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COMPARATIVE STUDY OF EXPRESSION OF ADAPTOR PROTEINS RUK/CIN85 AND CD2AP/CMS IN NORMAL AND TUMOR HUMAN UTERUS TISSUES

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Adaptor proteins play an important role in facilitating protein-protein interactions and subsequent formation of signalling networks. These proteins recruit binding partners to a specific location inside the cell, and also regulate their activity. Adaptor protein Ruk/CIN85 and its structural and functional homologue CD2AP/CMS are important components of different regulatory pathways involved in control of cell proliferation, adhesion, invasion and survival, and, thus, can play a role in uterine carcinogenesis. In this work, we set out a comparative study of expression of Ruk/CIN85 and CD2AP/CMS at the level of mRNA and protein in intact uterine tissues, as well as in benign and malignant uterine tumors of different histological types. In most cases, an increase of expression levels of Ruk/CIN85 full-length form mRNA and protein, as well as CD2AP/CMS protein, were observed in uterine tumors, comparing with surrounding normal uterine tissues. Characteristic feature of conditionally normal uterine tissues, as well as benign uterine lesions, was an elevated content of high-molecular mass Ruk/CIN85 forms of 140 and 130 kDa, while an increased expression level of low-molecular mass 40 and 30 kDa Ruk/CIN85 forms was observed in the malignant tissue samples. Our findings suggest that an abnormal expression patterns of adaptor proteins Ruk/CIN85 and CD2AP/CMS in uterine tumors, and, thus, the corresponding changes in the activity of downstream signalling pathways, might be involved in maintenance of the malignant phenotype.

***Key words**:* tumorigenesis, adaptor proteins, Ruk/CIN85, CD2AP/CMS, benign and malignant uterine tumors.

Abbreviations: Ruk/CIN85, regulator of ubiquitous kinase/Cbl-interacting protein of 85 kDa; CD2AP/CMS, CD2 associated protein/Cas ligand with multiple SH3 domains;

SH3 domain, Src homology 3 domain; PR, proline-rich; CC domain, C-terminal coiled-coil domain; RTK, receptor tyrosine kinase; PI3K, phosphoinositide 3-kinase; ERK/MAPK, extracellular signal-regulated kinase/mitogen-activated protein kinase; TGF-β, transforming growth factor beta; CAPZ, capping protein (actin filament) muscle Z-line; TXS, Triton X-100 soluble fraction; TTP, total tissue protein fraction; PBS, phosphate buffered saline.

INTRODUCTION

Adaptor proteins are involved in regulation of diverse signalling processes that control cellular proliferation, differentiation, survival, vesicular trafficking, adhesion and motility [14]. These proteins contain a variety of protein-binding modules linking together protein-binding partners and facilitating a creation of larger signalling complexes. Thus, adaptor proteins are positioned to regulate cell signalling in a spatial and temporal fashion [12].

Ruk/CIN85 and CD2AP/CMS belong to a family of ubiquitously expressed adaptor molecules. Different combinations of promoter utilization and splicing events create multiple *ruk/cin85* transcripts in various tissues and cell lines, and expression of some of these transcripts is regulated during development [3].In contrast, CD2AP/CMS is expressed as a single transcript that corresponds to mRNA encoding full-length form of Ruk/CIN85 with molecular mass of 85 kDa [17, 34].

The overall domain organization of Ruk$_l$/CIN85 and CD2AP/CMS is identical; they share 39% identity and 54% similarity in their amino acid sequences [8]. Both molecules are composed of three N-terminal SH3 domains, followed by a proline-rich region (providing binding sites for SH3 domain-containing proteins), an unstructured region of approximately 160 residues, enriched in Ser/Thr and C-terminal coil-coiled domain mediating homotypic and heterotypic interactions [17, 34]. The SH3 domains share similarities among themselves and between family members, and their overlapping functions were identified [8, 14, 33].

Ruk/CIN85 plays a role in various biological processes including control of receptor tyrosine kinase signalling [1, 9, 18, 27, 28, 32], rearrangement of actin cytoskeleton [30], neuronal and T cell apoptosis [5, 15, 26], herpes simplex virus 1 infection [21], adhesion [30] and invasion [25]. Overexpression of Ruk$_l$/CIN85 full-length form induces apoptotic cell death of primary neurons in culture [2, 15]. However, shorter molecular forms of Ruk/CIN85 block the pro-apoptotic effect of Ruk$_l$/CIN85, suggesting that expression of different combinations of Ruk/CIN85 proteins in cells could be involved in the regulation of their survival and other intracellular processes [2, 15].

