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Research Paper

Tumor-Specific Hyperactive Low-Molecular-Weight Cyclin E Isoforms Detection and Characterization in Non-Metastatic Colorectal Tumors

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ABSTRACT

Purpose: Several molecules involved in cancer biology have been studied as potential prognostic markers. Recently, overexpression of cyclin E and its low-molecular-weight (LMW) isoforms has been reported to be the most prominent prognostic marker in breast cancer, surpassing proliferation index, ploidy, and axillary nodal involvement. Furthermore, cyclin E and p53 are considered the main factors controlling the euploid equilibrium in human cells. We investigated the status of cyclin E and p53 in cell lines and tissue samples of colorectal cancer, one of the leading causes of death from a tumor in the Western world.

Experimental design: We analyzed colorectal cancer cells, from established cell lines and patient specimens, to determine the protein levels of cyclin E and p53, and to detect p53 and APC mutations, microsatellite and chromosome instability. In addition, we assessed the presence of cyclin E LMW isoforms and their enzymatic activity.

Results: Colorectal cancer cells expressed hyperactive LMW forms both in vitro and in vivo. These tumor-specific isoforms are correlated to genomic instability even in p53-proficient cells, and represented a constant feature in the tumors analyzed.

Conclusions: In colorectal cancer, the formation of cyclin E LMW forms is an early event leading to DNA-damage checkpoint-independent proliferation. Collectively, our results provide evidence that evaluation of LMW forms could represent a novel tool in the molecular characterization of colorectal tumors aimed at identifying sensitive prognostic factors and uncovering subsets of high-risk patients within the traditional categories.

INTRODUCTION

The deregulation of the cell cycle, particularly of the G_1/S phase boundary, is the leading mechanism in human cancer development. In normal cells, control of the G_1/S transition is ensured by the precisely timed accumulation and degradation of cyclin E, the regulatory subunit of the cyclin E/cdk2 kinase complex. Cyclin E transcription is induced during the mid G_1 phase of the cell cycle by the cyclin D/cdk4 complex via pRb phosphorylation. This event releases the transcriptional repression of cyclin E promoter exerted by a multiprotein complex containing E2F, pRb, histone deacetylase and the SWI/SNF chromatin-remodeling complex. At this point, cyclin E expression is maintained by an autonomous mechanism enhancing E2F-mediated transcription via cyclin E/cdk2-mediated pRb inactivation. Cellular abundance of cyclin E is then regulated by phosphorylation-mediated degradation by the hCDC4-dependent proteosome pathway. To date, two kinases have been identified to mediate this process, GSK3β and cdk2 itself. Once cyclin E/cdk2 complex is assembled, its kinase activity is regulated by the physical binding of the cdk inhibitors (CKIs) p21 and p27, and the pocket proteins p107 and p130 (reviewed in ref. 1).

Generation of cyclin E-deficient mice revealed that cyclin E functions are dispensable for proliferation of normal cells, while they are necessary for endoreplication, cell cycle reentry from the quiescent state and oncogenic transformation.[2] Indeed, cyclin E deregulation is involved in cancer formation and progression of several types of tumors, including those arising in the colon-rectum. The development of colorectal cancer depends upon two distinct pathways, both characterized by genetic instability. A small fraction (12–15%) of tumors presents microsatellite instability (MIN) at the nucleotide sequence level, whereas the others exhibit an abnormal chromosome number.[3] The MIN phenotype results from mismatch repair (MMR) deficiency,[4] while chromosomal instability (CIN) relies on at least cyclin E overexpression and hCDC4 inactivation.[5,6] The critical role of cyclin E in colorectal cancer cells is confirmed by the fact that it appears to be deregulated also in a significant percentage of MIN tumors.[7] Moreover, increasing protein levels of cyclin E are considered a marker for the transition from adenoma to adenocarcinoma, the key step in colorectal cancerogenesis.[8]

Several mechanisms could explain the high levels of cyclin E in colorectal cancer cells, some increasing the transcription rate, such as gene amplification[9] and up-regulation of E2F transcription factors,[10] others decreasing the degradation of the protein, as for inactivating mutation of hCDC4.[6] hCDC4 is the F-box protein that specifically recruits phosphorylated cyclin E to the ubiquitin-ligase complex, to label it for the destruction by the proteosome.[1] Accordingly, genetic inactivation of hCDC4 is sufficient to induce the CIN phenotype, due to a cyclin E-dependent defect in the execution of metaphase.[6]

