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ВПЛИВ ПОХІДНОГО МАЛЕІМІДУ І 5-ФТОРУРАЦИЛУ НА МОРФОЛОГІЧНИЙ СТАН ТОНКОЇ КИШКИ ЩУРІВ З ХІМІЧНО-ІНДУКОВАНИМ РАКОМ ТОВСТОЇ КИШКИ

Досліджено профіль експресії генів бета-дефенсинів людини 1-4 в клітинах Namalwa (лімфома Беркітта) та Jurkat (Т-клітинна лейкемія). Встановлено, що в клітинах Namalwa та Jurkat експресовано гени hBD-1, hBD-2 та hBD-3, але ген hBD-4 не експресовано. Інкубація клітин Namalwa та Jurkat з рекомбінантним hBD-2 в концентрації 0.1-1000 нМ мала наслідком значуще концентраційно-залежне пригнічення життєздатності вказаних клітин.

Ключові слова: бета-дефенсини людини, експресія, лінія клітин лімфоми, лінія клітин лейкемії.

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ВЛИЯНИЕ ПРОИЗВОДНОГО МАЛЕИМИДА И 5-ФТОРУРАЦИЛА НА МОРФОЛОГИЧЕСКОЕ СОСТОЯНИЕ ТОНКОЙ КИШКИ КРЫС С ХИМИЧЕСКИ-ИНДУЦИРОВАННЫМ РАКОМ ТОЛСТОЙ КИШКИ

Проанализирован профиль экспрессии генов бета-дефенсинов человека 1-4 в клетках Namalwa (лимфома Беркитта) и Jurkat (Т-клеточная лейкемия). Установлено, что в клетках Namalwa и Jurkat экспрессированы гены hBD-1, hBD-2 и hBD-3, но не экспрессирован ген hBD-4. Инкубация клеток Namalwa и Jurkat с рекомбинантным hBD-2 в концентрации 0.1-1000 нМ приводила к значительному концентрационно-зависимому угнетению жизнеспособности указанных клеточных линий.

Ключевые слова: бета-дефенсины человека, экспрессия, линия клеток лимфомы, линия клеток лейкемии.

UDK: 577.3.616-006.04:618

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IN VITRO TESTING FOR PREDICTION OF TOXITY AND FOR SCREENING OF POTENTIAL CARCINOGENS AND ANTI–CANCER DRUGS

Cell-based technologies can be used for screening of potential carcinogens and evaluation of stem cell quality for clinical applications. 3D spheroids can serve as intermediate in vitro/ in vivo model for evaluation of anti-carcinogenic effects of therapeutic substances.

Key words: mesenchymal stem cells, MCF7 cell line, cell transformation, cell differentiation, spheroids, transformed foci.

Introduction. Cell-based technologies have broad experimental applications including pre-clinical studies in drug discovery and in regenerative medicine. Multi-potent stem cells have many clinical applications due to their capacity to be expanded *in vitro,* differentiate into several lineages, and participate in tissue regeneration. Clinical application of stem cells requires quality control to avoid potential carcinogenic effects of non-differentiated cells. Cell transformation (foci formation) and cell differentiation assays can provide information about stem cell quality and also can be used to evaluate drug effects.

Standard two-dimensional cell cultures for testing effects of anticancer agents are simple and convenient. However, they have significant limitations in reproducing the complexity and pathophysiology of *in vivo* tumor tissue [31; 33; 34; 37; 38; 39; 46]. Three-dimensional culture systems are of increasing interest in cancer research since tissue architecture and the extracellular matrix (ECM) significantly influence tumor cell responses to micro–environmental signals [43].

Vinci et al. [45] discovered differential sensitivity to targeted agents between two-dimensional and three-dimensional cultures, and also demonstrated enhanced potency of some agents against cell migration and invasion compared to cell proliferation. 2D and 3D *in vitro* functional assays can enhance the biological relevance of early preclinical cancer studies. These assays will increase the translational predictive value of *in vitro* drug evaluation studies and reduce the need for *in vivo* studies by more effective triaging of compounds.

