Overexpression of lamin B1 induces mitotic catastrophe in colon cancer LoVo cells and is associated with worse clinical outcomes

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Abstract. Lamins are the major components of the nuclear lamina and play important roles in many cellular processes. The role of lamins in cancer development and progression is still unclear but it is known that reduced expression of lamin B1 has been observed in colon cancer. Thus, the aim of the present study was to elucidate the influence of LMNB1 upregulation on colon cancer cell line after treatment with 5-FU. The results indicate, that overexpression of LMNB1 induced dose-dependent cell death mainly by mitotic catastrophe pathway. Furthermore, after upregulation of this intermediate protein, lower expression of lamin A/C was observed. Moreover, we observed an increase in fluorescence intensity of nuclear β-catenin and decrease in cell-cell interaction area, that was connected with inhibition of colon cancer cells migration. We present the reorganization of actin filament and β-tubulin, because these cytoskeletal proteins are directly or indirectly linked with lamins, and analyzing publicly available mRNA data we show that patients with overexpression of LMNB1 are characterized by lower survival rates within the first 30 months from diagnosis. Summarizing our results, upregulation of LMNB1 induce mitotic catastrophe and only small percentage of apoptosis. Moreover, we showed inhibition of cell migration and promotion of cell-cell contact as a results of direct and indirect regulation of β-catenin, lamin A/C, actin and tubulin. However, it is possible that mitotic catastrophe cells in patients with colorectal cancer may be a reservoir of the cells responsible for faster disease progression, and further investigations are necessary to confirm this hypothesis.

Introduction

The nuclear lamins were classified as type V intermediate filament proteins that are critically important for the structure of the nucleus and are located between the inner nuclear membrane and peripheral heterochromatin (1). Based on sequence homologies, as well as biochemical properties, lamins were subdivided into types: A and B. In mammals, lamin A and C have been rated into A-type but lamin B1 and B2 have been classified as B-type lamins (2). Lamins, together with lamin-binding proteins, make up the nuclear matrix and are involved in many different functions, including nuclear stability, transcription, DNA replications and genome repair. Interactions of lamins with chromatin regulate gene expression, responsible for all such cellular processes as proliferation, differentiation and carcinogenesis (3,4).

The role of lamins in cancer development and progression is still unclear. However, overexpression of LMNB1 was shown in prostate, liver and pancreatic cancers, whereas downregulation in gastric and in several subtypes of lung cancer (2,3,5). Reduced expression of LMNB1 has been also observed in colon cancer, which is the most common group of malignant tumors throughout the world (4). Application of cytostatics plays an important role in colon cancer therapy, but different sensitivity to chemotherapeutics and development of drug resistance is frequent thus encouraging scientists to look for new therapeutic goal (6). Except for intermediate filaments (lamin B1), other elements of cytoskeleton could play an important role in the limiting of cancer cell proliferation.

Recently, the existence of actin in the nucleus has been accepted and it was shown as an additional element of multiple nuclear complexes which can actively enter and exit the nuclei (7,8). Nuclear actin is involved in chromatin remodeling, transcription and RNA splicing. Furthermore, together with actin-binding proteins, it forms the functional bridge between nuclear components and cytoplasm. A hypothesis states that one interconnected network connect the elements of the nucleus via the nuclear membrane to the cytoskeleton, cell adhesion

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molecules and the extracellular matrix (9). The recent identification of Actin-Binding site 1 (AB-1) within lamins A and B prompted us to test whether disability in the structure of nuclear lamina may have an impact on tumor cell adhesion and migration. One of the proteins participating in all these processes is β-catenin, which abnormally high expression in colorectal cancer has been detected. There is a multifunctional protein which can be located in cell membrane and function as a structural element of cadherin/catenin complex to modulate cell-cell adhesion and migration or detected in nucleus acts as an effector of the Wnt signaling pathway (10-12). It is known that actin is linked with cadherin/β-catenin with α-catenin. Additionally, lamin A and B bind directly to actin and thus it is assumed that the expression of LMNB1 has an influence on adhesion and migration of colon cancer cells.

The aim of the present study is to show dependence between the expression of lamin B1 and β-catenin as well as the influence of LMNB1 overexpression on cytoskeleton (F-actin, tubulin and lamin A/C) organization, cell death, cell cycle and migration of LoVo cells after treatment with 5-FU. Here, we show that upregulation of LMNB1 expression induced dose-dependent cell death mainly by mitotic catastrophe. Additionally, overexpression of this nuclear protein caused the inhibition of colon cancer cells migration which is connected with lower expression of lamin A/C and changes in expression of actin and β-catenin.

Materials and methods

Cell culture and treatment. LoVo, a human colon cancer cell line was purchased from the American Type Culture Collection (CCL-229; ATCC, Manassas, VA, USA). The cells were cultured in tissue culture flasks or 12-well plates (BD Biosciences, Franklin Lakes, NJ, USA) and grown as a monolayer at 37°C in a humidified CO2 incubator (5% CO2) in Dulbecco's modified Eagle's medium (DMEM; Lonza, Verviers, Belgium). The medium was supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA) and 50 µg/ml gentamycin (Sigma-Aldrich). LoVo cells were treated for 24 h with 0.01, 0.05, 0.1, 1, 2.5, 5 and 10 mM concentration of 5-fluorouracil (5-FU; Sigma-Aldrich) for MTT assay and 0.1, 1 and 5 mM for other experiments. The control cells were grown under the same conditions but without the addition of cytostatic. The LoVo culture was tested for mycoplasma based on rapid uptake of the dAPI (sigma-Aldrich) antibodies for 2 h at room temperature (RT). Next, the membranes were incubated with primary mouse monoclonal anti-lamin B1 (Invitrogen/Thermo Fisher Scientific) or mouse monoclonal anti-GADPH (Sigma-Aldrich) antibodies for 2 h at room temperature (RT). Next, the membranes were washed and incubated with a ready-to-use solution of alkaline phosphatase-conjugated anti-species IgG for 1 h in RT. The protein bands were visualized using a ready-to-use solution of BCIP/NBT substrate for alkaline phosphatase. The last step of this method was scanning of membrane and densitometry analysis of the bands using the Quantity One Basic software (ver. 3.6.5; Bio-Rad Laboratories, Hercules, CA, USA).

