Cullin-1 and -2 Protein Expression in Colorectal Cancer: Correlation with Clinicopathological Variables

OTHON MICHAEL1, DEMETRIOS MORIS2, STAMATIOS THEOCHARIS3 and JOHN GRINIATOS1

1First Department of Surgery, Laikon General Hospital, National and Kapodistrian University of Athens, Athens, Greece; 2Department of Surgery, Duke University Medical Center, Durham, NC, U.S.A.; 3Department of Forensic Medicine and Toxicology, Laikon General Hospital, National and Kapodistrian University of Athens, Athens, Greece

Abstract. Background/Aim: The cullin (CUL) family of proteins is involved in the ubiquitin-mediated degradation of proteins, regulating cell proliferation, cell-cycle control, migration, invasion and metastasis in the process of tumor progression. The aim of the present study was to examine if there is any correlation between the immunohistochemical (IHC) expression of Cullin-1 and -2 proteins in colorectal cancer tissue specimens with several clinicopathological variables. Materials and Methods: Between January 2012 and December 2014, 96 consecutive adenocarcinoma patients were submitted to oncological colectomy, as the first therapeutic option, with a curative intent. CUL-1 and -2 protein expression was examined with IHC on paraffin-embedded tissue sections. CUL-1 and -2 protein positivity, was correlated with patients’ age, gender, stage, histological grade, proliferative capacity (Ki-67 labeling index) and mutant p53 protein expression. The positivity for CUL-1, CUL-2, mutant p53 protein and Ki-67 index, was determined by the percentage of their IHC expression in the total number of cancer cells. Results: Choosing as a cut-off point for CUL-1 positivity the 10%, a statistically significant relationship of the expression of the mutant p53 protein (p=0.047) was noticed. Co-expression of CUL-1 and -2 in more than 10%, significantly correlated to the coexistence of adenomatous polyps along the large bowel (p=0.0329). Multivariate analysis of CUL-1 and -2 co-expression in more than 10% disclosed their expression as an independent factor for adenomatous polyps development in the large bowel (p=0.035, RR=2.1). Conclusion: CUL-1 overexpression may happen early in the process of carcinogenesis mainly affecting the vulnerable p53(+) large bowel cells, arresting them in the G1 phase of cell-cycle, while it may also induce the expression of CUL-2. Co-expression of CUL-1 and CUL-2, in the arrested (in G1 phase) large bowel cells, promotes carcinogenesis up to adenomatous polyp formation. Since no relationship between cullins expression and development of cancer on adenoma was found, the results of the present study may be useful explaining the initiation but not the progression of carcinogenesis in colorectal cancer. Further molecular and clinical studies are needed in order to delineate the clinical importance of these proteins in the management of colorectal cancer patients.

Several biological processes such as proliferation, differentiation, apoptosis, migration, invasion, signal transduction, transcription, cell-cycle progression and cell death, (1) depend precisely on the timely synthesis and degradation of key regulatory proteins (2).

While protein synthesis can be regulated at multiple levels, protein degradation is mainly controlled by the ubiquitin – proteasome system (UPS) (2, 3) which consists of two distinct steps: ubiquitination of targeted proteins by E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme and E3 ubiquitin ligase, (4) and subsequent degradation by the 26S proteasome (4, 5).

Among all E3 ubiquitin ligases, the SCF (SKP1-CUL1-F-box protein) E3 ligases are the largest family and are responsible for the turnover of many key regulatory proteins.
Similarly to other post-translational modifications, the process of ubiquitination is reversible, with the removal of ubiquitin from substrates regulated by deubiquitinating enzymes (7). Up to date, more than 600 E3 ubiquitin ligases and 100 de-ubiquitinating enzymes have been identified, forming a molecular network governing intracellular ubiquitination dynamics (8).

Dysregulation of the proteolytic system results in uncontrolled proliferation, genomic instability and cancer (1). Aberrant regulation of SCF E3 ligases is associated with various human diseases, such as cancers (2) while, misregulated expression of the members of the ubiquitination cascade, attributes a cancerous phenotype to various cells including enhanced proliferation, survival and metastatic potential (9).

Cullins (CUL) are a protein family acting as a matrix for E3-ubiquitin ligases. CUL1 is an essential scaffold of the SKP1-CUL1-F-box protein (SCF) E3 ubiquitin ligase complex, (6) which mediates the ubiquitination of proteins involved in cell-cycle induction and progression (3). CUL-1 is required for the developmentally programmed transitions from the G1 to the G0 phase of the cell cycle or the apoptotic pathway. Moreover, the mutant phenotype suggests that G1- to S phase progression is accelerated, overriding mechanisms for mitotic arrest and producing abnormally small cells (10). Cullin-2 (CUL-2) interacts with the trimeric von Hippel Lindau-elongin B-elongin C complex and plays an essential role in the degradation of hypoxia-inducing factor 1α by ubiquitination (11).

