µg aliquots of the cDNA were then subjected to RT-PCR. Based on probable splice sites, the following primers were used to investigate different regions whether alternative splice forms or deleted fragments of clusterin could be found (Table 1).

The PCR step was performed using Taq DNA polymerase (Invitrogen, Carlsbad, CA). As an internal control, GAPDH was amplified to ensure cDNA quality and quantity for each RT-PCR reactions. The amplified multiproducts were analyzed on 12-20 g/L agarose gels. Each PCR reaction was down triplicate.

Protein extraction and western blot
The tumor tissues and cell lines were lysed in the lysis buffer (50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 10 g/L TritonX-100, 1 g/L SDS, 1 mmol/L AEBSF, 20 µg/mL Approtinin and 20 µg/mL Leupeptin) for soluble protein extraction or in the extraction/labeling buffer (BD Biosciences, San Jose, CA) for whole proteins extraction, and then placed on ice for 20 min. Then they were sonicated and centrifuged at 12 000 g at 4 ℃ for 30 min. The supernatants were transferred and the protein concentration was measured by Bradford method[30].

Equal amount of proteins were separated on 100 g/L of SDS-PAGE gels and transferred to polyvinylidene difluoride (PVDF) membranes. After being blocked with 10 g/L non-fat milk, the membranes were incubated with anti-clusterin monoclonal antibody B-5 (sc-5289, Santa Cruz Biotechnology, Santa Cruz, CA) (1:1 000 dilution) at 4 ℃ overnight. After washing for 3 times, the membranes were incubated with rabbit anti-mouse IgG at room temperature for 1 h. The signals were developed with the ECL kit (Amersham Pharmacia Biotechnology, Piscataway, NJ) and using anti-α-tubulin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) as an internal loading control.

Rabbit anti-human clusterin polyclonal H-330 antibody (sc-8354, Santa Cruz Biotechnology, Santa Cruz, CA) was used to detect the truncated forms of clusterin in whole extracts from EC0156 and EC0132 cells and tissues. A 1:500 dilution of primary H-330 antibody was used, followed by a 1:3 000 dilution of horseradish peroxidase-conjugated anti-rabbit secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

Immunohistochemical analysis
The streptavidin-peroxidase method was used for the immunohistochemical staining of clusterin. Briefly, after deparaffinization in xylene and rehydration in grade ethanol, endogenous peroxidase activity was blocked by incubation with 30 mL/L hydrogen peroxide for 10 min. Tissue sections were then heated at 100 ℃ in citrate buffer (10 mmol/L, pH 6.0) to retrieve antigens for 10 min. After being incubated with anti-human clusterin monoclonal/polyclonal antibodies, clusterin was visualized by adding biotinylated secondary antibody and streptavidin-horseradish peroxidase (Zymed Laboratories, South San Francisco, CA). Counterstaining was performed with hematoxylin and 3,3' -diaminobenzidine used as a chromogen. Negative controls, made by PBS excluding mono- or polyclonal anti-human clusterin antibodies from the reaction, showed no specific staining and. Experiments performed using polyclonal and monoclonal anti-human clusterin antibodies from the same commercial source gave the same pattern of specific distributions. Cover slips were mounted with Eukitt (O. Kindler GmbH & Co., Freiburg, Germany).

RESULTS

N-terminal deletions of the clusterin transcription in ESCC
To detect the alterations of clusterin expression, a multi-region cDNA fragments of ESCC were analyzed by semi-quantitative

