Clusterin Expression in Normal Mucosa and Colorectal Cancer

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The gene Clusterin is a target for cancer therapy in clinical trials. The indication for intervention is up-regulated Clusterin expression. Clusterin has been reported to be deregulated in multiple cancer types, including colorectal cancer (CRC). However, for CRC the studies have disagreed on whether Clusterin is up- or down-regulated by neoplastic cells. In the present study we sought to clarify the expression and distribution of Clusterin mRNAs and proteins in normal and neoplastic colorectal tissue through laser microdissection, variant-specific real time RT-PCR, immunohistochemistry, immunofluorescence, Western blotting, and array-based transcriptional profiling. At the transcript level we demonstrated the expression of two novel Clusterin transcripts in addition to the known transcript, and at the protein level we demonstrated two Clusterin isoforms. Our analysis of normal epithelial cells revealed that among these, Clusterin was only expressed by rare neuroendocrine subtype. Furthermore our analysis showed that in the normal mucosa the majority of the observed Clusterin protein originated from the stromal compartment. In tumors we found that Clusterin was de novo synthesized by non-neuroendocrine cancer cells in ~25% of cases. Moreover we found that the overall Clusterin level in tumors often appeared to be lower than in normal mucosa due to the stromal compartment often being suppressed in tumors. Although Clusterin in normal neuroendocrine cells showed a basal localization, the localization in cancer cells was often apical and in some cases associated with apical secretion. Collectively our results indicate that Clusterin expression is very complex. We conclude that Clusterin expression is associated with neuroendocrine differentiation in normal epithelium and that the Clusterin observed in neoplastic cells is de novo synthesized. The cases with de novo synthesized Clusterin define a distinct subgroup of CRC that may be of clinical importance as anti-Clusterin therapeutics are now in clinical trials. Molecular & Cellular Proteomics 6: 1039–1048, 2007.

Over the recent years it has become increasingly clear that the gene Clusterin (CLU) and the proteins encoded by it are involved in carcinogenesis, tumor growth, and resistance to therapy (1). CLU has been reported to be involved in numerous physiological processes important for carcinogenesis and tumor growth, including apoptotic cell death, cell cycle regulation, DNA repair, cell adhesion, tissue remodeling, lipid transportation, membrane recycling, and immune system regulation (1, 2). In the literature the dominating view states that increased CLU expression confers cytoprotective capabilities to the tumor (1). The mechanisms for this are not fully understood; however, a recent study showed that intracellular CLU can inhibit apoptosis by interfering with Bax activation in mitochondria (3). It is generally accepted that CLU is a stress-inducible gene, induced for example by ionizing radiation and several chemotherapeutics (1). Increased CLU levels lead to inhibition of apoptosis and promote cancer cell survival in the face of therapeutic stimuli (1, 4). Recent preclinical and clinical phase I studies have demonstrated that inhibition of CLU expression using antisense oligonucleotides enhances the apoptosis induced by several conventional chemotherapeutic treatments (1, 4–6). CLU has been reported to be a nine-exon gene encoding three protein isoforms with distinct subcellular locations and functions (1, 7, 8): a prosurvival secretory form of ~80 kDa (glycosylated, proteolytically cleaved) (9–11), a prosurvival cytoplasmic form of ~60 kDa (non-glycosylated) (3), and a proapoptotic nuclear form of ~50 kDa (non-glycosylated) (12). It is unclear how the protein isoforms are produced from the CLU gene, and this remains a topic of debate. Current hypotheses focus on inclusion or exclusion of exon 2, which encodes an endoplasmic reticulum leader peptide. When included exon 2 targets the resulting protein for secretion (12, 13). Data from several public available database sources indicate the existence of other CLU mRNA variants, e.g. the National Center for Biotechnology Information (NCBI) reference sequence database (lists two CLU mRNA isoforms, neither has exon 2 excluded) and the Alternative Splicing Annotation Project database (predicts multiple alternative splicing CLU isoforms) (14). These other CLU transcripts could potentially be templates for the observed CLU proteins. Despite the...
publicly available data indicating the existence of multiple novel CLU transcripts, the task of evaluating the evidence for these transcripts and to validate their possible expression has not yet been addressed. Knowledge of all potential CLU transcripts is of utmost importance when designing oligos for silencing CLU translation using the novel antisense therapeutic strategy mentioned above.