Methods. Cells: Mouse mesenchymal stem cell line was obtained from the Tulane University Center for Gene Therapy under Material Transfer Agreement. These cells were originally derived from femurs and tibiae of C57BL mice. Cells were grown in IMDM (Invitrogen/GIBCO, CA) that was supplemented with 10% Premium Select FBS (Atlanta Biologicals, GA), 10% HS (Hyclone, UT), 100 U/mL penicillin, 100 µg/mL streptomycin (Invitrogen, CA). Colony formation and differentiation assays were performed periodically with iron oxide loaded and unloaded cells according to the instructions provided by the cell supplier. Briefly, cells were grown to 70-90% confluency in 6-well tissue culture plates, then incubated for 3 weeks in osteogenic or adipogenic differentiation medium, with medium change twice a week, and then stained for 10 min with 2 ml of 40 mM Alizarin red (pH 4.1; Sigma) for mineral deposits [36] or Oil Red-O (Sigma) for fat globules [18, 32], respectively. 3D transformed foci were comprised of cells that lost contact inhibition. We determined transformation frequency as ratio of foci number and the number of plated cells.

For tumor spheroid assay, we used MCF7 breast cancer cell line, kindly provided by Dr. I.Gut. We used modified standard method for multi-cellular spheroid generation [11]. Confluent cells were trypsinized, and single-cell suspension was seeded on low-adhesive substrate with density 1.0x105 cells/ml in growth medium with 0.24% carboxymethyl-cellulose (CMC). We generated spheroids in 6-wells test-plates (Nunclon, Belgium) and Petry dishes. Dishes with cells were placed on shaker with low rotation (150 rpm) for one hour. The number of live cells was determined in wells using MTT-colorymetric test. Cell count was performed after 48 hours incubation using a Trypan Blue dye [25]. Distribution of cells between different phases of the cell cycle and apoptotic index were assessed by flow cytometry [26]. Glucose absorption and LDG-

activity were tested by typical methods. Pro- and anti-carcinogenic effects evaluation was performed using cell proliferative index, spheroid size, and cell distribution between spheroid and adhesive fractions. Two types of culture media were used: complete (with 10 % FBS), and incomplete (without serum). As pro-carcinogenic factor, we used mitogen EGF. Herceptin (monoclonal antibody to HER2/neu receptor) was used as an anti-carcinogen.

Transfection:

MSC were transfected by electroporation with a recombinant plasmid pEF1□-GFP-IRES-CD4. To construct plasmid, the EGFP coding sequence was cloned into the pEF-1□ Myc/His vector along with an internal ribosome entry site (IRES) sequence coupled to the coding sequence of a truncated human CD4 (□CD4) receptor giving: pEF-1□-GFP-IRES-□CD4. Transfected cells were labeled with superparamagnetic MACS CD4-MicroBeads and isolated on MACSelect magnetic column. Stable pEF-1□-GFP-IRES-□CD4 expressing MSC clones were prepared in selection media with G418.□□CD4 receptor expression was evaluated immunocytochemically and by FACS using □CD4-PE antibodies [29].

Results and Discussion. In this study, we used: a) foci formation assay to compare frequency of transformation in control (non-transfected or transfected with buffer without DNA) and transfected mesenchymal stem cells and b) three-dimensional tumor spheroid assay for dynamic quantitative evaluation of pro- and anti-carcinogenic drug effects.

We evaluated frequency of transformation in culture of bone marrow derived murine mesenchymal stem cell line C57BL. Plasmid transfection resulted in stable and efficient GFP fluorescence; however, expression levels of CD4 were neither efficient nor stable possibly due to limited functionality of the IRES construct [29]. We also discovered that transfected MSC in long-term culture spontaneously formed foci of transformation with higher frequency than control cells (Figure 1C). Figure 1D,E demonstrates origination of transformed focus from GFP-expressing transfected cells. Subpopulations of genetically modified stem cells (transfected with the reporter gene) also changed their differentiation ability (Figure 2).