Western blot analysis. Semi-quantitative analysis of post-translational expression of lamin B1 was performed by using western blot analysis. After transfection, the LoVo cells were lysed with RIPA buffer (Sigma-Aldrich). Next, BCA protein assay kit (Thermo Fisher Scientific, Pierce Rockford, IL, USA) to the manufacturer's instructions. For determining the unspecific effect of the upregulation of LMNB1 overexpression, the cells were transfected with pCMV6-XL4 control plasmid vector (Origene). After transfection and growth in the medium for 72 h, the cells were used for further experiments. The efficiency of transfection was confirmed by western blot analysis.

Cell death analysis. The procedure was performed according to the manufacturer's protocol by using Tali™ apoptosis assay kit/Annexin V Alexa Fluor® 488 and propidium iodide (PI) (Invitrogen/Life Technologies). After trypsinization, the cells were centrifuged and resuspended in 100 µl Annexin binding buffer. Next, to each 100 µl of sample, 5 µl of Annexin V Alexa Fluor 488 was added. After 20 min of incubation in the dark, the cells were centrifuged, resuspended in Annexin binding buffer. Subsequently, 1 µl of PI to each sample was added and the cells were incubated at room temperature in the dark for 3 min. The data were analyzed using the FCS Express Research Edition software (ver4.03; De Novo Software, Los Angeles, CA, USA). The results revealed that the viable cells were Annexin V Alexa Fluor 488 and PI-negative; apoptotic cells were Annexin V Alexa Fluor 488-positive and PI-negative and Annexin V Alexa Fluor 488 and PI-positive.
whereas necrotic cells were Annexin V Alexa Fluor 488-negative and PI-positive.

**Cell cycle analysis.** For cell cycle analysis, the Tali Cell Cycle kit (Invitrogen/Life Technologies) was used according to the manufacturer's instructions. First, the treated cells were fixed in ice-cold 70% ethanol at 4°C and left at -25°C overnight. After the washing and centrifugation, the cells were resuspended in the Tali Cell Cycle Solution and incubated in the dark for 30 min. The data were determined using Tali image-based cytometer (Invitrogen/Life Technologies) and the percentage of cells in each phase of the cell cycle was designated using the FCS Express Research Edition software (version 4.03; De Novo Software).

**Fluorescent staining.** The LoVo cells with and without overexpression of LMNB1 after treatment with 0.1, 1 and 5 mM 5-FU were grown on glass coverslips. For β-tubulin labeling, after the 24 h incubation with cytotatic, the LoVo cells were prefixed for 10 min with bifunctional protein crosslinking reagent DTSP (1 mM 3,3′-dithiodipropionic acid; Sigma-Aldrich) which was diluted 1:50 in Hanks' balanced salt solution (HBSS; Sigma-Aldrich). Next, the cells were pre-extracted with TSB [0.5% Triton X-100 (Serva Electrophoresis GmbH, Heidelberg, Germany) in MTBS with the addition of DTSP (dilution 1:50) (microtubule stabilizing buffer: 1 mM EGTA, 10 mM PIPES, 4% poly(ethylene glycol); Sigma-Aldrich) for 10 min and rinsed with TSB (5 min)]. Then, the cells were fixed with 4% paraformaldehyde (Serva Electrophoresis GmbH) in MTBS for 15 min, washed with PBS (5x5 min) and incubated with 1% bovine serum albumin (BSA; Sigma-Aldrich) diluted in TBS (Tris-buffered saline) for 15 min. Tubulin was labeled using a mouse monoclonal antibody against β-tubulin (Sigma-Aldrich) and goat anti-mouse antibody TRITC (Sigma-Aldrich).

In the case of other immunofluorescence reactions, the cells were fixed with 4% paraformaldehyde in PBS, pH 7.4 (15 min, room temperature), incubated with 0.25% Triton X-100 (Serva Electrophoresis GmbH) for 5 min then blocked in 1% (w/v) BSA/PBS and double stained for proteins and F-actin, using antibodies and phalloidin conjugates in the following arrangement: i) rabbit anti-β-catenin (Sigma-Aldrich), anti-rabbit antibody-Alexa Fluor 488 (Invitrogen/Life Technologies), phalloidin-TRITC (Sigma-Aldrich); ii) mouse anti-lamin A/C (Sigma-Aldrich), anti-mouse TRITC (Sigma-Aldrich), Alexa Fluore-488 phalloidin (Invitrogen/Life Technologies); and iii) mouse anti-lamin B1 (Invitrogen/Life Technology), anti-mouse TRITC (Sigma-Aldrich).

