CLU and Colon Cancer. The Dual Face of CLU: From Normal to Malignant Phenotype

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The transition from normal to malignant phenotype implies the activation of some pathways that underlie the aberrant clone expansion. In some way, the conventional function of proteins involved in DNA repair, cell death/growth induction, vascularization, and metabolism is inhibited or shifted toward other pathways by soluble mediators that orchestrate such change depending on the microenvironment conditions. The adenoma–carcinoma sequence of the colon represents one of the most well studied and characterized models of human tumor progression. In this section, we focus our attention on defined pathways that underlie the initiation, promotion, and progression of colon cancer, conferring aggressiveness to the neoplastic cells. Clusterin (CLU) is a pleiotropic protein with a broad range of functions. It has recently drawn much attention because of its association with cancer promotion and metastasis. It is involved in prosurvival and apoptosis processes that are carried out by two different forms. sCLU is cytoprotective and its prosurvival function is the basis of the current Phase I/II clinical trials. In colorectal cancer an increase of sCLU expression occurs, whereas the nuclear proapoptotic form is downregulated. Several controversial data have been published on colon cancer discussing its role as tumor suppressor or prosurvival factor in colon cancer. Here, we report the dynamic interaction of the different forms of CLU with their partners DNA-repair protein Ku70 and proapoptotic factor Bax during colon cancer progression, which seems to be a crucial point for the neoplastic cell fate.

We also highlight that the appearance and the progressive increase of the sCLU in colorectal tumors correlate to a significant increase of CLU in serum and stool of patients. On the basis of results obtained by CLU immuno-dosage in blood and stool of colon cancer patients, we report that sCLU could represent a diagnostic molecular marker for colon cancer screening.

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I. INTRODUCTION: GENES AND PROTEINS IN COLORECTAL CANCER

Colorectal cancer is a significant cause of morbidity and mortality in Western populations. This cancer develops as a result of the pathologic transformation of normal colonic epithelium to an adenomatous polyp and ultimately an invasive cancer. The multistep progression requires years and possibly decades and is accompanied by a number of well-characterized genetic alterations. Chronic inflammation, as in inflammatory bowel disease, may predispose patients to malignancy.

Mutations in two classes of genes, tumor-suppressor genes and protooncogenes, impart a proliferative advantage to cells and contribute to development of the malignant phenotype (Gryfe *et al.*, 1997). Inactivating mutations of both copies (alleles) of the adenomatous polyposis coli (*APC*) gene, a tumor-suppressor gene on chromosome 5q, mark one of the earliest events in colorectal carcinogenesis. Germline mutation of the *APC* gene and subsequent somatic mutation of the second APC allele cause the inherited familial adenomatous polyposis syndrome (FAP). This syndrome is characterized by the presence of hundreds to thousands of colonic adenomatous polyps. If these polyps are left untreated, colorectal cancer develops. Mutation leading to dysregulation of the K-ras protooncogene is also an early event in colon cancer formation. Conversely, loss of heterozygosity on the long arm of chromosome 18 (18q) occurs later in the sequence of development from adenoma to carcinoma, and this mutation may predict poor prognosis. Loss of the 18q region is thought to contribute to inactivation of the DCC tumor-suppressor gene. More recent evidence suggests that other tumor-suppressor genes, DPC4 and MADR2 of the transforming growth factor beta (TGF-β) pathway, also may be inactivated by allelic loss on chromosome 18q. In addition, mutation of the tumor-suppressor gene p53 on chromosome 17p appears to be a late phenomenon in colorectal carcinogenesis. This mutation may allow the growing tumor with multiple genetic alterations to evade cell-cycle arrest and apoptosis.

Neoplastic progression is probably accompanied by additional genetic events, which are indicated by allelic loss on chromosomes 1q, 4p, 6p, 8p, 9q, and 22q in 25–50% of colorectal cancers. Moreover a third class of genes, DNA-repair genes, has been implicated in tumorigenesis of colorectal cancer. Study findings suggest that DNA mismatch repair deficiency, due to germline mutation of the hMSH2, hMLH1, hPMS1, or hPMS2 genes, contributes to development of hereditary nonpolyposis colorectal cancer (HNPCC). The majority of tumors in patients with this disease, and 10–15% of sporadic colon cancers display microsatellite instability (MSI), also know as the replication error positive (RER+) phenotype. These tumors

are characterized by genetic instability at microsatellite loci. Although colorectal cancer cells are characterized by specific microsatellite alterations, the same four different signaling pathways, WNT/Wingless pathway, K-ras pathway, TGF-β pathway and p53 pathway, could be implicated in tumor progression. These alterations contribute to the adenoma–carcinoma transition. Moreover changes in DNA methylation pattern, in sense of hypermethylation, have been shown to inactivate genes associated with DNA-damage responses and DNA repair, MLH1, MLH3 MSH6, and SFN (Loukola *et al.*, 2000; Taylor *et al.*, 2006), contributing to colon cancer development. The epigenetic hypermethylation instability is strictly linked to genetic instability.

