Экспрессия рецепторов соматостатина в адренокортикальных карциномах

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Обоснование. Адренокортикальный рак (АКР) — редкая злокачественная опухоль с ежегодной заболеваемостью 0,5—2 случая на 1 млн населения. Основным методом лечения АКР является хирургический. Если из-за распространения или прогрессирования опухоли ее полное удаление невозможно, используют митотан (o,p'DDD). При этом стабилизация и частичный эффект (по критериям RECIST) отмечен только в 48,7% случаев, что обусловливает необходимость поиска новых терапевтических мишеней.
Цель исследования — оценить экспрессию рецепторов соматостатина в адренокортикальных карциномах и опухолях коры надпочечника с неопределенным потенциалом злокачественности.
Материал и методы. Использован операционный материал опухолей коры надпочечников от 13 пациентов (4 мужчин и 9 женщин, в возрасте от 28 до 68 лет). Во всех случаях диагноз был верифицирован морфологически и иммуногистохимически (ИГХ): в 10 случаях имел место АКР (в 1 из них — метастаз АКР в печень), в 1 — онкоцитарный вариант АКР и в 2 — онкоцитома с неопределенным потенциалом злокачественности. Морфологическая оценка АКР проводилась в соответствии с критериями Weiss (для опухолей АКР) и Lin—Weiss—Bisceglia (для онкоцитарных новообразований коры надпочечников). ИГХ-исследование было выполнено с антителами к спектру тканеспецифических для надпочечника маркеров, а также Ki-67 и рецепторам к соматостатину 2-го и 5-го подтипов (РССТ2 и РССТ5).
Результаты. Экспрессия РССТ2 и/или РССТ5 была выявлена в 8 (61,5%) из 13 случаев АКР. Изолированная экспрессия РССТ2 отмечена в 4 случаях из 13, а РССТ5 — в 6 из 10, в 2 из 10 случаев наблюдалась коэкспрессия рецепторов обоих типов. Экспрессия РССТ наблюдалась как в АКР, так и в метастазе АКР в печень, а также в онкоцитарном варианте АКР.
Заключение. Экспрессия РССТ 2-го и/или 5-го подтипов в ткани АКР расширяет диагностические и прогностические возможности при данной патологии.

Ключевые слова: адренокортикальный рак, иммуногистохимическое исследование, рецепторы соматостатина.

Somatostatin receptor expression in adrenocortical carcinomas

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Background. Adrenocortical carcinoma (ACC) is a rare malignant tumor characterized by an annual incidence of 0.5—2 cases per million population. Surgery is the-first line treatment for ACC. When total tumor resection is not possible due to its proliferation or progression, mitotane (o,p'DDD) is used. In this case, stabilization and partial response (as assessed by RECIST criteria) was observed only in 48.7% of cases, necessitating the search for new therapeutic targets.
Objective — the study was aimed at assessing the somatostatin receptor expression in adrenocortical carcinomas and adrenal cortex tumors with uncertain malignant potential.
Material and methods. Surgical material from adrenocortical tumors of 13 patients (4 males and 9 females aged from 28 to 68 years) was used. In all cases, the diagnosis was verified by morphological and immunohistochemical (IHC) studies: ACC was detected in 10 cases (including 1 case of ACC liver metastasis), oncocytic carcinoma — 1 case, oncocytoma with uncertain malignant potential — 2 cases. Morphological assessment of ACC was carried out according to Weiss criteria (for ACC tumors) and Lin—Weiss—Bisceglia criteria (for oncocytic neoplasms of the adrenal cortex). IHC study was carried out with antibodies to the spectrum of adrenal cortex-specific markers, as well as Ki-67 and somatostatin receptors 2 and 5 (SSTR2 and SSTR5).
Results. The expression of SSTR2 and/or 5 was detected in 8 (61.5%) of 13 cases of ACC. Isolated SSTR2 expression was observed in 4 cases (4/13), while SSTR5 expression was observed in 6 cases (6/10). In 2 cases (2/10), co-expression of both receptor types was observed. SSTR expression was observed both in ACC and ACC liver metastasis, as well as in oncocytic ACC.
Conclusion. SSTR2 and/or 5 expression in ACC tissue expands diagnostic and prognostic capabilities for this pathology.