In normal epithelial cells the DNA-damage checkpoint plays a critical role in preventing genomic instability by regulating the cell cycle and DNA repair through several proteins, including the transcription factor p53.[11] Once stabilized and activated, p53 triggers the expression of a number of target genes, such as p21 and hCDC4 itself.[12,13] These two proteins represent the functional link between p53 and cyclin E, and the factors by which p53 inhibits cyclin E-related kinase activity. Moreover, cyclin E overexpression initiates a p53-dependent response that prevents excess cdk2 activity by inducing expression of p21. When either p53 or p21 are inactivated, the high levels of cyclin E become catalytically active and cause defects in S phase progression, centrosome amplification and then the CIN phenotype.[14,15]

As it has been recently shown, some types of tumors overexpress not only the full-length 50 kD cyclin E protein, but also up to five LMW isoforms (ranging in size from 33 to 45 kD).[16] These isoforms were previously identified in breast cancer cells, where their presence strongly correlates with poor survival,[17] but subsequently they were characterized also in ovarian and melanoma cells.[18,19] Their appearance has been suggested to be due to the proteolytic processing of the N-terminus of cyclin E, which is unique to tumor cell lines and tissues.[20] Functionally, cyclin E LMW forms are hyperactive, as compared to the full-length protein, in phosphorylating substrates and inducing progression from the G_1 phase to the S phase. These effects rely on the increased affinity of these LMW forms for cdk2 kinase and on the decreased sensitivity to the inhibition imposed by p21 and p27, despite of the intact physical binding with both CKIs.[20-23]

The full-length 50 kD cyclin E, named EL1, is present in both normal and tumor cells; conversely, only tumor cells have the machinery to process cyclin E into LMW isoforms. The EL4 isoform is generated by alternative translation at methionine 46, whereas cleavage at two distinct sites in the N-terminus of EL1 accounts for the two pairs of LMW forms. The first protease-sensitive domain spans the residues 40-45, whereas the second is located around D70. Both proteolytic products are then subjected to posttranslational modifications, such as phosphorylation or deacetylation, creating two closely migrating doublets, EL2/3 and EL5/6, respectively.[20]

In the present study we analyzed seven colorectal cancer cell lines and 20 primary adenocarcinomas. In all cell lines tested, the presence of cyclin E LMW forms correlated with both CIN and MIN phenotypes despite p53 status. To confirm that these isoforms were hyperactive, we compared cyclin E-related kinase activity of a Hct116 cell line with MDA-MB-157 breast cancer cells and human fetal skin fibroblasts (hFSF). Lastly, we identified at least one LMW form with variable expression in all tumors tested even in the absence of detectable EL1 levels. To complete the molecular analysis, we characterized these tumors for p53 status, APC mutations and MIN phenotype.

MATERIALS AND METHODS

Cell lines. hFSF were obtained by standard methods as we previously described.[5] The MDA-MB-157, Hct116, LoVo, Sw480, Sw48, CaCo2, DLD1 and Ht29 cell lines were obtained from the ATCC. All cell lines were grown in DMEM + 10% fetal bovine serum, avoiding confluency at any time.

Tumor samples. A total of 20 nonmetastatic sporadic colorectal tumors, paired with adjacent normal tissues, were selected. All the tumor tissue samples were obtained at the surgery and freshly frozen. Gross cryostat dissection was limited to areas consisting mainly of tumor cells. Germ-line DNA was available either from peripheral blood or normal adjacent colon tissue from each patient. High molecular weight genomic DNA was extracted from frozen tissues or blood according to the standard methods.

MIN analysis. MIN was evaluated as we previously described.[24] Briefly, we employed five polymorphic markers: BAT25, BAT26, D2S123, D5S346 and D17S250. Samples were considered positive for MIN when two out of five PCR products of tumor DNA showed the presence of novel bands that were not visible in the corresponding normal DNA.

Mutational analysis of p53 and APC. Exons of p53 were amplified as follows: exons 5, 6, 7, 8 and 9 using primers and polymerase chain reaction (PCR) amplification conditions given details elsewhere.[25] Moreover, APC gene was analyzed. The amplifications were performed using primers and PCR conditions for the fragments H and G of exon 15 previously reported.[26] Single-strand conformation polymorphism (SSCP) was performed as we described.[27] The PCR products were electrophoresed on ultrathin precast gels (GeneGel Excel; Amersham Biosciences AB, Sweden) and DNA bands visualized by silver staining. DNA showing a variant banding pattern were then sequenced using Sequenase version 2.0 kit (Big Dye Terminator ABI, Applied Biosystem, CA) and an ABIPRISM377 automated DNA sequencer (Perkin Elmer Corp., CA).

