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THE PREDICTIVE VALUE OF APOPTOSIS IN HUMAN THYROID NODULES AS A RISK FACTOR FOR MALIGNANCY

Komarevtseva K.V., Komarevtseva I.A.

Lugansk State Medical University

Комаревцева Е.В., Комаревцева И.А. Прогностическое значение уровня апоптоза в узловых тканях щитовидной железы как фактора риска малигнизации // Український морфологічний альманах. – 2009. – Том 7, №2. – С. 60-63.

На сегодняшний день существующие диагностические тесты оценки узловых образований щитовидной железы не могут дать объективных прогнозов в плане возможной малигнизации узлового зоба. В связи с этим, целью нашего исследования стало изучение уровня апоптоза по ДНК-фрагментации в узловых и окружающих тканях узлового зоба опухолевого и неопухолевого генеза. Всего в процессе работы мы обследовали 71 больного узловыми зобами щитовидной железы, из них 11 больных узловым, 25 больных многоузловым эутиреоидным зобом, 6 больных узловой формой хронического аутоиммунного тиреоидита Хашимото, 14 больных раком щитовидной железы; 8 больных с рецидивом многоузлового эутиреоидного зоба; 7 больных с рецидивом смешанного кистозно-узлового эутиреоидного зоба. Детекция апоптоза осуществлялась по выявлению ДНК-фрагментации в гомогенатах узла резецированной щитовидной железы с дифениламиновым реагентом по методу Messmer U.K. в нашей модификации. Во всех клинических группах уровень апоптоза в гомогенатах узлов был ниже, чем в окружающей ткани. Минимальный уровень ДНК-фрагментации наблюдался в гомогенатах узлов полученных у больных раком щитовидной железы: рак щитовидной железы < хронический аутоиммунный тиреоидит < многоузловой эутиреоидный зоб < узловой эутиреоидный зоб. В окружающих тканях показатели ДНК-фрагментации имели другую направленность, при этом, показатели ДНК-фрагментации в гомогенатах окружающей ткани у больных хроническим аутоиммунным тиреоидитом превосходили таковые в других клинических группах в 1,5-2 раза: хронический аутоиммунный тиреоидит > рак щитовидной железы > многоузловой эутиреоидный зоб > узловой эутиреоидный зоб.

Ключевые слова: узловой зоб щитовидной железы, апоптоз

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Сучасні діагностичні тести не можуть надати надійних прогнозів щодо можливої малігнізації вузлових пухлин щитоподібної залози. У зв'язку з цим, метою нашого дослідження стало вивчення рівню апоптозу за ДНК-фрагментацією в вузлових тканинах та тканинах вузлового оточення пухлинного і непухлонного генезу. Всього ми обстежили 71 хворого на вузловий зоб щитоподібної залази, з них 11 хворих вузловим зобом, 25 хворих багатовузловим еутіреоідним зобом,6 хворих вузловою формою хронічного аутоімунного тіреоідіту Хашимото,14 хворих на рак щитоподібної залази, 8 хворих з рецидивом багатовузлового еутіреоідного зобу, 7 хворих з рецидивом змішаного кістозно-вузлового еутіреоідного зобу. Детекція апоптозу відбувалося за наявністю ДНК-фрагментації у гомогенатах вузла щитоподібної залази з дифеніламіновим реагентом за методом Messmer U.K. у нашій модифікації. У всіх клінічних групах рівень апоптозу в гомогенатах вузлів був нижче, ніж у тканинах вузлового оточення. Мінімальний рівень ДНК-фрагментації відзначався в гомогенатах вузлов хворих на рак щитоподібної залози: рак щитоподібної залози < хронічний аутоімунний тіреоідіт Хашимото < багатовузловий еутіреоідний зоб < вузловий еутіреоідний зоб. У тканинах вузлового оточення показники ДНК-фрагментації мали іншу спрямованість, при цьому, показники ДНК-фрагментації в гомогенатах у тканинах вузлового оточення у хворих хронічним аутоімунним тіреоідітом Хашимото були вище ніж у інших клінічних групах у 1,5-2 разів: хронічний аутоімунний тіреоідіт Хашимото > рак щитоподібної залози > багатовузловий еутіреоідний зоб > вузловий еутіреоідний зоб.

Ключові слова: вузловой зоб щитоподібної залози, апоптоз

Komarevtseva K.V., Komarevtseva I.O. The predictive value of apoptosis in human thyroid nodules as a risk factor for malignancy // Український морфологічний альманах. – 2009. – Том 7, №2. – С. 60-63.