Keywords: adrenocortical carcinoma, immunohistochemistry, somatostatin receptors.

Background

adrenocortical carcinoma (ACC) is an uncommon neoplasm of the adrenal cortex that follows an aggressive clinical course with a poor prognosis.

The heterogeneous morphology of ACC is a frequent cause of diagnostic errors. Surgery is the first line treatment for patients with ACC. Nevertheless, delayed diagnosis, when the tumor process corresponds to III and IV grades, substantially limits good surgical outcomes. The recurrence rate after complete (R0) surgery is 85%, and the overall survival comprises only 18—48% and has not shown any significant tendency for improvement over the last 10—15 years [1—3].

Mitotane is used as adjuvant therapy for locally advanced ACC and in cases of disseminated tumoral process [4, 5]. In Russia, the efficacy of mitotane was tested in 57 cases of ACC [6]. The positive effect of stabilization

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and partial effect (according to RECIST criteria) were only noted in 48.7% of patients; the effect of therapy on patient life span was not studied.

A range of targeted drugs mediating their action through specific receptors have been proposed for medical therapy of ACC. However, their use is limited by a relatively low number of case reports and low efficacy in general [6, 7].

Hence, the limited potential and relatively low efficacy of surgery for ACC and the proposed medications dictate the need to search for new ways to improve treatment outcomes for patients with these neoplasms. One of these ways is molecular-biological studies that reveal new cellular targets for medical therapy of ACC.

The expression of somatostatin receptor (SSTR) in ACC tissue and the effect of synthetic somatostatin analogues on the growth of adrenocortical carcinoma cell lines in vitro has been recently reported [8, 9]. Nonetheless, the available data are ambiguous; this was the basis for our own research.

Purpose

to estimate the expression of SSTR subtypes 2 and 5 in ACC and adrenocortical tumors with uncertain malignant potential.

Mathods

Study design

An observational cross-sectional uncontrolled single-center pilot study included 13 patients aged 28—68 years with adrenocortical tumors.

Inclusion criteria

The study included patients older than 18 years with histological verification of ACC diagnosis or adrenocortical tumors with uncertain malignant potential.

A morphological diagnosis of adrenocortical carcinoma was established using criteria proposed by Weiss (for ACC) and Lin—Weiss—Bisceglia (for oncocytic tumors). The Weiss criteria for differential diagnosis of benign and malignant adrenocortical neoplasms include: nuclear atypia (Furman grade 3 to 4), mitotic rate higher than 5 per 50 high-power fields, atypical mitotic figures, clear cells comprising 25% or less of the tumor, diffuse architecture in more than 1/3 of the tumor, necrosis, venous invasion, sinusoidal invasion, and capsular invasion. Tumors with three or more of these criteria presented a malignant clinical outcome [10—12].

Criteria Lin—Weiss—Bisceglia for oncocytic neoplasms of the adrenal cortex are classified into major criteria (a mitotic rate of more than 5 mitoses per 50 high-power fields, atypical mitoses or venous invasion) and minor criteria (tumor size of >10 cm and/or mass >200 gr), necrosis, capsular invasion or sinusoidal invasion). The presence of any one of the major criteria indicates malignancy, the presence of one to four minor criteria is indicative of uncertain potential [10—12].

Tumor histogenesis was confirmed with immunohistochemical study (IHC) using tissue-specific markers: anti-melan-A and anti-inhibin-alpha antibodies. IHC typing using anti-synaptophysin and anti-chromogranin A antibodies was used for differential diagnosis of adrenocortical tumors from neuroendocrine neoplasms. Cases of only melan-A expression were assayed additionally with antibodies to HMB-45 and tyrosinase for exclusion of primary melanoma.

