Exp Oncol 2021
43, 2, 111–117

Experimental ONCOLOGY

5-FU RESISTANT COLORECTAL CANCER CELLS POSSESS IMPROVED INVASIVENESS AND β_{III}-TUBULIN EXPRESSION

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Background: **Elevated β_{III}-tubulin levels are associated with resistance to a broad spectrum of drugs in different carcinomas and with poor prognosis of various epithelial cancers. 5-Fluorouracil (5-FU) is a widely used standard drug in chemotherapeutic regimens for colorectal cancer treatment, although the resistance to 5-FU is a major obstacle to successful therapy.** ***Aim:*** **The aim of the study was to compare the invasive and adhesion properties and the expression levels of β_{III}-tubulin in a 5-fluorouracil (5-FU)-resistant colorectal cancer (CRC) cell line HCT116 and parental cells.** ***Materials and Methods:*** **The 5-FU-resistant cell line was established by continuous stepwise selection with increasing concentrations of 5-FU. Cell viability and properties were evaluated using MTT, adhesion and Transwell invasion assays, respectively. The expression of β_{III}-tubulin was revealed by immunoblot and immunofluorescence.** ***Results:*** **The derivative line is 25-fold more resistant to 5-FU and characterized by altered cell morphology. Twice as many cells of the 5-FU-resistant line fail to adhere as compared to the parental cell line. 5-FU-resistant cells are characterized by enhanced invasiveness, accompanied with the increased β_{III}-tubulin expression. In addition, we found that loss of β_{III}-tubulin expression was correlated with loss of 5-FU resistance.** ***Conclusion:*** **Our results indicate that even though 5-FU does not target microtubules, there appears to be a correlation between β_{III}-tubulin expression and resistance to 5-FU that is particularly important with regard to invasiveness. These findings indicate a possible contribution of β_{III}-tubulin to 5-FU resistance *in vivo*.**

Key Words: **colorectal cancer, 5-fluorouracil, chemoresistance, β_{III}-tubulin, invasiveness.**

DOI: 10.32471/exp-oncology.2312-8852.vol-43-no-2.16314

Colorectal cancer (CRC) is the fourth most common cancer-related cause of death in the world [1]. Failure of therapy is due to metastasis and chemoresistance of cancer cells, for instance, 5-year survival for a CRC patient with unresectable metastasis to distant organs treated with palliative chemotherapy is 2.2% [2]. 5-fluorouracil (5-FU) is a widely used standard drug in chemotherapeutic regimens for CRC treatment, although the resistance to 5-FU is a major obstacle to successful therapy [3]. Therefore, understanding the complex events underlying the development of resistance is essential. It was shown that 5-FU-resistant CRC cells were characterized by reduced apoptosis and a more aggressive growth phenotype, consistent with the observed up-regulation of apoptosis inhibitory genes, positive growth-regulatory genes, and metastasis genes and down-regulation of apoptosis-promoting genes [4–6]. For different epithelial cancer cell lines the connection between chemoresistance formation and more invasive phenotype was shown *in vitro* [7–10], with very limited data concerning the mechanism of such interrelation.

Tubulins form microtubules, which modulate fundamental cellular processes, such as cell division, movement, maintenance of cellular morphology, intracellular transport and active transport of cellular components throughout the cytoplasm, cell stress responses and others. Changes in microtubule stability and the expression of different tubulin isotypes as well as altered post-translational modifications have been reported for a range of cancers and have been correlated with poor prognosis and chemotherapy resistance [11]. β_{III}-tubulin is the most comprehensively examined isotype across a variety of cancers [12–15].