II. GENETIC INSTABILITY AND CONTROL OF DNA DAMAGE: DNA *DOUBLE-STRAND BREAKS* REPAIR

Genetic instability causes genetic heterogeneity, that is a peculiar feature of tumors and fundamental in cancer progression. The majority of tumors, with no exception for colorectal cancers, show no obvious familiar inheritance suggesting that multiple low penetrance genes segregating in the human population confer cancer susceptibility and resistance to environmental carcinogens. These low penetrance genes play a key role in DNA-damage repair, in apoptosis induction, in immune response efficiency, and may act combinatorially in a dosage-dependent manner, to confer predisposition of cancer insurgence. In fact, environmental insult or mutations that alter checkpoint genes involved in DNA-damage repair and survival pathways, could select cells that proliferate more quickly than those stopped to repair damage. Moreover, increased DNA synthesis is associated with extensive genetic damage. High levels of DNA synthesis together with chromosomal and MSI in tumors strongly suggest that alteration in DNA-repair machinery and apoptosis may contribute to uncontrolled and error-prone DNA synthesis.

As reported above, the efficiency of DNA repair is crucial to maintain the genome homeostasis, preventing malignant transformation and tumor insurgence (Difilippantonio *et al.*, 2000). Double-strand breaks (DSBs) are the most hazardous lesions occurring in the genome of eukaryotic organisms. These lesions could take place during DNA replication, meiosis, and immune system development. Not only colorectal cancer but also breast, endometrial, and gastric carcinomas display increased risk of development in subjects with germline mutations at the DNA–DSBs repair system (BRCA1, BRCA2, ATM, etc.).

The DSBs repair requires the homologous recombination (HR) and nonhomologous end joining (NHEJ). The NHEJ DSBs repair involves the activity of Ku70/80 protein heterodimer, sensor of the damage (Gottlieb and Jackson, 1993). In fact, the first character of the NHEJ is the DNA-dependent protein kinase (DNA-PK), a serine–threonine kinase consisting of a 470 kDa catalytic subunit (DNA-PKcs) and the regulatory protein, called Ku, which is composed of 70 and 86 kDa subunits. The heterodimer Ku, first described as a nuclear autoantigen, is a regulatory factor of DNA replication and transcription. The Ku heterodimer binds the ends of various types of DNA discontinuity, and is involved in the repair of DNA breaks caused by an incorrect DNA replication, V(D)J recombination, isotype switching, physiological oxidations, ionizing irradiation, and some chemotherapeutic drug effects (Blunt *et al.*, 1995; Jackson and Jeggo, 1995). The interaction of Ku with ends of DNA has been extensively studied. Ku binds with high affinity to free ends of double-stranded DNA as well as to nicked DNA hairpins and dumbbell structures *in vitro* and *in vivo* in nuclear extracts. The principal role of Ku proteins is to take care of the homeostasis of the genome being involved in telomere maintenance, regulation of apoptosis induction, specific gene transcription, DNA replication, and cell-cycle regulation. The function of this caretaker gene is to suppress chromosomal aberrations, translocation, and aneuploidy. Ku was originally reported to be a nuclear protein, consistent with its functions as a subunit of DNA-PK. However, several studies have revealed the cytoplasmic or cell surface localization of Ku proteins in various cell types (Prabhakar *et al.*, 1990). The subcellular localization of Ku70 and Ku86 changes during the cell-cycle progression (Koike *et al.*, 1999), and nuclear translocation of Ku70 precedes that of Ku86 in late telophase/early G1 phase. Furthermore, changes in subcellular localization of Ku could be controlled by various external growth-regulating stimuli (Fewell and Kuff, 1996). Recently, it has been demonstrated a Ku DNA-binding activity in the cytoplasmic compartment of highly invasive bladder and breast tumors and metastatic nodes (Pucci *et al.*, 2001), whereas the nuclear activity related to the DNA-repair system, was impaired. Experimental data further reported an inactivation of Ku DNA-binding activity, essential for genomic stability, in colon cancer progression models, in breast and in bladder carcinomas. A dysfunction of this protective activity let the aberrant cell clone growing. In highly infiltrative and metastatic tumors of the colon, breast and bladder, the impaired DNA-repair activity is due to the loss of Ku86 (Pucci *et al.*, 2001) and to the Ku70 shifting from the nucleus to the cytoplasm. The shift from the nucleus to the cytoplasm of the Ku70/80 proteins in tumor cells could represents a mechanism to inhibit cell death through the cooperative interaction with sCLU, giving rise to a new chemoresistant clone with a more aggressive phenotype.

