CELL TECHNOLOGIES IN BIOLOGY AND MEDICINE

Expression and Intracellular Localization of ACA and TRA-1-81 in Smooth Muscle Cell Tumors

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We studied the expression and intracellular localization of ACA and TRA-1-81 in smooth muscle cell tumors. The study was performed on tissue specimens obtained during surgery from patients with uterine leiomyoma and leiomyosarcoma (mean age 34 and 51 years, respectively). ACA was present in leiomyoma, leiomyosarcoma, and control myometrium. Intracellular expression of ACA varied in different types of tumors and was minimum in normal myometrium and maximum in leiomyosarcoma. Membrane localization of the protein is typical of common and cellular leiomyoma, while in the growth zones of mitotically active leiomyoma and leiomyosarcoma the reaction product was primarily located in tumor cell cytoplasm. TRA was detected in some leiomyosarcoma cells. Thus, ACA dysregulation was revealed in the growth zones of leiomyomas and in leiomyosarcomas, which manifested in enhanced expression of this protein and its detachment from the plasma membrane, which leads ACA translocation into the cytoplasm and nucleus of tumor cells and potentiates their proliferative activity.

Key Words: *ACA; TRA; leiomyoma; leiomyosarcoma*

Uterine smooth muscle cell tumors are presented by different types of leiomyomas (LM) and leiomyosarcomas (LMS). Uterine LM are the most prevalent tumors of the female reproductive system (it is diagnosed in 20-75% women over 30) [6]. LMS constitutes 1% of all malignant neoplasms of the uterus, most often at the age of 50-55 years [6]. Of particular interest is investigation of signal pathways potentiating tumor growth such as Ras-Erk/MAP and PI3K-Akt-mTOR. The PI3K-Akt-mTOR signal pathway [7] can be triggered by a recently discovered protein ACA.

ACA was fist isolated from human blood [1]. The important role of this protein in the regulation of human SC renewal has been proven in a number of studies. ACA is a GPI-bound protein widely presented in eukaryotes. GPI is a complex C-terminal structure in many eukaryotes consisting of a phosphoethanolamine linker, glycan core, and a phospholipid chain; it serves for binding of GPI protein to the outer side of the plasma membrane. GPI-anchored proteins perform different functions and participate in activation of intracellular signaling pathways involved into the immune response and pathogenesis of prion disease [1]. GPI-anchored proteins are associated with the plasma

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membrane lipid rafts and represent areas enriched with glycosphingolipids, cholesterol, and other lipoproteins. Lipid rafts make the membrane more rigid and stable and divide it into small domains for functionally specialized processes, *e.g.* signal transduction or vesicle transport [4,11]. The major part of lipid raft-associated signal pathways regulates cell growth. Disorders in the synthesis of GPI-structure lead to cell death and are lethal for mammals [5].

According to published data, disorders in the regulation of PI3/Akt kinase signal pathway play a special role in the pathogenesis of uterine LM and other tumors [7,9]. It is activated not only via ACA, but also by steroid hormones and growth factors and is probably involved into the pathogenesis of many gynecological diseases associated with increased risk of uterine and ovarian tumors [8]. Activation of ACA protein is followed by PI3K translocation into the cell membrane that triggers this signal pathway including phosphorylation of phosphoinositides (PIP) and conversion of PIP2 into PIP3. Due to this process, many proteins containing plextrin-homologous domains (a polypeptide sequence consisting of 120 amino acid residues found in some proteins participating in cascade intracellular signaling and in some cytoskeleton proteins), *e.g.* Akt, bind to PIP3 in the cell membrane and become membrane-associated proteins. Due to their membrane localization, kinases PDK1 and mTOR (mammalian target of rapamycin) can phosphorylate and activate Akt.

Apart from participation in the regulation of PI3/Akt signaling pathway, ACA modulates expression of TRA-1-81. TRA-1-81 is an epitope of podocalyxin, a transmembrane glycoprotein (sialomucin most closely related to CD34 and endoglycan) and embryonic SC marker [10,12]. TRA-1-81 is expressed in non-differentiated SC, embryonic germ cells, and embryonic carcinoma cells; it is activated in dedifferentiating cells.

We have not found published data about the role of ACA and TRA in the pathogenesis of LM and LMS.

Here we studied the expression and intracellular localization of ACA and TRA-1-81 in smooth muscle cell tumors.