Table 1 Primers and PCR reactions for multi-region amplifications of clusterin

Amplified fragments (bp)	Sense primers	Antisense primers	PCR conditions
1-1 350	5' -CCGGATCCTTATGATGA AGACTCTGCTGCTG-3'	5' -GCCTCGAGTCACTCC CGCTGCTTTTTG-3'	95 ℃ for 40 s, 62 ℃ for 40 s, and 72 ℃ for 90 s
1-1 056	5' -CCGGATCCTTATGATG AAGACTCTGCTGCTG-3'	5' -GTGTTGAGCATCTTCC ACTG-3'	95 ℃ for 30 s, 60 ℃ for 40 s, and 72 ℃ for 60 s
1-321	5' -CCGGATCCTTATGATGA AGACTCTGCTGCTG-3'	5' -CATCATGGTCTCATT GCACAC-3'	95 ℃ for 30 s, 56 ℃ for 30 s, and 72 ℃ for 30 s
437-1 056	5' -GAGCTCGCCCTTCTACTTCT-3'	5' -GTGTTGAGCATCTTCCACTG-3'	95 ℃ for 30 s, 56 ℃ for 30 s, and 72 ℃ for 30 s
437-1 350	5' -GAGCTCGCCCTTCTACTTCT-3'	5' -GCCTCGAGTCACTC CCGCTGCTTTTTG-3'	95 ℃ for 40 s, 60 ℃ for 40 s, and 72 ℃ for 60 s
1 120-1 350	5' -ACCTCACGCAAGGC GAAGACC-3'	5' -TCTCACTCCTCCCGGTGCTT-3'	95 ℃ for 30 s, 56 ℃ for 25 s, and 72 ℃ for 25 s
GAPDH	5' -ACCACAGTCCATGCCATCAC-3'	5' -TCCACCACCCTGTTGCTGTA-3'	

RT-PCR (Figure 1). The clusterin cDNA derived from the total RNA of both ESCC tissues and their matched dissected normal esophageal mucosa. The results of various fragments are shown in Figure 2. Comparing with the tumor matched normal epithelia of esophagus, the expressions of full-length and N-terminal fragment (1-320 bp) of clusterin were deleted obviously, however, there were no differences between the middle (437-1 056 bp) and C-terminal fragments (1 120-1 350 bp). By this approach, additionally using the primer containing the start codon of clusterin and the -3' end of the middle fragment obtained the same result with the one from full-length (1-1 056 bp), and also found the same expression level between the cancer tissue and normal epithelia from 437 to the end of clusterin (437-1 350 bp). The exceptional transcription area of clusterin was narrowed in the region of 1-437 bp, which contained the abnormal translation start site of the "death" form clusterin (nuclear clusterin) and omitted the region encoding the endoplasmic reticulum (ER)-targeting peptide of clusterin protein. These results indicated that clusterin was truncated at 5' end of gene.
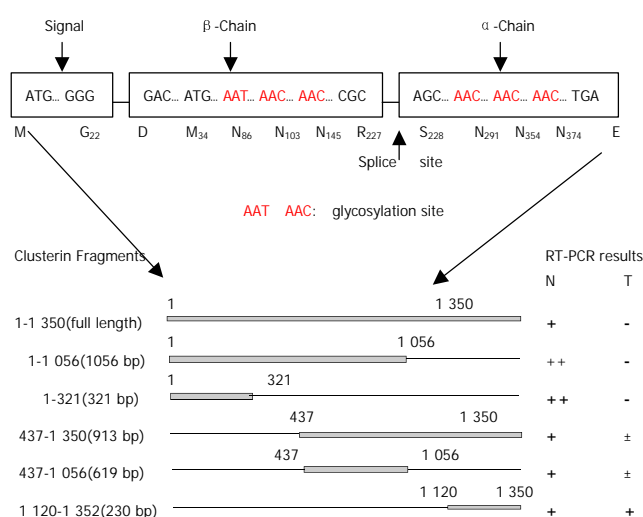

Figure 1 Schematic diagram of analyzed fragments of clusterin. A: structural map of the human clusterin protein. Letters and boxes signify the following: ER-targeting signal, endoplasmic reticulum-targeting hydrophobic leader peptide; splice site, α/β-cleavage site (amino acid 205 and 206) of cytoplasmic -60 ku pre-secreted clusterin which was glycosylated and cleavaged into α and β chains to form an 80 ku matured secretory clusterin protein; rectangles, hydrophobic leader peptide (N-terminal 66 bp encode aa M1 to G22, 22 aa), β-chain (5' end) and α-chain (3' end); Red letters, the glycosylation sites. B: Schematic diagram of analyzed fragments of clusterin and their RT-PCR results.
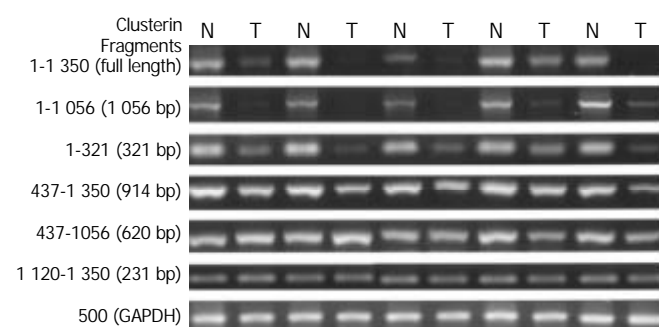

Figure 2 N-terminal truncated clusterin was analyzed by RT-PCR. *GAPDH* was used as internal control. N: tumor matched far edge normal esophageal mucosa. T: ESCC specimens. The expression of full-length and different of clusterin fragments

were analyzed comparing with the tumor matched normal esophageal mucosa. The truncated region was narrowed down at the 5' end of clusterin (1-437 bp).