CD2AP/CMS is required for rapid activation of PI3K and ERK/MAPK pathways by TGF-β [29]. Direct interactions between CD2AP/CMS, nephrin, and podocin, and between CD2AP/CMS and the podocyte-specific actin-bundling protein synaptopodin, are essential for slit diaphragm integrity [16]. CD2AP/CMS was shown to localize in highly dynamic actin structures at the leading edge of cells and membrane ruffles, and have a role in cytoskeleton polarization associated with the activation of T cell receptors [10]. CD2AP/CMS associates with potassium-iodide-sensitive filamentous actin (F-actin) in the lysates of purified kidney glomeruli [37]. CD2AP/CMS associates with anillin, a component of the actin-rich cleavage furrow, at the midbody during cell division [23]. At the molecular level, CMS is linked to F-actin indirectly by binding to the focal adhesion protein p130Cas and Src-family kinases, cortactin, and the CAPZA and CAPZB heterodimer. Moreover, Ruk/CIN85 has been also shown to bind the focal adhesion kinase [14].

Some proteins have been demonstrated to interact with both Ruk/CIN85 and CD2AP/CMS, whereas others bind only one of them. Experimental data obtained by different groups have shown similar functions [8, 14] as well as antagonizing functions [34] for Ruk/CIN85 and CD2AP/CMS. It was reported that (Ruk/CIN85)/(CD2AP/CMS) balance is involved in the orchestrated signal transduction response [34].

Tumorigenesis is a multistep process that involves genetic alterations driving a progressive transformation of normal cells to the malignant phenotype. It is characterized by a dysregulation of numerous molecular pathways, such as cell cycle progression, angiogenesis, and apoptosis, that represent rational targets for the development of selective therapeutic approaches.

Uterine cervix and uterine corpus carcinomas are a significant cause of death for women suffering from gynaecologic malignancies [31]. The aim of our study was to compare the expression levels of Ruk/CIN85 and CD2AP/CMS in tissue samples of uterine fibromyoma, cervical dysplasia, stromal tumors, endometrial hyperplasia, body cancer, cervical cancer, sarcoma, and conditionally normal uterus.

MATERIALS AND METHODS

Sample collection. Lesion samples with corresponding control samples were obtained from 31 women who underwent initial surgery at the Danylo Halytsky Lviv National Medical University. The diagnosis was verified upon clinical and/or pathological criteria. Based on these criteria, the samples were divided into several groups representing control tissue (n=8), benign tumors (fibromyoma: n=3; cervical dysplasia: n=1; stromal tumor: n=4; endometrial hyperplasia: n=2), malignant tumors (body cancer: n=12; cervical cancer: n=4; sarcoma: n=2). The main clinical characteristics of patients are summarized in the Table. Just after removal, samples were macrodissected by the morphologist, rapidly frozen in liquid nitrogen, and stored at -130°C until use.

Samples preparation. Triton X-100 soluble (TXS) fraction was obtained by using lysis buffer (10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 50 mM NaF, 1 mM Na_3VO_4, 5 mM benzamidine, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 μg/ml pepstatin). Total tissue protein (TTP) fraction was obtained using guanidine thiocyanate buffer (4 M GTC, 25 mM sodium citrate, pH 7.0, 0.5% N-lauroylsarcosine, 0.1 M 2-mercaptoethanol). GTC-extracts were centrifuged at 12,000 g for 30 min at 4°C; phenol-chloroform interphase was precipitated with 2-propanol and protein pellets were dissolved in 50 mM Tris, pH 6.8, 2% SDS [6].

Northern blot-analysis. A modified protocol described by Chomczynski and Sacchi [7] was used. Briefly, uterine tissue (100 mg) was homogenized in 2.0 ml of a solution containing 4 M guanidine thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarcosyl, 0.1 M 2-mercaptoethanol. Then, 0.2 ml of 2 M sodium acetate (pH 4.0), 2.0 ml of phenol (water saturated) and 0.4 ml of chloroform were added and the homogenate was mixed. Prior to centrifugation at 10,000 g for 20 min, the solution was chilled on ice for 15 min. After centrifugation and precipitation by isopropanol, the resulting RNA pellet was dissolved in 100% formamide and stored at -70°C until use. Total RNA (20 μg) was subjected to electrophoresis in denaturing formaldehyde 1.25% agarose gel, and then transferred onto Hybond-N nylon membrane („Amersham"). RNA was fixed by the ultraviolet cross-linking. The original 3E7 clone was used as a probe for Northern hybridization.