MATERIALS AND METHODS

The study was performed on operation material (specimens obtained after myomectomy and hysterectomy) from 31 patients of reproductive age with LM and 3 patients with LMS. The mean age of LM and LMS patients was 34 and 51 years, respectively.

During macroscopic examination of the operation material, samples of LM and LMS (4-10 fragments from different sites) and adjacent myometrium were isolated. A part of the material was frozen at -25°C and cryostat sections were sliced (Microm HM 550 cryostat, Thermo Scientific). The other part was fixed in 10% neutral formalin and embedded in paraffin.

Histological and immunohistochemical methods and immunofluorescent microscopy were used. Histological study was performed on paraffin and stepwise frozen sections stained with hematoxylin and eosin. Immunohistochemical assay (peroxidase labeling) was performed routinely on frozen and paraffin sections using primary antibodies to ACA, TRA-1-81 (ACA CELL Biotech, Heidelberg, Germany, patent No. ER 1745126 A1), SMA, CD117, Ki -67 (Dako Cytomation), and progesterone and estrogen receptor (Dako Cytomation). Immunofluorescent analysis was performed on frozen sections using secondary antibodies carrying a fluorescent label (Goat anti-mouse IgG DyLightb 488 N/A; Thermo Scientific) and on paraffin sections using peroxidase-labeled antibodies (EnVision™ Detection Systems Peroxidase/DAB, Rabbit/Mouse, Dako Cytomation). Tissue structure was studied under an Olympus BX61 microscope equipped with an Olympus DP72 camera. Immunohistochemical reaction for ACA and TRA-1-81 was evaluated semiquantitatively (score); the percent of cells positively stained for SMA, CD117, Ki-67, PR, and ER was also determined. Semiquantitative evaluation of reaction intensity was performed using a 6-point scale (2 points – <20% stained cells, 4 points – 20-40% stained cells, 6 points – >40% stained cells). For ACA, the staining intensity and localization of the reaction product (membrane, cytoplasm, and nucleus) was assessed.

The results were processed using Mann–Whitney test.

RESULTS

According to the results of morphological analysis and WHO classification (2007) [6], all LM were divided into the following histological types: common, cellular, and mitotically active. The most predominant type were common LM (16 cases) consisting of mature leiomyocytes separated by fibrovascular septae that contained hyaline and sclerotic foci. Proliferation index was 0% (Ki-67 assay). In leiomyocytes, high expression of progesterone and estrogen receptors was observed (Fig. 1, *a*, *b*, Fig. 2).

Cellular LM (10 cases) contained densely packed leiomyocyte bundles separated by thin fibrovascular septae. Proliferative index was 2% (Ki-67 assay); intensive expression of estrogen and progesterone receptors was observed (Fig. 2).

Mitotically active LM (5 cases) differed from cellular LM by higher mitotic index (5%, Ki-67 index). The expression of progesterone and estrogen receptors remained at a high level (Fig. 1, *c*; Fig. 2).