Conditions of analysis

The morphological verification of diagnosis with IHC assay was performed at the Pathologicoanatomic Department of the Moscow Regional Research Clinical Institute n.a. M.F. Vladimirskiy. The histological specimens and IHC reaction were assessed independently by two pathologists with subsequent discussion of the results and collegiate conclusion.

Length of study of the study

This study was performed from 2016 to 2017.

The main result

The main result of the study was the presence or absence of SSTR 2 and/or 5 subtype expression in the adrenocortical tissue of the tumor.

Analysis in subgroups

The patients were separated into SSTR2+ and SSTR5+ subgroups according to the expression of SSTR2 or SSTR5. In case of SSTR2 and SSTR5 co-expression, the patients were not separated into an individual group because of small case numbers (2 cases) and these two cases were included in SSTR2+ (1 case) and SSTR5+ subgroups (1 case).

Methods of outcome registration

The expression of somatostatin receptors in tumor cells was assessed using a scoring system proposed by M.Volante et al.: 0 scores — absence of immunoreactivity, 1 score — pure cytoplasmic immunoreactivity, 2 scores — complete or partial membranous reactivity in <50% of tumor cells, irrespective of the presence of cytoplasmic staining and 3 scores — circumferential membranous reactivity in >50% of tumor cells, irrespective of the presence of cytoplasmic staining. Cases with a score of 2—3 were considered as positive [13] **(Fig 1)**.

The expression was assessed and the Ki-67 index was estimated using a standard technique (the percentage of Ki-67-positive nuclei per 100 nuclei of tumor cells was counted per 500 tumor cells in high-power fields, objective x400).

IHC assay was performed using an automated Ventana Benech Ultra system (Roche, Switzerland) accord-

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ing to standard protocols using antibodies to melan-A (clone A103; dilution 1:100; CellMarque, United States), inhibin-alpha (clone MRQ-63; dilution 1:100; CellMarque, United States), synaptophysin (clone MRQ-40; dilution 1:100; CellMarque, United States), chromogranin A (clone LK2H10; dilution 1:100; CellMarque, United States), Ki-67 (clone MIB-1; dilution 1:100; Dako, United Kingdom), SSTR2 (clone EP149; dilution 1:100; Epitomics, United States), SSTR5 (clone IDUMB4, dilution 1:100; Epitomics, United Kingdom) using the REVEAL Biotin-FreePolyvalent DAB kit for visualization (Roche, Switzerland).

Ethics committee

All the patients signed a voluntary informed consent on the use of biological samples for scientific purpose and for processing of personal data. The study protocol was approved by the Ethics Committee of the Moscow Regional Research Clinical Institute n.a. M.F. Vladimirskiy, December 15, 2016 (meeting protocol No. 10).

Statistical analysis

Estimation of sample size: the sample size was not estimated preliminary.

Statistical analysis of data was performed using the STATISTICA software for Windows v.12.0 (StatSoft, United States). Quantitative data are given as median (Me), mode (Mo) and quartile (25 and 75 percentiles, Q25—75), qualitative data — as percentage.

The differences between two independent groups for quantitative variables were compared using Mann-Whitney test; for qualitative variables — using two-tailed exact Fisher's test. The correlation between the variables was estimated using the Spearman correlation coefficient (r_s). A $p<0.05$ value was significant.

Results

Subjects (participants) of the study

The study included 13 patients (4 men and 9 women) aged 28 to 68 years with adrenocortical tumors (Me=46; [38;55]).

Adrenocortical neoplasms were 10 primary ACC (among these ACC there was 1 case of adrenal oncocytic carcinoma), 1 ACC liver metastasis, and 2 adrenal oncocytomas with uncertain malignant potential. In 3 cases, ACC demonstrated glucocorticoid overproduction with Cushing syndrome; there was 1 case of concurrent glucocorticoid and androgen overproduction and 8 cases of non-hormone producing tumors (6 carcinomas, 2 oncocytomas). The functional activity associated with a neoplasm was impossible to assess reliably in 1 case **(Table 1)**.