The elevated levels of β_{III}-tubulin are associated with poor prognosis of different epithelial cancers [11, 12, 15]. In addition to promoting resistance to tubulin-targeted agents, β_{III}-tubulin influences sensitivity to non-tubulin-targeted agents [12, 15]. The clinical observations of patients with solid tumors are supported by numerous *in vitro* studies where altered β_{III}-tubulin expression confers resistance to a broad spectrum of drug classes [11, 16–18]. However, the underlying mechanisms of the involvement of β_{III}-tubulin in inducing resistance to anti-tumor drugs have yet to be explained and are likely to be very complex.

The aim of this study was to characterize the changes in invasive and adhesion properties and expression of β_{III}-tubulin of the 5-FU-resistant CRC cell line HCT116 in comparison with the parental cell line. In this study, we establish a 5-FU resistant cell line HCT116-5-FU using stepwise increasing drug concentrations and short exposures. We confirmed its association with decreased adhesiveness and increased invasive phenotype, accompanied with the increased β_{III}-tubulin expression.

Submitted: October 27, 2020.

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Abbreviations used: 5-FU — 5-fluorouracil; CRC — colorectal cancer.

MATERIALS AND METHODS

Cell culture. HCT116 human CRC line (ATCC CCL247) was grown in RPMI-1640 medium (Lonza, Switzerland) supplemented with 25 mM HEPES, 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin and 5-10% fetal bovine serum (FBS) (HyClone™, Fisher Scientific Co. LLC, USA) (complete media) in a humidified atmosphere with 5% CO_2 at 37 ˚C.

To obtain the resistant cell line, the exponentially growing HCT116 cells for 48 h were treated with 5-FU (Merck KGaA, Germany) in complete culture medium followed by recovery drug-free periods until the cells achieved exponential growth, using 5-FU concentrations ranged from 0.04–0.80 mM. Drug treatment continued until cell line demonstrated stable resistance to 5-FU (about 10 months) as assessed by MTT assay. The parental HCT116 cells were also serially passaged as an untreated control along with the resistant derivatives. For experiments with the loss of resistance, cells were incubated in growth medium without 5-FU during 2 months and controlled for 5-FU resistance by MTT assay and for the β_{III}-tubulin content by Western blotting.

MTT assay. HCT116 cells or 5-FU-resistant derivate (5×10^3/well) were plated in 96-well culture plates and incubated overnight. Cells were treated with indicated concentrations of 5-FU for 48 h. The number of viable cells was determined by MTT assay using MTT reagent (Carl Roth, Germany) as described [19]. The percent viability was calculated from three wells as

$$\frac{\text{absorbance of drug-treated cells}}{\text{control absorbance}} \cdot 100\%$$

The drug concentration that resulted in a 50% cell growth inhibition (IC_{50}) was determined graphically using cell survival curves in which the percentages of survived cells were plotted as a function of 5-FU concentration.

Adhesion assay. Twenty-four well plates were coated with bovine collagen type I (Gibco, Thermo Fisher Scientific Inc, USA) at a concentration of 50 μg/ml at room temperature for 2 h. Cells ($2 \cdot 10^5$ per well) in complete media were allowed to attach for 30 min at 37 ˚C in a humidified atmosphere with 5% CO_2. Non-attached cells from each well were counted manually under a microscope and the cell adhesion rate (%) was calculated according to the formula:

$$\left(1 - \frac{\text{number of non-adherent cells}}{\text{total cell number}}\right) \cdot 100\%$$

The experiments were repeated five times in triplicates.

Invasive assay. Cell invasion was performed by the modified Boyden chamber method in 24-well plates with culture inserts with 8 μm pore size (Costar Corning, USA), coated with bovine collagen type I (Gibco, Thermo Fisher Scientific Inc, USA) or matrigel (Becton Dickinson and Company, USA) according to the manufacturer's recommendations. Cells ($5 \cdot 10^4$ cells/ml) were resuspended in RPMI-1640. The upper chamber was loaded with 100 μl of cell suspension and the lower chamber was loaded with 600 μl of RPMI-1640 with 10% FBS. After incubation for 24 h at 37 ˚C in 5% CO_2, cells that penetrated to the lower chamber were fixed and stained with 4% paraformaldehyde and 0.2% crystal violet. Cells were counted under a microscope; five microscopic fields were randomly selected for cell counting. Each assay was performed in triplicate.

