Comparative Analysis of Cells with Combined Apoptosis and Proliferation Markers in Thyroid Tissue Specimens from Patients with Cancer, Adenoma, and Autoimmune Diseases

S. P. Kazakov, T. N. Zabotina*, O. V. Korotkova*, A. K. Dzabarov*, and I. A. Kazantseva**

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Combined phenotypes of cells with membrane and intracellular expression of apoptosis and proliferation regulation markers (p53, bcl-2, CD95, CD95L, Ki-67) were studied by flow cytometry of cell suspension from thyroid tissue specimens from patients with autoimmune diseases, adenoma, and thyroid cancer. The incidence of cell groups with phenotypes p53/Ki-67, p53/CD95, bcl-2/Ki-67, bcl-2/CD95, CD95/Ki-67, p53/CD95L, CD95/CD95L, and bcl-2/CD95L was evaluated and the density of receptor distribution on/in each cell group are presented. Patients with autoimmune diseases had high incidence of cells with phenotypes p53/Ki-67, p53/CD95, bcl-2/Ki-67, bcl-2/CD95, CD95/Ki-67, p53/CD95L, CD95/CD95L, and bcl-2/CD95L; cells with the bcl-2/CD95 phenotype were the most incident. Patients with thyroid adenoma had high levels of cells with p53/CD95L phenotype, while patients with thyroid cancer had significantly lower levels of p53 expression in the p53/CD95L cell group. The density of CD95L receptors on CD95/CD95L-positive cells was 4-7-fold higher in patients with thyroid tumors; the density of CD95L receptors on CD95/CD95L cells was maximum in thyroid adenoma and minimum in thyroid cancer. These data indicate differences in the expression of apoptosis and proliferation markers in thyroid adenoma, cancer, and autoimmune diseases. Analysis of the expression of these markers in the above diseases can be useful for differential diagnosis.

Key Words: *thyroid cancer; p53; bcl-2; CD95; CD95L; Ki-67*

Flaws in the knowledge of the pathogenesis of autoimmune diseases and tumors of the thyroid impede the development of highly informative methods for differential diagnosis and monitoring of therapy of patients with these diseases [1-4,6]. The role of apoptosis and proliferation markers in the development of autoimmune and tumor processes in the thyroid has been in the focus of attention in recent years [5,7-10].

MATERIALS AND METHODS

Thirty-seven patients (32 women and 5 men) were examined: 9 with autoimmune diseases of the thyroid (AIDT; Hashimoto and Graves diseaes), 12 with thyroid adenoma (TA), and 16 with thyroid papillary carcinoma (TPC). The mean age of the patients was 53.0±14.0 years.

N. N. Burdenko Military Clinical Hospital, Ministry of Defense of the Russian Federation; *N. N. Blokhin Russian Cancer Center, Russian Academy of Medical Sciences, Moscow; **M. F. Vladimirsky Moscow Regional Institute, Moscow, Russia. ***Address for correspondence:*** biochimia@mtu-net.ru. N. E. Kushlinsky

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Specimens of thyroid tissue were collected during surgery. Cell suspensions were prepared by common methods (0.25% trypsin solution and 1 mM EDTA in PBS) and brought to a final concentration of $1\text{-}2\times10^6$ cell/ml. The membrane and intracellular receptors were stained with monoclonal antibodies Bcl-2-FITC, CD95-PE-Cyc5, CD95L-PE-Cy5 (CALTAG), and p53-FITC, Ki-67-PE (Beckman Coulter, BD Biosciences). The resultant suspension was analyzed in three-color protocols on a COULTER EPICS XL-MCL flow cytofluorometer (Beckman Coulter). At least 100,000 cells in the gating zone were evaluated. The numerical data in the form of LMD files were analyzed using CXP 2.2 analytical software. The percentage of eight small group of cells (p53/Ki-67, CD95/p53, bcl-2/Ki-67, CD95/bcl-2, CD95/Ki-67, CD95L/p53, CD95L/CD95, CD95L/bcl-2) and distribution densities of membrane and intracellular receptors with combinations of apoptosis (CD95, CD95L, bcl-2, p53) and proliferation (Ki-67) markers in these cell groups were evaluated. Total densities of membrane and intracellular receptor expression were evaluated in arbitrary units by the mean fluorescence intensity (MFI) proportional to the channel number measured in the logarithmic mode.