Main results of the study

This study included 9 ACC with Weiss scores from 3 to 8 (Me=5; Mo=5; 25;75Q [4, 6]), 1 oncocytic carcinoma (1 major criterion — atypical mitosis), 2 oncocytomas with uncertain malignant potential (1 and 2 minor criteria; in the first case — sinusoidal invasion, in the second case — sinusoidal invasion and capsular invasion) and 1 case of ACC liver metastasis. In IHC study, the expression of tissue-specific marker melan-A was revealed in 100%, inhibin-alpha — in 54% tumors. Synaptophysin was expressed in 90%; the expression of chromogranin A was not revealed in any tumor.

The Ki-67 proliferation index of ACC varied from 5 to 30% (Me=21; [8;24]) and it was 6% and 12% in oncocytomas with uncertain malignant potential **(Table 1)**. The expression of SSTR 2 was revealed in 8 tumors (8/13; 61.5%): in four samples — 1 score and in four samples — 2 scores. The expression with a score of 3 was not revealed in any case **(Table 1)**.

The expression of SSTR2 was assessed in 13 cases and that of SSTR5 — in 10.

The expression of SSTR5 was revealed in 9 (9/10; 90%) samples with 1 score — in 3 cases, with 2 scores — in 1 case, and with 3 scores — in 5 cases **(Table 1)**.

Since the expression with a score of 2 and 3 is diagnostically significant, the reaction with anti-SSTR2 antibodies was regarded positive in 4 cases (31%) and anti-SSTR5 antibodies — in 6 (60%). Co-expression of both types of SSTR was revealed in 2 cases **(Table 1)**.

The expression of SSTR was diffuse in most tumors (60% cases): 20% of patchy and 20% of focal SSTR expression (in individual cells or small groups). In two latter cases, the expression was considered as positive since >30% cells in high-power field were immunopositive **(Table 1)**.

The expression of SSTR was detected both in primary ACC, including adrenal oncocytic carcinoma and in ACC liver metastasis **(Fig. 2)**.

Discussion

Summary of the main study result

This pilot study on 13 patients has shown that the presence of SSTR expression in ACC of different morphological patterns, including in adrenal oncocytic carcinoma, and in ACC liver metastasis. The tumors demonstrated more frequent and marked expression of SSTR5 than SSTR2.

Discussion of the main study result

The recommended tissue-specific markers of the adrenal cortex include SF1 (steroidogenic factor-1), inhibin-alpha and melan-A. Melan-A and inhibin-alpha were used in this study. Our data showed the expression of melan-A in 100% of assessed tumors, whereas the expression of inhibin-alpha was only observed in 54%. Weissferdt et al. studied the immunophenotype of 40 ACC and positive staining was observed for inhibin-alpha in 92.5%, synaptophysin — in 72.5%, and melan-A — in

Clinical-morphological characteristics of the patients

Case number	Clinical data				Morphological findings				
	Gender	Age, years	Hormonal activity	Diameter of a tumor, cm	Morphological diagnosis	Scores	SSTR2	SSTR5	Ki-67, %
1	f	38	Cushing syndrome	8.5	ACC	8*	2	0	23
2	m	44	NHPT	11	ACC	4*	0	1	8
3	m	28	ND	ND	ACC	5*	2	2	24
4	f	44	Cushing syndrome	8.5	ACC	6*	1	1	30
5	f	49	NHPT	11	ACC with focuses of oncocytic differentiation	5*	1	3	7
6	f	38	NHPT	15	ACC	5*	0	3	16
7	f	45	Cushing syndrome	3.5	ACC	3*	1	3	8
8	f	56	NHPT	9	ACC	6*	1	1	21
9	m	36	NHPT	5	ACC	4*	2	ND	26
10	f	60		ACC liver metastasis	Cushing syndrome + virilism	2*	2	3	24
11	f	40	NHPT	4.5	Oncocytic ACC	1 major criterion **	0	3	5
12	m	55	NHPT	8	Oncocytoma with uncertain malignant potential	1 minor criterion**	0	ND	6
13	f	68	NHPT	7	Oncocytoma with uncertain malignant potential	2 minor criteria**	0	ND	12