Silencing CLU translation is of interest for the treatment of all cancers that overexpress CLU, including bladder, breast, lung, and potentially also colorectal cancer (CRC) (1). However, previous studies on CLU expression in CRC have disagreed on whether CLU is up-regulated (7, 15) or down-regulated by the cancer cells (16). There are many possible explanations for the inconsistent observations, e.g. analysis of different CLU variants caused by the use of different antibodies or by PCR assays targeting different isoforms. Another explanation could be the lack of knowledge of the cell types producing the observed CLU signal. The importance of the latter was exemplified in a recent study of prostate cancer (17) where immunohistochemistry (IHC) analysis revealed CLU to be almost undetectable in carcinoma cells despite being highly expressed in peritumoral fibroblasts, a conclusion unlikely to have been reached by applying crude sample techniques such as Western blotting or real time RT-PCR.

Another issue is the regulation of CLU expression. In neoplasia a well known mechanism of gene regulation involves genomic alterations. Genomic abnormalities of the short arm of chromosome 8, where the CLU locus is located (8p22), are commonly observed in CRC (18) and thus could potentially explain the reported CLU deregulation.

The objectives of the present study addressed the issues raised above and were as follows: 1) to identify potential CLU mRNA variants and validate their expression in colorectal tissue, 2) to investigate the expression levels of validated CLU transcripts in normal epithelia and cancer cells, 3) to resolve the controversy regarding the expression of CLU by neoplastic colorectal tissue, and (4) to investigate whether loss of genomic integrity at the CLU locus is associated with the deregulation of CLU observed in CRC.

**EXPERIMENTAL PROCEDURES**

**Patients**—Seven normal mucosa samples and two adenocarcinoma samples were used to validate the expression of CLU mRNA variants by RT-PCR. IHC was performed on an independent set of 93 formalin-fixed and paraffin-embedded samples. This set comprised a colon carcinoid tumor, 15 pairs of matched normal colorectal mucosa and adenocarcinoma specimens (conventional histological sections from six stage I, three stage II, and six stage III adenocarcinomas), and 62 colorectal specimens (two normal colon mucosa, nine adenoma, 10 stage I adenocarcinoma, 10 stage II adenocarcinoma, 10 stage III adenocarcinoma, 10 stage IV adenocarcinoma, and 11 liver metastasis) located in a human colon carcinoma tissue microarray (COSCA 912-5-OL, BioCat, Heidelberg, GE). The International Union Against Cancer “Tumor, Node, Metastasis” staging system was used for staging of the adenocarcinomas. Formalin-fixed and paraffin-embedded sections from two of the 15 pairs of normal and adenocarcinoma samples used for IHC were also used for immunofluorescence analysis.

Fresh frozen tissue from all 15 pairs of matched normal mucosa and adenocarcinoma were submitted to laser microdissection by laser-microdissected samples and used for quantitative real time RT-PCR analysis. Furthermore eight of the 15 sample pairs were also investigated by Western blot analysis. An independent set of 119 samples (17 normal mucosa and 102 adenocarcinomas) were used for transcriptional profiling as described previously (19).

**Validation of CLU Transcript Variant Expression in Colorectal Tissues by RT-PCR and Sequencing of the PCR Products**—To validate the expression of predicted CLU transcript variants in colorectal tissues, cDNA was synthesized from 1 μg of total RNA from each of seven normal mucosa and two cancer samples. The oligo(dT)-initiated cDNA synthesis was performed using the Superscript II reverse transcriptase kit (Invitrogen). Variant-specific PCRs, using the cDNAs as templates, were performed using the Expand High Fidelity PCR System (Roche Applied Science). Variant-specific primer pairs were constructed by combining specific forward primers located in the unique exons 1 (1a, 1b, or 1c) with a common reverse primer located in exon 4 (see Fig. 1, A and B). Primer sequences are given in Supplemental Table 1. The resulting PCR products were excised from the agarose gel and purified using the QIAquick Gel Extraction kit (Qiagen, Valencia, CA). The excised products were then used as templates for direct bidirectional sequencing using the BigDye Terminator kit (Applied Biosystems, Foster City, CA) and an ABI 3100 Genetic Analyzer (Applied Biosystems). Sequencing was performed using the same primers as used for RT-PCR.