Down-regulation of nuclear clusterin and secreted clusterin in ESCC

According to the results of RT-PCR, anti-clusterin antibodies were used to detect the expression of clusterin protein both in the tissues and cell lines of ESCC (Figure 3). The predominant form of clusterin in tumor tissues was the secreted heterodimeric glycoprotein with MW of 75-80 ku (37-40 ku smear in reducing SDS-PAGE), which was down-regulated in ESCC tissues compared with the normal epithelia of esophagus (100%, 21/21), but was absent in the ESCC cell lines (EC0516 and EC0132). The results confirmed our previous data that the clusterin gene is generally down-regulated in esophageal cancer[7]. Meanwhile, an about 50 ku nuclear clusterin was appeared in both the ESCC tissues (100%, 21/21) and cell lines (100%, 2/2), and sometimes in normal epithelia. It was highly expressed in the tumor tissues and cells. Additionally, an about 35 ku band was detected by anti-clusterin antibody H-330 in the EC0132 cell line. Under these experimental conditions, it must be noticed that, as shown in Figure 3, the monoclonal antibody used (Clusterin-β, B-5 from Santa Cruz Biotechnology, Santa Cruz, CA) might react with the carboxy terminus of clusterin β chain of human origin and not react with uncleaved nuclear clusterin. However, the polyclonal antibody used (Clusterin-α/β, H-330 from Santa Cruz Biotechnology Inc., Santa Cruz, CA) recognized the major part of clusterin from 120 to 449 amino acids at the carboxy terminus of clusterin-α/β chains, including the 60 ku precursor (cytoplastic clusterin), from which all clusterin isoforms are supposed to be derived, uncleaved nuclear clusterin (-50 ku) and the matured secreted clusterin (-37-40 ku, cleaved in –α and –β chains).
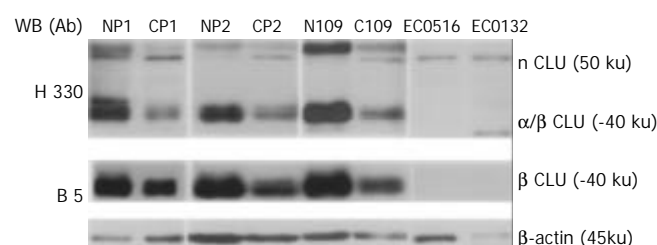

Figure 3 Western blot analysis of expression of multiple forms of clusterin in ESCC. nCLU: the nonglycosylated uncleaved nuclear clusterin; α/β CLU: cleaved and glycosylated secreted clusterin protein; β CLU: cleaved and glycosylated clusterin β chain; B-5: monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) reacted with the C-terminus of clusterin-β of human origin and did not react with the nuclear clusterin; H-330: polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) recognized the major part of clusterin from 120 to 449 aa at the C-terminus of clusterin-α/β chains including unleaved nuclear clusterin (-50 ku and the matured secreted clusterin (-37-40 ku); Np: pooled normal esophageal mucosa from 10 cases; Cp: pooled ESCC tumor tissues from 10 cases; N109: normal esophageal mucosa from a single individual; C109: ESCC tumor tissue from a single individual. β-actin was used as loading control.