Western blotting. Proteins were extracted from the cells by lysis in RIPA buffer in the presence of protease inhibitors. The lysates were centrifuged (15 min at 14,800 g at 4 ˚C) and proteins were denatured by boiling for 5 min in loading buffer, separated by electrophoresis using 12.5% SDS-PAGE and blotted onto nitrocellulose membrane (Whatman Protran nitrocellulose membranes, Sigma-Aldrich Co. LLC, Germany). The membrane was blocked with blocking buffer (2% bovine serum albumin (BSA) in phosphate buffer saline (PBS)-Tween 1%) for 2 h and incubated overnight at 4 ˚C with the anti-human β_{III}-tubulin antibody 5G8 (Thermo Fisher Scientific Inc, USA) at 1:1000 or anti-human β-actin antibody at 1:1000 (Abcam, UK) as loading control. Anti-mouse immunoglobulin G, poly-horseradish peroxidase-conjugated antibody (EnVision, DAKO, Agilent Technologies, Inc., USA) was used as a secondary antibody and an immunoblott was developed with enhanced chemoluminescent (ECL) substrate (clarity Western ECL substrate, BioRad, Ireland, UK) and visualized using a Kodak Image Station 2000R and the ImageJ 1.47v (NIH, USA) software.

Immunofluorescence microscopy. Cells were grown on glass cover slips for about 70% confluent monolayer in complete RPMI-1640 medium. They were fixed for 10 min with ice-cold methanol and permeabilized with ice-cold acetone for 2 min at –20 ˚C. Cover slips were blocked for 30 min in PBS containing 2% BSA, and incubated with the anti-human β_{III}-tubulin mouse antibody 5G8 at 1:1000 overnight at 4 ˚C in a humid chamber. After incubation with Alexa Fluor 546-conjugated secondary antibody (Life Technologies, USA) for 2 h, cells were stained with DAPI for nuclear staining, mounted with ProLong Gold Antifade-Mountant (Life Technologies, USA) and then visualized by a fluorescence microscope Leica DM5000B.

Statistical analysis. Data are provided as means ± SEM, or means ± SD, n represents the number of independent experiments. Levels of statistical significance were evaluated by using non-parametric Mann–Whitney test or Wilcoxon matched pairs test. Differences were considered statistically significant when *p*-values were less than 0.05.

RESULTS

We generated 5-FU-resistant cell lines from parental HCT116 cells by serial passage of HCT116 cells with repeated exposure to increasing 5-FU concentrations. These cells were designated as HCT116-5-FU. A MTT assay was performed to compare the viability of the 5-FU-resistant HCT116 and parental HCT116 cells.

The IC_{50} values for the parental and derivative cells were determined as 0.012 and 0.3 mM, respectively (Fig. 1, *a*). These data indicate that the derivative line was 25-fold more resistant to 5-FU than was the parental HCT116 cell line.

The 5-FU-resistant cells were morphologically distinct from their parental cell line HCT116 (Fig. 1, *b*). Light microscopic analysis of the cell phenotype confirmed that the parental HCT116 cells were uniform in shape and grew with tight cell–cell junctions in monolayer culture. However, HCT116-5-FU cells appeared to have spindle-cell morphology with increased formation of pseudopodia and enlarged intercellular separation signifying loss of intercellular adhesion (Fig. 1, *b*).

To determine if the acquisition of 5-FU resistance induces changes in the adhesive ability and invasive potential of the cells, we compared HCT116 and HCT116-5-FU cells in adhesive and ECM (extracellular matrices) gel-coated Transwell invasive assays.