On the other hand, knockdown of CUL-1 inhibits cell growth, proliferation, migration and invasion (mainly by up-regulating p27 expression) (12, 13) in cases of melanoma (12), gastric cancer (13), lung cancer (14), breast cancer (15) and skin cancer (16), by arresting cells in the G1 phase (16).

The aim of the present study was to examine if there was any correlation between the immunohistochemical (IHC) expression of CUL-1 and CUL-2 proteins in colorectal cancer tissue specimens to several clinicopathological variables.

### Materials and Methods

**Patients.** From 2011 onwards, all patients who were referred to our Department for further investigation and treatment, having been diagnosed with colorectal cancer, were prospectively collected. Demographics, clinical data, adjuvant or neo-adjuvant therapies, type of operation, postoperative complications, histological findings and follow-up, were recorded.

All patients suffered from colorectal cancer and had undergone colonoscopy and biopsies for histological confirmation of the disease. All of them were submitted at least to computer tomography (CT) of thorax and abdomen for staging of the disease, while patients suffering from rectal tumors were further submitted to magnetic resonance imaging (MRI) of the pelvis for loco-regional staging of the disease (17).

Excluding patients (i) who were diagnosed with histological types other than adenocarcinoma, (ii) who were operated on for palliation, (iii) who were classified as suffering from locally advanced disease and referred for neo-adjuvant therapies, (iv) who were diagnosed as stage IV, even though a curative resection was achieved and (v) who suffered from multiple distant metastases and referred for systematic chemotherapy, a total of 96 consecutive adenocarcinoma patients were submitted to oncological colectomy, as a first therapeutic option, with curative intent, between January 2012 and December 2014.

The pathological stage of the disease was based on the 7th TNM Classification, (18) while tumor grade was based on the WHO classification (19).

**Immunohistochemical (IHC) staining.** Tissue blocks were extracted from the surgical specimen and subjected to immunohistochemical (IHC) staining. Paraffin embedded biopsy specimens were used. The method used is as follows: One 4-μm-thick section was cut from 1 representative paraffin block of each case. The sections were floated onto salinized glass slides, dried out at 37°C overnight, and then kept at 60°C for 1 hour, before de-paraffinization in xylene and rehydration through graded ethanol. All sections were subjected to microwave heating at 850 W for 22 min in pH 6.0 citrate buffer and cooled in running water. The antibodies used were mouse cullin-1 and 2 (Novus Biological, Littleton, CO, USA), pRB (Santa Cruz Biotechnology, Dallas, TX, USA), Ki-67 (DAKO, Poland, Warsaw), dilution 1:50.

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**Table I. Clinicopathological characteristics of the enrolled patients.**

| Gender   | Male | Female |
|----------|------|--------|
|          | 43   | 53     |

| Age       | Median + Interquartile Range (IR) |
|-----------|----------------------------------|
|           | 70 (63-77)                       |

| Location of the primary tumor | Cecum-Ascending colon | Transverse colon | Descending-Sigmoid colon | Rectum |
|-------------------------------|----------------------|-----------------|--------------------------|--------|
|                               | 15                   | 5               | 47                       | 29     |

| Grade | 1 | 2 | 3 |
|-------|---|---|---|
|       | 21| 62| 13|

| Stage | A | B | C1 | C2 |
|-------|---|---|----|----|
|       | 23| 33| 23 | 17 |

| Cancer on adenoma | Yes | No |
|-------------------|-----|----|
|                   | 18  | 78 |

| Coexistence of adenomas | Yes | No |
|-------------------------|-----|----|
|                         | 29  | 67 |

| Vascular invasion | Yes | No |
|-------------------|-----|----|
|                    | 24  | 72 |

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at room temperature for 1 hour. IHC staining was carried out using an HRP polymer detection envision method (DAKO EnVision+ System, Poland, Warsaw). Diaminobenzidine (DAB) was used as chromogen and sections were counterstained with Harris' hematoxylin. Appropriate positive and negative controls omitting the primary antibodies were included with each slide run.

Table II. Univariate analysis between expression of cullin-1 ≥10% and evaluated parameters.