**Antibodies**—Four different antibodies against human CLU were used: Clone 41D, a mouse monoclonal anti-human CLU antibody (Upstate, Charlotteville, VA); H-330, a rabbit polyclonal anti-human CLU antibody (Santa Cruz Biotechnology, Santa Cruz, CA); CLU-9, a mouse monoclonal anti-human CLU antibody (Alexis Corp., Lausen, Switzerland); and B-5, a mouse monoclonal anti-human CLU antibody (Santa Cruz Biotechnology). Detailed information can be found in Supplemental Table 2.

As a marker of neuroendocrine differentiation, we used a monoclonal mouse anti-human Chromogranin A antibody (LKH210, ab8204) from Abcam. As loading control for Western blotting, we used a monoclonal mouse anti-human β-actin antibody (catalog number A-1978, clone AC-15, Sigma-Aldrich).

**Immunohistochemistry**—IHC was performed as described previously (20). In brief, paraﬁn was removed followed by blocking of endogenous peroxidase activity, heat-induced epitope demasking, and blocking to prevent unspeciﬁc binding. Then primary antibodies were applied at the following dilutions: CLU clone 41D, 1:600; CLU H-330, 1:100; CLU CLU-9, 1:100; CLU B-5, 1:100; and Chromogranin A (CgA), 1:10. Detection was performed using the Envision system (DakoCytomation, Glostrup, Denmark). Finally the sections were counterstained with hematoxylin.

**Scoring of CLU IHC Staining**—The TMA and histological sections were reviewed independently by two observers (C. L. Andersen and K. Birkenkamp-Demtroeder). The presence of cytoplasmic and secretory CLU was evaluated. To avoid misleading border staining, border areas were excluded from evaluation.

Criteria for scoring of cytoplasmic CLU were as follows. Only the epithelial cells of the normal mucosa sections were scored; likewise only the cancer cells were evaluated in the tumor sections. Sections were scored positive if immunoreactivity was seen in 10% or more of the target cells and negative if seen in less.

Criteria for scoring of secretory CLU were as follows. Only acini secretion was scored. Sections were scored positive if immunoreactivity was seen in 10% or more of the luminal acini and negative if seen in less.
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*Immunofluorescence Microscopy—* Immunofluorescence microcopy was performed as described previously (21). After deparaffinization, sections were incubated with a mixture of CLU H-330 rabbit polyclonal antibody (1:50 dilution) and monoclonal mouse anti-human CgA antibody (1:10 dilution). CgA is a well known marker of neuroendocrine differentiation (20). Bound primary antibodies were detected with AlexaFluor 488-conjugated goat anti-rabbit IgG highly cross-adsorbed (1:1,000; Molecular Probes Inc., Eugene, OR) and AlexaFluor 546-conjugated goat anti-mouse IgG1 (1:400; Molecular Probes Inc.). DNA was visualized by 4,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich). Images were captured using a charge-coupled device camera (Quantix, Photometrics, Tucson, AZ) mounted on a Leica DMRXA epifluorescence microscope (Leica, Wetzlar, Germany) with appropriate filters and operated via SmartCapture software (Digital Scientific, Cambridge, UK).

**Western Blotting—* Western blot analysis was performed as described previously (20). From eight pairs of adenocarcinoma and adjacent normal mucosa 10 μg of total protein were loaded on 12% NuPAGE gels (Invitrogen) and blotted to PVDF-plus membranes. After blocking with 5% skimmed milk, CLU protein was detected using three different specific anti-CLU antibodies at the following dilutions: Clone 41D, 1:500; CLI-9, 1:1500; and B-5, 1:200. As loading control, three different specific anti-CLU antibodies at the following dilutions: Clone 41D, 1:500; CLI-9, 1:1500; and B-5, 1:200. As loading control, parallel immunoblotting using a β-actin monoclonal antibody diluted to 0.05 μg/ml was performed.