SDS-PAGE and Western blot-analysis. Tissue extracts were prepared as described above, boiled in 2x Laemmli's sample buffer [19], and electrophoresed in gradient (5–18%) SDS-polyacrylamide gel. Then proteins were electrophoretically transferred onto nitrocellulose membrane („Amersham") in a buffer containing 25 mM Tris, 192.5 mM Glycine, 20% methanol at 250 mA for 2 hrs [35]. After blocking with PBST (PBS/0.05% Tween-20) containing 5% dried fat-free milk, membranes were incubated with the anti-CD2AP (Santa Cruz Biotech., USA), anti-Ruk/SH3A or anti-Ruk/CC [22] (dilution 1:2,000 in blocking solution) primary antibodies, followed by incubation with secondary anti-mouse or anti-rabbit IgGs conjugated with horseradish peroxidase („Amersham"). The membrane was washed three times with PBST and twice with PBS, and subjected to ECL detection (Amersham Biosciences, USA). Monoclonal anti-Ruk/SH3A antibody raised against the first SH3A domain of Ruk/CIN85 recognizes full-length form of Ruk/CIN85. Polyclonal anti-Ruk/CC antibodies raised against C-terminal coiled-coil region of Ruk/CIN85 recognize all Ruk/CIN85 multiple molecular forms [22]. Protein expression was quantified by using GEL-PRO Analyzer 32.

RESULTS AND DISCUSSION

Study of *ruk/cin85* transcripts expression in benign tumors and malignant uterine samples. Samples of conditionally normal tissues and uterine tumors were analyzed by the Northern blot analysis. The expression levels of *ruk/cin85* mRNA transcripts were normalized according to 18S rRNA.

The main 3.5 kb *ruk/cin85* transcript which encodes the full-length form of Ruk/CIN85 with molecular mass of 85 kDa was detected in the samples of total RNA isolated from uterine tissues. In the analyzed samples, both 2.5 kb (ruk_m) and 1.5 kb (ruk_s) transcripts were expressed at significantly lower level in comparison with 3.5 kb (ruk_l) transcript. High expression level of 3.5 kb *ruk/cin85* transcript was revealed in analyzed benign uterine tumors (Fig. 1, Table). In most cases, an increase of 3.5 kb *ruk/cin85* transcript expression level was observed in malignant uterine body and uterine cervix samples in comparison with the surrounding normal uterine tissue (Fig. 1, Table).

Expression of specific genes is not always followed by synthesis of functional proteins. Therefore, at the next stage we analyzed the expression patterns of Ruk/CIN85 multiple molecular forms in TXS and TTP fractions of conditionally normal uterine tissues, as well as uterine lesions. Notably, TTP extraction gives a possibility to solubilize additional proteins that are tightly associated with cell cytoskeleton and nuclear matrix.

Multiple immunoreactive bands, which correspond to proteins with apparent molecular mass of 140, 130, 85, 56, 40 and 34 kDa, were revealed in TXS fraction of benign uterine lesions using polyclonal anti-Ruk/CC antibodies (Fig. 2). According to current experimental data, some of these multiple molecular forms of Ruk/CIN85 detected with anti-Ruk/CC antibodies (full-length form – 85 kDa, a form without first SH3A domain – 70 kDa, and a form without two SH3 domains – 56 kDa) could be a result of alternative splicing of Ruk/CIN85 pre-mRNA transcript. Other forms may be a result of posttranslational modifications caused by the ubiquitilation (140, 130 and 100 kDa) and limited proteolysis (40 and 34 kDa) [36]. High content of the full-length form was revealed in TXS fraction of uterine benign lesion samples (Fig. 2, Table). The characteristic feature of benign uterine lesions was high content of p140 and p130 forms. Interestingly, Ruk/CIN85 multiple molecular