Immunoblot assay. Levels of full-length cyclin E and LMW isoforms were evaluated by immunoblot analysis of lysates prepared from the frozen tissues and cell pellets, as previously described.[28,5] Briefly, cell pellets were homogenized by sonication in 1 volume of sonication buffer (50 mM Tris-HCl ph 7.4, 0.25 M NaCl, 1 mM dithiothreitol) at 4°C with following centrifugating at 15000g for 20 min at 4C. Then 50 μg of proteins from each sample were electrophoresed in a 10% SDS-polyacrylamide gel (SDS-PAGE) and transferred to Nitrocellulose membrane (Biorad). The blots were blocked overnight at 4°C in Blotto (5% non-fat dry milk in 20 mM Tris, 137 mM NaCl, and 0.25% Tween). After six 10 min washes in TBS-T (20 mM Tris, 137 mM NaCl, and 0.05% Tween), the blots were incubated in primary antibodies for 1 h. The primary antibodies used were: monoclonal antibody HE12 to cyclin E (1:500; Santa Cruz Biotechnology, CA), actin monoclonal antibody (1:500; Santa Cruz Biotechnology, CA), and p53 monoclonal antibody (1:500, Santa Cruz Biotechnology, CA). All antibody dilutions were made in Blotto. After primary antibody incubation, the blots were washed and incubated with the appropriate goat anti-mouse horseradish peroxidase conjugate (1:5000) in Blotto for 1 h, then washed and developed with the ECL chemiluminesence reagent (Amersham) as directed by the manufacturer. Equivalent amount of protein from the control cell line (breast-cancer cell line MDA-MB-157) was included on the gel as internal laboratory standard.

The protein levels in the immunoblots were measured by densitometric scanning of the corresponding bands with the use of Quantity One software (Biorad) and normalized with the intensity of the actin band.

Plasmids and GST-proteins. The construct to generate the GST-Rb (379–928) fusion protein was already described elsewhere.[29] The plasmid was expressed in bacteria and the protein product was purified as we described above.[30]

Sequential kinase assay. Immunoprecipitation and kinase assay were performed as we described elsewhere.[29] Briefly, MDA-MB-157, Hct116 and hFSF cell were harvested and lysed in Lysis Buffer [50 mM Tris, 5 mM EDTA, 250 mM NaCl, 50 mM NaF, 0.1% Triton, 0.1 mM Na3VO4, and 10 mg/ml aprotinin, leupeptin and phenylmethylsulphonyl fluoride (PMSF)]. The protein concentration was determined by Bradford assay

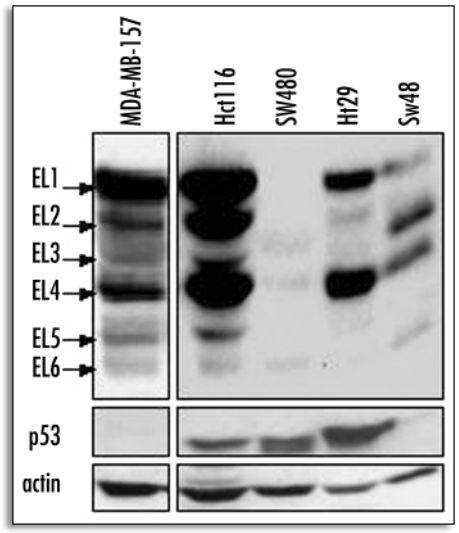

Figure 1. Cyclin E LMW isoform detection in colorectal cancer cell lines. 50 μg of total cell extracts of the indicated cell lines were probed with anti-cyclin E, anti-p53 and anti-actin antibodies. The relative mobility of cyclin E and its LMW forms are labeled EL1 (50 kD), EL2 (44kD), EL3 (43kD), EL4 (40kD), EL5 (35 kD) and EL6 (33 kD). The protein levels detected by the immunoblot analysis were measured by densitometric scanning of the corresponding bands and then normalized with the intensity of the actin band. The data obtained are presented in Table 1.