The purpose of our research became studying a level of apoptosis on DNA-fragmentation in multinodular goiters and thyroid adenomas and the nonnodular part of thyroid tissue. Thyroid specimens were obtained from 71 patients with thyroid nodules (multinodular goiters, Hashmoto's thyroiditis, thyroid carcinomas) who underwent total or pertial thyroidectomy at our clinic. For quantification of apoptotic events, we measured fragmented DNA contents from total thyroid extracts by using the diphenylamine method. The extent of apoptosis was significantly associated with the type of thyroid lesion, both proliferative (multinodular goiter) and neoplastic (malignant). Low levels of apoptosis were detected in multinodular goiters and thyroid adenomas (thyroid carcinomas < Hashmoto's thyroiditis < multinodular goiters) by the diphenylamine assay. In contrast, high levels of thyrocyte apoptosis were found in the nonnodular part of thyroid tissue (Hashmoto's thyroiditis > thyroid carcinomas > multinodular goiters).

Key words: thyroid nodular goiters, apoptosis.

Nonular goiter is one of the most common endocrine diseases. The pathogenesis of nodule formation has been intensively studied [1, 5].

Thyroid nodules are frequently found in patients with multinodular goiters, Hashmoto's thyroiditis, thyroid carcinomas. Presence of these nodules raise concern about co-existent thyroid carcinoma. The reported incidence of nodules in thyroid disease varies from to 40-50%. The incidence of malignancy in these nodules ranged from 15-20%. The incidence varies in different series and seems to increase in recent reports mainly due to employment of ultrasonography in diagnostic work-up and increasing popularity of total thyroidectomy for surgical treatment [3].

Pathogenic relationship between multinodular goiters and thyroid carcinoma is still obscure. While some authors have reported that carcinoma in the background of multinodular goiters behaves more aggressively, others have not found so. There is yet no consensus on preoperative work-up and need for surgery in these cases. Though, carcinoma is the major concern, there are other causes of nodules in the thyroid diseases [8].

Increasing evidence suggests that apoptosis plays an important role in the pathogenesis of autoimmune and proliferative thyroid diseases, and that the apoptotic pathways involved are complex and highly regulated [7, 10].

Apoptosis plays a critical role in the development and homeostasis of multicellular organisms. An increased rate of apoptosis is involved in the pathogenesis of several degenerative diseases. Conversely, inhibition of apoptosis has been implicated in autoimmune diseases and carcinogenesis [6, 7].

The specific pathogenesis of nodular goiter and the role of apoptosis in goitrogenesis are not known.

The aim of this study was to establish differential apoptotic criteria between multinodular goiters and thyroid adenomas and the nonnodular part of thyroid tissue. Thyroid specimens were examined for biochemical quantification of cellular apoptosis and morphological differen-

tiation by DNA fragmentation, staining with H33342/ fluorescence microscopy, and light microscopy and phase-contrast microscopy.

Subjects and methods. In all patients thyroid nodules were detected by ultrasound. Hot and cold thyroid nodules were characterized by scintigraphy. All preoperatively identified nodules were also identified at surgery and postoperatively by histology. All patients were euthyroid at surgery. The diagnosis was based on clinical criteria and confirmed by appropriate laboratory tests (TSH, T_3, T_4).

The large majority, 85.5% were women and mean age was 40 years. All patients had had goiter for more than 5 years. Thyroid specimens were obtained from 56 patients with thyroid nodules (multinodular goiters, Hashmoto's thyroiditis, thyroid carcinomas) who underwent total or pertial thyroidectomy at our clinic. Normal thyroid tissue was obtained from patients at thyroidectomy from the uninvolved, contralateral lobes of thyroids resected for tumors or the nonnodular part of multinodular goiters.

Hoechst Staining Assay. Intact cells were stained with Hoechst 33342 (5 μg/ml) and propidium iodide (10 μg/ml) for 10 min and analyzed in a fluorescence microscope (Zeiss Axioskop) with excitation at 360 nm.

Quantitative DNA fragmentation analysis. **F**ollicular thyroid cell DNA fragmentation was assayed with the diphenylamine assay [9]. The percentage of DNA fragmentation was calculated as the ratio of the DNA content in the supernatant to the amount in the pellet.

DNA Gel Electrophoresis. Fragments of the nonnodular thyroid tissue and nodules were homogenized, fixed in 70 percent ethanol, and incubated in 40 μl of phosphate–citrate buffer (pH 7.8) for one hour. The supernatant was concentrated by vacuum and digested with RNase (1 mg per milliliter) and proteinase K (1 mg per milliliter). Samples were subjected to electrophoresis on 1 percent agarose gel containing 5 μg of ethidium bromide per milliliter.