The data were statistically processed using nonparametric Mann–Whitney methods. The differences were considered significant at $p<0.05$.

RESULTS

The relative content of cell groups and distribution densities of the main receptors (proteins) with combinations of at least two apoptosis and proliferation markers were analyzed in patients with AIDT, TA, and TPC (Table 1).

The content of p53/Ki-67 cells was the maximum in patients with AIDT (5.74±3.33%), minimum in TPC (0.96±0.37%), and medium in patients with TA (1.17±0.60%). The patients with TPC and AIDT differed significantly by the levels of p53/Ki-67 cells (p=0.0001) and distribution densities of p53 (p=0.0001) and Ki-67 (p=0.002). A relationship between TA and AIDT groups by the levels of p53/Ki-67 cells was detected (p=0.001). Statistically significant difference between TPC and TA groups was revealed for the density of p53 expression (p=0.032) in this cell group.

The content of p53/CD95 cells was maximum in tissues from patients with AIDT (9.81±2.10%), the minimum in TA (2.57±2.02%), and somewhat higher in TPC (2.72±2.24%). Significant differences between TPC and AIDT patients were detected for the content of p53/CD95 cells (p=0.0001) and density of receptors/proteins on/in cells of this group: p=0.0001 for p53 and p=0.0001 for CD95. The counts of p53/CD95 cells (p=0.0001) and CD95 receptor expression density (p=0.0001) on p53 cells differed significantly in patients with TA and AIDT. No appreciable differences in the counts of p53/CD95 cells in patients with TPC and TA were detected.

The maximum levels of bcl-2/Ki-67 cells were found in patients with AIDT (7.02±1.29%), minimum (almost undetectable) in TPC patients (0.42±0.08%), and somewhat higher levels in TA patients (0.56±0.27%). The levels of cells of this phenotype (p=0.0001) and the distribution densities of proteins (p=0.0001 for bcl-2 and p=0.0001 for Ki-67) differed significantly in patients with TPC and AIDT. Patients with TA and AIDT differed significantly by the levels of bcl-2/Ki-67 cells (p=0.0001). The densities of bcl-2 protein expression in cells with bcl-2/Ki-67 phenotype differed significantly (p=0.0001) in patients with TPC and TA.

The maximum level of cells with bcl-2/CD95 markers (Fig. 1) was detected in patients with AIDT (47.2±1.82%), low levels in patients with TPC and TA (1.19±0.59 and 1.87±0.57%, respectively).

Patients with TPC and AIDT differed by the levels of bcl-2/CD95 cells (p=0.0001). Significant differences in the levels of bcl-2/CD95 cells (p=0.0001) and bcl-2 protein expression density (p=0.019) were found between the TA and AIDT groups. The groups with TPC and TA differed significantly (p=0.02) by the densities of bcl-2 protein expression on CD95/bcl-2 cells.

The level of cells with CD95/Ki-67 markers was the minimum in patients with TA (0.24±0.21%) and differed slightly from that in patients with TPC (0.29±0.23%). The highest level of these cells (3.00±1.82%) was found in patients with AIDT. The levels of cells carrying CD95/Ki-67 markers differed significantly (p=0.0001) in the TPC and AIDT groups. The TA and AIDT patients also differed significantly (p=0.0001) by the levels of CD95/Ki-67 cells. No differences in the quantitative composition of CD95/Ki-67 cells in TPC and TA patients were detected.