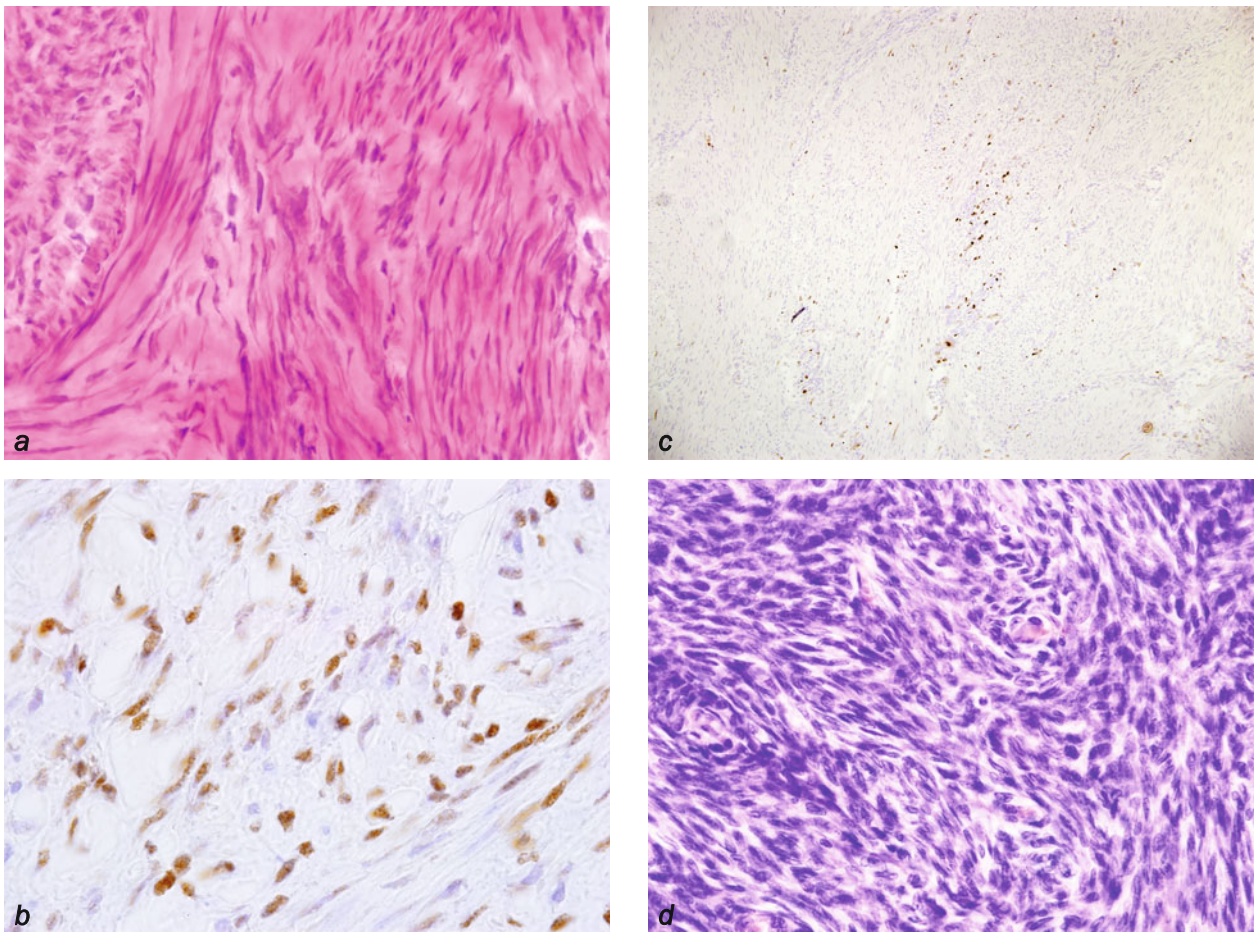

Fig. 1. Morphology of uterine LM and LMS. *a*) common LM; hematoxylin and eosin staining, ×600; *b*) progesterone receptors in common LM; immunohistochemical staining, ×600; *c*) Ki-67 in mitotically active LM; immunohistochemical staining, ×200; *d*) LMS; hematoxylin and eosin staining, ×600.

Growth zones [6] typical of cellular and mitotically active LM were presented by perivascular cuffs consisting of spindle-shaped cells often with sings of smooth muscle cell differentiation (are positively stained with SMA). In these zones, Ki-67- and CD117 (c-kit)-positive cells were primarily concentrated (Fig. 3).

LMS (3 cases) consisted of bundles of atypical cells with signs of smooth muscle cell differentiation (SMA-positive) infiltrating the adjacent tissues. Index of mitotic activity (Ki-67 index) was 35%. However, the expression of steroid hormone receptors was low (Fig. 1, *d*; Fig. 2). Some tumor cells expressed CD117 (c-kit).

In the control myometrium, proliferation index was 0%, the expression of estrogen and progesterone receptors was not high and CD117 (c-kit) was expressed in solitary cells (Fig. 2).

ACA was detected in all tumor types and in the control myometrium. It was located in cells and extracellular matrix. In cells, ACA was seen as fine granules on the cell membranes and in the cytoplasm; it was also detected in the nuclei of some cells.

The intracellular ACA expression varies in different samples: it was minimum in normal myometrium and maximum in LMS; its content in common LM was lower than in cellular LM and its content in cellular LM was lower than in mitotically active LM (Fig. 4).

Moreover, localization of ACA in cells varied in different pathologies (Fig. 5, *a-c*). Membrane localization of the protein was typical of common and cellular LM; in mitotically active LM, cells with cytoplasmic localization of the reaction product appeared (primarily in the growth zones). In LMS, the reaction product was presented by coarse granules in the cytoplasm of tumor cells (Fig. 5, *e*).