Results. In all patients thyroid nodules were detected by ultrasound. Thyroid nodules were characterized by scintigraphy. All preoperatively identified nodules were also identified at surgery and postoperatively by histology according to the WHO criteria.

Histologically (photo 1), the enlarged gland is characterized by proliferation of several components of thyroid follicles, such as thyrocytes, fibroblasts, endothelial cells and parafollicular cells, the presence of infiltrated lymphocytes, enlarged blood capillaries, and compenents of extracellular matrixes, such as amyloid and collagen. Among these features, increased number of thyrocytes is the most common. Cells with feathered cytoplasmic edges occurred with statistically significantly different frequency between bench specimens and patient goiter specimens. Cytomorphologic features that showed differences between specimens from goiters and normal thyroid tissue included the presence of microfollicles, prominent nucleoli, abundant cytoplasm, the number of cells with paravacuolar granules, the presence of Hurthle cells, and the presence of cells with feathered cytoplasmic edges. The results of the current study indicate that there are cytologic differences between specimens from normal thyroid and from goiterous nodules. The presence of Hurthle cells, prominent nucleoli, and cells with abundant cytoplasm favored the diagnosis of nodular goiter, whereas large numbers of cells with paravacuolar granules favored a determination of histologically normal thyroid. These differences may aid in the distinction of normal thyroid tissue from hyperplastic goiters in cytologic specimens.

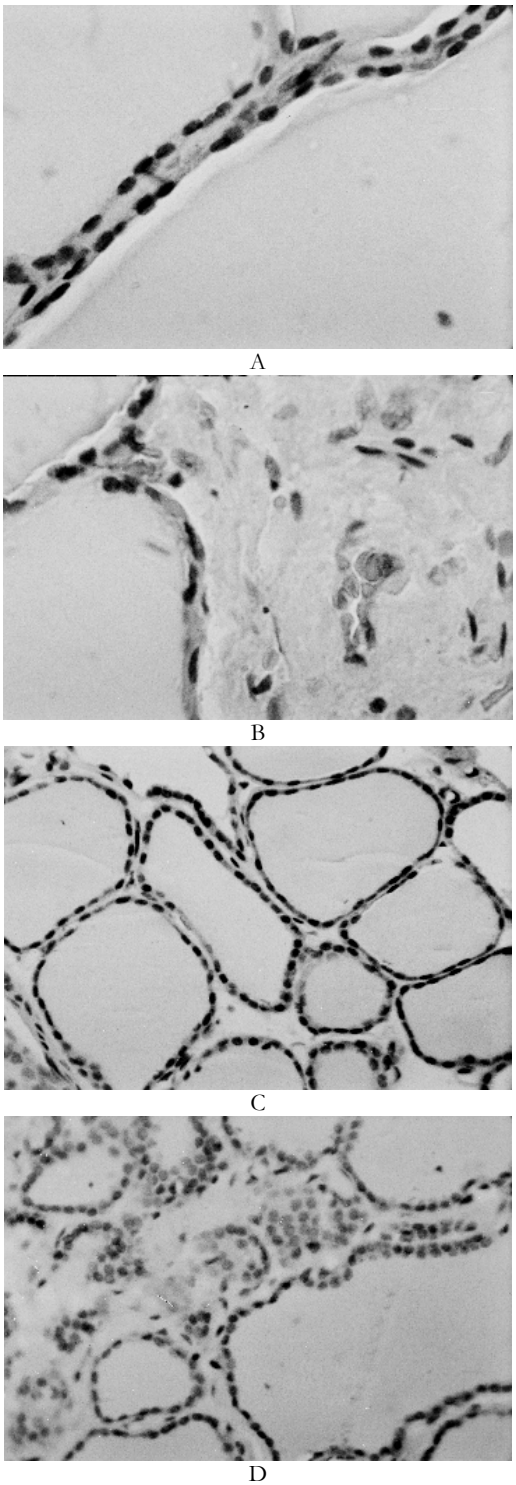


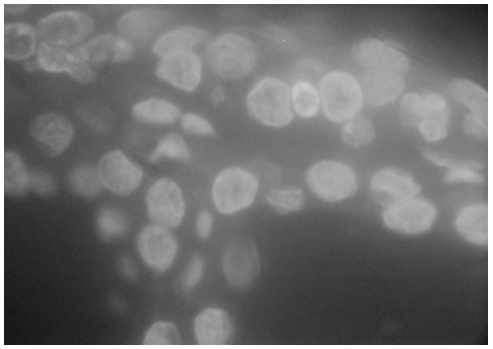
Photo 1. Photomicrographs of Specimens of Thyroid Nodules. A, B - thyroid carcinomas, C, D - multinodular goiters

The tissue mass is, however, modulated by not only cell proliferation but also cell death. Examination of thyrocyte proliferation under experimental conditions showed that the actual number of cells found at the end of the experiment is less than expected based on the proliferative activity. This discrepancy has been interpreted as a result of an extensive cell loss within the thyroid gland.