The highest levels of cells carrying p53/CD95L markers (Fig. 2) were found in patients with AIDT (9.46±1.61%). A lesser level of p53/CD95L cells (3.33±0.54%) was found in TA patients and the minimum level (2.18±0.59%) in TPC patients. Patients with TPC and TA differed significantly by the levels of p53/CD95L cells (p=0.005) and density of p53 expression in these cells (p=0.02). Patients with TPC and AIDT differed significantly by the levels of p53/CD95L cells (p=0.0001) and density of p53 in p53/CD95L cells (p=0.0001). Patients with TA and AIDT differed significantly by the levels of p53/CD95L cells (p=0.0001) and by the density of p53 distribution in p53/CD95L cells (p=0.001).

TABLE 1. Cell Group Levels and Receptor Densities in and on the Cells with Apoptosis and Proliferation Marker Combinations in Thyroid Tissue in Different Thyroid Diseases

Parameter	Groups of patients		
	AIDT (n=9)	TA (n=12)	TPC (n=16)
Percentage of p53/Ki-67 cells	5.74±3.33	1.17±0.6*	0.96±0.37*
p53 density	9.94±0.68	8.74±1.68	5.91±1.14*+
Ki-67 protein density	3.68±1.69	2.50±3.55	1.61±0.16*
Percentage of p53/CD95 cells	9.81±2.10	2.57±2.02*	2.72±2.24*
p53 density	9.83±0.56	8.53±1.31	7.88±0.93*
CD95 receptor density	2.77±0.08	3.71±0.43*	4.79±1.36*
Percentage pf bcl-2/Ki-67 cells	7.02±1.29	0.56±0.27*	0.42±0.08*
bcl-2 protein density	8.47±0.53	6.11±3.10	2.76±0.24*+
Ki-67 protein density	2.98±0.05	2.04±3.49	1.23±0.22*
Percentage of bcl-2/CD95 cells	47.20±1.82	1.87±0.57*	1.19±0.59*
bcl-2 protein density	9.08±0.57	10.91±0.70*	9.12±1.04+
CD95 receptor density	5.98±0.89	7.52±3.80	6.33±1.65
Percentage pf CD95/Ki-67 cells	3.00±1.82	0.24±0.21*	0.29±0.23*
CD95 receptor density	9.15±2.83	6.52±2.18	7.31±0.81
Ki-67 protein density	2.75±0.58	2.55±0.57	2.32±0.28
Percentage of p53/CD95L cells	9.46±1.61	3.33±0.54*	2.18±0.59*+
p53 density	9.14±0.39	6.87±0.55*	5.41±0.76*+
CD95L receptor density	6.42±0.29	5.96±4.27	4.91±2.14
Percentage of CD95/CD95L cells	8.04±1.04	0.85±0.52*	0.95±0.61*
CD95 receptor density	5.30±2.11	8.27±5.05	7.80±5.31
CD95L receptor density	8.62±3.53	61.77±11.92*	37.51±11.97*+
Percentage of bcl-2/CD95L cells	12.19±1.62	2.35±0.83*	2.59±0.55*
bcl-2 protein density	10.95±0.58	11.17±3.87	10.08±0.46
CD95L receptor density	2.37±0.22	5.14±0.52*	6.40±0.44*+

Note. p<0.05 compared to: *AIDT group, +TA group.

The level of cells with CD95/CD95L membrane markers was the minimum (0.85±0.52%) in patients with TA. This value was higher in patients with TPC (0.95±0.61%) and virtually 8-fold higher in AIDT patients (8.04±1.04%). Patients with TPC and TA differed significantly by the expression of CD95L receptors (p=0.007) in CD95/CD95L cells. Significant differences were detected in the levels of CD95/CD95L cells (p=0.0001) and density of CD95L receptor expression on CD95/CD95L cells (p=0.0001) in patients with TPC and AIDT. Patients with TA and AIDT differed significantly by the levels of CD95/CD95L cells (p=0.0001) and density of CD95L receptor expression on CD95/CD95L cells (p=0.001).