III. CLONAL EXPANSION: APOPTOSIS INHIBITION

A. Clusterin (CLU), a Multifunctional Protein Influenced by the Cellular Context

The cooperative interactions among proteins involved in DNA repair, apoptosis induction, and the influence of the microenvironment on their activity play a central role to understand the mechanisms that underlie the clonal expansion. Partners and regulatory proteins of Ku activity are evidenced in the last few years. In this view, CLU and the balance between its different forms has been shown to be one of the main player involved in colon cancer progression, being the regulator of Ku70/80 DNA double-strand breaks repair and Bax-dependent apoptosis induction.

CLU expression is markedly upregulated both *in vitro* and *in vivo* in response to various cell stress conditions. These include heat shock, UV radiation, oxidative stress, and pathologic states, such as neurodegenerative disorders, multiple sclerosis, atherosclerosis, myocardial infarction, and cancer. The presence of different CLU protein isoforms (nCLU and sCLU) and their functions within the cell was a much debated question.

Nuclear clusterin (nCLU) (XIP8), was firstly described as an X-ray-induced Ku70-binding protein (KUBs) that signals cell death (Leskov *et al.*, 2003; Yang, *et al.*, 2000). Its role in apoptosis induction has been further described (Leskov *et al.*, 2003; Pucci *et al.*, 2009a,b). In normal cells, after an irreversible cell damage, nCLU cooperates with Ku70 to induce apoptotic death, activating the translocation of Bax to mitochondria. Confocal microscopy experiments revealed an apparently inactive nCLU form in the cytoplasm of nonirradiated cells (Yang *et al.*, 2000) that translocates to the nucleus after ionizing radiation, colocalizing with nuclear Ku70/86 heterodimer involved in DNA repair and apoptosis induction (Yang *et al.*, 1999, 2000). Data on the preferential induction of the proapoptotic clusterin form after ionizing radiation (Leskov *et al.*, 2003), suggest that the transcription of one of the two mRNA forms is closely linked to the cellular state and could be influenced by intracellular and extracellular *milieu* (such as cytokines, growth factors, and stress-inducing agents) (O'Sullivan *et al.*, 2003; Pucci *et al.*, 2004a,b, 2009a,b; Reddy *et al.*, 1996; Yang *et al.*, 1999).

Ku70 DNA end-joining protein has been shown to suppress apoptosis by sequestering Bax from mitochondria. The regulation of its sequestering interaction with Bax would be regulated by Ku70 acetylation state. It has been found that the acetylation of lysine at the C-terminus of the protein is sufficient to completely block the ability of Ku70 to suppress Bax-mediated apoptosis (Cohen *et al.*, 2004). The regulation of the proapoptotic factor Bax is relevant for the development and progression of cancer (Evan and

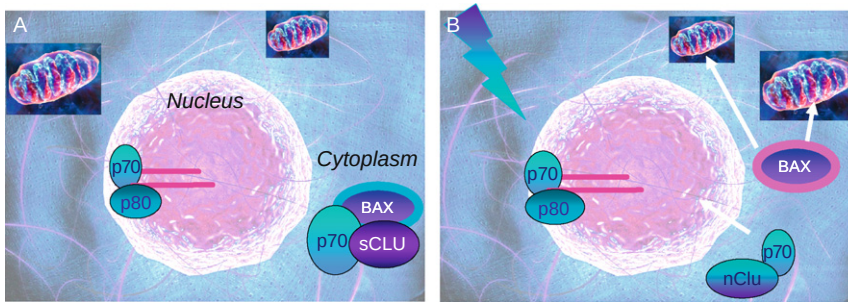

Fig. 1 Ku70/80–CLU–Bax: Physiological interactions. (A) Bax is localized inactive in the cytoplasm in normal, undamaged cell interacting with the Ku70 protein C-terminus. This status determines its inability to give rise to apoptotic event. sCLU stabilizes the Ku70–Bax interaction in the cytoplasm acting as cytoprotectant. (B) After DNA damage inducing DNA double-strand breaks repair (UV treatment, ionizing radiation, etc.) Ku70 allows the translocation of Bax to the mitochondria.

Vousden, 2001). Following its activation, Bax homodimerizes translocating into the mitochondrial membrane and leading to the release of death-promoting factors such as cytochrome *c*, in the cytoplasmic compartment. Bax is localized physiologically inactive in the cytoplasm in normal, undamaged cells interacting with the Ku70 protein C-terminus (Fig. 1).

This status determines its inability to homodimerize and give rise to apoptotic key events. Overexpression of Ku70 *in vitro* blocks the Bax-induced apoptosis under some variety of stimuli in epithelial cells. After a UV treatment inducing DNA damage, the DNA double-strand-breaks repair sensor Ku70 allows the translocation of Bax to the mitochondria and its homodimerization after its sterical modification. This important function of Ku as regulator of Bax-mediated release of several death-promoting factors is in agreement with its role as caretaker in the nucleus. On the other hand, CLU seems to play an important role in cell survival pathways and in cell death escape, stabilizing the Ku70–Bax interaction in the cytoplasm that in pathological condition could lead to the survival of the aberrant cell clone. Overall, the dynamic interaction among CLU, Ku70, and Bax seems to have an important role in both tumor insurgence and its progression (Pucci *et al.*, 2009a,b) (Fig. 2).