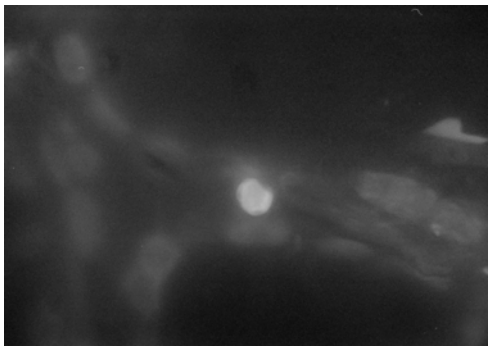
At present, the type of eukaryotic cell death is classified into apoptosis and necrosis.

Tissue homeostasis requires a proper balance between cell proliferation and cell death. Apoptosis occurs

through an evolutionarily conserved cellular program that eliminates infected or unnecessary cells and cells produced in excess or having genetic damage. Aberrant apoptosis is involved in the pathogenesis of many human diseases: abnormal cell death results in excessive parenchymal cell loss, but decreased cell death contributes to the development of hyperplasias and neoplasias.



A



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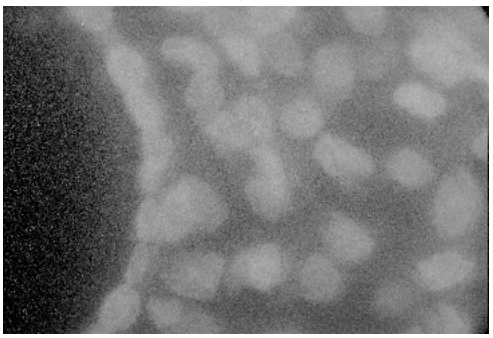

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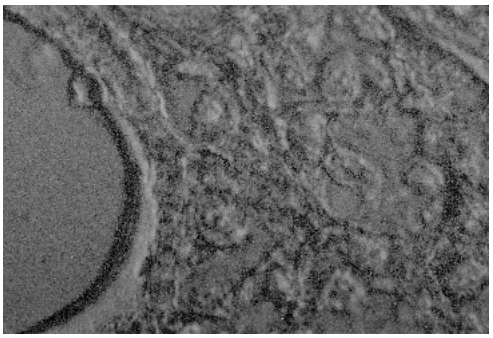

D

Photo 2. Apoptosis of thyrocytes determined by Hoechst 33342 dye staining. With Hoechst staining, viable cells were observed with intact nuclei (A), and apoptotic cells (B) with fragmented or condensed nuclei. Apoptotic nuclei were observed in the area of multinodular goiters (A, B) and thyroid adenomas (C, D-phaso-contrast microscopy) and the nonnodular part of thyroid tissue. Apoptotic nuclei were rarely found in the area of fibrosis, where many collagen fibers were dominant. In normal thyroid glands, apoptotic nuclei were occasionally found in follicular cells.

Apoptosis is of interest because the presence of apoptotic cells in abnormal thyroid glands can be detected by histological examination. Apoptosis was assessed through staining with H33342 (photo 2) and quantification of the percentage of apoptotic nuclei (400 cells counted per sample). Under these conditions, the Hoechst dye stained all nuclei, whereas propidium iodide stained the nuclei of only necrotic cells with disrupted plasma membranes. With Hoechst staining, viable cells were observed with intact nuclei, and apoptotic cells with fragmented or condensed nuclei.

Apoptotic nuclei were observed in the area of multinodular goiters and thyroid adenomas and the nonnodular part of thyroid tissue. Most cell types undergoing apoptosis in the inflamed tissues were macrophages/histiocytes and lymphocytes, but not multinucleate giant cells. Apoptotic nuclei were also found in regenerating follicular cells, which took place as a group in an insular pattern in the fibrous stroma and showed hypertropic cytoplasms and enlarged nuclei with prominent nucleoli. Apoptotic nuclei were rarely found in the area of fibrosis, where many collagen fibers were dominant. In normal thyroid glands, apoptotic nuclei were occasionally found in follicular cells.