**Real Time RT-PCR—* Quantitative real time RT-PCR for the CLU mRNA variants was performed on an ABI PRISM® 7000 Sequence Detection System (Applied Biosystems) using the relevant (TaqMan or SYBR Green) Master Mix (Applied Biosystems). For normalization the gene Ubiquitin C (UBC) was used. We have demonstrated previously the suitability of UBC as a normalization gene for analysis of normal mucosa and colorectal cancer specimen sample sets (22). We applied laser microdissection to procure ~100% pure normal epithelial and cancer cell populations from fresh frozen tumor and adjacent normal mucosa biopsies of five CRC patients (two stage I, two stage II, and one stage III). From each sample ~10,000 cells were dissected. Total RNA was extracted from the pairs of matched normal epithelia and cancer cells using the RNeasy microkit, including on-column DNase digestion (Qiagen). RNA integrity was evaluated by Bioanalyzer analysis using Pico chips (Agilent Technologies, Palo Alto, CA). Only samples with two clear ribosomal bands were included.

The presence of the CLU mRNA variants were quantified using variant-specific primer/probe sets. The overall CLU level (the sum of all three variants) was measured using a primer/probe set targeting all three variants in normal colorectal mucosa and cancer specimen sample sets (22). The sequences of primer and probes can be found in Supplemental Table 3. The specificity of each primer set was validated by Basic Local Alignment Search Tool (BLAST) searches (www.ncbi.nlm.nih.gov/BLAST/) and electronic PCR (29). Furthermore agarose gel electrophoresis confirmed the individual primer sets to generate only a single amplicon of the expected size. The normalization gene UBC was investigated using a SYBR Green assay. The primers for the UBC assay have been published previously (22).

Each measurement was performed in triplicate, and no-template controls were included for each assay. Relative expression values were obtained using a dilution curve consisting of four 10-fold serial dilution points. The dilution curve was created using a cDNA pool containing 2 μl of each of the test cDNAs.

**Loss of Heterozygosity and DNA Copy Number Analysis Using Single Nucleotide Polymorphism Arrays—* Previously we have analyzed 15 pairs of matched germ line (blood) and laser-microdissected CRC samples by Affymetrix 10K XbaI SNP arrays (18). Briefly matched cancer and germ line DNA was extracted using the PUREGENE® DNA extraction system (Gentra Systems, Minneapolis, MN). The Single Primer Assay Protocol (labeling, hybridization to the 10K XbaI SNP array, washing, staining, and scanning) was performed according to the manufacturer’s instructions (Affymetrix, Santa Clara, CA). Loss of heterozygosity (LOH) and DNA copy number information was extracted from the arrays as described previously (18).

**Sequencing of the Genomic CLU Locus—* The integrity of the genomic CLU locus was analyzed by bidirectional sequencing using the BigDye Terminator kit (Applied Biosystems) and the ABI 3100 Genetic Analyzer (Applied Biosystems). Fragments representing each exon and the adjacent intron-exon boundaries were generated by PCR. The sense and antisense primers applied for generation of the PCR fragments were also used for sequencing. Primer details can be found in Supplemental Table 4. Importantly the splice junctions of all exons were included in the sequence analysis. Sequencing covered all 11 exons (1a, 1b, 1c, and 2–9) in Fig. 1.

**Transcriptional Profiling—* The transcriptional profiling data used in the present study have been published previously by our group (19). In brief, total RNA from 17 normal mucosa samples and 102 colorectal adenocarcinomas (one Dukes A, 36 Dukes B, 64 Dukes C, and one Dukes D) were transcriptionally profiled using Affymetrix HG-U133A GeneChips arrays. The expression data were GC_RMA-normalized using ArrayAssist software (Stratagene, La Jolla, CA).

**RESULTS**

In Silico Analyses of CLU mRNA and EST Sequences to Identify Potential CLU mRNA Variants—To identify novel CLU variants, we inspected the CLU entries in various alternative splicing databases (The AceView Genes, www.ncbi.nlm.nih.gov/IEB/Research/Acembly; the Alternative Splicing Annotation Project (ASAP) (14)). To evaluate the sequence support for the predicted alternative splice variants we inspected the sequences in the CLU UniGene cluster Hs.436657 (90 mRNA and 4873 EST sequences as of June 2006). These inspections revealed multiple possible CLU mRNA variants of which three stood out by having substantially more sequence support than the rest. We termed them CLU34, CLU35, and CLU36. These three variants all contained nine exons. They each had a unique exon 1 and shared the remaining exons 2–9 (Fig. 1A). CLU34 and CLU35 correspond to the two CLU transcript isoforms predicted by the NCBIR Reference Sequence project, NM_001831 and NM_203339, respectively. The sequences of all three variants are given as supplemental information. Surprisingly we found no sequence support in the UniGene cluster for the "skipping of exon 2" variant observed in cDNA from the MCF7 breast cancer cell line by Leskov et al. (12).