IV. CLU IN COLORECTAL CANCER PROGRESSION: sCLU AND APOPTOSIS ESCAPE

Cell survival and cell death represent key processes in cancer development and progression. These processes could be both regulated by the balanced expression of the different CLU forms involved in antagonistic action that

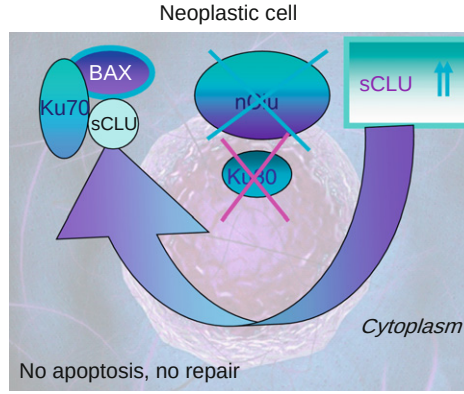

Fig. 2 Ku70–Bax–CLU pathological interaction. Apoptosis escaping. The shift of clusterin forms production, the loss of ku80, and the cytoplasmic relocalization of ku70 are related to cell death inhibition and cancer progression.

turns the cell fate. Hence a large number of studies have focused their interest on CLU in tumors and tumor progression models and its controversial role in cancer progression was ruled out focusing on the CLU different forms functions and their action in normal and in neoplastic cell processes.

Evidence of the upregulation of CLU expression in intestinal tumors was reported by Chen *et al.* (2003). The authors investigated the relationship between CLU expression, APC function, cell proliferation, and apoptosis. CLU gene was identified as upregulated in murine and human colon cancer. Wild-type and B6-Min mice were investigated, the last carrying the multiple intestinal neoplasia (Min) mutation in the adenomatous polyposis coli (*APC*) gene. This line provides an experimental model of human familial intestinal cancer progression. Loss of tumor suppressor APC function initiates tumorigenesis in the intestine. The APC protein is involved in the degradation of β-catenin within the cytoplasm, thus the loss of WT APC antigen leads to enhanced levels of cytoplasmic β-catenin protein. A strong positive association was found between elevation of CLU expression and loss of APC function in tumor cells. The authors found CLU expression much stronger in murine tumors than in normal tissues. Tumor cells are normally poorly differentiated during uncontrolled proliferation. Lack of differentiation factors in most tumor cells with elevated CLU expression suggested that CLU could be a sensitive and stable histological indicator for murine and human intestinal tumors representing a useful diagnostic marker for colon cancer disease. Elevated CLU expression was maintained in both murine and human invasive adenocarcinomas indicating that this protein plays a role in the maintenance and/or progression of tumors. High levels of

CLU were also detected in normal human colon crypts adjacent to the adenomas and adenocarcinomas, whereas they failed to reveal CLU in normal crypts far from the tumors and in tumor-free colonic tissues. Recent reports suggest the apparent dichotomy of function may be related to two different isoforms, one secreted and cytoplasmic, the other nuclear. To clarify the functional role of CLU in regulating apoptosis, Bettuzzi and his collaborators examined its expression in human colon cancer tissues and in human colon cancer cell lines. They additionally explored its expression and activity using models of *APC*- and chemotherapy-induced apoptosis (Chen *et al.*, 2004).

They found a decrease of CLU RNA and protein levels in colon cancer tissues largely devoid of wild-type *APC* when compared with matched normal tissue controls, suggesting a means for invasive cancers to avoid apoptosis. Conversely, induction of apoptosis by expression of wild-type *APC* or by treatment with chemotherapy led to increased clusterin RNA and protein levels localizing to apoptotic nuclei. They observed that transient transfection of CLU to colon cancer cell lines directly enhanced basal and chemotherapy-induced apoptosis. CLU-induced apoptosis was inhibited by antisense CLU and was found to be highly dependent on p21 but not p53 expression, yet a deficit in p21 can be subverted by CLU transfection. Collectively, these data support the hypothesis that nCLU function is proapoptotic when induced by *APC* or chemotherapy in the context of p21 expression. Absent of p21, CLU in not induced, and apoptosis is significantly inhibited. These data support a potential therapeutic role for CLU in enhancing chemotherapy-induced apoptosis and in promoting apoptosis in cells deficient in p21.

Other findings were reported by Thomas-Tikhonenko *et al.* (2004). He demonstrated that Myc-transformed epithelial cells model downregulated CLU and that CLU could inhibit cell growth *in vitro* and prevent carcinogenesis *in vivo*. Indeed, in this experimental model, CLU transient overexpression decreased cell accumulation in Myc-transduced colonocytes suggesting a potential role of CLU as tumor suppressor. The debated role of CLU in colon cancer lately was attributed to the differential expression of CLU forms displaying antagonistic functions (nCLU and sCLU) conciliating the "tumor suppressor" and the "tumor promoting" role of this protein in cancer.