Note: ACC — adrenocortical carcinoma; NHPT — non-hormone producing tumor; SSTR2 — somatostatin receptors subtype 2; SSTR5 — somatostatin receptors subtype 5; Ki-67 — proliferation index Ki-67 (clone MIB-1); ND — no data; *Weiss scoring; ** Lin—Weiss—Bisceglia scoring

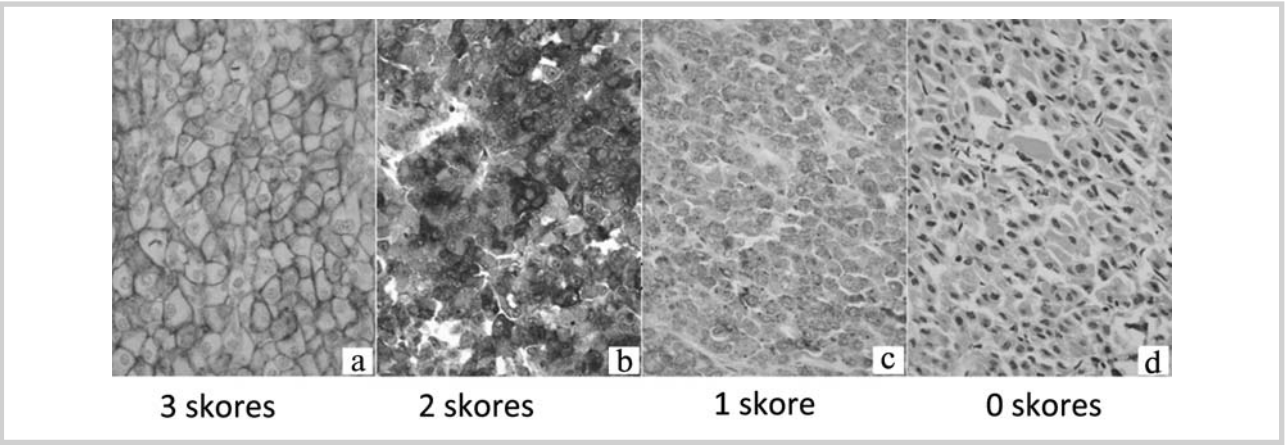

Fig. 1. **Assessment of somatostatin receptor expression in scores in adrenocortical tumors. A. 3 scores: circumferential membranous reactivity in >50% of tumor cells (x400); B. 2 scores: membranous-cytoplasmic reactivity, mostly incomplete membranous expression in <50% of tumor cells (x400); C. 1 score: pure cytoplasmic immunoreactivity (x400); D. 0 scores: absence of immunoreactivity (x400).**

65% cases [14]. It is suggested that the differences in the expression of tissue-specific markers are associated with different numbers of case descriptions and distinct histogenesis of adrenocortical neoplasms. In the normal adrenal cortex, positive staining is mainly confined to the zona reticularis [15].

The most widely used system for morphological diagnosis of ACC is based on the Weiss scoring scale for assessing microscopic criteria and estimation of the total index [12]. Oncocytic ACC is a rare adrenal carcinoma which has been insufficiently studied. Oncocytomas are adrenocortical tumors with diffuse architecture, large cells with abundant dense eosinophilic cytoplasm, patchy nuclear atypia and prominent nucleoli [12, 16]. The scoring scale of morphological evaluation of malignancy proposed by Weiss is not suitable for diagnosis of adrenal oncocytic carcinomas and the Lin—Weiss—Bisceglia system of morphological stratification was specifically proposed to define oncocytic ACC [12]. According to these criteria, the tumors in our study were verified as ACC in 9 cases, oncocytic carcinoma — in 1 case, oncocytoma with uncertain malignant potential — in 2 cases.