TRA was seen as cytoplasmic staining; it was detected only in solitary LMS cells and only in the cytoplasm (Figs. 4 and 5, *f*).

In different types of uterine LM and in LMS, inhomogeneous expression and localization of ACA were detected. The highest expression detected in LMS correlated with Ki-67 index of proliferative activity. It can be hypothesized that ACA stimulates the

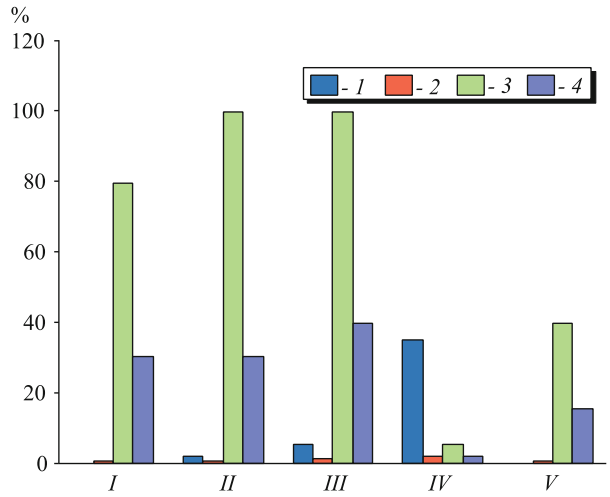

Fig. 2. Immunohistochemical (average) expression of Ki-67 (*1*), CD117 (*2*), progesterone receptors (*3*), and estrogen receptors (*4*). Here and in Fig. 4: *I*: common LM, *II*: cellular LM, *III*: mitotically active LM, *IV*: LMS, *V*: myometrium.

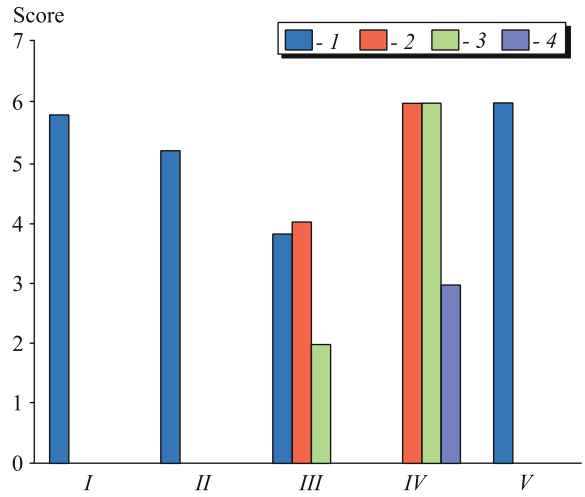

Fig. 3. Growth zones in LM. *a*) perivascular growth zones; *b*) Ki-67 in vascular endothelium and perivascular leiomyocytes; *c*) ACA in vascular endothelium and perivascular leiomyocytes; *d*) c-kit in vascular endothelium and perivascular leiomyocytes. Hematoxylin and eosin staining (*a*), immunohistochemical staining (*b-d*). Magnification: *a*) ×200, *b-d*) ×1000.