We found that HCT116-5-FU cells have a decreased capacity for attachment to collagen-treated plastic (n = 5, $p = 0.043$) (Fig. 2, *a*) and displayed enhanced invasiveness to a collagen- or matrigel-treated 8.0 μm pore size membrane in comparison with parental cells (n = 3; $p = 0.001$ and $p = 0.03$, respectively) (Fig. 2, *b*).

Previously we showed a preferential localization of β_{III}-tubulin in the invading epithelium in CRC specimens [20]. According to the current understanding, β_{III}-tubulin may represent a biomarker of the cancer aggressiveness, the response to therapy and poor outcome in a broad spectrum of epithelium cancers, regardless of chemotherapy composition [21–28]. We studied the expression of β_{III}-tubulin in parental and 5-FU resistant cell lines. The Western blot analysis demonstrated that HCT116-5-FU cells expressed increased β_{III}-tubulin protein relative to the parental HCT116 cells (Fig. 3, *a*). The immunofluorescence assay also revealed that resistant cells had increased contents of β_{III}-tubulin expression compared to HCT116 (Fig. 3, *b*).

It is important to note that the level of β_{III}-tubulin expression by HCT116-5-FU cells decreased during the long period of culturing in the absence of 5-FU. At the same time, resistance to 5-FU was lost as confirmed by the MTT-test (Fig. 4).

DISCUSSION

5-FU is widely used for treatment of digestive system cancers and it is a first-line chemotherapeutic agent for CRC treatment, preferentially used in combination with other chemotherapeutic drugs such as irinotecan [29]. The detailed mechanism of the clinical response to 5-FU chemotherapy is still unclear as can-

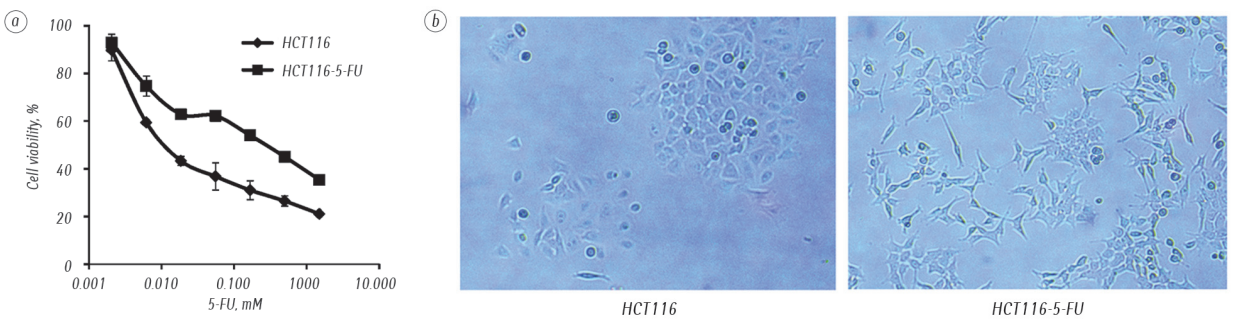

Fig. 1. HCT116-5-FU cells are resistant to 5-FU-induced cytotoxicity and have morphological changes in comparison with parental cell line HCT116: *a* — cells were treated with increasing concentrations of 5-FU (up to 1.5 mM) for 48 h, and cell viability was assessed by MTT and reported as percentage viability. Results are expressed as mean ± SD of triplicate. Similar results were obtained in three independent experiments; *b* — Acquisition of 5-FU resistance induces changes in cell morphology in HCT116-5-FU cells. Cells were examined using phase contrast microscopy (original magnification, × 200). The left panel shows the parental HCT116 cells and right panel — the HCT116-5-FU cells