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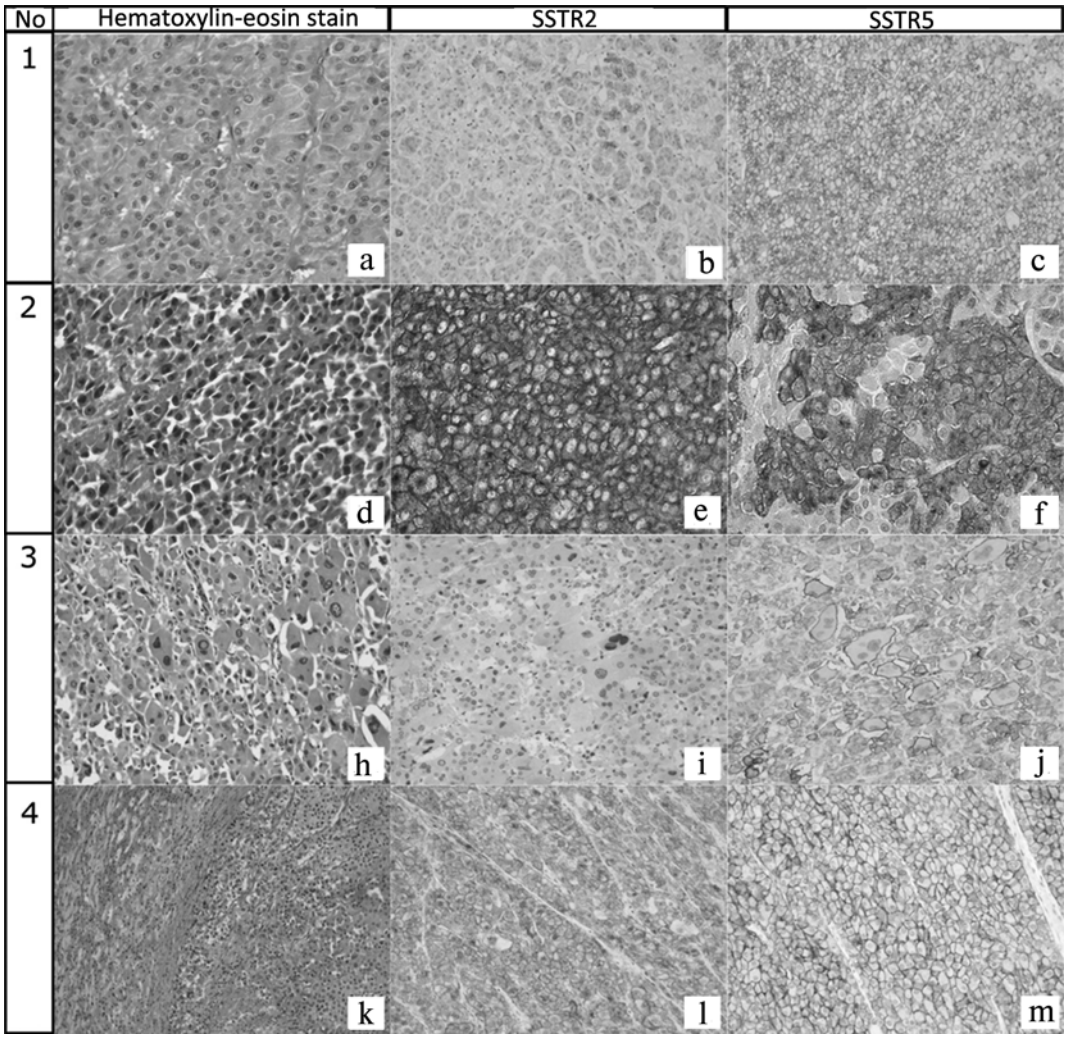