Table 1 **Molecular characterization of colorectal cancer cell lines**

Cell Line	MIN	CIN	p53m[a]	p53[b]	EL1[b]	LMW forms[b]
hFSF	-	-	-	+/-	+/-	-
Sw48	+	-	-	+/-	+	+
Hct116	+	-	-	+	+++	+++
DLD1	+	-	+	++	++	++
LoVo	+	+	-	+	++	++
CaCo2	-	+	-	+/-	++	++
Sw480	-	+	+	++	+/-	+
Ht29	-	+	+	+++	++	+++

[a]p53m, p53 mutations. [b]Densitometric normalization of protein expression. –, not expressed; +/-, detectable; +, expressed; ++, +++, levels of overexpression.

(Biorad), following the manufacturer's instructions and by using BSA as a standard. Cell extracts (200 μg) were used for immunoprecipitation with a monoclonal antibody HE12 to cyclin E (Santa Cruz Biotechnology, CA), (negative control: IgG). The immuno-complexes were pulled down with protein G-sepharose beads and washed three times with Lysis Buffer and twice with Lysis Buffer containing 400 mM NaCl. The complexes were then equilibrated in kinase assay buffer (minus ATP) (20 mM HEPES pH 7.4, 10 mM Mg Acetate). The kinase assay was performed in a volume of 20 μl, using 5 μCi/sample of γ-ATP (Amersham). 0.1 μg of GST alone and GST-pRb (379-928) were added to the samples and incubated for 30 min at 30°C. The reactions were stopped by adding 5x Laemmli Buffer and the samples were resolved on a 13% SDS-PAGE and subjected to autoradiography. For quantitation, the protein bands were excised, and the radioactivity of each band was measured by scintillation counting.

RESULTS

Cyclin E LMW isoforms are related to both CIN and MIN phenotypes in colorectal cancer cell lines. The deregulation of cyclin E expression in colorectal cancer cells was reported to be involved in both CIN and MIN tumor phenotypes.[5,7] Recently, the characterization of cyclin E LMW forms in breast tumors shed new light on cyclin E's role in cell transformation and cancer progression,[17] and prompted us to investigate the presence of LMW forms in colon cancer cells by employing an antibody specifically recognizing cyclin E C-terminus, the common region present in all six isoforms (EL1-6).

For this purpose we compared breast cancer MDA-MB-157 cells, expressing all LMW forms of cyclin E, with seven colorectal cancer cell lines, three presenting the CIN phenotype (CaCo2, Sw480, Ht29), three the MIN phenotype (DLD1, Hct116 and Sw48), and one both CIN and MIN (LoVo).[3,5,31] Immunoblot analysis revealed the presence at least of three cyclin E isoforms in both MIN and CIN cell lines tested, whereas euploid human fetal skin fibroblasts (hFSF) presented only EL1 expression (Fig. 1 and Table 1). Interestingly, aneuploid Sw480 cells, which in our previous study seemed to escape the relationship between cyclin E deregulation and genomic instability,[5] were uncovered to express EL3, EL4 and EL6 (Fig. 1 and Table 1). This finding suggests that the ability of LMW forms to induce the CIN phenotype could exceed that of cyclin E full-length as was demonstrated for breast tumors, where the presence of EL2/3 and EL5/6 significantly correlated with an increased number of chromosomal structural aberrations compared to EL1 alone.[22] Furthermore, we detected the presence of cyclin E LMW forms in tumor cell lines exhibiting the mutator phenotype, confirming the involvement of cyclin E in the MIN pathway.[7] Altogether, these data suggest that in colorectal cancer, cyclin E deregulation has a key role in escaping the DNA-damage checkpoint control of the integrity of the genome.

In normal skin fibroblasts, prolonged and deregulated cyclin E expression paradoxically inhibits cdk2 kinase activity through the induction of p53-mediated expression of p21.[14] Due to this cellular mechanism, bladder cancer cells need the overexpression of cyclin E together with the inactivation of p53 (or p21) to reach the CIN phenotype.[15] Interestingly, in colorectal cancer cells the presence of the cyclin E LMW forms correlates to genomic instability even in Sw48, Hct116, LoVo and CaCo2 p53-proficient cells (Fig. 1 and Table 1). These data seem to be in accordance with the critical impact of LMW forms on cell homeostasis already described in breast cancer cells even if p53 and p21 were significantly induced.[22]