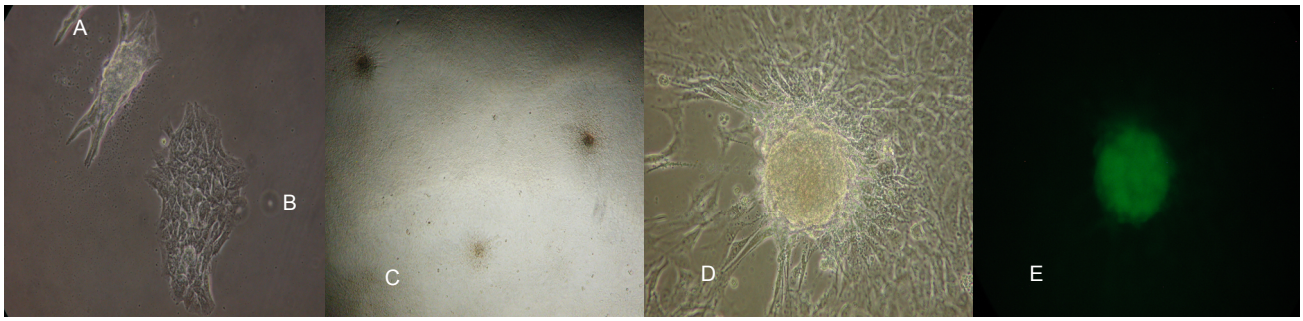
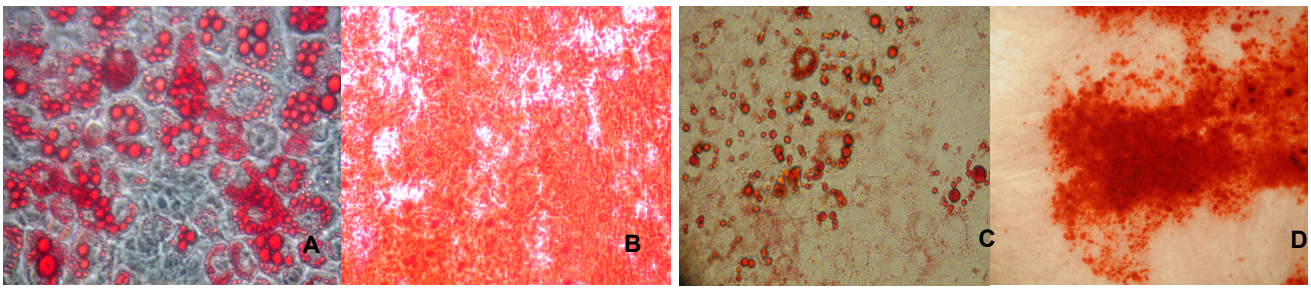


Figure 1. Spontaneous transformation in transfected murine MSC: A – transformed focus and B-normal growth; C – cell monolayer with three transformed foci; D, E – transformed focus on monolayer of MSC transfected with reporter gene: D – white light; E – green fluorescence of GFP



Passage 5; control **Passage 14; transfected cells**

Figure 2. Differentiation assay of mouse MSC in adipogenic (A,C) or osteogenic (B,D) medium: A, C – Oil Red-O staining for fat globules; B, D – Alizarin Red staining for mineral deposits

Thus, we demonstrated that genetically engineered stem cells in long-term culture are unstable: they may change their behavior and transform at higher frequency. Our findings indicate importance of bio-safety studies for long-term cell imaging and for efficient stem cell clinical applications [27; 28]

Foci formation assay is also applicable to drug screening for evaluation of drug carcinogenic potential and to discover drugs that prevent initiation of transformation. By identifying the potential for *in vivo* toxicity early, this assay helps to minimize the likelihood of late stage drug failure and allows drug development resources to be allocated toward advancing those candidates with the best chances for success.

To study pro- and anti-carcinogenic effects, we used 3D MCF7 cell culture. Many studies have highlighted significant differences between two-dimensional and three-dimensional cultures [9, 48]. 3D spheroids better reflect the *in vivo* tumor microenvironment in terms of cellular heterogeneity, nutrient and oxygen gradients, cell- cell interactions, matrix deposition and gene expression profiles [4; 7; 8; 9; 12; 48].