Fig. 2. **The expression of somatostatin receptors in ACC. No.1 (A—C). Solid-trabecular ACC (hematoxylin-eosin stain, x200); B. Patchy cytoplasmic expression of SSTR2 (1 score) (x200); C. Diffuse circumferential membranous expression of SSTR5 (3 scores) (x100). No. 2 (D—F). D. Solid ACC (hematoxylin-eosin stain, x200); E. Diffuse cytoplasmic and incomplete membranous expression of SSTR2 (2 scores) (x200); F. Patchy incomplete membranous-cytoplasmic expression of SSTR5 (2 scores) (x200). No. 3 (H—J). H. Oncocytic ACC (hematoxylin-eosin stain, x200); I. Expression of SSTR2 is absent (0 scores) (x200); J. Diffuse complete membranous expression of SSTR5 (3 scores) (x200). No.4 (K—M). K. ACC liver metastasis (hematoxylin-eosin stain, x100); L. Diffuse incomplete membranous-cytoplasmic expression of SSTR2 (2 score) (x200); M. Diffuse circumferential membranous expression of SSTR5 (3 scores) (x200).**

Liver metastasis from ACC was diagnosed in 1 case based on anamnesis data and IHC data with tissue-specific markers. As has been demonstrated in practice, morphological diagnosis and evaluation of the malignancy grade of oncocytic adrenocortical carcinomas pose a significant challenge; in some cases, an inaccurate diagnosis can result in improper management of the patients.

The detection results of different SSTR subtypes in ACC depend on the method used for their identification (polymerase chain reaction, IHC). The results of IHC method are affected by the chosen antibodies (manufacturer, clonality), detection systems, and assessment criteria in IHC reaction. To illustrate, Germano et al. observed the expression of SSTR2 in 29% (17/58) and that of SSTR5 — in 35% (19/55) of ACC [9]. An earlier paper by Mariniello et al. revealed SSTR2 in 100% (6/6) of ACC and SSTR5 — in 83% (5/6) [8]. Unger et al. demonstrated the expression of SSTR2 in 12% of 25 ACC and SSTR5 — in 24% [17]. In our study, a diagnostically significant expression of SSTR was revealed in 61.5% (8/13) tumors; in addition, their co-expression was noted (SSTR2+/SSTR5+) in 2 cases. The expression of SSTR5 was observed 1.5 times more frequently than that of SSTR2; however, this difference was not significant ($p=0.28$), which can be accounted for by a small sample size. Noteworthy, the expression of SSTR5 was significantly more marked ($p=0.048$) with a score of 3 in 5 cases, whereas the maximum level of SSTR2 expression had

a score of 2. It is suggested that a lower frequency of SSTR2 expression in the tumors under study is associated with the effect of hypercorticism. De Bruin et al demonstrated that in human neuroendocrine tumor cell lines the expression of SSTR2 was strongly down-regulated by excessive glucocorticoid levels and SSTR5 expression was less significantly downregulated. The same study also noted the reappearance of SSTR2 expression within 2—4 days upon glucocorticoid withdrawal [18]. However, in our group, Cushing syndrome was confirmed with laboratory methods in 4 patients with ACC, the expression of SSTR2 was revealed in 2 of these patients.

Therefore, according to most studies, SSTR5 is more frequently expressed than SSTR2 in ACC in contrast to neuroendocrine tumors of different locations, which display a significant dominance of SSTR2 with a score of 2 and 3 [19].