Several experimental data have shown a strong correlation between a differential shift of the two CLU isoforms and tumoral progression. Our report (Pucci *et al.*, 2004a,b) provided the first link among the unbalanced overexpression of sCLU form, the disappearance of nCLU form, and colorectal cancer progression. In fact, immunohistochemical analysis, performed on 30 bioptic and surgical samples of colorectal tumors, showed a nuclear localization of CLU in normal colonic mucosa, and a complete loss of nCLU in the advanced stages of colon cancer (Dukes C, D). In addition, the progression toward the advanced stages of cancers led to an overexpression

of the highly glycosylated cytoplasmic form. In particular, colonic adenomas presented positive staining both in the nuclei and in the cytoplasm and its expression was significantly increased, as compared with normal mucosa. sCLU expression strongly increased in noninvasive carcinomas (Dukes stage A, B). The immunohistochemical observation of highly aggressive and metastatic tumors (Dukes C, D) showed that CLU could also be released in the extracellular space.

Western blot analysis displayed the presence of different CLU isoforms using an anti-CLU α-chain antibody. psCLU precursor form was present both in normal and tumoral tissues. The nCLU form was evident in normal mucosa, whereas it was completely lost in the tumoral tissues. The 40 kDa CLU, corresponding to the secreted form (sCLU), was present in normal tissues and it was overexpressed in the cancer samples. Moreover, the apoptotic index was inversely related to the increase of sCLU expression and to the tumor stage. In addition, *in vitro* experiments confirmed that in colon cancer cell CLU was extracellularly released and that the form released in the extracellular space corresponded to the sCLU.

In vitro experiments were performed to determine whether the translocation of the CLU from the cytoplasm to the nucleus could be modulated by a cytostatic and proapoptotic treatment, restoring the physiological balance of the two CLU isoforms. *In vitro* studies confirmed a shift of the different isoforms after cytostatic treatment in colon cancer cells, related to the apoptotic induction. The cytostatic treatment with somatostatin in colon carcinoma cells (Caco2) induced a strong increase of nCLU in the nucleus. In addition, e*x vivo* isolated cells from normal mucosa and colorectal cancer tissues of the same patients confirmed the restore of nCLU isoform following antiproliferative treatment, concurrent to apoptosis induction. Overall, the overexpression of the sCLU in the cytoplasm of highly infiltrating tumors (and metastatic nodes), was due to a shift of CLU forms expression in cancer cells driven by exogenous growth regulatory factors.

A. CLU–Ku–Bax Localization in Colon Cancer

In view of the emerging role of CLU, Ku70, and Bax interactions in tumor development and progression, the expression, localization, and physical interaction of Bax, Ku70, Ku86 were also investigated in human colorectal cancers ($n = 50$) (Pucci *et al.*, 2009a,b) (Fig. 3). A tumor-specific modulation of these protein factors was found in human colon cancer. Bax showed only faint cytoplasmic staining in normal mucosae (70% of controls), whereas it was overexpressed in the cytoplasm of quite all carcinomas ($P = 0.04$). Ku70 staining was strongly positive in the nuclei of normal mucosa aside the neoplasia. In node-negative carcinomas, Ku70 expression

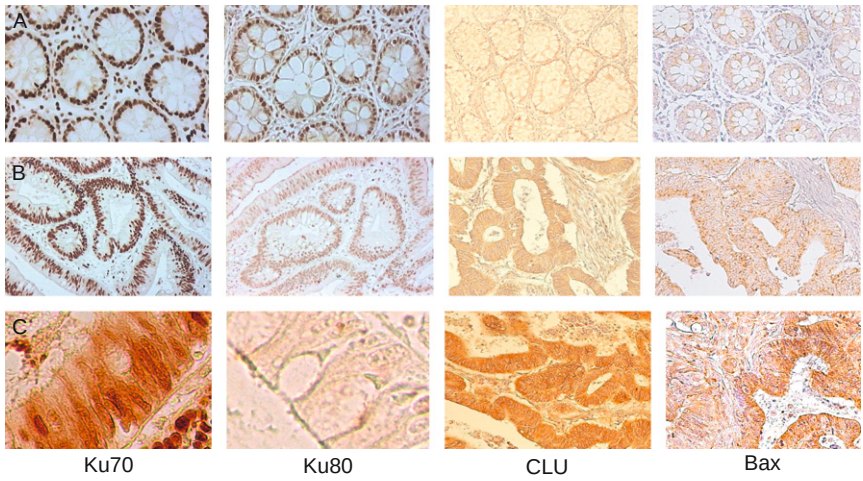

Fig. 3 Tumor-specific modulation of ku70/80, CLU and Bax proteins in human colon cancer. *Bax* showed faint cytoplasmic staining in normal mucosae (A) and it was overexpressed in the cytoplasm of all carcinomas (B, C). *Ku70* staining was strongly positive in the nuclei of normal mucosa (A). In node-negative carcinomas (B) Ku70 expression slightly decreased and it localized mainly in the nucleus. In node-positive carcinomas (C) Ku70 staining was distributed between nucleus and cytoplasm. The expression of *Ku86* was positive in the nuclei of control tissues (A). Nuclear Ku86 expression was strongly decreased in node-negative tumors (B). No staining for Ku86 was found in the nucleus or in the cytoplasm of node-positive carcinomas (C). *CLU* isoforms expression was reported in Pucci *et al.* (2004a,b).