Using protein sequences based on the largest open reading frame, the subcellular localization of all three variants was predicted using PSORT II (23). Both CLU34 and CLU35 yield the same open reading frame starting in exon 2, right in front of an endoplasmic reticulum localization leader sequence; thus, the resulting proteins were predicted to be secreted. CLU36, on the other hand, was predicted to be localized to the nucleus.

All Three CLU mRNA Variants Are Expressed in Normal Colorectal Mucosa and Cancer—Variant-specific RT-PCR followed by sequencing of the products confirmed the expression of all three variants in normal colorectal mucosa and cancer (Fig. 1B). We did not observe any additional bands in the three variant-specific RT-PCRs; thus we found no evi-
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A

<table>
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B

<table>
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<td>A</td>
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<tr>
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</tr>
<tr>
<td>10 2 3 4 5 6 7</td>
<td>452</td>
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C

Fig. 1. Identification of multiple CLU transcript variants. Identification, verification, and quantification of three CLU transcript variants are shown. A, three CLU transcript isoforms were identified. The existence of all three variants is supported by several alternative splicing databases and multiple sequences in the CLU UniGene cluster Hs436657. At the mRNA sequence level the three variants differ only by the first exon. B, all three variants were found to be expressed in tumors and normal mucosa of the colon. Isoform-specific RT-PCR was performed, the resulting products were cut from the gels, and the exon structure was verified by DNA sequencing. C202 and N202 and T271 and N271 represent tumor and adjacent normal mucosa from patients 202 and 271, respectively. The N pool is a pool of cDNA generated from normal mucosa from five independent individuals. C, variant-specific quantitative real time RT-PCR revealed a non-uniform expression pattern of isoform CLU34 in matched normal epithelia and cancer and a uniform pattern for isoform CLU35 that was always down-regulated in the cancer cells. An average of all three variants was measured using the CLU overall primer/probe set located in exons 7 and 8, which are common to all variants. The CLU overall expression pattern was very similar to that of CLU34, indicating that CLU34 is the dominating CLU transcript in epithelial and cancer cells of the colon and rectum. The real time RT-PCR analyses were performed on ~100% pure cell populations (normal epithelia or cancer cells) laser-microdissected from normal mucosa and tumor tissue biopsies of five patients (patient numbers 326, 336, 368, 496, and 497). Real time RT-PCR data were normalized to the expression level of UBC. The whisks on the bars indicate 1× S.D. 

To confirm that the observed CLU expression pattern was
not just a reflection of a specific antibody targeting a specific CLU protein variant, the analysis was repeated using three other CLU antibodies. All four antibodies, one polyclonal and three monoclonal antibodies targeting different parts of the CLU protein, yielded similar IHC results (Supplemental Fig. 1) indicating that the observed expression pattern was general and genuine.

The Normal Epithelial Cells Expressing CLU Are Characterized by Neuroendocrine Differentiation—Their low number and distribution along the crypt axis along with the subcellular localization of the CLU protein made us hypothesize that the CLU-positive epithelial cells were neuroendocrine epithelial cells. To test this hypothesis, we performed IHC with CLU and CgA, an often used marker of neuroendocrine differentiation (20), on consecutive sections of normal colon mucosa specimens. CLU appears to be expressed by a subset of the CgA-positive cells (arrows). C, CLU expression in a carcinoid tumor (cancer cells with neuroendocrine differentiation). D–G, co-localization of CLU and CgA by IF. D, CLU IF (CLU-positive cells are indicated by arrows). E, CgA IF (CLU-positive cells are indicated by arrows). F, DAPI counterstain of the nuclei, illustrated in grayscale to enhance the contrast (CLU-positive cells are indicated by arrows). G, overlay of CLU, CgA, and DAPI fluorescence. The overlay illustrates that a fraction of the CgA-positive cells are also CLU-positive. Cells concomitantly positive for CLU and CgA are indicated by arrows; cells only positive for CgA are indicated by arrowheads.