Cell nuclei were stained with DAPI (Sigma-Aldrich). The slides were mounted in Aqua-Poly/Mount (Polysciences, Warrington, PA, USA) and examined using CL1 laser-scanning confocal microscope system (Nikon, Tokyo, Japan) or Nikon Eclipse E800 fluorescence microscope and NIS-Elements 4.0 software (Nikon).

The measurement of fluorescence intensity of lamin B1 in LoVo cells with and without overexpression of LMNB1 was performed on confocal images. The fluorescence intensity measurement was executed using the ImageJ software (Ver. 1.51j8, National Institute of Health, Bethesda, MD, USA).

The measurement of fluorescence intensity of junctional protein (β-catenin) in LoVo cells with and without overexpression of LMNB1 after treatment with 0.1, 1 and 5 mM 5-FU was performed on confocal images acquired at the brightest signals at cell-cell interaction areas. The fluorescence intensity measurement of nuclear F-actin and β-catenin was executed using Nikon EZ-C1 software (Ver 3.90, Gold; Nikon).

**Wound healing assay.** The LoVo cells with and without overexpression of LMNB1 after treatment with 0.1, 1 and 5 mM 5-FU were grown in 6-well plates. The cell monolayer was then scratched using a sterile pipette tip. For imaging of cell migration during wound healing assay live cell imaging microscope (Carl Zeiss, Oberkochen, Germany) was used.

**Analysis of publicly available datasets.** To analyze LMNB1 mRNA expression in colorectal adenocarcinoma, we obtain data from TCGA Data Portal Open-Access directory via the cBio Portal (13). Overall survival analysis was then performed using the GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA).

**Statistical analysis.** The data are shown as mean ± SEM. A two-way ANOVA analysis was performed for wound healing, fluorescence intensity, cell death and cell cycle data between cells transfected with control vector and plasmid with cloned cDNA of LMNB1. A Kruskal-Wallis test was used to evaluate the differences in mean values between cells treated with cytostatic in comparison to the control in a group of cells with normal expression of LMNB1 and transfected with plasmid with cloned cDNA of LMNB1 independently. A one sample t-test compares the mean with a hypothetical value, thus it was used for the analysis of MTT data. The differences between the groups were considered significant at P<0.05. GraphPad Prism 6.0 software was used for statistical analyses. Statistically significant differences between cells treated with cytostatic in comparison to the control in a group of cells with normal expression of LMNB1 are marked by *. In turn, statistically significant differences within cells with overexpression of LMNB1 are presented in the figures as # and the differences between the cells transfected with control vector and plasmid with cloned cDNA of LMNB1 are shown with $ symbol.

**Results**

**The effect of 5-FU on the viability of LoVo cells.** MTT analysis was performed to select appropriate doses of the cytostatic drug. This experiment indicated that 5-FU exhibited a cytostatic effect on the growth of LoVo cell line and cell viability was decreased in a dose-dependent manner (Fig. 1). Twenty-four hours of treatment of cells with 0.01, 0.05 and 0.1 mM doses of 5-FU decreased viability to 94.06, 87.8 and 87.5%, respectively. Following treatment of LoVo cells with the higher doses (1, 2.5, 5 and 10 mM) survival rate was even lower and amounted to 73.1, 65.1, 63.6 and 26.3%, respectively. The results were statistically significant in comparison to the control (P<0.05) (Fig. 1). On the basis of results obtained from MTT analysis, for further experiments, 5-FU was used in concentrations of 0.1, 1 and 5 mM.
Western blot analysis of LMNB1 expression. The upregulation of LMNB1 expression in LoVo cells transfected with pCMV6-XL4 expression plasmid vector with cloned cDNA of LMNB1 was examined by using the western blotting method (Fig. 2A). The densitometric analysis confirmed an increase in the post-translational expression of LMNB1 in the control and cells exposed to 0.1, 1 and 5 mM 5-FU, as compared to cells transfected with control vector (Fig. 2B). Analysis of the relative to GADPH band intensity showed 1.17-, 1.18-, 1.21- and 1.41-fold increase in the post-translational expression of LMNB1 in the control and cells treated with 5-FU at doses 0.1, 1 and 5 mM, respectively (Fig. 2B). Additionally, Fig. 2C shows an increase of lamin B1 fluorescence intensity in cells transfected with pCMV6-XL4 expression plasmid vector with cloned cDNA of LMNB1 (Fig. 2C).

The effect of LMNB1 upregulation on cell death. The type of 5-FU-induced cell death was analyzed by dual staining with Annexin V and PI. In cells without upregulated expression of LMNB1, statistically significant differences in the percentage of live cells were observed following treatment with 1 and 5 mM 5-FU, as compared to the untreated control. The percentage of live cells in control cells and after treatment with 0.1 mM 5-FU was similar and amounted to 92.9 and 93%, respectively. However, when the 1 and 5 mM doses were used, the fraction of live cells was significantly decreased in comparison to the control to 84 and 78.8% (Fig. 3A). After upregulation of LMNB1 statistically significant decrease in the percentage of live cells was observed following their treatment with 5-FU. The mean values for live cells after 0.1, 1 and 5 mM 5-FU treatment were 76.4, 76.2 and 71.5%, respectively (Fig. 3B). A statistically significant decrease was also observed in the mean percentage of live cells between transfected cells with and without altered expression of LMNB1 after treatment with the same doses of 5-FU (Fig. 3C). In the LoVo cells without upregulated LMNB1 expression, a statistically significant increase from 4.5 to 9.8% and to 10.8%, in the percentage of apoptotic cells was observed following treatment with 1 and 5 mM 5-FU, respectively (Fig. 3D). Furthermore, much higher percentage of apoptotic cells was observed in cells with upregulated LMNB1 expression (Fig. 3E). As shown in Fig. 3E, the population of...
apoptotic cells increased from 6.7 to 12, 13.1 and to 24.2% in the control and after treatment with 0.1, 1 and 5 mM 5-FU, respectively. Moreover, statistically significant differences were noted between cells with and without elevated expression of LMNB1 after incubation with 0.1 and 5 mM 5-FU (Fig. 3F).