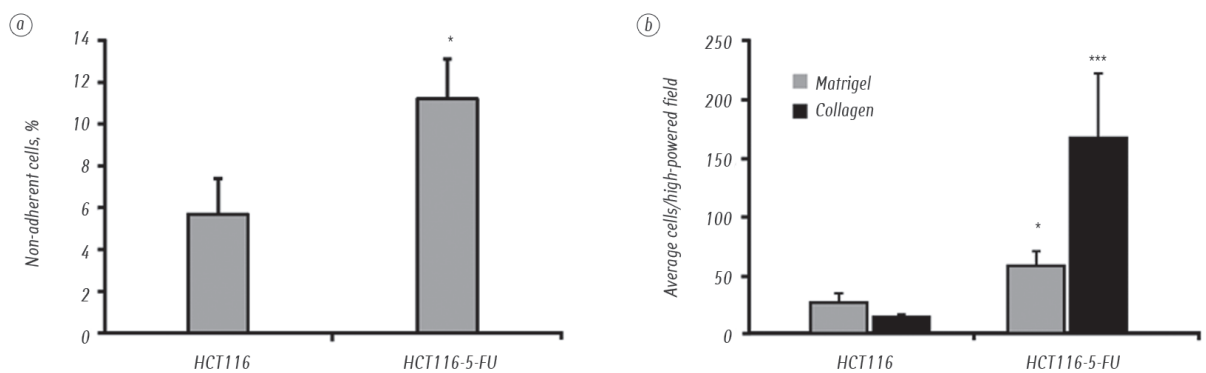

Fig. 2. HCT116-5-FU cells have altered adhesive and invasive capacity: *a* — quantitative analysis of cell adhesion to collagen-treated plastic. The percentage of non-attached cell after 30 min of incubation is presented as mean and SEM (error bars) for n = 5; *b* — quantitative analysis of cells invading through collagen or matrigel–coated Transwell chambers. Data are presented as mean and SEM (error bars) for n = 3. *$p < 0.05$; **$p < 0.001$

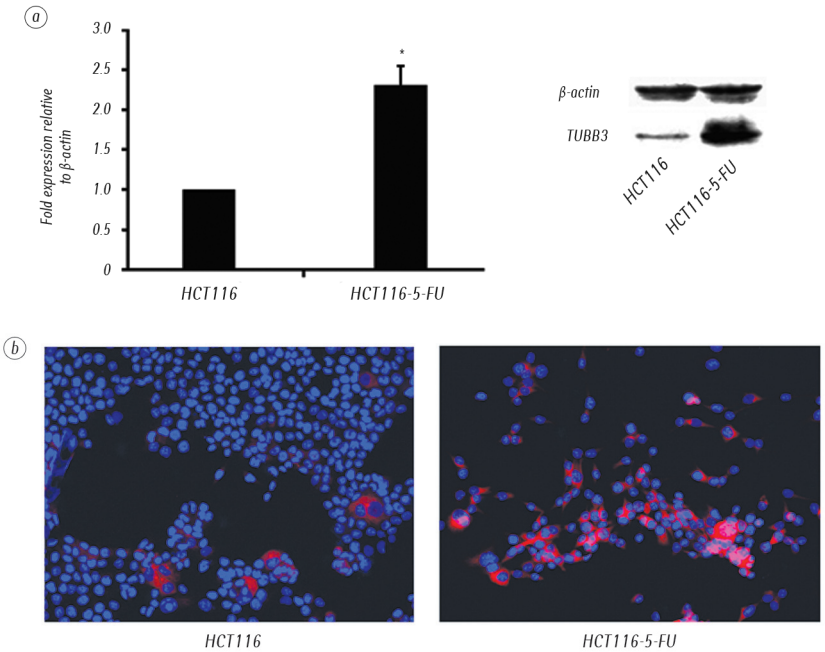

Fig. 3. HCT116-5-FU cells have increased expression of b_{III}-tubulin: *a* — Immunoblotting of β_{III}-tubulin protein expression by HCT116-5-FU and HCT116. Expression was normalized to β-actin. Results are presented as mean and SEM for n = 3, $p < 0.05$, compared with parental HCT116 cells (left) and as representative immunoblot of HCT116-5-FU and HCT116 cells (right); *b* — Immunofluorescence staining for the expression and cellular localization of β_{III}-tubulin (red). DAPI was used as a nuclear stain (blue). Original magnification, × 400