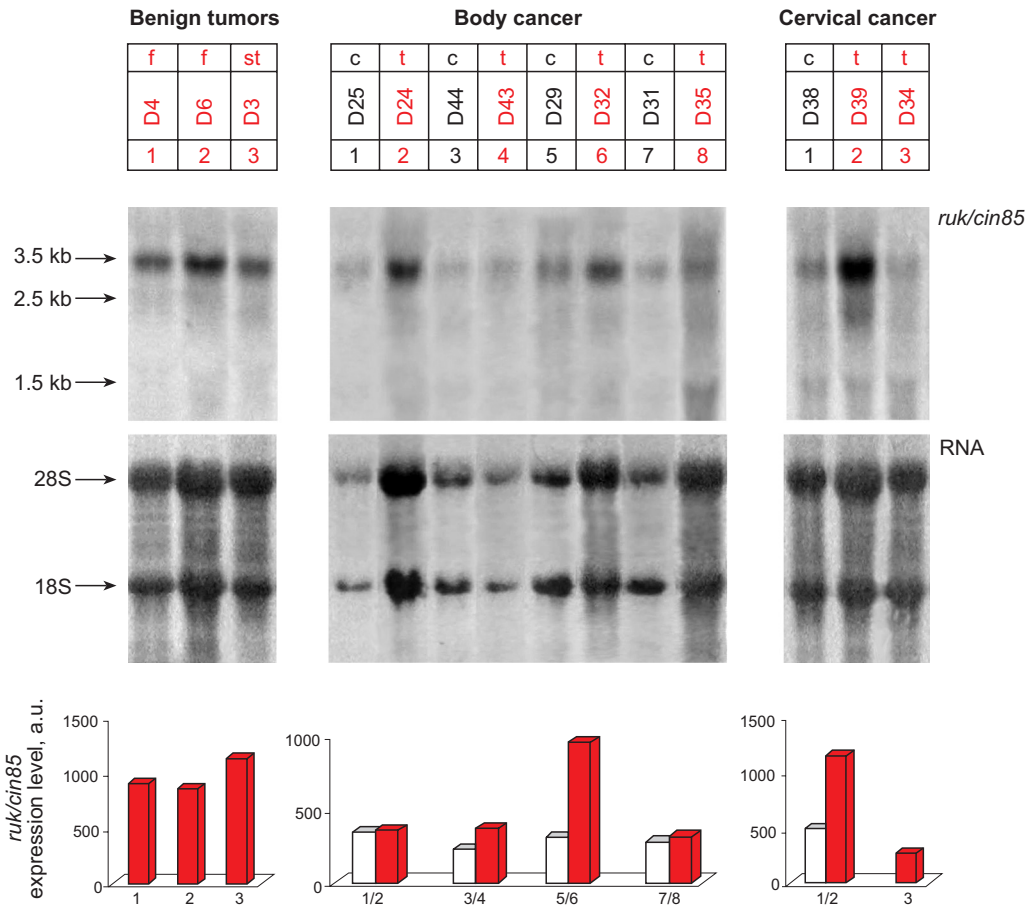

Fig. 1. Northern blot-analysis of *ruk/cin85* mRNA transcripts in samples of uterine lesions: benign tumors, body cancer, cervical cancer. Expression level of 3.5 kb *ruk/cin85* transcript is presented in arbitrary units (a.u.). f – fibromyoma, st – stromal tumor, c – normal uterine tissue, t - tumor

Рис. 1. Нозерн-блот-аналіз мРНК транскриптів *ruk/cin85* у зразках пухлин матки: доброякісні пухлини, рак тіла, рак шийки. Рівень експресії *ruk/cin85* транскрипту розміром 3,5 тпн представлений в умовних одиницях (a.u.). f – фіброміома, st – стромальна пухлина, c – контроль, t – пухлина

forms pattern of benign lesions was very similar to that detected in the conditionally normal uterine tissues (p140, p130, p85, p56, p40 and p34 forms). The same patterns of expression of Ruk/CIN85 multiple molecular forms were detected in the TXS fraction of most uterine body and cervical tumor samples, however, some level of polymorphism of p140 content was present (Fig. 2).

Polymorphism of Ruk/CIN85 full-length form expression was revealed in the TTP fraction of the benign uterine lesions. An increase of p85 content in the TTP fraction was detected in the uterine body tumors, as well as in uterine cervix carcinoma samples in