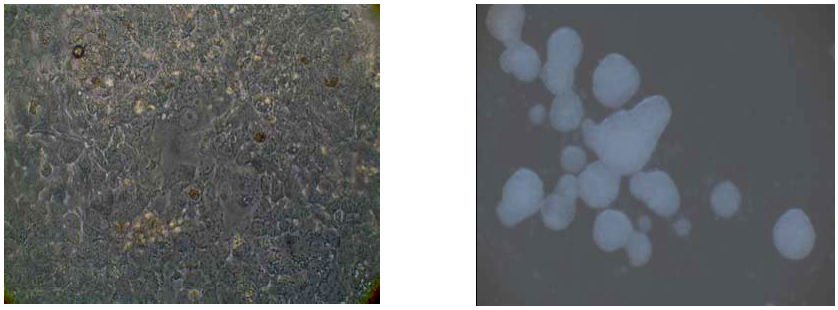
To model solid tumors more effectively, several three-dimensional (3D) culture systems have been established: whole perfused organs, tissue explants,

scaffold/microcarrier-based cultures, hollow-fiber bioreactors, organotypic cultures (multicellular spheroids and cellular multilayers) and gel/matrix-based cultures [2; 14; 19; 24; 30]. Of these, the multicellular tumor spheroid model is the best characterized and most widely used.

Tumor spheroids are heterogeneous cellular aggregates that, when greater than 500 μm diameter, are frequently characterized by hypoxic regions and necrotic centers [15; 41]. Three-dimensional spheroids are therefore considered valid models to recapitulate features of tumor microregions, neovascular domains or micrometastases [9; 20].

Sutherland et al. first applied this technique in cancer research [41]. Since then, several methods have been used to generate tumor spheroids: spontaneous aggregation [5], spinner flasks [47], rotary cell culture systems [24; 44], poly-2-hydroxyethyl methacrylate (poly-Hema)-coated plates [17; 51], hanging drops [6], liquid overlay on agar [10; 22; 49], low binding plates [42; 50], gel/matrix-based culture [21], polymeric scaffolds [8], or micropatterned plates [13; 23]. Each method has advantages and limitations [19; 24], and simple, standardized and rapid protocols appropriate for routine preclinical drug development studies within academic or pharmaceutical labs are lacking. Vinci et al. screened and classified a diverse collection of human tumor cell lines for their ability to form spheroids [45]. Spheroid formation depends on the cell type, cell density at seeding, rotation speed, type of culture medium, FTS concentration, and incubation time.

In this study, we used MCF7 cell line representing highly malignant human breast tumors and characterized by an optimal three-dimensional structure (Figure 3).



A **B**

Figure 3. 2D (A) and 3D (B) growth of MCF7 cells *in vitro*

Breast cancer is very invasive and highly metastatic tumor. The breast carcinomas development involves a set of complex phenotypic alterations in breast epithelial cells and in surrounding tissues [3]. MCF7 is a well known and widely used breast cancer cell line, which can be propagated as monolayer and as spheroid system. Architecture of three-dimensionally propagated MCF-7 cells is very similar to avascular tumor areas. The gradient of diffusion in cell aggregates leads to reduced proliferation rates and increased drug resistance.

Gene expression profiles of cells in spheroids are more similar to natural tumors, than profiles of the same cells in monolayer culture. Spheroid morphology is often resembles real tumor morphology.

We used 3D model of tumor growth to study pro- and anti- carcinogenic effects of serum deprivation. Figure 4 illustrates those effects. We evaluated spheroids' number, size, and shape, ratio of adhesive and suspension fractions, proliferation rate, number of apoptotic and necrotic cells, and cell cycle progression. Some of those parameters are summarized in Table 1. This system also allows for evaluation of drug effects on biochemical parameters reporting metabolic status of cells during spheroid growth under different conditions. Comparison of those parameters in 2D (monolayer) and 3D (spheroids) culture of MCF7 cells under serum starvation conditions demonstrates differential reactivity of those systems. 2D and 3D MCF7 cell cultures reacted differently to serum deprivation (Table 1).

***Table* 1. Serum starvation effects on cells in spheroid (3D) and monolayer (2D) culture.**

Apoptotic cells %		Necrotic cells %		Cells in G_0/G_1 (%)		LDG-activity		Glucose absorption	
2D	3D	2D	3D	2D	3D	2D	3D	2D	3D
Complete culture media (with 10 % FBS)									
19.0±1.3	14.2±1.7	11.4±1.7	17.0±2.5	56.3±1.5	41.0±1.0	0.18±0.01	0.12±0.01	0.19±0.01	0.07±0.01
Serum free medium (without FBS)									
39.2±7.3	22.4±3.9	33.5±2.8	20.4±2.2	54.1±1.4	67.5±1.7	0.27±0.02	0.32±0.03	0.29±0.01	0.23±0.01

LDG-activity showed in μKat/1000 cells; glucose absorption in μM/1000 cells.