Cyclin E LMW isoforms are hyperactive. To examine the enzymatic activity of the tumor-specific cyclin E LMW forms compared to that of EL1 alone, we performed a kinase assay using immunoprecipitated cyclin E complexes of hFSF, breast cancer MDA-MB-157 and Hct116 colorectal cancer cells. Under logarithmic growth conditions, hFSF expressed only a moderate amount of cell cycle-regulated EL1[5] (Table 1), while MDA-MB-157 expressed high levels of EL1 and EL4 together with appreciable levels of EL2, and a lower amount of EL3, EL5 and EL6 (Fig. 1). Under the same conditions, Hct116 expressed very high levels of EL1, EL2 and EL4, with relatively high levels of EL3, EL5 and EL6 (Fig. 1). For the sequential kinase assay, we employed antibody, able to detect all cyclin E isoforms (EL1-6) (Fig. 1), and then we tested the immunoprecipitated enzymatic activity on the well-known cyclin E substrate GST-pRb.[20] Cancer cells showed an activity significantly higher than normal cells (Fig. 2), and increased phosphorylation of pRb seemed to be related to the relative amount of cyclin E LMW forms (see Fig. 1 and 2). These results, in accordance with those previously published,[20-22] support the notion that cyclin E LMW forms are more active than the full-length protein.

Detection of LMW forms of cyclin E in non-metastatic tumors from colorectal cancer patients. In breast cancer, high levels of LMW forms correlated strongly with disease-specific survival surpassing proliferation index, ploidy, and even axillary nodal involvement.[17] In fact, among patients with stage I tumors, only those with high levels of LMW forms died of breast cancer within five years after diagnosis.[17] These findings indicate that evaluation of cyclin E LMW forms can represent the most efficient marker for the clinical management of cancer patients, uncovering a new category

of high-risk subjects within the traditional classes sorted by standard clinical and histopathological parameters.

Presently, the assessment of prognosis in colorectal cancer patients is based on grade and TNM evaluation, but also in the lower-risk classes there are a variable percentage of unfavorable, disease-specific events. To evaluate the role of LMW forms as prognostic markers in the clinical assessment of colorectal cancer patients, we decided to test whether our findings in established cell lines were confirmed by in vivo analysis. For this purpose we selected 20 nonmetastatic adenocarcinomas: 15 node-negatives (one pT1N0M0, two pT2N0M0, 12 pT3N0M0) and five node-positives (two pT3N1M0, three pT3N2M0).

Initially, we decided to start the analysis with an immunoblot to search for cyclin E LMW forms. As presented in Figure 3 and Table 2, EL1 was detected in 17 tumors (85%), EL2 in eight (40%), EL3 in 19 (95%), EL4 in 17 (85%), EL5 in 16 (80%), and EL6 in ten (50%). Indeed, all tumors tested expressed variable levels of at least one LMW form, independently of tumor site, local invasion, nodal status and differentiation grade. One out of 20 (5%) tumors did not express either EL2 nor EL3, while neither EL5 nor EL6 were detected in four samples (20%). These five tumors (25%) showed a limited local invasion without affecting lymph nodes, and represented one third (33%) of all the node-negative cases. Conversely, all patients with lymph node involvement presented both proteolytic products of cyclin E. Interestingly, six LMW form-expressing tumors exhibited low levels or even absence of EL1, suggesting that the proteolytic processing does not depend on the cellular amount of the protein, but on the tumor-specific protease activity. Moreover, considering the two different proteolytic products, EL2/3 and EL5/6, it appears that the equilibrium between each doublet is shifted from EL2 to EL3, and EL6 to EL5, respectively. This evidence is intriguing because indicates that the main forms, EL3 and EL5, are then modified by two different or even opposite concentration-dependent processes, such as phosphorylation/dephosphorylation or acetylation/deacetylation.

To further characterize these tumors, we investigated the status of microsatellite DNA repeats to identify cancers with MIN phenotype. The analysis was carried out on normal and tumor tissue DNA by amplification of two mononucleotide repeats (BAT 25 and 26) and three dinucleotide repeats D2S123, D5S346 and D17S250. Three tumors (15%) exhibited the MIN phenotype, and all of them overexpressed cyclin E LMW forms (Fig. 3 and Table 2) in accordance with the molecular features of Sw48, Hct116 and DLD1 cells (see Table 1).