Moreover, a statistically significant increase in the percentage of necrotic cells was observed in cells without overexpressed LMNB1 after treatment with 1 and 5 mM (to 6.2 and 10.3%, respectively) (Fig. 3G). Statistically significant increase in the percentage of necrosis was observed only after treatment of LoVo cells with upregulated expression of LMNB1 with 1 mM 5-FU (Fig. 3H). Comparison between the cells with normal and overexpressed expression of LMNB1 showed a statistically significant difference in the percentage of necrotic cells only after their treatment with 5 mM 5-FU (Fig. 3I).

The effect of LMNB1 upregulation on cell cycle distribution. The cell cycle was analyzed using image-based cytometer. In the LoVo cells with normal expression of LMNB1, a statistically significant increase from 19.6 to 35.5 and to 42.1% in the mean percentage of cells with DNA content corresponding to G0/G1 phase was observed following treatment with 0.1 and 1 mM 5-FU, respectively (Fig. 4B). The population of cells in S-phase was 53.4, 43.2, 31.5 and 38.7% in the control and cells treated with 0.1, 1 and 5 mM doses of 5-FU, respectively (Fig. 4C). After treatment with 1 and 5 mM 5-FU, a statistically significant increase in the number of cells in the G2/M phase was noted (Fig. 4D). Moreover, there was a significant increase in the percentage of polyplloid cells (from 1.5 in the control to 5.1 and 6.5% after treatment with 0.1 and 5 mM 5-FU, respectively) (Fig. 4E). Additionally, an increase the percentage of the cell with sub-G1 DNA was noted in cells treated with 0.1 and 1 mM of 5-FU (from 4.2 to 6.5 and 10.3%, respectively) (Fig. 4A). The upregulation of LMNB1 resulted in a statistically significant increase of cells in the G0/G1-phase after treatment with 0.1 and 1 mM 5-FU (from 30.9 to 54.3 and 54.4%, respectively) (Fig. 4B). However, a statistically significant decrease in S-phase was observed (from 49.7 in
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The control to 25.6, 30.3 and 18.9% after treatment with 0.1, 1 and 5 mM 5-FU, respectively) (Fig. 4C). Moreover, 1 and 5 mM of 5-FU induced an increase of polyploidy (Fig. 4E). The increased sub-G1 phase of cell cycle was observed after

The quantitative analysis of cell cycle in LoVo cells with and without overexpression of LMNB1 after treatment with 5-FU. (A) Percentage of cells in sub-G1 phase. (B) Percentage of cells in G0/G1 phase. (C) Percentage of cells in S phase. (D) Percentage of cells in G2/M phase. (E) Percentage of cells with DNA content >4N. *Statistically significant differences between cells treated with 5-FU in comparison to the control in a group of the cell without upregulated LMNB1, P<0.05. †Statistically significant differences within cells with upregulated LMNB1, P<0.05. ‡Statistically significant differences between cells without upregulated LMNB1 and with upregulated LMNB1, P<0.05.

Figure 4. The quantitative analysis of cell cycle in LoVo cells with and without overexpression of LMNB1 after treatment with 5-FU. (A) Percentage of cells in sub-G1 phase. (B) Percentage of cells in G0/G1 phase. (C) Percentage of cells in S phase. (D) Percentage of cells in G2/M phase. (E) Percentage of cells with DNA content >4N. *Statistically significant differences between cells treated with 5-FU in comparison to the control in a group of the cell without upregulated LMNB1, P<0.05. †Statistically significant differences within cells with upregulated LMNB1, P<0.05. ‡Statistically significant differences between cells without upregulated LMNB1 and with upregulated LMNB1, P<0.05.

Figure 5. Fluorescence analysis of β-tubulin (red) and F-actin (green) organization in LoVo cells with and without overexpression of LMNB1 in control cells and after treatment with 0.1, 1 and 5 mM 5-FU. Nuclei were counterstained with DAPI. Bar, 50 µm.
showed that tail domains of type A and B can directly bind to actin filaments. Additionally, these authors suggested that particularly lamin A might influence on the nuclear actin concentration and thereby impact transcription, chromatin remodeling and actin polymerizable. Furthermore, the normal shape of nucleus and regularity of nuclear processes are dependent on interactions between microtubules and lamin networks (15). Although, lamins are the nuclear proteins but play also an important role in cell migration by the link with β-catenin, which undergo nucleocytoplasmic distribution and participate in both Wnt signaling pathway and cell-cell adhesion (16). It turned out also, that lamins may be involved in cell movement through actin filaments and linker of nucleoskeleton and cytoskeleton complex (17).