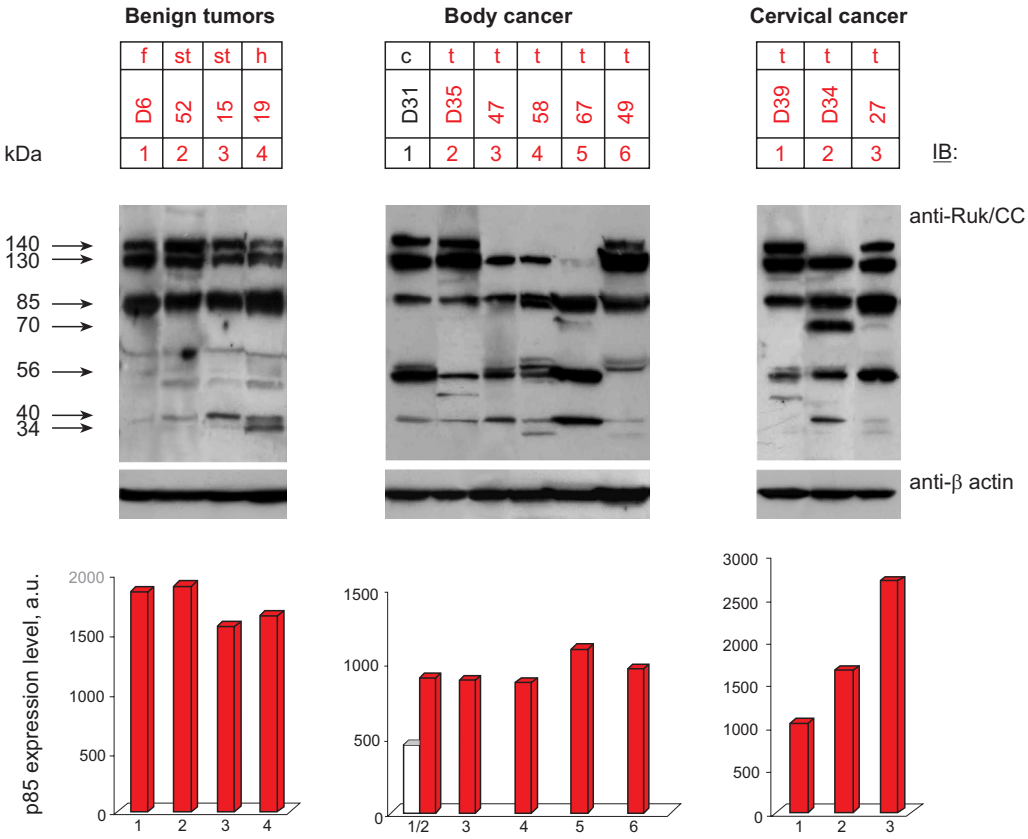

Fig. 2. Ruk/CIN85 content in the TXS fraction extracted from uterine lesions: benign tumors, body cancer, cervical cancer. p85 content is presented in arbitrary units (a.u.). IB – immunoblotting; f – fibromyoma, st – stromal tumor, h – endometrial hyperplasia, c – normal tissue, t – tumor

Рис. 2. Вміст Ruk/CIN85 у TXS фракції, екстрагованої з пухлин матки: доброякісні пухлини, рак тіла, рак шийки. Вміст p85 представлений в умовних одиницях (a.u.). IB – імуноблотинг; f – фіброміома, st – стромальна пухлина, h – гіперплазія ендометрію, c – контроль, t – пухлина

comparison with corresponding control samples. By using anti-Ruk/CC antibodies in Western-blot-analysis, the immunoreactive bands corresponding to proteins with apparent molecular mass of 140, 130, 100, 85, and 50 kDa were detected in samples of the normal uterine tissue. The patterns of Ruk/CIN85 multiple molecular forms in analyzed samples of the uterine cancer (body of uterus) differ from that in normal uterine tissues, by a predominant decrease in content of high-molecular mass forms (p140, p130, p100) and the appearance of p70 form. An additional feature of Ruk/CIN85 patterns in the uterine body carcinoma was an increase in content of low-molecular mass forms (p40, p34). Similar peculiarities in the patterns of Ruk/CIN85 expression were revealed in control and cancer uterine cervix samples (Fig.3).