The status of APC and p53, both genes frequently mutated in CIN tumors, was evaluated. The APC protein is an upstream regulator of cyclin E transcription through a pathway involving β-Catenin, c-Myc, cyclin D and cdk4.[5] We found three mutations (15%) in the hot-spot mutation cluster region of the APC sequence,[26] which affected tumors overexpressing cyclin E and its LMW forms (Fig. 3 and Table 2). Lastly, we detected only two mutations in the hot-spot region of the p53 sequence (Table 2),[25] but immunoblot analysis revealed another five tumors presenting altered p53 expression (Fig. 3 and Table 2). Three of them (425, 431 and 432) expressed a mutated or abnormally modified p53, whereas 423 and 622 presented higher levels of protein product. p53 overexpression often represents a hallmark of gene mutation[32] (Table 2) or the paradoxical response of homeostatic feedback loops in tumor cell circuitry that maintain an appropriate balance between growth-promoting and inhibitory factors.[33] The tumors (35%) showing an altered p53 status overexpressed at least one LMW form (Fig. 3 and Table 2).

DISCUSSION

Cancer is one of the most common diseases affecting hundreds of thousands of people worldwide every year. A better understanding of the characteristics of a tumor would aid physicians in the prognostic evaluation of a particular patient and in the selection of the best available therapeutic strategy; consequently, only those patients who would likely benefit from adjuvant chemotherapy would receive it.

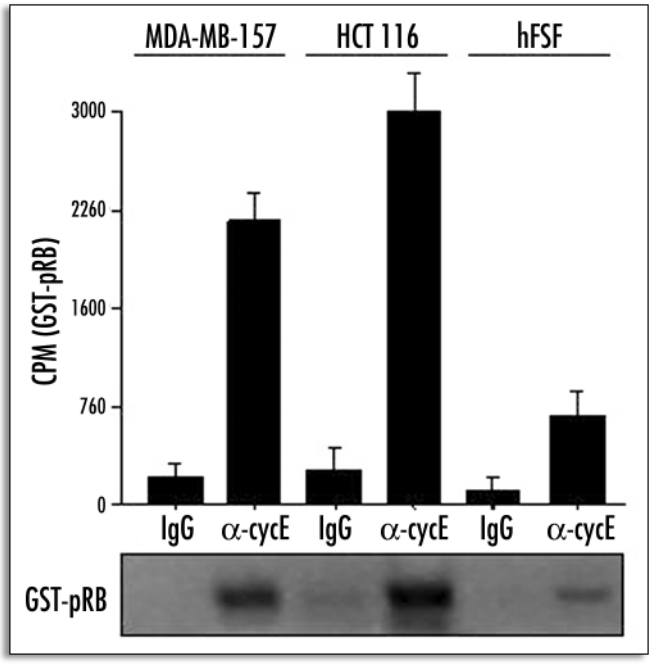

Figure 2. Cyclin E LMW forms are hyperactive. To evaluate kinase activity, equal amounts of proteins (250 μg) from total cell lysates were prepared from each indicated cell line and immunoprecipitated with anti-cyclin E antibody and control IgG. GST-pRb (379-928) was used as substrate in the kinase assay, and GST alone as negative control (data not shown). For each kinase reaction, the resulting autoradiogram of the GST-pRb (379-928) SDS-PAGE is presented together with quantitation of incorporated γ-P^{32} measured by scintillation counting.

As such, there is an urgent need for sensitive and specific prognostic indicators. A multitude of molecules involved in cancer biology have been studied as potential prognostic markers. Recently, in breast cancer, overexpression of cyclin E and its LMW forms has been reported to be the most prominent prognostic factor surpassing axillary nodal involvement.[17] This means that small and node-negative tumors, despite the tumor grade, could kill the patient if the cancer cells express LMW forms. Moreover, it has been proven that estrogen-receptor-positive breast tumors overexpressing LMW forms do not respond to antiestrogen treatment.[22] These findings suggest that detection of LMW forms is a powerful diagnostic tool in clinical oncology.

Colorectal cancer is one of the leading causes of death from a tumor in the Western world. Prognostic evaluation is currently based on histological appearance, and there are no molecular markers internationally recognized as standard predictor factors. We analyzed nonmetastatic adenocarcinomas to assess whether LMW forms were present in cancer cells, particularly in the initial phases of tumorigenesis. Collectively, our data indicate that all tumors analyzed displayed the proposed elastase-like activity able to process cyclin E full-length protein in LMW isoforms (Fig. 3 and Table 2). When attention was given separately to each proteolytic product, it was possible to identify four node-negative tumors not expressing either EL5 or EL6, and one not expressing EL3 or EL2. This fact, together with the observation that EL6 and EL2 were never expressed alone, but always concurrently with a higher amount of EL5 and EL3, respectively, suggests that the last two isoforms are the main products of cyclin E processing. Furthermore, we can speculate that EL6 and EL2 are originated from post-translational modifications imparted by