The fluorescence staining of β-tubulin showed 5-FU-induced changes in the organization of microtubules and mitotic spindle morphology (Fig. 5). In the control cells without upregulated LMNB1, β-tubulin was organized in a regular and dense network of long tubules, which radiated from the microtubule-organizing centers (MTOCs) (Fig. 5). Together with 5-FU doses and especially after 5 mM 5-FU, microtubules formed a less dense network consisting of shorter fibers, as compared to the control (Fig. 5). Moreover, in shrunken cells, a significantly higher fluorescence of tubulin was noted (Fig. 5). The morphology of LMNB1 upregulated cells underwent changes due to cytoskeletal reorganization (Fig. 5), and these changes escalated following treatment with 5-FU. In control cells with induced overexpression of LMNB1, the organization of microtubules was similar to control cells without upregulated LMNB1 (Fig. 5). However, after exposure to 5-FU, giant and multinucleated cells with a phenotype resembling mitotic catastrophe were observed (Fig. 5). In these cells, microtubule network was well-developed, but fibers were shorter and in some cases thicker and/or less regular (Fig. 5). 5-FU induced also changes in the organization of actin cytoskeleton in cells with upregulated expression of LMNB1 (Figs. 5 and 6A). Control cells without upregulated LMNB1 were characterized as highly-developed microtubule structure were not observed (Figs. 5 and 6A). In turn, after upregulation of LMNB1 and treatment with 5-FU reorganization of actin filaments occurred (Figs. 5 and 6A). They exhibit a diffuse network of F-actin with short actin fibers and/or small, punctate accumulations within the cytoplasm and cortical area, also in regions of cell-cell junctions (Figs. 5 and 6A). After treatment with 5-FU, in smaller cells, they underwent accumulation in the cortical region, but significant changes in microfilament structure were not observed (Figs. 5 and 6A). In turn, after upregulation of LMNB1 and treatment with 5-FU reorganization of actin filaments occurred (Figs. 5 and 6A). They exhibit a diffuse network of F-actin with short actin fibers and/or small, punctate accumulations within the cytoplasm or in the cortical region of cells (Figs. 5 and 6A). Analysis of intensity of nuclear F-actin fluorescence showed a statistically significant increase in fluorescence intensity after treatment of cells with normal expression of LMNB1 with 0.1, 1 and 5 mM of 5-FU in comparison to the control cells (Fig. 6B). Statistically significant increase of nuclear actin fluorescence was observed also in cells with overexpressed LMNB1 following treatment with 5 mM of 5-FU, as compared to the control and between cells with and without upregulated LMNB1 (Fig. 6B).
The effect of LMNB1 upregulation and 5-FU on fluorescence of β-catenin was also investigated (Fig. 7A). Fluorescence double staining of β-catenin and F-actin in LoVo cells transfected with control plasmid showed the dose-dependent decrease of fluorescence β-catenin at the cell periphery area (Fig. 7A). In control cells and cells after treatment with 0.1 and 1 mM of 5-FU colocalization of β-catenin and actin filaments was seen. Furthermore, the β-catenin was presented as a small punctate accumulation in the cytoplasm and in the nuclear area. Additionally, after treatment of these cells with 5 mM of 5-FU, an increase in accumulation of nuclear β-catenin was observed (Fig. 7A). After overexpression of LMNB1 and treatment with 0.1 and 1 mM of 5-FU, statistically significant increase of β-catenin fluorescence intensity in cell-cell interaction area was observed, as a comparison to the cells transfected with control plasmid and treated with the same dose of 5-FU (Fig. 7B). On the other hand, the fluorescence intensity of nuclear β-catenin was radically increased in LoVo cells transfected with control plasmid and after treatment with 5 mM of 5-FU, but in the cells with upregulated LMNB1 expression the nuclear intensity of β-catenin increased following treatment with the lowest dose of 5-FU and reduced following treatment with higher concentrations of 5-FU (Fig. 7B). The fluorescence intensity of nuclear β-catenin in the cells with upregulated LMNB1 and treated with 5 mM of 5-FU was lower than in the control (Fig. 7B). In control cells with upregulated expression of LMNB1, β-catenin was located in cell-cell interaction area and colocalized with cortical actin filaments. Additional, granules of β-catenin in the cytoplasm were stained (Fig. 7A). Moreover, following treatment with the lowest dose of 5-FU, β-catenin was presented similarly to results obtained in control cells (Fig. 7A). The incubation of LoVo cells with upregulated LMNB1 with 1 and 5 mM of 5-FU resulted in changes in β-catenin distribution. This protein was located in the periphery of the cells and on the nucleus area, but the intensity of fluorescence was lower as compared to the control and cells treated with 0.1 mM of 5-FU (Fig. 7A).

The effect of LMNB1 cDNA transfection on migration of LoVo cells. Wound healing assay is a commonly accepted and well-developed method to measure cell migration in vitro. Live-cell imaging system was used to record the process of wound closure and cell migration. Documentation was done every 10 min and lasted 36 h. In all cases of the study scratch in the monolayer were still observed (Fig. 9). After 24-h incubation