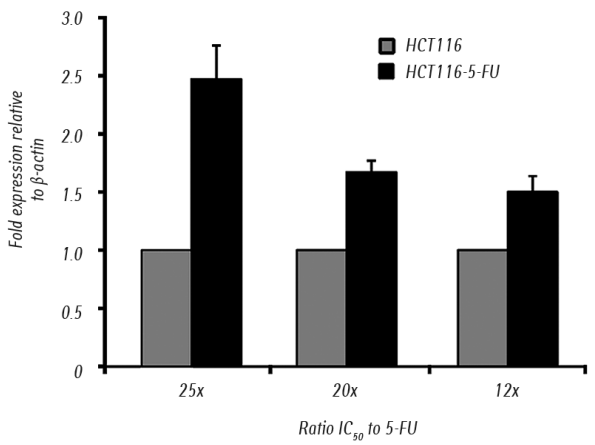

Fig. 4. Immunoblotting of b_{III}-tubulin protein expression by HCT116-5-FU depending on 5-FU resistance, presented as ratio IC_{50} to 5-FU of resistant cells (HCT116-5-FU) to parental cell HCT116. HCT116-5-FU cells were cultured without drug for 0 day (resistance indicated as 25x), for 1 month (indicated as 20x) and for 2 months (indicated as 12x). HCT116 cells were also serially passaged as an untreated control along with the resistant derivatives. Expression was normalized to β-actin. Results are shown as mean ± SD of triplicate. Similar results were obtained using two independent cell culturing

cer cells gain resistance to 5-FU over time, which is a serious problem for 5-FU-based chemotherapy. Although several key biochemical pathways involved in 5-FU resistance have been investigated [30, 31], its definitive mechanism in CRC is still unclear. A better understanding of how residual tumor cells survive after chemotherapy and what molecular alterations cause or correlate with resistance could ultimately provide new, more effective therapeutic strategies for patients diagnosed with CRC. In this study, we obtained a 5-FU-resistant CRC cell line HCT116 and investigated the changes in the cellular behaviors and associated expression of β_{III}-tubulin marker in comparison with parental cells.

We found that cells acquiring resistance to 5-FU have undergone certain morphological changes — formation of spindle-shaped cells, pseudopodia and loss of cell-cell adhesion, together with enhanced invasive and decreased adhesive ability. Such changes at the cellular level *in vitro* suggests a cascade of events at the tumor level *in vivo* including loss of adhesion and tight junctions, dissociation of epithelial layer into separate cells and local invasion, which is the first and key step in the metastatic process [32].

In agreement with our findings, changes in cell morphology and cell invasiveness were also reported for acquired resistance to 5-FU in other cancer cells, namely, breast [8] and squamous cell carcinoma [9]. Moreover, resistance to drugs of different mechanisms of action in a broad spectrum of cancer cell lines leads to the formation of similar changes in cells [8–10, 33] indicating that chemotherapy induces the same survival mechanisms in different epithelial cancer, contributing to the formation of tumor cells of high-grade malignancy.

In the present report, we showed that these changes in cellular behavior of 5-FU-resistant CRC cells were accompanied by elevated expression of β_{III}-tubulin. Microtubule alterations are thought to influence cellular responses to chemotherapeutic and micro-environmental stressors, thereby contributing to a broad-spectrum chemotherapy resistance. In clinical studies, the correlation between poor response to treatment and high β_{III}-tubulin expression, for example, in uterine serous carcinoma [21] and

ovarian cancer in response to taxane/platinum [22, 23], gastric [24] and breast cancer in response to taxane [25, 26], non-small cell lung cancer in response to tubulin-binding agents [27, 28] was shown.