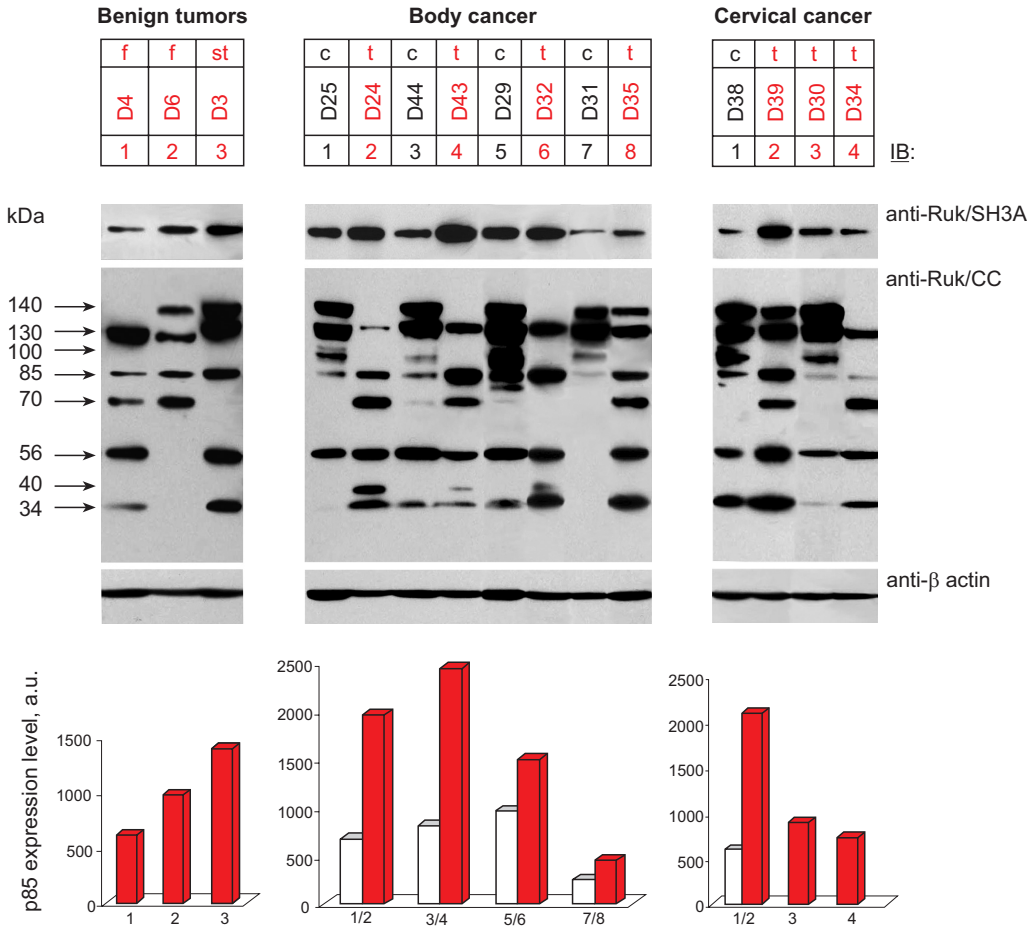

Fig. 3. Ruk/CIN85 content in the TTP fraction extracted from uterine lesions: benign tumors, body cancer, cervical cancer. p85 content is presented in arbitrary units (a.u.). IB – immunoblotting; f – fibromyoma, st – stromal tumor, h – endometrial hyperplasia, c – normal tissue, t – tumor

Рис. 3. Вміст Ruk/CIN85 у ТТР фракції, екстрагованої з пухлин матки: доброякісні пухлини, рак тіла, рак шийки. Вміст p85 представлений в умовних одиницях (a.u.). IB – імуноблотинг; f – фіброміома, st – стромальна пухлина, h – гіперплазія ендометрію, c – контроль, t – пухлина

Since adaptor protein CD2AP/CMS is a structural and functional homologue of Ruk/CIN85, we investigated an expression level of CD2AP/CMS protein in uterine lesions in comparison with adjacent normal tissues. A comparatively high expression level of CD2AP/CMS was detected in the benign tumors. Similarly to Ruk/CIN85, up-regulation of CD2AP/CMS was detected in most samples of the uterine body and uterine cervix tumors.

Although the present study has certain limitations, the obtained results demonstrate the same directivity in Rukl/CIN85 and CD2AP/CMS expression levels in most uterine carcinomas studied in comparison with surrounding conditionally normal tissues. At the