Figure 7. Fluorescence analysis of β-catenin and F-actin and fluorescence intensity of β-catenin measured in nuclear and cell-cell interaction areas in LoVo cells. (A) Fluorescence analysis of β-catenin (green) and F-actin (red) organization in LoVo cells with and without overexpression of LMNB1 in control cells and after treatment with 0.1, 1 and 5 mM 5-FU. Nuclei were counterstained with DAPI. Bar, 50 µm. (B) Fluorescence intensity of β-catenin measured in nuclear and (C) cell-cell interaction areas in LoVo cells with and without overexpression of LMNB1 in control cells and after treatment with 0.1, 1 and 5 mM 5-FU. *Statistically significant differences between cells treated with 5-FU in comparison to the control in a group of the cell without upregulated LMNB1, P<0.05. $Statistically significant differences within cells with upregulated LMNB1, P<0.05. #Statistically significant differences between cells without upregulated LMNB1 and with upregulated LMNB1, P<0.05.
with a cytostatic, the inhibition of cell migration in LMNB1 upregulated cells in comparison to cells without overexpression of LMNB1 was observed (Fig. 9). As shown in Fig. 9C, the wound area at 24 h after treatment with 5-FU was 20.22% in cells transfected with control plasmid. Moreover, following the treatment with 0.1, 1 and 5 mM 5-FU, the percentage reduction in wound size was observed and amounted to 26.8, 21 and 4.9%, respectively. Furthermore, the results were statistically significant in cells treated with 0.1 and 5 mM of 5-FU as a comparison to the control (Fig. 9C). Additionally, the cell mobility measured as wound area was less in cells with overexpressed LMNB1 as compared to cells transfected with control plasmid and amounted to 17.2, 22.3% (0.1 mM of 5-FU), 18.3% (1 mM 5-FU), 4.1% (5 mM 5-FU) in the control and cells treated with 0.1, 1 and 5 mM 5-FU, respectively (Fig. 9C). In cells transfected with control plasmid and treated with 5 mM dose of cytostatic in 12, 24 and 36 h migration test, a statistically significant decrease in cell migration was observed, as compared to the control. In turn, in these cells incubated with 0.1 mM of 5-FU after 24 and 36 h of increase of cells migration was noted (Fig. 9A). The same dependence was observed in cells with overexpressed LMNB1 (Fig. 9B). As shown in Fig. 9D, the inhibition of migration of cells with overexpression of LMNB1 following treatment with 0.1 mM 5-FU was noted (Fig. 9D).

The effect of LMNB1 expression on survival of patients with colorectal adenocarcinoma. Using the data obtained from TCGA Data Portal Open-Access directory via cBioPortal database we elucidated prognostic value of LMNB1 mRNA expression in patients with colorectal adenocarcinoma. The results showed that there are no significant differences between patients with low and high expression of LMNB1 in long perspective. However, patients with upregulated LMNB1
were characterized by lower survival rates within the first 30 months (P=0.0419) (Fig. 10).

**Discussion**

Carcinogenesis, as a multistep process in which many components could be modified, is a target of scientists all over the world. In this study, we presented the role of lamin B1 in the regulation of pathogenesis and progression of colorectal cancer, which is the most common malignancy and a major cause of cancer-related deaths (4). Many authors have shown that expression of LMNB1 in colon cancer is decreased, and can be related to high aggressive of this tumor (4,18). Chemotherapeutic treatment of colorectal cancer is based on 5-fluorouracil (5-FU) which is an anticancer drug interfering with nucleoside metabolism through incorporation into RNA and DNA. This leads to cell death (6,19,20). After treatment of LoVo cells without altered expression of LMNB1 with 5-FU, we observed induction of apoptosis. Similar, apoptotic effect was presented for these types of cells by Das et al (21) and Wang et al (22,23). As it was shown by Mhaidat et al (24) in colorectal cancer, 5-fluorouracil induced apoptosis mediated by protein kinase C-δ and by activation of caspase-9. Moreover, 5-FU can modulate some members of the Bcl-2 protein family and induces cell death (25). Additionally, it is possible to increase the apoptotic potential by combination of 5-FU with other substances. An example of such research are studies presented by Wang et al (23), in which they observed enhanced apoptotic effect of 5-FU by SM-1 in LoVo cells. SM-1 is a novel, proapoptotic agent, which directly activates caspase-3 and induces the cell death process on the higher level. Furthermore, Milczarek et al (26) presented that vitamin D analogs enhance the anticancer effect of 5-FU in mouse colon cancer model. Thus, the intensity of cytotoxic effect can be enhanced by manipulation of gene expression, which results in direct or indirect impact on proliferation, death or migration of cells. It was presented by Chen et al (27) that after silencing HIF-1α, the level of apoptotic cells increased after the treatment of cells with adriamycin, irinotecan, vincristine and 5-FU. Similarly, Li et al (28) indicated that human colorectal cancer HCT116 cells after downregulation of caveolin-1 are more sensitive to 5-FU. In the present study, we also observed a slight increase in the percentage of apoptotic cells following treatment with all doses of 5-FU in cells with upregulated LMNB1, as compared to the control. Except apoptosis, a small percentage of necrotic and high number of mitotic catastrophe cells were observed. Necrosis was present also in colorectal cancer cells by Hamam et al (29) but they suggested that the higher level of necrotic and apoptotic cells was observed after treatment of cells with a combination of 5-FU and CUDC-907, than with 5-FU alone. In recent years, many studies presented that autophagy is linked to 5-FU resistance in colon cancer cells and inhibition of this process could be promising apoptotic effect of 5-FU (30,31). This dependence is connected with the regulation of apoptotic proteins Bcl-2 and Bcl-xL by 5-FU, which influence the autophagy-promoting Beclin1VP34 complex (32,33). It is also presented that p38MAPK signaling pathway has a very important role in controlling the balance between autophagy and induced by 5-FU apoptosis (34). Necroptosis is another type of cell death, which is indirect induced by 5-FU (35). In turn, overexpression of LMNB1 induced by β-Asarone increased cellular senescence cell population and inhibited colon cancer formation (36). In the present study, we observed mitotic catastrophe. After upregulation of LMNB1 in LoVo cells, a dose-dependent increase of cells with DNA content >4N were noticed, what testifies of polyploidy cells.