Very high levels of bcl-2/CD95L cells (12.19±1.62%) were found in patients with AIDT. The levels of these cells were lower in patients with TPC and TA (2.59±0.55 and 2.35±0.83%, respectively). Patients with TPC and TA differed significantly by the density of CD95L receptor distribution on bcl-2/CD95L cells (p=0.004). The significance of differences in the counts of bcl-2/CD95L cells (p=0.0001) and in the expression density of CD95L receptors on bcl-2/CD95L cells (p=0.0001) in TPC *vs.* AIDT patients has been confirmed. Relationships between TA and AIDT patients for the levels of bcl-2/CD95L cells (p=0.0001) and densities of CD95L receptor distribution on bcl-2/CD95L cells (p=0.0001) have been proven.

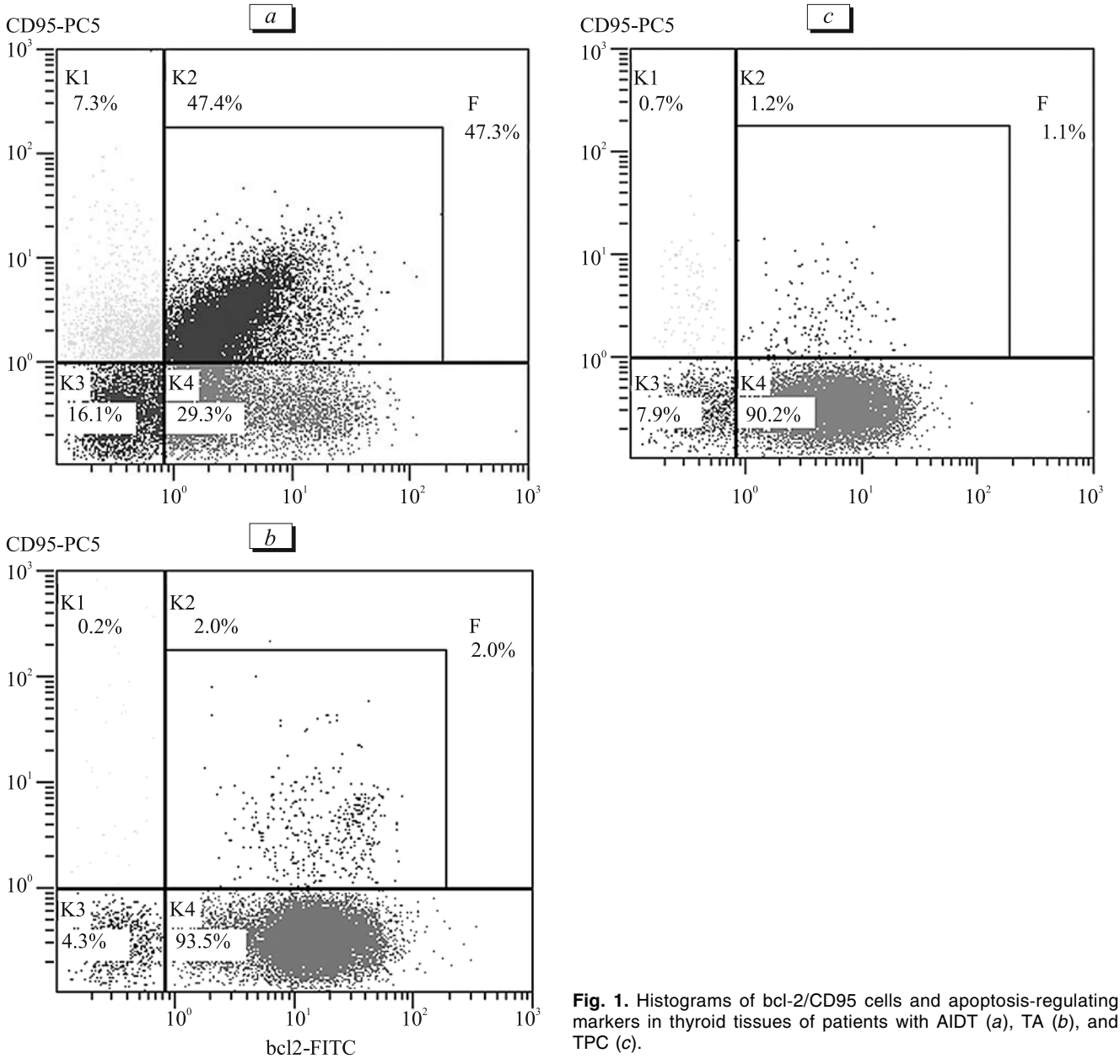

Fig. 1. Histograms of bcl-2/CD95 cells and apoptosis-regulating markers in thyroid tissues of patients with AIDT (*a*), TA (*b*), and TPC (*c*).

Hence, evaluation of the percentage of cells expressing combined apoptosis and proliferation markers provides additional information about the levels of thyroid tissue cells presumably essential for the pathogenesis of AIDT, TA, and TPC. The levels of all 8 cell groups were significantly higher in patients with AIDT. The cell group with bcl-2/CD95 phenotype was particularly interesting, because the level of these cells was 25 times higher than in the total group of patients with thyroid tumors. Hence, quantitative analysis of cells with p53/CD95L phenotype can be used for the diagnosis of benign and malignant tumors of the thyroid.

Measurements of the levels of cells expressing combined apoptosis and proliferation markers provides additional information on the levels of cells presumably significant for the pathogenesis of this organ's diseases. Analysis of these cell groups revealed an increase in their levels in tissues from patients with AIDT, TA, and TPC, this increase being usually paralleled by reduction of total density of receptor distribution in the studied cell group. The most significant criterion for comparative characterization of these cell groups in thyroid diseases is a higher level of combined phenotype cells in thyroid tissues, paralleled by higher density of receptor expression in the studied cells.

Comparative analysis of the values in patients with AIDT and thyroid tumors (TA and TPC) showed some characteristic features of the quantitative composition of combined phenotype cells and density of