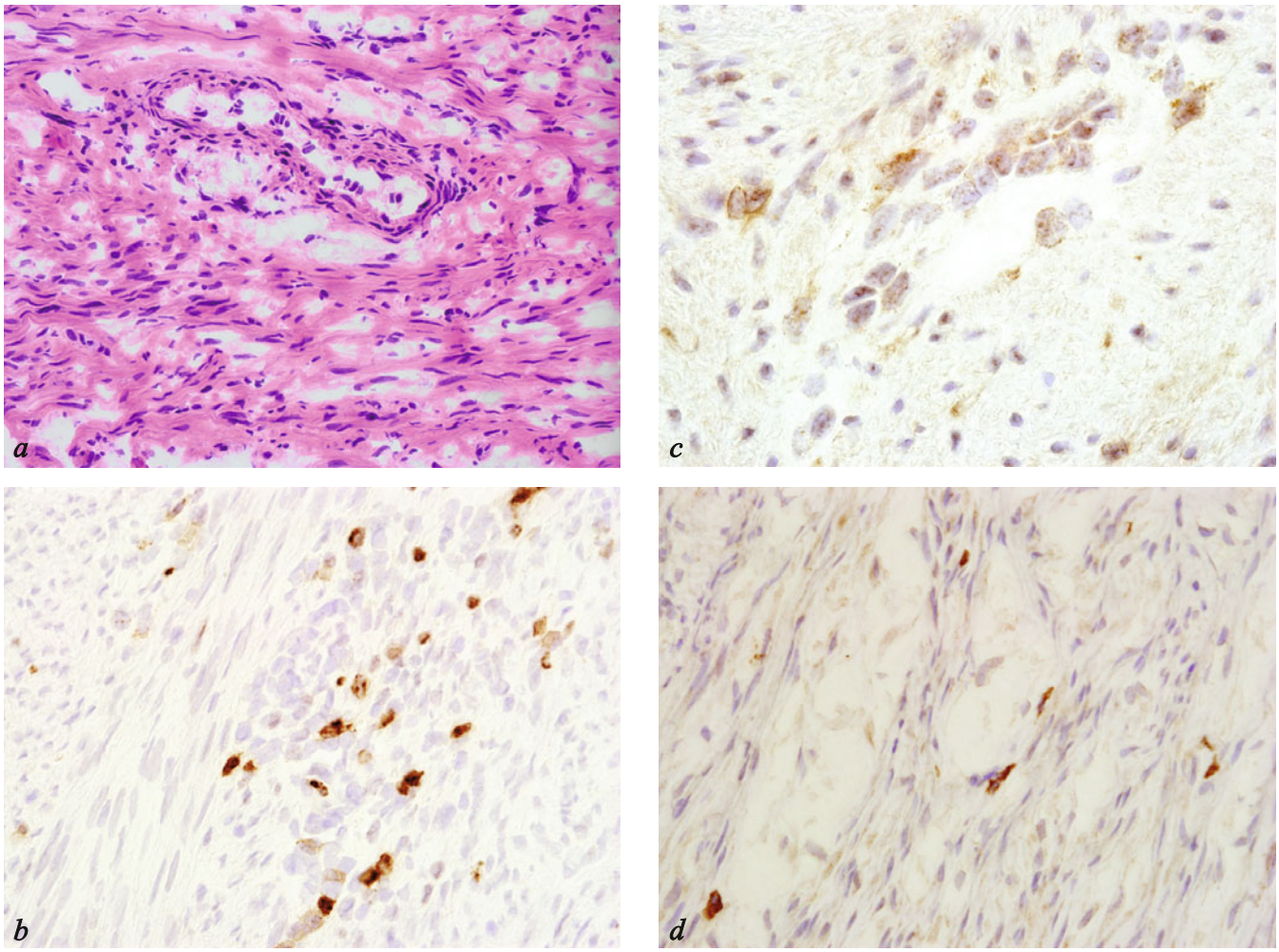

Fig. 4. Immunohistochemical (average) expression of ACA and TRA in various tissues. *1*) ACA, cytolemma; *2*) ACA, cytoplasm; *3*) ACA, nucleus; *4*) TRA cytoplasm.