Fluorescence microscopic analysis confirmed the results. Induction of mitotic catastrophe was also shown by Yoshikawa et al (37) in colorectal carcinoma cells (SW480 and COLO320DM) after treatment with 5-FU. Aforementioned scientists observed a dual effect of 5-FU, which is dependent on the dose of the cytostatic. At the lower dose (100 ng/ml) the cells were arrested in G2/M phase and mitotic catastrophe was observed. Following treatment with a higher dose of 5-FU, the cells underwent apoptosis. In our previous study, we also presented that induction of cell death with doxorubicin in

Figure 10. LMNB1 mRNA expression in patients with colorectal adenocarcinoma. (A) Low LMNB1 mRNA expression associated with lower overall survival in the first 30 months of disease. (B) No significant differences in overall survival in longer perspective. Probabilities of overall survival of total 188 patients with colorectal adenocarcinoma were analyzed by Kaplan-Meier survival analysis.
CHO AA8 cells is dose-dependent. On the other hand, down-regulation of cofilin-1 caused lack of apoptosis and following treatment with doxorubicin only mitotic catastrophe was observed (38). Changes in cell cycle involved not only a dose-dependent increase of cells with DNA content >4N but also in G0/G1, S and G2/M phases. Similarly to Chen et al (39), after treatment of control cells with 0.1 and 1 mM of 5-FU accumulation in G0/G1 and significantly decreased the population of S-phase cells was shown. It is known that in comparison with sensitive cancer cells, the higher population of 5-FU-resistant cancer cells were arrested in G0/G1 cell cycle phase (40) but we observed the increase of cell population in G0/G1 following treatment of cells with upregulated LMNB1 with 0.1 mM of 5-FU, but during their incubation with 1 and 5 mM of 5-FU, the population of cells in this phase decreased in the interest of sub-G1 and >4N, and in the case of 5 mM to G2/M also. Nie et al (41) after downregulation of Rac1 showed arrested LoVo cells in G0/G1 phase and induced apoptosis. The sub-V1 peak has been identified as the apoptotic fraction and in our study the sub-G1 population square with the analysis of apoptotic cells presented by dual staining with Annexin V and PI. Differences were observed following treatment with 5 mM of 5-FU in control cells and the cells with upregulation of LMNB1, but low level of the sub-G1 peak did not conform to the lack of apoptosis (42). Apoptosis has been marked as DNA degradation into oligonucleosomal-length fragments, but in the literature, there are many examples where particularly for apoptosis internucleosomal DNA degradation and fragmentation is not observed. In this case identified apoptotic cells based on cellular DNA content is worse way than Annexin V/PI assay or TUNEL analysis (43-45).

As mentioned above, after treatment with the highest dose of 5-FU, we observed a dramatic increase in >4N cell fractions. Moreover, immunofluorescent staining showed increased number of cells with visible hallmarks of mitotic catastrophe. 5-FU alone has limited potential to induce endoreduplication alone but it has been proven that can act synergistically with other drugs. Colorectal cancer cell lines treated with 5-FU with Dual Histone Deacetylase and Phosphatidylinositol 3-Kinase Inhibitor (CUDC-907) exhibited enhanced cell polyploidization (29). The lamin B1 plays a crucial role in the maintenance of nuclear structure and function. The decrease in LMNB1 expression results in disturbed chromatin condensation, improper heterochromatin localization and altered gene expression and splicing. It is possible that overexpressed LMNB1 drives to endoreduplication and allows cells to start another S phase without mitotic division (46). The presence of polyploidy coincides with the appearance of cells which show morphological aspects of mitotic catastrophe. The role of mitotic catastrophe is still discussed but the most interesting aspect of this phenomenon possibly is its pro-survival character. Mitotic catastrophe is commonly observed in p53 mutated tumor cells which avoid cell cycle checkpoints. Studies showed that polyploid giant cells have the ability to chromatid exchange and probably the production of a fully repaired chromosome. Moreover, homologous chromosomes from the giant cell can be relocated and paired which is the step to depolyploidization (47,48). Silencing of LMNB1 affects DNA damage and repair pathway. Disregulated proteins such as BRCA1 and RAD51 after downregulation of LMNB1 caused non-efficient DNA repair via both non-homologous end joining and homologous repair (49). Additionally, depletion of lamin B1 prevented induction of RAD51 expression after IR radiation. Proteasome inhibitor MG132 repealed this effect which suggests that lamin B1 stabilizes RAD51 by preventing proteasome-mediated degradation in cells with IR-induced DNA damage. Moreover, cells with depleted levels of LMNB1 were characterized by higher radiosensitivity and lower survival rates (50). We hypothesize that overexpression of LMNB1 drives to enhanced ability of DNA repair which in turn allows these ‘catastrophic cells’ to return to the normal cell cycle avoiding cell death and provides survival.