Fig. 2. CLU expression in the normal colon. Investigation of CLU protein expression in normal colon by immunohistochemistry is shown. A, only a very small fraction of the epithelial cells express CLU (arrows). B, an enlargement of a part of the section shown in A. C, CLU protein aggregates are often found in the basal part of lamina propria (arrows). D, CLU is expressed and secreted by cells located in the germinal center of lymphoid nodules (arrow). E, parasympathetic ganglia in the lamina propria express and secrete CLU (arrow). F, CLU is expressed by endothelial cells (arrow), and characteristic CLU protein aggregates are found in the periphery of arteries probably corresponding to tunica adventitia (arrowhead). G, negative control (no primary antibody).

Fig. 3. CLU expression by epithelial cells with neuroendocrine differentiation. IHC and immunofluorescence (IF) reveals co-localization of CLU and CgA, a marker of neuroendocrine differentiation, expression in a subset of colon epithelial cells. A and B, examples of CLU and CgA IHC on consecutive sections of normal colon mucosa specimens. CLU appears to be expressed by a subset of the CgA-positive cells (arrows). C, CLU expression in a carcinoid tumor (cancer cells with neuroendocrine differentiation). D–G, co-localization of CLU and CgA by IF. D, CLU IF (CLU-positive cells are indicated by arrows). E, CgA IF (CLU-positive cells are indicated by arrows). F, DAPI counterstain of the nuclei, illustrated in grayscale to enhance the contrast (CLU-positive cells are indicated by arrows). G, overlay of CLU, CgA, and DAPI fluorescence. The overlay illustrates that a fraction of the CgA-positive cells are also CLU-positive. Cells concomitantly positive for CLU and CgA are indicated by arrows; cells only positive for CgA are indicated by arrowheads.
Expression of Clusterin in normal colorectal mucosa (n = 17) and in benign and malignant colorectal tumors (n = 75)

All sections were scored blinded and independently by two observers (C. L. Andersen and K. Birkenkamp-Demtröder). There was an excellent interobserver agreement indicated by $\kappa$ values of 0.899 ($p < 0.0001$) and 0.851 ($p < 0.0001$) for the cytoplasmic and secretory scores, respectively. The few cases showing disagreement were re-evaluated, and consensus was reached before interpretations were made. The results show a significant increase in frequency of cytoplasmic CLU expression in adenomas and adenocarcinomas (5% level, one-tailed Fisher exact test). The increase in frequency of acini secretion was not significant at the 5% level (one-tailed Fisher exact test) for either tumor category.

<table>
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<th>Cases with CLU expression*</th>
<th>Normal mucosa</th>
<th>Adenoma</th>
<th>Primary adenocarcinoma</th>
<th>Liver metastases</th>
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<td>0 (0%)</td>
<td>11 (20%)</td>
<td>2 (18%)</td>
</tr>
<tr>
<td>Acini secretion</td>
<td>0 (0%)</td>
<td>6 (60%)</td>
<td>10 (19%)</td>
<td>2 (18%)</td>
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</table>

* The first value listed represents n.

Fig. 4. CLU protein expression in colorectal adenocarcinomas. Investigation of CLU protein expression in colorectal adenocarcinomas by immunohistochemistry is shown. Illustrated are representative examples of CLU staining patterns. A, no CLU staining. B, uniform cytoplasmic CLU staining. C, cytoplasmic CLU staining in the apical part of the cancer cells. D, cytoplasmic expression and apical secretion of CLU by the cancer cells. E, negative control (no primary antibody).
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Fig. 5. In the majority of cases the overall CLU level is lower in adenocarcinoma than normal mucosa. IHC, Western blotting, and gene expression profiling of whole (including stroma) normal mucosa and adenocarcinoma biopsies reveal that the overall CLU protein level in adenocarcinomas is often lower than in matching normal mucosa. A, CLU IHC on a representative pair of tumor and matched normal mucosa. The reduction in the overall CLU level appears because the CLU-positive stromal compartments of the normal mucosa are lost in the tumor. B, Western blotting indicates that the level of the 40-kDa isoform is lower in adenocarcinoma than normal mucosa (similar results were obtained with CLU antibodies CLI-9 and B-5, results not shown). In the adenocarcinoma samples the 40-kDa band splits into two, probably reflecting a change in the level of glycosylation. C, array-based CLU expression profiling of 17 normal mucosa and 102 adenocarcinoma samples shows that the overall CLU mRNA level on average is 3-fold (log2 scale, 8-fold on linear scale) lower in adenocarcinomas than normal mucosa samples. The probe set 208791_at targets exons 6–9, which are common to all three CLU mRNA isoforms; thus the measured intensities correspond to the overall CLU mRNA level on the tumor. We conclude that colorectal cancers often lose one CLU allele, whereas the integrity of the remaining allele apparently remains intact.