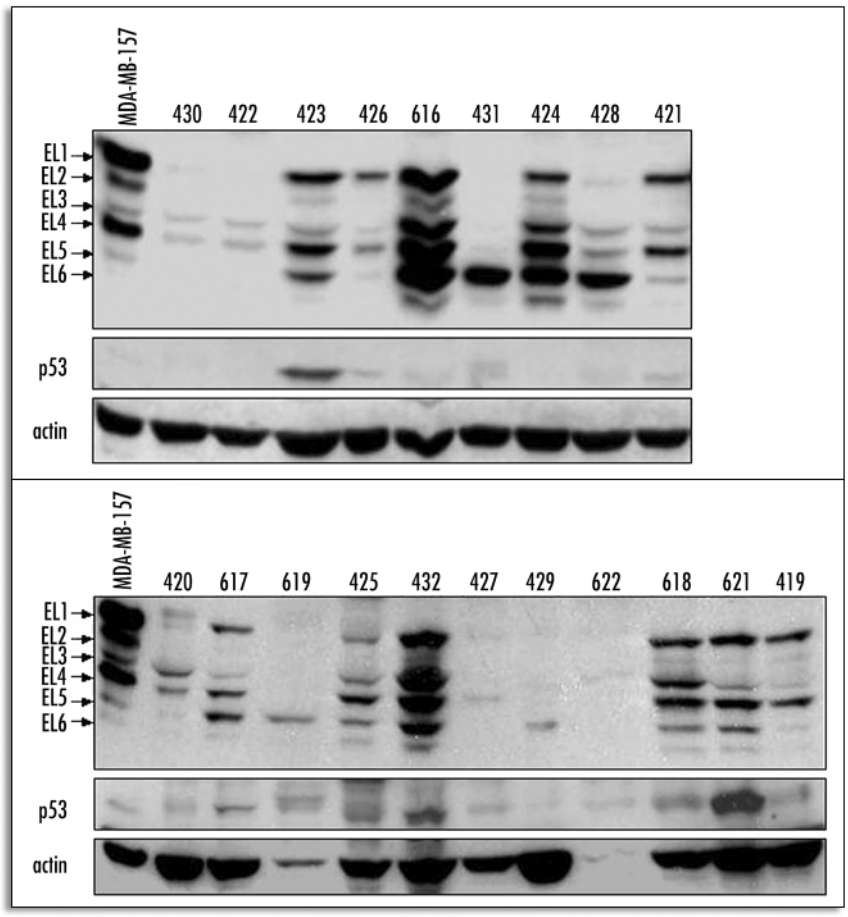

Figure 3. Presence of cyclin E LMW forms in non-metastatic tumors of colorectal cancer patients. Immunoblot assay of 50 µg of total cell lysates from tumor tissues probed with anti-cyclin E, anti-p53 and anti-actin antibodies. Cyclin E and its LMW forms relative bands are indicated and labeled as EL1 (50 kD), EL2 (44kD), EL3 (43kD), EL4 (40kD), EL5 (35 kD) and EL6 (33 kD). The protein levels detected by the immunoblot analysis were measured by densitometric scanning of the corresponding bands and normalized with the intensity of the actin band. The data obtained are presented in Table 2.

cellular pathways attempting to counteract hyperactivity of LMW forms.