As far as we are aware, there is no data supporting a predictive value for β_{III}-tubulin in CRC. However, here we have shown that the significantly increased expression of β_{III}-tubulin in 5-FU-resistant CRC HCT116 cells is associated with increased invasiveness, suggesting that such a correlation may be relevant to CRC patients. To our knowledge, this is the first study reporting the overexpression of β_{III}-tubulin in CRC cells with acquired resistance to 5-FU characterized by enhanced invasiveness.

Considering all evidence to date regarding the properties of β_{III}-tubulin and its role in cancer cells, it is possible to speculate about how β_{III}-tubulin may work in colon cancer. It should be noted that each of these features has been strongly conserved in the evolution of vertebrate β_{III}-tubulin for at least half a billion years, suggesting that they are functionally highly significant.

Firstly, β_{III}-tubulin has Ser239 instead of Cys239 present in the other tubulin β_I-, β_{II}-, β_{IVa}- and β_{IVb}-isotypes. The sulfhydryl of Cys239 is extremely sensitive to oxidation, which inhibits microtubule assembly [34, 35]. The replacement of this sulfhydryl group in β_{III}-tubulin allows it to function in cells with high levels of free radicals and reactive oxygen species such as aggressive metastatic cancers [36–40], since it was shown that reactive oxygen species inhibit microtubule assembly [41–43].

Secondly, the replacement of Ser124 in the β_I-, β_{II}-, β_{IVa} — and β_{IVb}-tubulin isotypes with Cys124 in β_{III}-tubulin and its very close position with respect to Cys127 and Cys129, present in all vertebrate β-tubulin isotypes and, in fact, in almost all of the eukaryotic β-tubulins allow forming a sulfhydryl cluster that could perhaps help to scavenge free radicals. This role has been proposed and discussed but never directly demonstrated [12, 44]. β_{III}-tubulin could play such a role in CRC as well as in other cancers. It should be noted that such a role does not necessitate that β_{III}-tubulin be part of a microtubule.

Thirdly, β_{III}-tubulin has an unusual C-terminal region — residues 431–450, where β_{III}-tubulin has a serine that can be phosphorylated [45] and ends with a basic residue. Recent discoveries have shown that β_{III}-tubulin plays a role in signaling in pro-survival pathways [13, 25, 30, 46–50] and this is consistent with its unusual and highly conserved structure. The presence of a highly negatively charged C-terminal domain could easily protect the β_{III}-tubulin molecule from both microtubule and free-floating form and interact with signaling molecules.

In discussing the functional significance of the C-termini of the tubulin as a possible participant of signaling pathway, the most obvious prediction is that the unique C-terminus of β_{III}-tubulin could be part of a unique signaling pathway. The potentially important signaling role of β_{III}-tubulin is confirmed by the fact that β_{III}-tubulin undergoes both transcriptional and post-transcriptional regulation [30, 51]. In the recent paper by Parker *et al*. [52], the role of β_{III}-tubulin as part of a signaling pathway was demonstrated, with an emphasis on its role in regulating glucose metabolism in non-small cell lung cancer. It is probable that β_{III}-tubulin is in a signaling pathway with MDR, as Li *et al*. [16] have suggested. It is certainly possible that β_{III}-tubulin has a signaling role in CRC, although what that role could be is not clear.

And finally, the fourth distinctive feature of β_{III}-tubulin is that it forms much more dynamic microtubules than is the case for microtubules formed from either β_{II}-tubulin or β_{IV}-tubulin [53]. The ability to form very dynamic microtubules would be important in cells that are changing their shape, dividing rapidly and migrating, all of which would apply to metastatic cells. These observations can explain the overexpression of β_{III}-tubulin in CRC. Moreover, the elevation of β_{III}-tubulin in embryonic tissues [54, 55] or in stem cells even in those that are not neuronal [56, 57] can be explained by the necessity of rapid growth and morphological changes.