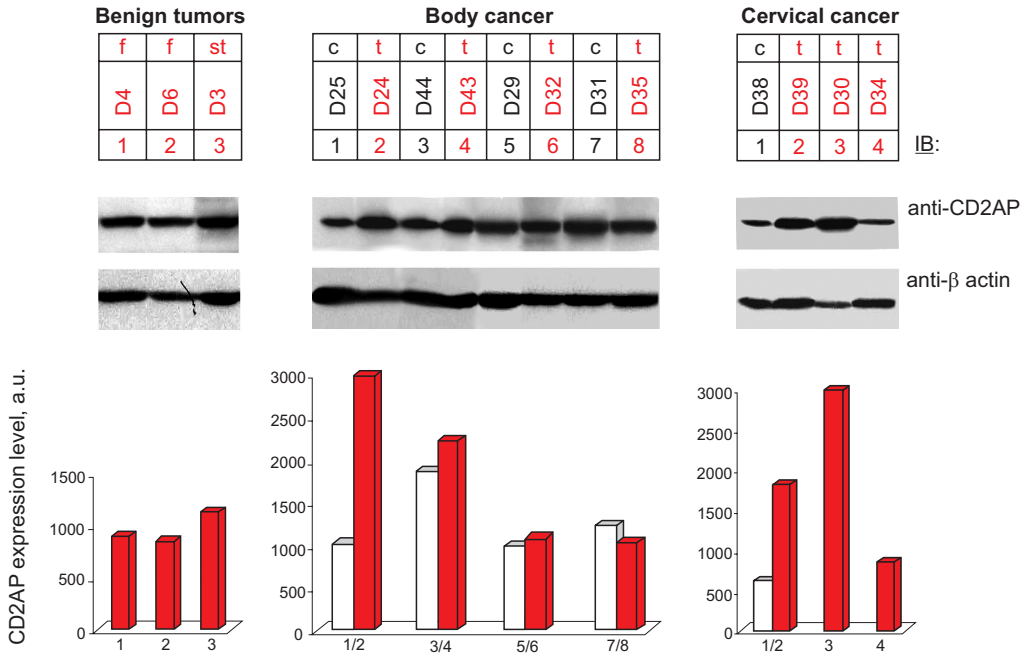

Fig. 4. CD2AP/CMS content in the TTP fraction extracted from uterine lesions: benign tumors, body cancer, cervical cancer. CD2AP/CMS content is presented in arbitrary units (a.u.). IB – immunoblotting; f – fibromyoma, st – stromal tumor, c – normal tissue, t – tumor

Рис. 4. Вміст CD2AP/CMS у ТТР фракції, екстрагованої з пухлин матки: доброякісні пухлини, рак тіла, рак шийки. Вміст CD2AP/CMS представлений в умовних одиницях (a.u.). IB – імуноблотинг; f – фіброміома, st – стромальна пухлина, c – контроль, t – пухлина

same time, there is an essential heterogeneity both in the fold of adaptor proteins elevation and their ratio between different samples pairs. These features may be related to the tumors type and grade as well as sensitivity to anti-tumor therapy that needs further investigation.

CONCLUSION

Numerous molecular studies showed that PI3K-AKT-mTOR, RAS-MEK-ERK and SRC-FAK signalling pathways are implicated in the development of uterine malignancies [4, 11, 13, 24, 38]. Taking into account that (Ruk/CIN85)/(CD2AP/CMS) adaptor proteins are key components of these signalling networks, we suggest that their abnormal expression patterns in uterine tumors might be involved in the maintenance of malignant phenotype and, additionaly, may represent a novel molecular target for chemotherapeutics.

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Table. Clinical data, *ruk/cin85* transcripts expression and (Ruk/CIN85)/(CD2AP/CMS) proteins content in the TXS and TTP fractions

Таблиця. Клінічні дані пацієнтів, рівень експресії транскриптів *ruk/cin85* і вміст білків (Ruk/CIN85)/(CD2AP/CMS) у фракціях TXS і TTP

	Type	No	RNA				Protein		
			ruk/cin85				Ruk EB	Ruk GTC	CD2AP GTC
			4.5	3.5	2.5	1.5	p85	p85	p83
Control tissues		d19		168	86	115	1100	1473	2210
		d23 (d22)		118	76	94	305		
		d25 (d24)		341	143	175		675	1018
		d29 (d32)		310	70	40	1300	963	986
		d31 (d35)		276	78	77	450	250	1235
		d38 (d39)		476	330	231	1038	600	638
		d40 (d39)		299	80	50			
		d44 (d43)		225	115	105		814	1867
Benign tumors	Fibromyoma	d4		851	71		1031	500	900
		d6		800	41	115	1851	800	852
		32					1966		
	Cervical dysplasia	17		887	182	219	840		
	Stromal tumor	d3		1200	140	119		1130	1131
		9		500					
		15					1551		
		52					1900		
	Endometrial hyperplasia	14					1436		
		19					1650		
Malignant tumors	Body cancer	d24 (d25)		358	37	88		1956 ↑	3000 ↑
		d35 (d31)		317 ↑	469	574	900	448 ↑	1029 ↑
		d32 (d29)		962 ↑	150	109		1508 ↑	1077 ↑
		d43 (d44)		361 ↑	95	127		2445 ↑	2237 ↓
		1					183		
		36		168	121	63	1395		
		39	528	376	328	304	96		
		47	1533	968	1606	1272	889		
		49					966		
		58		969	374	70	870		
		59	775	688	529	459	1005		
		67					1097		
	Cervical cancer	d30						900	2990
		d34		266	194	166	1659	750	850
		d39 (d38)		1120 ↑	190	155		2100 ↑	1811 ↑
		27					2700		
	Sarcoma	72		1008	326	119	1714		
		55					2518		

Levels of transcript expression and protein content are presented in the arbitrary units.