Germano et al. assessed 58 ACC and showed that the presence of SSTR5 is associated with the worst prognosis [9]. We estimated the correlation of SSTR2 and SSTR5 expression with the Ki-67 proliferation index. High Ki-67 values are a poor prognostic factor. The Ki-67 proliferation index varied from 23 to 26% in ACC expressing SSTR2 and from 5 to 24% — in ACC expressing SSTR5. The expression of SSTR5 negatively correlated with the Ki-67 index value (r_s = -0.32), whereas the expression of SSTR2 correlated with higher values of this index (r_s =0.625). These contradictory data can be due to the small sample size and difficulties in Ki-67 estimation. In addition, recent papers showed substantial variations for Ki-67 index values in ACC estimated by different authors and for estimations made by one pathologist [20].

The assessment of the status of somatostatin receptors in ACC and adrenocortical tumors with uncertain malignant potential is a preparatory step for searching of new targets and treatment options for ACC. Further studies with inclusion of a larger number of cases are needed.

The use of somatostatin analogues in patients with ACC has long been regarded as a method to correct hormonal overproduction. According to some data, somatostatin analogues significantly reduce hormones production; this effect was absent according to other data [21,22]. Each study analyzed a subset of ACC that was very heterogeneous in histology and immunophenotype. Herewith, the probability of diagnostic errors is high that highlights the need for very accurate verification of adrenocortical tumors.

Somatostatin analogues due to their anti-proliferative action have recently emerged as a treatment option in ACC. Downregulation of proliferative activity in tumor cells mediated by SSTR upregulation can be associated with the inhibition of the secretion of growth factors and different trophic hormones (STH, IGF1, insulin, gastrin, epidermal growth factor) by tumor cells and the extracellular matrix [23]. Upregulation of SSTR2 can trigger apoptosis in tumor cells in a p53-independent manner. Activation of SSTR2 and SSTR5 can arrest cell cycle through the induction of cyclin-dependent kinase inhibitor 1B (p27 ^{Kip1}), Rb hypophosphorylation, downregulation of cyclin/cdk2 and guanylyl cyclase [24].

Somatostatin agonists can also affect angiogenesis in a tumor and inhibit the release of proangiogenic factors and expression of relevant receptors, such as the vascular endothelial growth factor (VEGF) and its receptor -2 (VEGFR-2), fibroblast growth factor (bFGF), growth hormone/insulin-like growth factor-I (IGF-1) [24] and others.

The application of somatostatin analogues in ACC, alone or combined with other drugs, was considered by many authors. Ziegler et al. showed a significant influence of RC-160 (vapreotide) on growth and survival of tumor cells in SW-13 culture (mitotane-insensitive ACC) [25]. However, Mariniello et al. assessed the influence of pasireotide (SOM 230) on ACC cell lines (primary culture and SW13 and H295R lines) and concluded that somatostatin analogue inhibited hormone secretion in ACC cells but had no evident effect on cell viability [8]. A later paper by Germano et al. also did not reveal the effect of pasireotide on cell viability in mitotane-sensitive (H295R) and mitotane-insensitive (SW13) ACC cell lines. Meanwhile, mitotane and pasireotide induced a synergistic inhibition of SW13 (mitotane-resistant) cell growth. Conversely, the combinations among mitotane and pasireotide produced antagonistic effects on mitotane-induced growth inhibition on H295R mitotane-sensitive cell line [9].

Limitations of the study

The team of the authors admits that the small sample size had certain effect on the evidence of these results. Nevertheless, the specificity of the reaction with anti-SSTR antibodies gives no doubts as to the quality of IHC assay and the presence of SSTR, at least, in certain part of ACC.

Conclusion

the presence of SSTR in ACC and adrenocortical tumors with uncertain malignant potential has been revealed. The expression of SSTR5 dominated in these tumors. The data can be a basis for further study of SSTR expression in carcinomas of the adrenal cortex to analyze its correlation with morphology and immunohistochemistry of the tumors and prognostic role.

Additional information

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Conflict of interest. The authors declare that they have no evident and potential conflicts of interest related to the publication of this paper.

Participation of the authors: Voronkova I.A. — accomplishment of histological and immunohistochemical assays and data assessment, data processing, paper writing and preparation of illustrations; Gure-

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