Intracellular localizations of clusterin in ESCC

Immunohistochemistry analysis was performed in the tissue sections of ESCC specimen and matched normal counterparts using the same antibody (B 5, Santa Cruz Biotechnology, Santa Cruz, CA) as previously used for Western blot analysis (Figure 4). In normal esophageal mucosa, clusterin protein localized prevalently to the stroma of esophageal mucosa, while the squamous epithelial cells and basal lamina were negative. Very
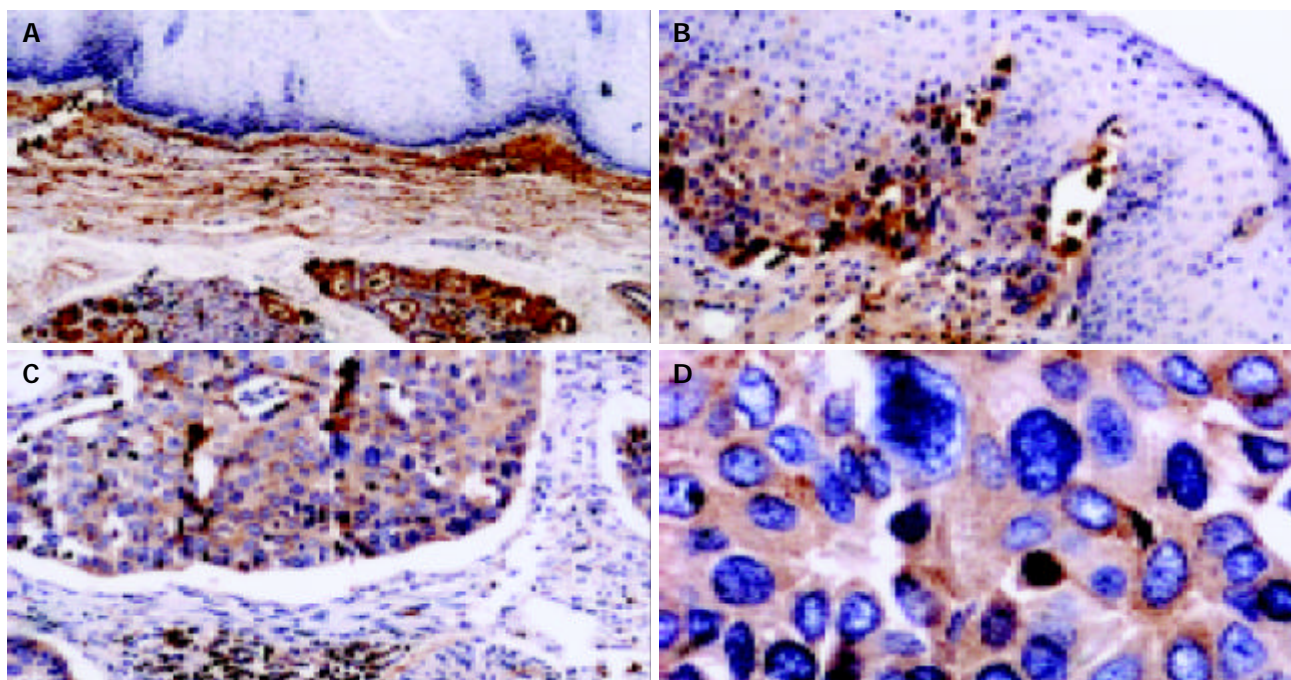
Figure 4 Detection of clusterin protein in tissue sections of human ESCC by immunohistochemistry. In normal esophageal mucosa, the expression of clusterin in squamous epithelial cells and basal lamina was negative, while clusterin immunostaining was visualized obviously in the stroma of epithelial mucosa (A, 100×); clusterin was positive in middle dysplasia, while the vast majority of stromal cells were negative in normal squamous epithelia of esophagus (B, 100×); clusterin was strong positive in the remnants of stromal extracellular matrix invaded by tumor cells in well-differentiated ESCC (C, 100×). D show that stromal and basal membrane are almost completely disrupted and invaded by tumor cells. (D, same section, different field of that of C, 400×). Counterstaining was performed with hematoxylin.

high levels of clusterin expression were found in the connective tissue of the lamina *propria* of epithelial mucosa, in which clusterin was confined to what appeared to be the remnants of the extra cellular matrix right under the basal membrane of epithelia, the cytoplasm of esophageal glandular cells, plasma membrane and cytoplasm of duct glandular epithelia, and plasma membrane and cytoplasm of lymphocytes in lymphatic follicle were detected positive by IHC (Figure 4A). During tumorigenesis the intracellular localizations of clusterin was translocated from stroma to the squamous epithelial cells at very early states. It was even found positive in middle dysplasia, a kind of very early lesion of esophagus versus negative in normal squamous epithelia (Figure 4B). However, clusterin and its pre-matured form were distributed in cancerous epithelia of ESCC (the antibody B 5 is able to detect the 60-ku secretory clusterin precursor by Western blot analysis, data not show here) and then, disappeared in stromal mucosa of esophagus (Figure 4C and D).