Cytoskeletal components (microtubules, microfilaments and intermediate filaments), especially their organization and changes, are strictly connected with the state of cells. The cytoskeleton is also involved in many cellular processes such as mitosis, proliferation, migration and cell death (51,52). In the control cells transfected with control plasmid, microtubules were present as a regular, dense network of long microtubules, which radiated from the microtubule-organizing centers (MTOCs). Following treatment with a cytosstatic, especially with the highest dose microtubules, formed a less dense network with shorter fibers or were depolymerized in apoptotic cells. In our previous study, we showed significant thickening of microtubule bundles which was particularly evident in the giant multinucleated cells after 10 and 20 mM of caffeine treatment of H1299 cells. Furthermore, in shrunken cells β-tubulin underwent depolymerization and the network of microtubules was not observed (53). Similar observation was presented by Pawlik et al (54). As shown, after the exposure of H1299 cells to phenethyloiothiocyanate, the rearrangement of tubulin was observed (54). Here, actin filaments were present in the cytoplasm and in the cell-cell interaction areas. In turn, 5-FU resulted in disorganization of actin cytoskeleton in cells with upregulated LMNB1. In enlarged multinucleated cells expanded F-actin cytoskeleton was located mainly in the form of small granules and/or short fibers within the cytoplasm or in the cortical region of cells. A well-developed actin cytoskeleton in cells with the phenotype of mitotic catastrophe was also observed in other studies (54-56). In apoptotic cells, actin filaments are located in the whole cytoplasm and accumulated near the plasma membrane. A number of previous studies presented that actin polymerization and accumulation at the cell periphery is indispensable for plasma membrane blebbing and apoptotic bleb formation (57-61). Thus, actin cytoskeleton, located in the cortical region in shrunken cells is very important during the apoptotic process. However, not only cytoplasmic actin is crucial in this cell death, because F-actin presence in the nucleus of different cell lines following treatment with cytostatics has been implicated in chromatin remodeling during both apoptosis and mitotic catastrophe (38,62,63). In this study, we observed the high intensity of nuclear F-actin fluorescence in LoVo cells with overexpression of LMNB1 and following treatment with 5 mM of 5-FU. This correlated with a number of cells with mitotic catastrophe phenotype. These observations are connected with our previous study, where we showed F-actin in the transcriptionally active regions of the cell nucleus during active cell death. Additionally, it was presented that F-actin colocalized with SATB1 protein, which
is one of the most important nuclear proteins involved in chromatin organization (64-66).

As evidenced by the above study, nuclear F-actin plays an essential role in cell death, but cortical actin filaments maintain the shape, polarity of cells and are involved in cell-cell adhesion (67). Adhesion proteins form the complexes of intercellular junctions by interaction with the cytoskeleton (67-69). β-catenin is a multi-functional protein, which is an element of adherent junctions and acts as a transcriptional activator in Wnt signaling pathway (70,71). Additionally, β-catenin is indirect, by α-catenin links to actin filaments (72). Stability of cell-cell adhesions depends on the proper organization of actin filaments, which may impact on cell migration and invasion.

In the study presented by Gagat et al (73), the stabilization of F-actin through overexpression of tropomyosin-1 in EA.hy926 cells increased junctional β-catenin expression and thereby preserved endothelial barrier function against oxidative stress conditions. Here, the fluorescence intensity of β-catenin located in cell-cell interaction areas in LoVo cells with an elevated level of LMNB1 following treatment with all doses of 5-FU was significantly higher in comparison to the cells with a naive expression of LMNB1 and the treatment with the same doses of cytostatic. This result can suggest that junctions between the cells with overexpressed LMNB1 are stronger, with consequences for the control of cancer cell dissociation and spread. Tumor invasion and metastasis frequently coincide with the loss of cell-cell adhesion proteins, mainly cadherin, but the key to the adhesive activity is the interaction between these proteins, cadherin and actin cytoskeleton (74,75). On the other hand, in cells transfected with control plasmid, a dose-dependent increase of nuclear β-catenin was observed. However, after upregulation of LMNB1 inverse results were observed. This observation agrees with Han et al (10). They showed that downregulation of β-catenin inhibited the invasion and migration of LoVo cells. Additionally, we also present here the slight inhibition of cell migration in cells with upregulated LMNB1.

Very important in the aspect of the regulation of migration and tumor invasion is lamin A/C. In the present study, we observed a high level of fluorescence intensity of lamin A/C in control cells treated with all doses of 5-FU. In turn, upregulation of LMNB1 decreased the fluorescence of lamin A/C in all doses of 5-FU. Willis et al (76) proposed that the high risk of colorectal cancer-related mortality is connected with an expression of lamin A. Lamin A increases invasive potential of the tumor due to its interaction with actin filaments. Dynamics of these interactions caused the loss of cell adhesion and led to increased cell motility.

Then, using the data obtained from TCGA Data Portal Open-Access directory via cBioPortal we investigated if patients with overexpression of LMNB1 mRNA were characterized by lower survival rates. However, there are no significant differences in long-term overall survival but a significantly lower survival rate in patients with high expression of LMNB1 was observed in the first 30 months. As we described above overexpression of LMNB1 was associated with the appearance of the cells with morphological hallmarks of mitotic catastrophe. Mitotic catastrophe plays a dual role in the cell cycle. This mechanism eliminates cells which failed proper mitosis and limits proliferation. Simultaneously, cells which undergo mitotic catastrophe are more resistant to cytostatic drugs and are capable of repairing damaged DNA to return to the normal cell cycle. It is possible that mitotic catastrophe cells in patients with colorectal cancer may be a reservoir of the cells responsible for faster disease progression, but further investigations are necessary to confirm this hypothesis.

In summary, this is the first report which shows the influence of upregulation of LMNB1 in colon cancer cells. Based on the current results we may conclude that the overexpression of LMNB1 in LoVo cells causes induction of mitotic catastrophe by 5-FU, intensification of cell-cell junctions and limits migration of cells.

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