Cells in 2D system are more sensitive to serum starvation than in 3D cultures. Cell viability is significantly higher in 3D system. Levels of necrotic and apoptotic cells in 2D culture are twice higher in comparison to 3D culture. Serum-free medium has no detectible effects on morphology of 3D cultured cells. The LDG-activity is increased in both culture systems under serum-free conditions. In serum-free medium, Glucose absorption level increases more significantly in 3D cultures. All parameters demonstrate increased cell survivability in spheroid system.

EGF has anti-apoptotic effect on cells in both systems in contrary to Herceptin that has pro-apoptotic effect (Figure 4). EGF increases proliferative pool in MCF7 cells. Herceptin has the opposite effect on MCF7 cell proliferation. Spheroid number and size in standard field of view was also different in the medium containing both agents (EGF and Herceptin) in comparison to those parameters in control (Table 2).

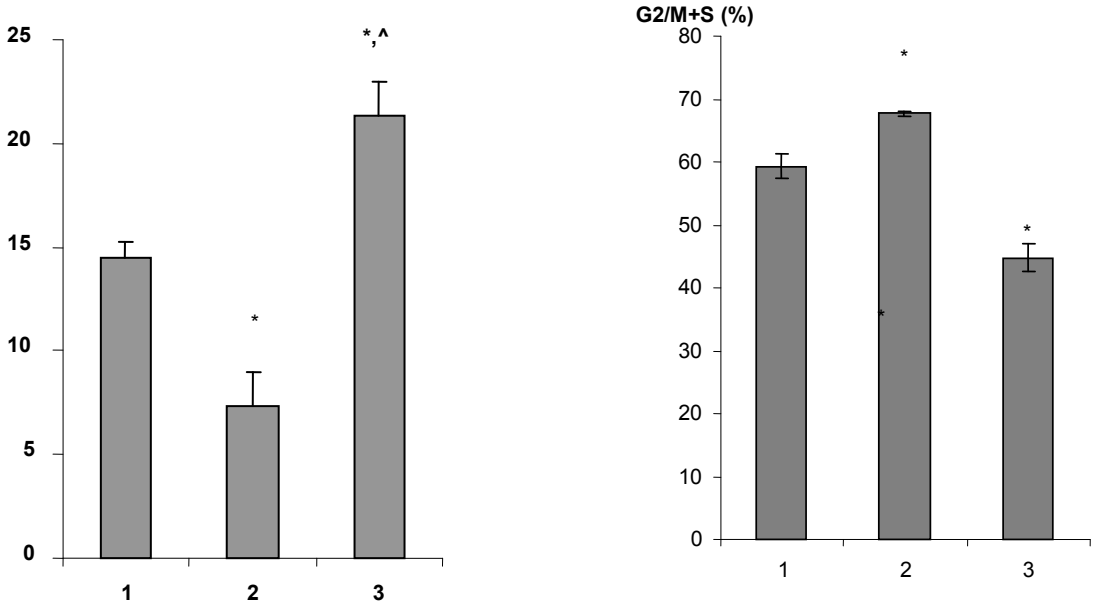
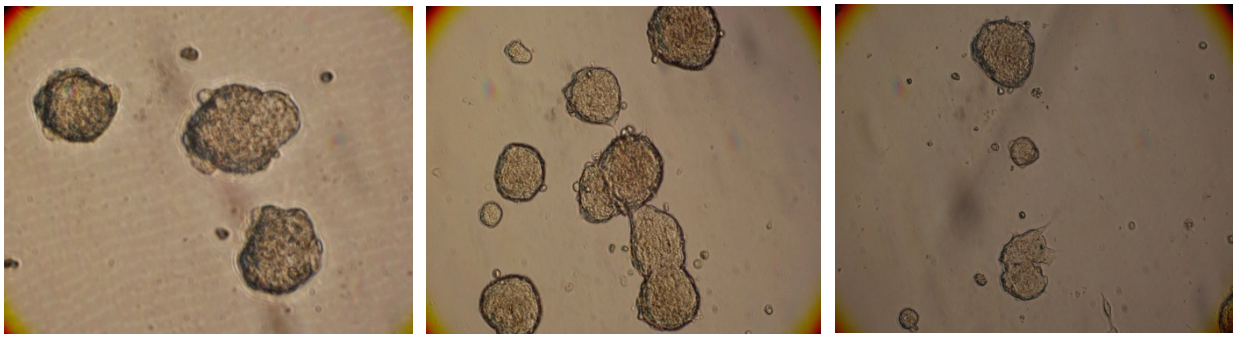