DISCUSSION

Based on our previous data, clusterin is markedly down-regulated in both serum and tissues of ESCC[7]. To further clarify the mechanisms of clusterin alterations during tumorigenesis, we first analyzed the gene transcription using semi-quantitative RT-PCR by amplifng different regions from 5' end to 3' end of the gene. We found the same expression results from 437 to 1 350 bp of clusterin between cancer and their matched normal. However, comparing with the cDNA reverse-transcripted from the total mRNA of normal epithelial mucosa, using the cDNA of tumor tissues, no fragment was amplified completely by the N-terminus of 5'-primer which contains the start codon located in the first exon (see underline sequences of the primer 5'-CCGGATCCTTATGATGAAGACTCTGCTGCTG-3'). Those suggested that there was an N-terminal deletion or miss splicing located on 1-437 bp that induced a wrong transcription

forms of the clusterin in ESCC. A miss splicing site repeated in breast cancer cell line MCF 7 was right in this area[29]. At protein expression level, this region is naturally corresponding to the β chain of clusterin protein. According this rationale, an N-terminal truncated form of clusterin protein would be also detectable. The different protein forms of clusterin in ESCC also describe the biogenesis of multi-transcription forms of clusterin.

The significance of alterations of clusterin gene expression during tumorigenesis remains a mystery. Our data indicated that human esophageal epithelial cells synthesized at least two forms of clusterin, secretory and nuclear clusterins, and their expression levels were changed during tumorigenesis. The secretory clusterin was down-regulated both in serum and tissues of ESCC; while, nuclear clusterin was induced in tumor cells. We also found a 60 ku secretory clusterin precursor protein in ESCC and normal epithelial cells (data not shown). Previous data indicated the existence of a mature about 55 ku nuclear clusterin protein, as a cell death protein and lack ER-signaling peptides, which did not appear to be either glycosylated or cleaved at its α/β site, a site cleaved during maturation of the 60 ku secretory clusterin precursor protein[12,28,29]. Yang et al[27], described nuclear clusterin was induced by relatively high levels of cytotoxic stress in direct proportion to lethality after growth stimulation of ionizing radiation (IR). Utilization of an in-framed secondary downstream AUG as a translation start site was proposed to result in the synthesis of an about 50 ku precursor nuclear clusterin protein that resided in cytoplasm of undamaged cells by confocal microscopy. Overexpression of this nuclear clusterin, even without IR treatment, caused cell death[12]. In our immunoblotting results, an about 50 ku nuclear clusterin was detected in both ESCC tissues and cell lines, and occasionally in normal esophageal epithelia using H-330 anti-clusterin antibody. This form of nuclear clusterin could not be detected with B-5 anti-clusterin antibody. As Leskov et al.[29] used, H-330 anti-clusterin antibody recognizes the major part of clusterin from 120 to 449 amino acid at the

carboxy terminus of clusterin α/β chains, including the 60 ku precursor and a -49 ku nuclear clusterin precursor (pnCLU). These results were consistent with our findings by RT-PCR that deletion at 5' end or appearance of a splicing at different sites of clusterin gene resulted in N-terminal truncated forms of clusterin transcription. These abnormal forms of clusterin undergo alien post-translational modification compared to secretory clusterin.

Nuclear clusterin is associated with Ku70[8], a DNA double-strand break repair protein. Its Ku70 binding activity was localized to the C-terminal coiled-coil domain of nuclear clusterin[30]. The C-terminal coiled-coil domain of nuclear clusterin was the minimal region required for Ku binding and apoptosis. We also found that Ku70 altered in ESCC (data not shown). Although we do not yet know whether nuclear clusterin binding to Ku70 is essential for lethality, we could speculate that enhanced clusterin binding to Ku70 may hinder the formation of Ku70/Ku80 heterodimer, consequently interfere with non-homologous DNA repair, resulting in genomic instability or cell death.

Our studies suggested, on the other hand, the alterations of the localizations and intracellular localizations of clusterin. It was translocated from stroma to the epithelial cells by disrupting the basal membrane of epithelial mucosa during carcinogenesis.

In general, our results demonstrated that the alterations in the expression, proteolysis and intracellular localizations of clusterin and an N-terminus truncated form of clusterin were found in ESCC. Its possible roles in cell survival, cell death and neoplastic transformation remain an additional debate.

ACKNOWLEDGEMENTS

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