DISCUSSION

In the present study, we have provided evidence for the existence of three CLU transcript isoforms, termed CLU34, CLU35, and CLU36, of which the last two are novel. We demonstrated that all three variants were expressed by normal and neoplastic human colorectal tissue. The three variants all have nine exons; each of them has a unique exon 1, while they share exons 2–9. CLU34 corresponds to the CLU variant commonly reported in the CLU literature; it encodes the secreted CLU protein isoform (1, 12, 24, 25). The two other transcript isoforms are novel in respect to the CLU literature. In the literature, an alternative splicing variant of CLU34 has been reported, leading to skipping of exon 2 and, supposedly, the generation of the CLU protein isoform localizing to the nucleus (12). We found no support for this skipping of exon 2 variant in the alternative splicing databases or in our inspection of the ~4,900 sequences in the CLU UniGene cluster. Moreover our experimental search for CLU mRNA variants expressed in colorectal tissues (illustrated in Fig. 1) showed no signs of a skipping of exon 2 variant. Possibly this variant is not expressed by colorectal tissue, or it could be an artifact of the MCF7 breast cancer cell line where it was discovered (12).

Surprisingly sequence analysis of the CLU34 and CLU35 variants predicted them to yield one and the same secreted CLU protein. Although based on our data, we cannot determine whether the CLU34 and CLU35 variants give rise to the same protein, our real time RT-PCR data indicate that their expression levels are regulated by different factors/mechanisms and, thereby, that they have different biological functions. To explain our real time data we hypothesize that CLU34 and CLU35 are either 1) differentially expressed within the same cells or 2) expressed by different epithelial cell
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In line with the latter, it is interesting to note that disappearance of CLU35 expression coincides with disappearance of cells with neuroendocrine differentiation. Possibly the CLU35 variant is expressed predominantly by neuroendocrine cells. Sequence analysis of the CLU36 variant predicted its subcellular localization to be the nucleus. Interestingly our real time RT-PCR analysis indicated very low expression levels of this variant in both normal epithelia and cancer cells, implying no or at least very low nuclear levels of CLU protein, which was indeed what we observed by IHC.

Although some reports have indicated CLU to be up-regulated in neoplastic colorectal tissues (7, 15), others have indicated down-regulation (16). This apparent discrepancy is not unique for studies investigating CLU expression in CRC as it has also been reported for prostate cancer (1). Unawareness of the different mRNA and protein variants is, at least in part, a likely cause of the inconsistencies. Our novel knowledge of three unique transcript variants enabled us to address this problem. We performed our variant-specific real time RT-PCR on ~100% pure normal epithelia and cancer cell subpopulations procured by laser microdissection. Thereby we ensured not only that we measured the variant of interest but also that we did so in the right cell population. This issue has only rarely been taken into account in previous studies on CLU expression. Interestingly only one of the variants showed a uniform dysregulated pattern in cancer cells: CLU35 was uniformly down-regulated in all five investigated cancer specimens. Based on these data, we hypothesize that either down-regulation of CLU35 is advantageous for the cancer cells, or the reduction in CLU35 is caused by the tumor-associated disappearance of the cell type expressing CLU35.

To characterize CLU expression in the normal mucosa we turned to IHC analysis. Unfortunately neither of the CLU antibodies we tested allowed us to distinguish the individual CLU protein isoforms: in Western analysis they all produced bands of ~40 and ~60 kDa. However, an important finding of the IHC analysis was that the majority of CLU-expressing cells in the normal mucosa were located in the stromal compartment. CLU was observed in fibroblasts, endothelial cells, germinal center cells, and nerve cells and as protein aggregates in the basal part of lamina propria and encircling the periphery of arteries. Surprisingly we found that only a minority (<1%) of the epithelial cells expressed CLU. This finding was in contrast to a previous study on CLU expression in colorectal tissues that indicated expression of nuclear CLU in all normal epithelial cells and a shift in subcellular localization to the cytoplasm in tumors (7). Importantly our analysis included both the CLU antibodies (CLU-31D and H-330) applied in the aforementioned study. Despite this, we never observed nuclear expression in normal epithelial cells. For this discrepancy we have no explanation; however, our observations are supported by another study of CLU expression in normal and neoplastic colorectal tissue in which the authors did not observe any nuclear CLU either (15).