slightly decreased and it localized in the nucleus, while 11 out of 28 cases displayed a cytoplasmic staining as well. In node-positive carcinomas, Ku70 staining was not altered in total amount, compared with node-negative tumors, but it was distributed between nucleus and cytoplasm. In all cases, Ku70 was positive in the cytoplasmic compartment. The expression of Ku86 was positive in the nuclei of control tissues. Nuclear Ku86 expression was strongly decreased in A–B stage tumors. No staining for Ku86 was found in the nucleus or in the cytoplasm of node-positive carcinomas (C–D stages). Interestingly, Ku86 expression was lost in metastatic nodes. CLU isoforms expression confirmed previous data (Pucci *et al.*, 2004a,b).

Double immunofluorescence analysis showed that strong nuclear Ku70 staining in normal mucosa and faint Bax staining in the cytoplasm. Advanced stage carcinomas (C–D stage) showed increased levels of Ku70 and Bax and CLU proteins. Triple immunostaining and confocal analysis demonstrated the Ku70–CLU–Bax colocalization in the cytoplasm. This data suggests that in highly aggressive tumours the interaction of Ku70 and CLU with Bax permanently inhibits Bax activation and its subsequent

heterodimerization and translocation into the mitochondria. This condition in advanced tumor stage leads to apoptosis escape. *In vitro* experiments, reported also in chapter "CLU and tumor microenvironment" of this volume, demonstrated that in colon cancer progression this physical interaction among Ku70–CLU and Bax are not irreversible and it is strongly influenced by the tumor microenvironment, suggesting that apoptosis escape could be related to exogenous factors, such as IL-6 and VEGF and TGF-β present in the extracellular *milieu* of the tumoral mass (Pucci *et al.*, 2009a,b).

As previously mentioned, a physiological growth regulatory factor such as Somatostatin induces apoptosis after 24 h of treatment in colon cancer cell line Caco-2, determining the release of Bax from sCLU and Ku70 (Pucci *et al.*, 2004a,b). In addition, Somatostatin treatment induced also the shift of CLU forms production inducing the upregulation of the proapoptosis nCLU. An antithetic effect was obtained treating Caco-2 with IL-6 or VEGF165a, microenvironmental factors involved in tumor progression and metastasis. In fact a strong upregulation of sCLU production and an increase in Ku–CLU–Bax binding were observed, confirming that these interactions that regulate the Bax-dependent cell death could be driven by exogenous and endogenous factors that could be determine the cell fate.

From these findings, it seems that the differential shift of CLU isoform production, the loss of Ku80, and the cytoplasmic relocalization of Ku70 and sCLU overexpression are related to cell death inhibition and colorectal cancer progression.

Others studies focused on CLU different forms production and their function in colon cancer. The study of Chen *et al.* (2004) highlighted the function of nCLU in colorectal cancer tissues and colon cancer cell lines. nCLU RNA and protein levels were decreased in colon cancer tissue, compared with normal mucosa as means of apoptosis escaping. The author analyzes APC status associated with CLU. Most colon cancer lack functional APC protein and the data suggest diminished CLU expression in these samples. The expression of WT *APC* or chemotherapy treatment associated to increased levels of CLU and apoptosis. Apoptosis induced by CLU was p21 dependent. In addition, it was shown that the depletion of sCLU did not affect significantly the growth rate. Data of Chen are consistent with results reported by Pucci *et al.* Chen T. analyzed in particular the nuclear form at protein level (60 kDa by Western blot analysis). Also the primers used to detect mRNA levels matched for the splicing isoform of nCLU variant.

Xie *et al.* (2005) also confirmed the overexpression of cytoplasmic staining of CLU, on human tissue microarrays which contained 85 advanced colorectal cancer (Dukes B, C, and D). A significant positive correlation between overexpression of CLU and clinical stage was observed ($P < 0.01$). Nevertheless the same authors failed to detect the nuclear staining neither in normal nor in neoplastic colonocytes. They also showed an inverse relation between the

cytoplasmic Clu overexpression and the apoptotic index (TUNEL assay). In fact, the frequency of high apoptotic index was significantly higher in tumors with a normal expression of CLU, than that in cases which overexpress CLU ($P < 0.01$). In addition, the cell proliferation in colorectal cancer (evaluated with ki-67 expression) positively correlated with CLU expression.