All four of these issues contribute to the complexity of cancer therapy and to the contradictory effects of β_{III}-tubulin on resistance to anti-tumor drugs. Taking into account all of the above, we should mention that there is no data that 5-FU interacts directly with microtubules. Nevertheless, each model described above could conceivably explain the correlation that we have observed. We hope that further experiments will be able to clarify the hypotheses presented here and lead to improved chemotherapeutic regiments in CRC.

We showed for the first time the up-regulation of β_{III}-tubulin, the cytoskeleton microtubular component, in 5-FU resistant cancer cell line that demonstrated increased invasive potential and altered cell morphology. Our data being in agreement with broad clinical studies on predictive value of β_{III}-tubulin revealed its association with chemotherapy resistance both to tubulin-targeted and non-tubulin-targeted agents. Our findings of β_{III}-tubulin accumulation in cancer cells during resistance formation suggest that β_{III}-tubulin may serve not only as a marker of poor response to chemotherapy, but also as a potential target for the prevention of the chemoresistance formation.

ACKNOWLEDGMENTS

This study was funded by the Ministry of Health of Republic of Belarus (grant № 1.2.42) and International Scientific and Technology Center (ISTC №B-1636).

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest regarding the publication of this paper.

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РЕЗИСТЕНТНІ ДО 5-ФТОРУРАЦИЛУ КЛІТИНИ КОЛОРЕКТАЛЬНОГО РАКУ ХАРАКТЕРИЗУЮТЬСЯ ПІДВИЩЕНОЮ ІНВАЗИВНІСТЮ ТА ЕКСПРЕСІЄЮ β_{III}-ТУБУЛІНУ

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Стан питання: Підвищені рівні β_{III}-тубуліну пов'язані зі стійкістю до широкого спектру ліків та асоціюються з поганим прогнозом при різних типах епітеліального раку. 5-фторурацил (5-ФУ) — стандартний препарат, що широко використовується в хіміотерапевтичних схемах лікування хворих на колоректальний рак, однак стійкість до 5-ФУ є основною перешкодою для успішної терапії. ***Мета:*** Метою дослідження було порівняння інвазивних і адгезивних властивостей і рівнів експресії β_{III}-тубуліну в клітинній лінії HCT116, стійкій до 5-ФУ, та вихідних клітинах HCT116. ***Матеріали і методи:*** Лінія клітин HCT116, стійких до 5-ФУ, була отримана шляхом безперервного покрокового відбору зі зростаючими концентраціями 5-ФУ. Життєздатність клітин оцінювали за допомогою MTT-тесту, визначали адгезивні властивості та оцінювали інвазію в лунках із вставками Transwell. Експресію β_{III}-тубуліну виявляли методами імуноблотингу та імунофлюоресценції. ***Результати:*** Похідна лінія була у 25 разів більш стійка до 5-ФУ і характеризувалася зміненою морфологією клітин. Удвічі більше клітин 5-ФУ-стійкої лінії не проявляли адгезії порівняно з вихідною клітинною лінією. 5-ФУ-стійкі клітини характеризувалися посиленою інвазивністю, що супроводжувалася підвищеною експресією β_{III}-тубуліну. Крім того, ми виявили, що втрата експресії β_{III}-тубуліну корелювала з втратою резистентності до 5-ФУ. ***Висновок:*** Наші результати свідчать, що навіть незважаючи на те, що дія 5-ФУ не спрямована на мікротрубочки, імовірно, існує кореляція між експресією β_{III}-тубуліну і резистентністю до 5-ФУ, і що це особливо важливо по відношенню до інвазивності. Ці дані вказують на можливий внесок β_{III}-тубуліну в стійкість до 5-ФУ *in vivo*.

Ключові слова: колоректальний рак, 5-фторурацил, резистентність до хіміопрепаратів, β_{III}-тубулін, інвазивність.