Our further characterization of the nature of the few CLU-positive epithelial cells led us to conclude yet another piece of novel information, namely that the CLU-positive cells constituted a subfraction of the rare neuroendocrine cells. This is very interesting as previous studies have shown CLU to be stored in secretory granules of other neuroendocrine secretory tissues and released upon stimulated exocytosis (26–28). This raises the possibility that the CLU protein we often observed in the basal part of the lamina propria was secreted by the CLU-expressing neuroendocrine cells. Many studies have indicated that CLU is a stress-related protein induced by many different stress factors including ionizing radiation, heat, cytokines, and chemotherapy (1, 2). Thus, stress could be the stimulus causing the neuroendocrine cells to release CLU into the extracellular space.

We also investigated a series of tumors by IHC. These tumors represented the full spectrum of CRC pathogenesis, including benign adenomas, adenocarcinomas, and metastases. In the majority of tumors (75%) the cancer cells did not express CLU. In the remaining 25%, we found CLU de novo synthesized (hence up-regulated) by non-neuroendocrine differentiated cancer cells. These 25% of cases may account for the few tumors that have expression values in the range of normal cells as seen in Fig. 5C. The de novo CLU synthesis was observed in tumors covering all stages of CRC pathogenesis from the earliest adenoma to advanced liver metastases. Interestingly the de novo synthesized CLU was often secreted.

Not surprisingly, the stromal compartments of the tumor specimens were often dramatically suppressed compared with normal mucosa. Hence in 75% of the tumor specimens without CLU-expressing cancer cells, the overall level of CLU appeared lower than in normal mucosa. This observation, which was confirmed both at the protein and mRNA level by Western blotting and array-based transcriptional profiling of complete biopsies, is a further explanation of the discrepancies among the previous studies on CLU expression in colorectal tissues. Collectively our data indicate that CLU expression is involved in tumorogenesis and progression of ~25% of CRCs.

How CLU expression is regulated in normal colorectal epithelial cells and especially in cancer cells is unknown. However, a common mechanism to distort the transcriptional regulation of oncogenes and tumor suppressor genes involves alterations of their genomic loci. To investigate the integrity of the CLU locus, we performed LOH and DNA copy number analysis on a series of CRC specimens. Based on the CLU protein expression levels revealed by our IHC data we expected the majority of tumors to have two CLU alleles and up to 25% of tumors to show a slight amplification. Much to our surprise, we reached a completely different result, namely that the majority (67%) of tumors showed loss of one CLU allele.

To investigate whether biallelic inactivation of the CLU locus was the explanation for CLU not being expressed in ~75% of
tumors, we next analyzed the integrity of the remaining alleles by sequencing. In all cases the integrity of the remaining allele was intact. Unfortunately our analyses did not reveal the LOH and copy number status of the CLU-expressing tumors. However, collectively our data revealed that all investigated tumors had at least one intact CLU allele and thus the potential to express CLU.

Seen from a clinical perspective the finding that CLU is de novo synthesized in 25% of CRCs is very interesting. It is well accepted that CLU up-regulation confers cytoprotective and prosurvival measures to the cancer cells and that this in turn facilitates tumor progression (1). Hence we suggest that the CRC patients with de novo CLU expression should be targets for treatment with the novel CLU antisense therapy possibly in conjunction with conventional treatment regimens. This suggestion is in line with 1) a recent review of the clinical implications of CLU expression that advocated silencing of CLU expression in all cancers that overexpress CLU (1) and 2) the multiple preclinical and clinical phase I trials that have shown CLU antisense therapy to sensitize prostate, breast, lung, bladder, and kidney cancer cells to conventional chemotherapeutics (1,4–6). A recent CLU review has urged for caution when using CLU antisense therapy (1). Our data indicate this is not a major issue for the treatment of CRC as we pinpointing the patients likely to benefit from the antisense CLU expression level using ordinary IHC methodology, thus CRC patients can easily be subclassified according to chemo-sensitivity in prostate cancer. Vancouver experience from discovery to clinic. Int. J. Urol. 12, 785–794


Clusterin Expression in Colorectal Tissue