In light of the above, sCLU overexpressed in highly aggressive tumors and metastatic nodes, being correlated to cell matrix formation, cell membrane remodeling, and cell–cell adhesion, could represent a potential predictive marker for colon carcinoma aggressiveness.

V. CLU AS A NEW BIOMARKER FOR COLON CANCER SCREENING

At present, colon cancer is second only to lung cancer in men and to breast carcinoma in women, for incidence and mortality in western countries. The higher incidence per age is observed between the sixth and seventieth decade, while 60% of the patients survive up to 5 years.

The most important reason for the low percentage of recoveries is due to the fact that when the primary tumor is removed, a high number of patients have already developed micrometastases, principally at liver. Therefore, methods for early screening are requested.

Genetic counseling, predictive molecular testing, and when indicated, endoscopic surveillance at appropriate intervals should be offered to individuals from families at high risk for colorectal cancer (HNPCC or FAP).

At present, the early diagnosis protocols (secondary prevention) consist of rectal exploration, determination of fecal occult blood, and rectosigmoidoscopy periodically performed on individuals of 45 years of age and older and nonsymptomatic. Periodic pan-colonoscopy is the only procedure for early diagnosis of neoplasia on individuals with positive familiar history for colorectal cancer (CRC), on patients with already a neoplasia or affected by syndrome with a high risk of neoplasia insurgence, that are part of the so-called "at risk population." Randomized controlled trials (RCTs) have shown that annual or biennial screening in asymptomatic people over the age of 50 years using fecal occult blood test (FOBTs), can reduce CRC mortality by 15–33%. Nevertheless FOBT, utilized for early colon carcinoma diagnosis in clinical practice, yields frequent false-negative and false-positive results that lower screening effectiveness and raise program costs. On the basis of the above, new molecular pathogenetic markers, that would overcome the restrictions of the invasive methods used at present such as colonoscopy, are needed to improve the efficacy, sensitivity, and specificity of the

early diagnosis test. Moreover, molecular markers would help to stratify more selectively the cohort of patients who really need colonoscopy.

The use of CLU as a diagnostic marker in some pathological conditions such as type II diabetes and several coronary pathologies has already been described (Trougakos *et al.*, 2002). There were just few previous attempts to determine CLU by ELISA in tumoral pathologies, specifically in the blood of prostate carcinoma patients (Morrissey *et al.*, 2001). Moreover, CLU level in blood and urine has been demonstrated to be a potential marker for bladder and for kidney tumors, being directly related to the dimension of the neoplasia (Stejskal and Fiala, 2006). In a recent paper, we highlighted that the appearance and the progressive increase of the CLU cytoplasmic isoform in tumors correlated to a release of CLU in the extracellular space. In this paper, we demonstrated that sCLU upregulated in the neoplastic colonocytes was also secreted in the intestinal lumen (Pucci *et al.*, 2009a,b). In an *ex vivo* experiment, isolated cells of healthy and neoplastic colonic mucosa were collected and after 72 h sCLU-level culture supernatant was determined. A significant increase of CLU level (2.9 times) was found in the culture supernatant of tumoral cells, compared to normal colonocytes of the same patient. The increased release of sCLU in tumoral cell supernatant confirmed that the overexpression previously observed *in situ* was strongly correlated to an increase of CLU release.

Furthermore, in order to investigate if CLU release from colon cancer cells could effectively affect the total amount of the circulating protein, human colon cancer cells, Caco-2, were underskin injected in nude mice. Before inoculating Caco-2 cells, blood was collected from each mouse in order to evaluate the endogenous basal level of CLU before tumor cells injection. Mice were sacrificed at the day 15th, 20th, and 25th after tumor injection, in order to evaluate CLU level in relation of tumor size. Blood was collected, tumor was removed, and tumor size was evaluated. The level of CLU was significantly increased in blood of tumor-injected mice as compared to uninjected mice; moreover, an increased level of CLU was correlated to the dimension of the tumors suggesting its potential value as new biomarker for colorectal cancer screening.

sCLU level was evaluated in the serum and stool samples of CRC patients and age-matched controls.

The Dot blot analysis on human sera from colorectal cancer patients (CRC, $n = 35$) and no cancerous subjects (controls, $n = 25$) displayed statistically significant differences in CLU levels. In fact, CLU concentration was 82.8 ± 26.9 μg/ml in CRC cancers and 57.8 ± 19.3 μg/ml in controls (CRC vs. controls: $P = 0.0002$).

In order to avoid the interference of the increased level of CLU in blood due to other nontumoral or tumoral diseases (cancer of breast, prostate, testicle, ovary, SNC, hemo-lymphopoietic system), the level of CLU was

determined in stool of the colorectal cancer patients. Dot blot analysis of fecal extracts from cancer patients ($n = 28$) as compared to controls ($n = 25$), provided significant differences with mean values of 47.5 ± 19.6 and 26.8 ± 12.8 μg/g, respectively (CRC vs. controls: $P < 0.000$). A significant correlation between CLU values in stool and colorectal cancer stages was found ($P = 0.05$).