The increased ability to phosphorylate substrates (Fig. 2) seems to depend on the excised N-terminal domain spanning the amino acids (aa) 1-75, since EL2/3 originates by cleavage at 40–45 residues and EL5/6 around D70.[20] This region is involved in cyclin E regulation, as several pathways converge on it. First, optimal hCDC4-mediated degradation depends on at least four phosphorylation sites, three on the C-terminus, and one on the N-terminus, T77 of EL1.[34,35] Whereas cdk2 and GSK3β were identified as the enzymes responsible for the C-terminal phosphorylations, the kinase acting on T77 is still unknown.[35] Therefore, the N-terminal deletions could represent an obstacle in the degradation of LMW forms that bypasses cell cycle regulation,[28] either by eliminating a kinase recognition motif close to the phosphorylation site[30] or by interfering with the right folding of the entire N-terminus. Moreover, this domain is involved in the interaction with p21 and p27 to modulate cyclin E/cdk2 kinase activity.[1] Cell cycle, differentiation, senescence, DNA-damage response and apoptosis require a tightly regulation of the cyclin/cdk complexes.[1] Crystallographic studies revealed that CKIs bind cyclin/cdk complexes as an extended structure interacting with both partners. On the cyclin, it binds in a hydrophobic patch within the conserved cyclin box domain.[36] On the cdk, it binds and rearranges the N-terminus and also inserts into the catalytic cleft, mimicking ATP.[37] The overall inhibitory effect of p21 and p27 is abolished by LMW forms despite the intact physical binding.[23] suggesting that N-terminal deletions somehow alter the spatial configuration of the multiprotein complex. This property explains LMW form hyperactivity and its role in uncontrolled tumor proliferation despite hormonal therapy.[22] It is likely that the absence of p21 inhibition, and possibly of the hCDC4-mediated degradation, is also responsible for the deficiency of p53 response in LMW-expressing tumors. Human cells, during the transformation and cancer progression, activate a p53-dependent DNA-damage network that elicits growth arrest and DNA repair or programmed cell death.[38] The involvement of cyclin E LMW forms in the onset of genomic instability, both CIN and MIN (Figs. 1 and 3, Tables 1 and 2), indicates that they interfere with DNA-damage checkpoint, allowing mutated cells to bypass the restriction point and transmit their tumoral phenotype to daughter cells.

Future studies are awaited to obtain new insights into cyclin E LMW form production and functions, a fundamental step toward the identification of novel prognostic tools and therapies based on their early detection and inactivation, for anti-protease treatment and the design of small molecules specifically targeting cyclin E isoforms. Our laboratory is currently working on the completion of a study on a large group of patients with more than five years follow-up to assess whether the evaluation of LMW forms is a reliable prognostic factor in colorectal cancer.

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Table 2. Clinical and molecular evaluation of the colorectal adenocarcinomas

N°	Age	Site	TNM	Grade	MIN	APCm[a]	p53m[b]	p53[c]	EL1[c]	EL2[c]	EL3[c]	EL4[c]	EL5c	EL6[c]
425	52	rectum	pT1N0M0	well	-	-	-	+*	+	+/-	+	++	+	+
430	59	rectum	pT2N0M0	well	-	-	-	+/-	+/-	-	+	+	-	-
421	46	rectum	pT2N0M0	well	-	-	-	+/-	++	-	+	++	+	-
431	72	rectum	pT3N0M0	mod	+	-	-	+/-*	-	-	+/-	+/-	+++	+/-
616	78	transv	pT3N0M0	mod	+	-	-	+/-	+++	+	+++	+++	+++	+
432	76	ascend	pT3N0M0	mod	+	+	-	+*	+++	+	+++	+++	+++	+
426	88	descend	pT3N0M0	mod	-	+	-	+/-	+	-	+/-	+	+/-	-
419	67	sigmoid	pT3N1M0	mod	-	+	-	+	++	+/-	+	++	+/-	-
621	64	rectum	pT3N0M0	mod	-	-	+	+++	++	+/-	+	++	+	+
427	71	rectum	pT3N0M0	mod	-	-	-	+/-	+/-	-	+/-	+	-	-
422	73	rectum	pT3N0M0	mod	-	-	-	-	-	-	+	+	-	-
622	58	transv	pT3N0M0	mod	-	-	-	++	++	-	+++	-	-	-
424	74	ascend	pT3N0M0	mod	-	-	-	-	++	+	++	+++	+++	+
420	72	sigmoid	pT3N0M0	mod	-	-	-	+/-	+	-	+	+	+/-	-
619	61	rectum	pT3N0M0	poor	-	-	+	+++*	+	-	-	-	+++	-
618	65	rectum	pT3N1M0	well	-	+	-	+	++	+/-	++	++	+	+
617	82	ascend	pT3N1M0	mod	-	-	-	+	+	-	+	+	+	+/-
429	58	rectum	pT3N2M0	mod	-	-	-	+/-	+/-	-	+/-	-	+	-
423	60	rectum	pT3N2M0	poor	-	-	-	++	++	+	+	++	+	+/-
428	77	rectum	pT3N2M0	poor	-	-	-	+/-	+/-	-	+	+	+++	+

[a]APCm, *Adenomatous Poliposis Coli* (APC) mutations. [b]p53m, p53 mutations. [c]Densitometric normalization of protein expression. –, not expressed; +/-, detectable; +, expressed; ++, +++, relative levels of overexpression.*SDS-PAGE migration pattern suggestive of mutated or abnormally modified protein product.

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