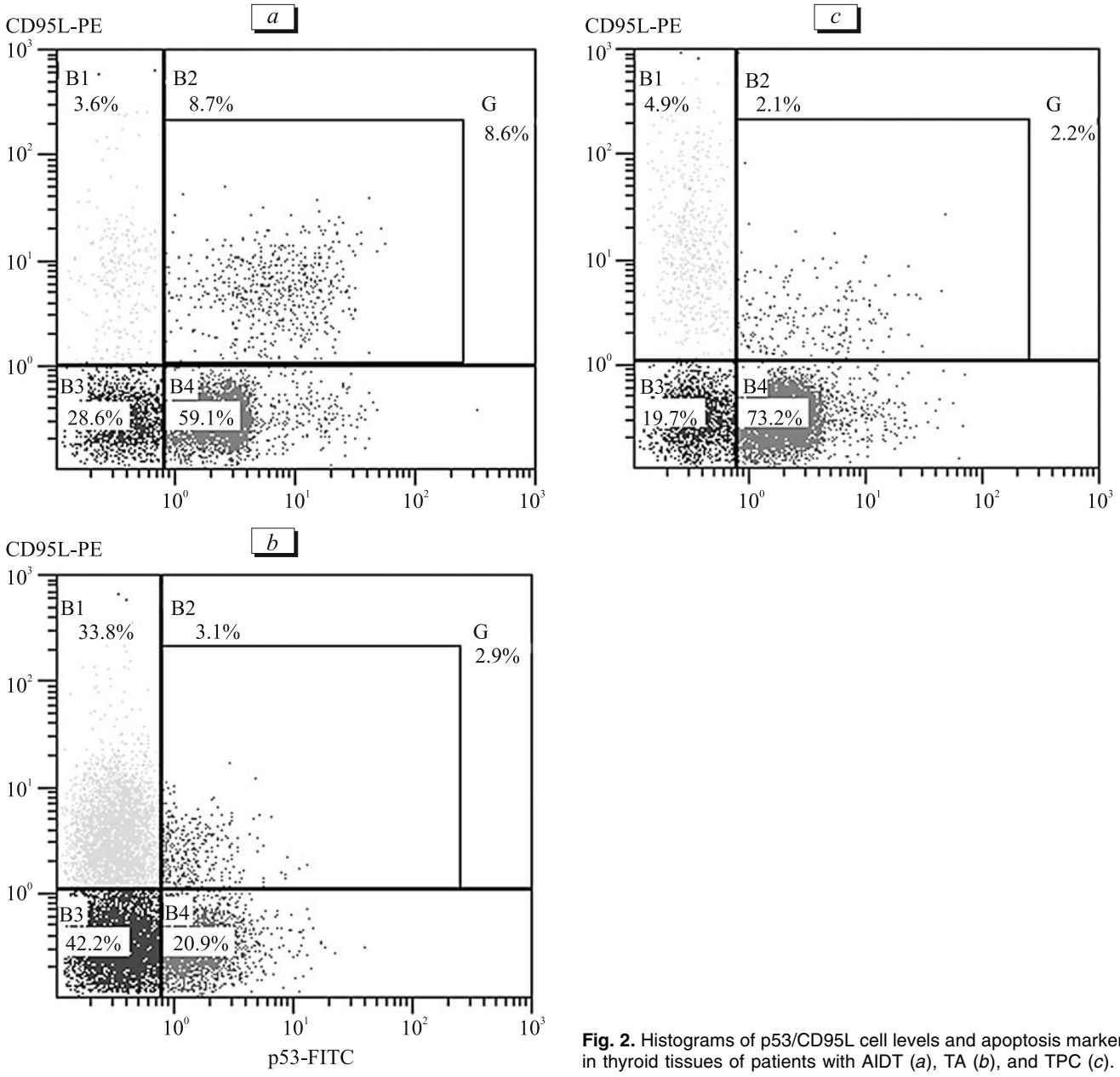

Fig. 2. Histograms of p53/CD95L cell levels and apoptosis markers in thyroid tissues of patients with AIDT (*a*), TA (*b*), and TPC (*c*).

the receptor distribution in them. It was found that the levels of all groups of combined phenotype cells (p53/Ki-67, p53/CD95, bcl-2/Ki-67, bcl-2/CD95, CD95/Ki-67, p53/CD95L, CD95/CD95L, bcl-2/CD95L) in patients with AIDT were 3-25 times higher than in patients with thyroid tumors. The greatest differences (more than 25 times) between patients with AIDT and tumors were found for the bcl-2/CD95 cell group.

Patients with TA and TPC differed only by the level of p53/CD95L-positive cells. A slight increase in the percentage of p53/CD95L cells in TA patients in comparison with that in TPC suggests this parameter for additional differential diagnosis of malignant/benign tumors of the thyroid. The density of p53 expression in this cell group was significantly lower in TPC compared to TA patients. One more characteristic feature of patients with thyroid tumors was a 4-7-fold higher density of CD95L receptors on CD95/CD95L-positive cells, the density of CD95L receptors on CD95/CD95L cells being maximum in TA patients and minimum in TPC patients.

Hence, studies of new small groups of cells with combined apoptosis and proliferation markers in patients with AIDT, TA, and TPC showed the most characteristic groups of these cells for each of the studied diseases and detected high expression of receptors and proteins in them. The increase of the number of combined phenotype cell groups and density of mem-

brane receptor and intracellular protein distribution in patients with the studied thyroid diseases suggests characteristic, but heretofore unknown pathogenetic mechanisms of apoptosis and proliferation processes associated with the development of autoimmune process or blastomogenesis in this organ. The detected cell groups can be used for additional differential diagnosis of AIDT, TA, and TPC.

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