These results demonstrated that sCLU efficiently discriminates between colorectal cancer disease and nonneoplastic controls. In fact, the receiver operating characteristic (ROC) curves provided several cut off points to show the trade-off between sensitivity and specificity, at different cut off values. For Dot blot assay in blood, the optimal cutoff corresponded to 55.6% sensitivity and 100% specificity, whereas the stool test reached 66.7% sensitivity and 84% specificity at the selected cut off value, as reported above.

In addition, a recent report confirmed that increased levels of sCLU correlated with poor survival in a population of 251 CRC patients, stage II. Recently, Kevans *et al.* (2009) studied and reported the same by tissue microarray and immunohistochemistry. The adverse outcome of stage II colorectal cancer correlated with epithelial and stromal sCLU immunostaining in tumor tissues.

Taken together, these data suggest a potential role of sCLU as a biomarker for colon cancer screening and relapse of the disease.

VI. CONCLUSIONS AND FUTURE PERSPECTIVES

Despite the original hypothesis that CLU is a marker for programmed cell death, several experiments and clinical studies have demonstrated conflicting findings on the role of CLU in tumors. Experimental results obtained in SCID mice injected with CLU transfected human renal carcinoma cells indicate that CLU overexpression may contribute both to enhance cancer cell survival, preventing apoptosis, and to increase the metastatic potential. Moreover, *in vitro* studies showed that CLU overexpression stimulates cell motility and invasive ability in human renal cell line.

Recent findings on the opposite function of CLU different forms contributed to clarify the conflicting data on its function inside and outside the cell. Collectively these data suggest that sCLU upregulation plays a protective role against apoptosis induced by various kinds of stimuli and thereby may confer an aggressive phenotype during cancer progression. The observation on CLU expression throughout the different steps of colon carcinoma progression demonstrated the presence of the nuclear form in the nuclei of the normal mucosa. As the nuclear form has been demonstrated to be involved

in cell-cycle regulation and apoptosis induction this result suggests that in a normal cell proliferative state of the colonic mucosa this protein could be probably involved in cell-cycle regulation and apoptosis induction involving the regulation of Bax activation.

In colon cancer, the upregulated sCLU isoform is extracellularly released both in blood and in stool and a sensitive method was assessed to detect it, highlighting its value as new biomarker for a noninvasive colon cancer screening. There is a consensus that CRC screening is effective to prevent this disease in many cases. Due to CRC screening, the incidence of this tumor has dropped in recent years. There is less consensus regarding optimal screening strategies, as sensitivity, specificity, and patient acceptance limit current options. To overcome these barriers a range of approaches, including proteomics-based testing, stool genetic testing, radiological imaging, and enhanced endoscopies have been the focus of intense research. Presently, colonoscopy with a sensitivity of 97% and a specificity of 98% for colon cancer and a 90% sensitivity for adenomas of at least 1 cm diameter is considered the gold standard for colon cancer diagnosis and offers the potential to both diagnose and remove premalignant lesions, but it is associated with patient discomfort, complications, variable sensitivity given through the experience of the endoscopies and high costs.

A useful diagnostic assay must be sensitive, must detect cancer at the onset and it must have a high specificity to minimize false positives that necessitate expensive and invasive examination. Stool testing, unlike other conventional screening approaches, is noninvasive and requires no cathartic preparation. New stool tests for CRC diagnosis have been recently developed displaying a higher sensitivity as compared to FOBT, whereas specificity is still to be defined. In particular, specificities of about 95% have been reported for tests based on detection of genetic mutations occurring in the tumoral tissues but not in the early stage and these are not present in all cases. On the other hand, markers such as calprotectin, may represent both a marker of cancer disease and of bowel inflammation, leading to nearly 30% false positive results. Recently a high-specific serum testing for colon cancer-specific antigen 2 and 4 (CCSA-2 and -4) has been proposed, but the limitation of this test is that not all colon cancers may express the NMP CCSA-2 and -4 (20–30%) and therefore a multiple marker testing is needed.

Data obtained by stool analysis by Pucci *et al.* clearly point out that the increase of CLU in cancer patients is significant not only compared to healthy subjects but also compared to patients affected by systemic or bowel inflammatory pathologies and benign lesions of the colon. Moreover, data obtained by Dot blot in stool of cancer patients showed a positive correlation between sCLU values and stage of disease.

Furthermore, data on animal model point out that the increase of sCLU level correlates with tumor size, suggesting a role of sCLU as a new marker

of onset, prognosis, and relapse of colon cancer. Hence, these results suggest the potential applicative role of CLU detection to improve the effectiveness and efficiency appeal for large-scale clinical cancer screening. Moreover, studies on the molecular mechanisms that regulate the activation of CLU promoter and CLU isoforms shifting could provide new molecular targets for specific antineoplastic therapies.

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