Figure 4. Effect of EGF and Herceptin on cell cycle and apoptotic index: 1 – control; 2 – EGF; 3 – Herceptin

***Table* 2. Effgect of EGF and Herceptin on the spheroid parameters**

MCF7 3D growth	Area of spheroids (мкм^{2}x10^3)	Spheroid number in the standard field of view	Cell distribution between adhesive and spheroid fraction (%)
control	3,3±1,7	5,1±1,3	15:85
EGF	5,7±0,8*	9,9±2,9*	24:76
Herceptin	2,7±1,4	4,1±1,1	33:67



Control **Pro-carcinogenic effect (EGF)** **Anti-carcinogenic effect (Herceptin)**

Figure 5. Pro- and anti-carcinogenic effects of EGF and Herceptin

Conclusions. *In vitro* testing on primary cells and cell lines can allow investigators to preview *in vivo* responses, thus facilitating design of better dosing strategies and optimization of animal and Phase I clinical studies. Cell transformation assay and spheroid model provide useful tools for evaluation of potential carcinogenic risk and early toxicity and for screening of anti-carcinogenic drugs. Expansion of preclinical testing to incorporate assays that better predict desired effects or potential toxicities earlier in the drug development process has obvious advantages for the selection of successful lead candidates.

Acknowledgements:

We thank the group of Dr. Prockop for providing mouse mesenchymal stem cells and Dr. Gut from Ludwig National Cancer Institute for sharing MCF-7 breast adenocarcinoma cells.

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Received to editorial board 10.07.13

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ПРОГНОСТИЧНЕ ТЕСТУВАННЯ ТОКСИКОЛОГІЧНОГО, ПРО- ТА АНТИКАНЦЕРОГЕННОГО ВПЛИВУ В СИСТЕМІ *IN VITRO*

Досліджено вплив похідного малеіміду (1-(4-Cl-бензил)-3-Cl-4-(CF_3-феніламіно)-1H-пірол-2,5-діону; MI-1) і 5-фторурацилу (5FU) протягом 6 тижнів на слизову оболонку тонкої кишки щурів на тлі 1,2-диметилгідразин-індукованого раку товстої кишки. Встановлено, що MI-1 і 5FU зменшують пошкодження слизової оболонки тонкої кишки, викликаних впливом канцерогену. Позитивний ефект MI-1 є більш вираженим, ніж 5FU.

Ключові слова: похідне малеіміду, 5-фторурацил, 1,2-диметилгідразин, рак товстої кишки, тонка кишка.

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ПРОГНОСТИЧЕСКОЕ ТЕСТИРОВАНИЕ ТОКСИЧЕСКОГО ПРО-И АНТИКАНЦЕРОГЕННОГО ВЛИЯНИЯ В СИСТЕМЕ *IN VITRO*

Исследовано влияние производного малеимида (1-(4-Cl-бензил)-3-Cl-4-(CF_3-фениламино)-1H-пиррол-2,5-дион, MI-1) и 5-фторурацила (5FU) в течении 6 недель на слизистую оболочку тонкой кишки крыс с 1,2-диметилгидразин-индуцированным раком толстой кишки. Установлено, что MI-1 и 5-фторурацил уменьшают повреждения слизистой оболочки тонкой кишки, вызванные воздействием канцерогена. Положительный эффект от MI-1 является более выраженным, чем 5FU.

Ключевые слова: производное малеимида, 5-фторурацил, 1,2-диметилгидразин, рак толстой кишки, тонкая кишка.