| Parameter                        | Cullin-1 ≥10% (n=75) | Cullin-1 <10% (n=21) | p-Value |
|----------------------------------|---------------------|---------------------|---------|
| Gender                           | Male                | 31                  | 12      |         |
|                                  | Female              | 44                  | 9       |         |
| Age (Median+IR)                  | 70 (63.5-77)        | 69 (63-80)          |         |
| Location of the primary tumor    | Right colon         | 17                  | 3       |         |
|                                  | Left colon          | 36                  | 11      |         |
|                                  | Rectum              | 22                  | 7       |         |
| Size of the primary tumor (mm)   | (Median+IR)         | 50 (40-70) mm       | 75 (50-90) mm |         |
| Grade                            | 1                   | 16                  | 5       |         |
|                                  | 2                   | 49                  | 13      |         |
|                                  | 3                   | 10                  | 3       |         |
| Stage                            | I                   | 18                  | 5       |         |
|                                  | II A                | 10                  | 5       |         |
|                                  | IIB                 | 13                  | 5       |         |
|                                  | III A               | 21                  | 2       |         |
|                                  | IIIB                | 13                  | 4       |         |
| Nodal Infiltration               | Yes                 | 33                  | 6       |         |
|                                  | No                  | 42                  | 15      |         |
| Cancer on adenoma                | Yes                 | 15                  | 3       |         |
|                                  | No                  | 6018                |         |         |
| Coexistence of adenomas          | Yes                 | 24                  | 5       |         |
|                                  | No                  | 51                  | 16      |         |
| Vascular invasion                | Yes                 | 18                  | 6       |         |
|                                  | No                  | 57                  | 15      |         |
| Cullin-2 expression              | Positive            | 47                  | 15      |         |
|                                  | Negative            | 28                  | 6       |         |
| Cullin-2 expression (%)          | (Median+IR)         | 60 (37.5-72.5)      | 35 (20-45) | 0.003   |
| Ki-67 (%)                        | (Median+IR)         | 20 (10-37)          | 16 (3-25) |         |
| Mutant p53 (%)                   | (Median+IR)         | 43.5 (2.25-68.75)   | 2 (2-58) | 0.04    |

The percentage of cells expressing CUL-1, CUL-2, mutant p53 protein and Ki-67 index as assayed by IHC was determined and all examined parameters were correlated with patient’s age, gender, stage of the disease, tumor histological grade, tumor proliferative activity (Ki-67 labeling index) and mutant p53 protein expression.

Table III. Univariate analysis between co-expression of cullin-1 and cullin-2 in percentage ≥10 and evaluated parameters.

| Parameter                        | Cullin 1 +2 pos (n=47) | Cullin 1 1+2 neg (n=49) | p-Value |
|----------------------------------|------------------------|-------------------------|---------|
| Gender                           | Male                   | 20                      | 23      |         |
|                                  | Female                 | 27                      | 26      |         |
| Age (Median+IR)                  | 69 (63-76.5)           | 70 (63-78)              |         |
| Location of the primary tumor    | Right colon            | 11                      | 10      |         |
|                                  | Left colon             | 23                      | 20      |         |
|                                  | Rectum                 | 13                      | 19      |         |
| Size of the primary tumor (mm)   | (Median+IR)            | 50 (30-65) mm           | 52 (40-70) mm |         |
| Grade                            | 1                      | 11                      | 10      |         |
|                                  | 2                      | 31                      | 31      |         |
|                                  | 3                      | 5                       | 8       |         |
| Stage                            | A                      | 10                      | 13      |         |
|                                  | II A                   | 7                       | 8       |         |
|                                  | II B                   | 9                       | 9       |         |
|                                  | III A                  | 11                      | 12      |         |
|                                  | IIIB                   | 10                      | 7       |         |
| Nodal infiltration               | YES                    | 20                      | 19      |         |
|                                  | No                     | 27                      | 30      |         |
| Cancer on adenoma                | Yes                    | 10                      | 8       |         |
|                                  | No                     | 37                      | 41      |         |
| Coexistence of adenomas          | Yes                    | 19                      | 10      |         |
|                                  | No                     | 28                      | 39      | 0.0329  |
| Vascular invasion                | Yes                    | 11                      | 13      |         |
|                                  | No                     | 36                      | 36      |         |
| Mutant p53 (%)                   | (Median+IR)            | 50.5 (14-70) mm         | 12 (2-66) | 0.064   |
| Ki-67 (%)                        | (Median+IR)            | 25 (12-38) mm           | 17 (4-27) | 0.070   |

Statistical analysis. For the correlation between CUL and the clinicopathological variables the chi-square test was used. p-Value with statistical significance was set at p<0.05. For statistical analysis the SPSS (version 18) statistical package, was used.
Results

There were 96 patients with a median age of 70 years (IR: 63-77 years), who underwent oncological colectomy for colorectal adenocarcinoma. The clinicopathological characteristics of the patients enrolled, are presented in Table I.

Univariate analysis between CUL-1 expression to several clinicopathological variables. Choosing as a cut-off point for CUL-1 positivity the 10% (Table II), 75 specimens (78%) were characterized as positive and statistically significantly related to the expression of mutant p53 protein \((p=0.04)\) and the co-expression of CUL-2 \((p=0.003)\).

By setting the cut-off limit for CUL-1 expression to 30%, 36 specimens (37.5%) were characterized as positive. The statistical significance to the CUL-2 co-expression was preserved \((p=0.02)\), although a slight decrease of the statistical significance between CUL-1 expression and the mutant p53 protein expression was observed \((p=0.07)\).