↑↓ – up- or down-regulation of Ruk/CIN85 in uterine tumor in comparison with conditionally normal tissue

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ПОРІВНЯЛЬНЕ ДОСЛІДЖЕННЯ ЕКСПРЕСІЇ АДАПТЕРНИХ БІЛКІВ RUK/CIN85 ТА CD2AP/CMS У НОРМАЛЬНИХ І ПУХЛИННИХ ТКАНИНАХ МАТКИ ЛЮДИНИ

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Адаптерні білки відіграють важливу роль у білково-білкових взаємодіях, що забезпечують формування сигнальних мереж. Ці білки не лише залучають зв'язувальні білки-партнери до специфічних компартментів усередині клітини, але також регулюють їхню активність. Адаптерний білок Ruk/CIN85 та його структурний і функціональний гомолог CD2AP/CMS є важливими компонентами численних регуляторних шляхів, залучених до контролю проліферації, адгезії, інвазії та виживання клітин, і тому вони можуть відігравати важливу роль у канцерогенезі матки. У цій роботі представлено результати порівняльного дослідження експресії Ruk/CIN85 та CD2AP/CMS як на рівні мРНК, так і на рівні білка, в інтактних тканинах матки та в доброякісних і злоякісних пухлинах матки різного гістологічного типу. У більшості випадків виявлено зростання рівнів експресії мРНК і білка повнорозмірної форми Ruk/CIN85 та білка CD2AP/CMS у пухлинах матки порівняно з оточуючою їх нормальною тканиною. Характерною особливістю умовно нормальних тканин матки, як і доброякісних пухлин, є високий вміст високомо-

лекулярних форм Ruk/CIN85 розміром 140 та 130 кДа, тоді як зростання вмісту низькомолекулярних форм Ruk/CIN85 розміром 40 та 30 кДа виявлено у зразках злоякісних пухлин. Результати проведених досліджень дають змогу припустити, що аномальна експресія адаптерних білків Ruk/CIN85 і CD2AP/CMS у пухлинах матки й відповідні можливі зміни функціональної активності розташованих нижче сигнальних шляхів можуть бути залучені до підтримання злоякісного фенотипу.

Ключові слова: канцерогенез, адаптерні білки, Ruk/CIN85, CD2AP/CMS, доброякісні та злоякісні пухлини матки.

СРАВНИТЕЛЬНОЕ ИЗУЧЕНИЕ ЭКСПРЕССИИ АДАПТЕРНЫХ БЕЛКОВ RUK/CIN85 И CD2AP/CMS В НОРМАЛЬНЫХ И ОПУХОЛЕВЫХ ТКАНЯХ МАТКИ ЧЕЛОВЕКА

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Адаптерные белки играют важную роль в белок-белковых взаимодействиях, обеспечивающих формирование сигнальных сетей. Эти белки не только собирают вместе связующие белки-партнеры в специфических компартментах внутри клетки, но и вовлечены в регуляцию их активности. Адаптерный белок Ruk/CIN85 и его структурный и функциональный гомолог CD2AP/CMS являются важными компонентами многочисленных регуляторных путей, вовлеченных в контролирование пролиферации, адгезии, инвазии и выживания клеток и поэтому могут играть важную роль в канцерогенезе матки. В работе представлены результаты сравнительного изучения экспрессии Ruk/CIN85 и CD2AP/CMS как на уровне мРНК, так и на уровне белка, в интактных тканях матки и в доброкачественных и злокачественных опухолях матки разных гистологических типов. В большинстве случаев обнаружено повышение уровней экспрессии мРНК и белка полноразмерной формы Ruk/CIN85 и белка CD2AP/CMS в опухолях матки по сравнению с окружающими их нормальными тканями матки. Характерной особенностью условно нормальных тканей матки, как и доброкачественных опухолей, является высокое содержание высокомолекулярных форм Ruk/CIN85 размером 140 и 130 кДа, в то время как повышенное содержание низкомолекулярных форм Ruk/CIN85 размером 40 и 30 кДа обнаружено в образцах злокачественных опухолей. Результаты проведенных исследований позволяют предположить, что аномальная экспрессия адаптерных белков Ruk/CIN85 и CD2AP/CMS в опухолях матки и возможные изменения активности находящихся ниже сигнальных путей могут быть вовлечены в поддержание злокачественного фенотипа.

Ключевые слова: канцерогенез, адаптерные белки, Ruk/CIN85, CD2AP/CMS, доброкачественные и злокачественные опухоли матки.

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