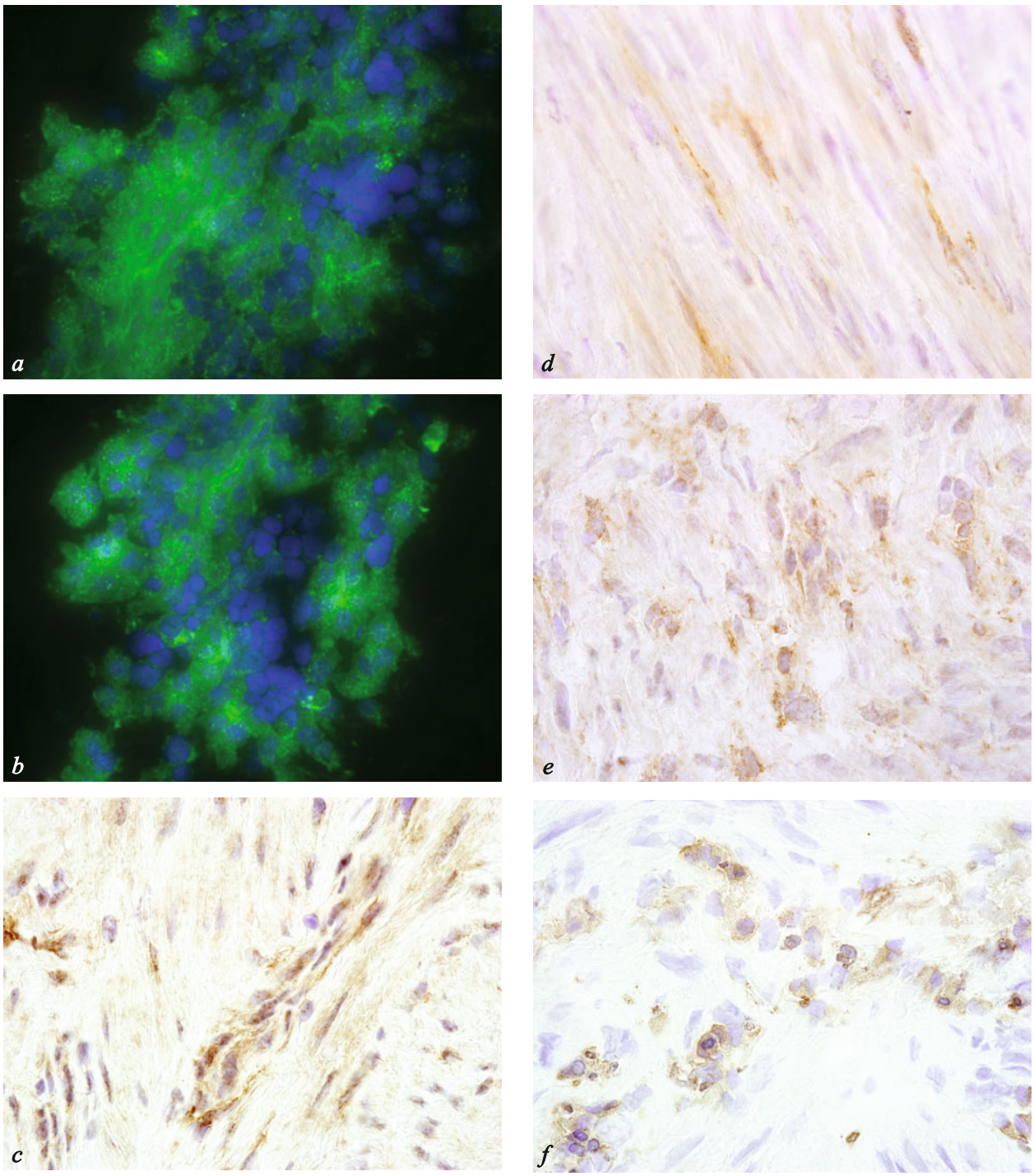

Fig. 5. ACA and TRA in uterine LM and LMS, ×1000. *a*) ACA in common LM (membrane staining; immunofluorescence); *b*) ACA in LMS (cytoplasmic staining, immunofluorescence); *c*) ACA in growth zone (membrane and immunohistochemical staining); *d*) ACA in common LM (immunohistochemical staining); *e*) ACA in LMS (immunohistochemical staining); *f*) TRA in LMS (immunohistochemical staining).

growth of tumor cells.

Protein localization in tissues and cells is of particular interest. In the tissue, the protein was localized in cuffs (growth zones); at the cellular level, membrane localization of the protein was observed in normal myometrium, and in common and cellular LM, while cytoplasmic localization was typical of mitotically active LM and LMS. It can be assumed that ACA activation is followed by its detachment from the membrane and translocation into the cytoplasm, where it triggers the mitotic signaling pathway PI3K-Akt-mTOR.

Tissue localization of ACA in growth zones also confirms its role in LM and LMS growth, which can

be related to cells with signs of stem cells (expression of CD117 – c-kit). The highest expression of ACA in LMS correlated with high expression of TRA-1-81.

These findings can be useful for the development of a new method of targeted therapy of LM and LMS based on the blockade of PI3K-AKT-mTOR signal pathway of tumor growth [3,9].

Thus, LM and LMS are characterized by dysregulation of ACA and TRA-1-81. ACA dysregulation manifests in enhanced expression of this protein during tumor growth, detachment from the plasma membrane and translocation into the cytoplasm and nuclei of tumor cells, which potentiates proliferative activity of tumor cells.

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