Univariate analysis between CUL-2 expression to several clinicopathological variables. By setting the cut-off limit for CUL-2 expression to 10%, 62 specimens (64.5%) were considered positive. No statistically significant differences were observed between CUL-2 >10% expression and the examined clinicopathological variables.

By increasing the cut-off limit for CUL-2 expression to 30%, 44 specimens (46%) were considered positive. A statistically significant correlation of its expression to the mutated p53 protein was noticed \((p=0.047)\).

Univariate analysis between CUL-1 & 2 expressions ≥10% to several clinicopathological variables. By setting the cut-off point for both CUL expressions to 10%, 47 specimens (49%), were considered positive (Table III). A statistically significant correlation between their co-expression to the coexistence of adenomatous polyps along the large bowel \((p=0.0329)\), as well as a nearly statistically significant correlation to mutant p53 protein expression \((p=0.064)\) and Ki-67 expression \((p=0.07)\) were observed.

Multivariate analysis of CUL-1 and -2 co-expression. Multivariate analysis (Table IV) of CUL-1 and -2 co-expression in more than 10% disclosed their expression as an independent factor for adenomatous polypl development along the large bowel \((p=0.035, RR=2.1)\).

Discussion

The present study disclosed that 78% of the colorectal cancers analysed, expressed CUL-1. The prognostic significance of CUL expression in colorectal cancer has been studied by Wang et al. (20) who found that high CUL-1 expression was positively associated with a larger primary tumor diameter and lymph node metastasis, revealing that high CUL1 expression was an independent unfavourable prognostic factor for colorectal cancer patients. Similarly, Jiang et al. (21) addressed that high CUL4B expression was significantly associated with the depth of tumor invasion, lymph node metastasis, distant metastasis, histological differentiation, vascular invasion, and advanced tumor stage, while patients with CUL4B-positive tumors, had a higher recurrence rate and a poorer survival compared to those with CUL4B-negative tumors, finally concluding that CUL4B expression was an independent factor for determining colon cancer prognosis after surgery. Both studies (20, 21) addressed that CUL expression was significantly upregulated in colorectal tumor tissue compared to the paired normal mucosa, both in vitro and in vivo.

The present study did not provide similar results, however disclosed that CUL-1 expression was statistically significantly related to the expression of mutant p53. Since the expression of mutant p53 protein represents a well-known independent dismal prognostic factor for sporadic colorectal cancer (22, 23) and TP53 mutant cancer cells tend to be more resistant to a range of cytotoxic drugs, (24) the present study indirectly indicates a potential unfavorable prognostic role for CUL-1.

Although CUL-1 expression did not directly correlate to CUL-2 expression, the present study addressed that CUL-1(+) tumors overexpress CUL-2, an also indirect
finding for inductiveness of CUL-1 on CUL-2 expression. A potentially clinically interesting observation of the present study is that neither the expression of CUL-1 or CUL-2 nor their co-expression, promoted carcinogenesis on an adenomatous polyp. However, multivariate analysis disclosed CUL-1 and CUL-2 co-expression as an independent factor favoring adenomatous polyp development in the large bowel.

Inactivation or loss of the TP53 gene is a prerequisite for tumor growth (25). Mutant p53 not only loses its anti-tumor transcriptional activity, but also often acquires oncogenic functions to promote tumor proliferation and invasion (26). The present study indicates that in these vulnerable mutant p53(+)+ large bowel cells, CUL-1 overexpression happens at an early stage of carcinogenesis arresting cells in the G1 phase (16) and may also induct the expression of CUL-2. Co-expression of CUL-1 and CUL-2 promotes carcinogenesis further, to adenomatous polyp formation in the large bowel. In the absence of hereditary syndromes, adenomatous polyps represent a predisposing but not a definite causative factor for colorectal cancer development. Further research regarding the malignant progression from adenoma status requires cellular and molecular pathways unable to be explained based only on clinical observations.

Conclusion

The results of the present study indicate that CUL-1 overexpression may happen early in the process of carcinogenesis mainly affecting the vulnerable p53(+) large bowel cells, arresting them in the G1 phase of cell-cycle, while it may also induct the expression of CUL-2. Co-expression of CUL-1 and CUL-2 in G1 phase-arrested large bowel cells promotes carcinogenesis. Since no relationship between cullin expression and development of adenocarcinoma was found, the results of the present study may be useful in explaining the initiation but not the progression of carcinogenesis in colorectal cancer. Further molecular and clinical studies are needed in order to delineate the clinical importance of these proteins in the management of colorectal cancer patients.

Conflicts of Interest

None.

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Received December 2, 2017
Revised December 29, 2017
Accepted January 3, 2018