The histological examination was corroborated with biochemical quantification of apoptosis.

DNA "ladders" (photo 3), indicative of apoptotic internucleosomal DNA fragmentation (agarose gel electrophoresis of DNA), were clearly visible in agarose gels of DNA from the nonnodular part of thyroid tissue. Ladders were also present in the DNA from thyroid tissue multinodular goiters. No ladders were observed with DNA from thyroid tissue of thyroid adenomas and Hashmoto's thyroiditis.

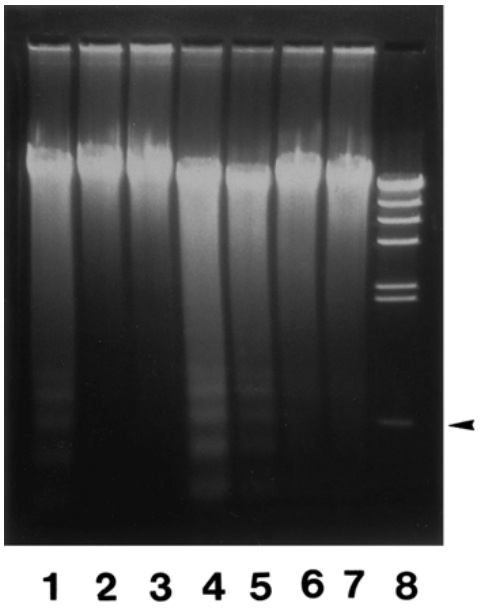

Photo 3. Agarose gel electrophoresis of thyroid tissue DNAFor quantification of apoptotic events, we measured fragmented DNA contents from total thyroid extracts by using the diphenylamine method.

The extent of apoptosis was significantly associated with the type of thyroid lesion, both proliferative (multinodular goiter) and neoplastic (malignant). Low levels of apoptosis were detected in multinodular goiters and thyroid adenomas (thyroid carcinomas < Hashmoto's thy-

roiditis < multinodular goiters) by the diphenylamine assay.

In contrast, high levels of thyrocyte apoptosis were found in the nonnodular part of thyroid tissue (Hashmoto's thyroiditis > thyroid carcinomas > multinodular goiters) [2].

The percentage of fragmented DNA increased with statistically significantly different frequency between the nonnodular thyroid tissue and patient goiter specimens:

- multinodular goiters (29,2±4,1% - in the nonnodular thyroid tissue and 18,6±4,8% - in the goiter specimens);
- Hashmoto's thyroiditis (43,6±8,2% - in the nonnodular thyroid tissue and 12,2±2,6% - in the goiter specimens);
- thyroid carcinomas (46,4±6,7% - in the nonnodular thyroid tissue and 7,8±1,3% - in the goiter specimens).

Discussion. A proper balance between cell proliferation and cell death is required to maintain tissue homeostasis. Normal thyroid cells are resistant to all the known death ligands, despite the constitutive expression of their respective death receptors but can be sensitized to death induction by these ligands using proinflammatory cytokines. In contrast to normal cells, the majority of goiter cell populations are not sensitized to TRAIL- or Fas-mediated apoptosis by cytokine pre-treatment [4]. This suggests a functional decrease in death receptor-mediated apoptotic activity in goiter-derived primary thyroid cells and indicates that there is an altered regulation of these death pathways in goiter cells. Moreover, the sensitivity to TRAIL-induced cell death inversely correlated with the goiter size. This raises the possibility that TRAIL is important in maintaining normal thyroid cell populations and TRAIL resistance contributes to the development of nodular goiter [10].

Goitrogenesis is a very slow process, unlike cancer, which is caused by increased cell proliferation. It is possible that a decrease in cell death can contribute to the accumulation of cells in goiter nodules. On the other hand, an imbalance between thyroid cell proliferation and cell death may be crucial for goiter formation or cancer development and progression [6]. In human thyroid goiter, Fas-mediated apoptosis is suppressed, leading to thyroid cell hyperplasia. Furthermore, malignant thyroid cells may escape immune attack by over expressing Fas ligand and inducing apoptosis in the invading immune cells. However, the exact mechanisms involved in the regulation of apoptosis in thyroid disease remain unclear. Further investigation is needed that may provide new strategies in the prevention and treatment of these diseases.

Apoptosis can be a useful indicator of enhanced risk, to be considered in the overall diagnostic process.

THE LITERATURE:

1. Апоптоз и патоморфоз опухолей почек /Комаревцев В.Н., Комаревцева И.А., Фильчуков Д.А., Головченко Н.Н., Харченко В.В. //Лікарська справа. – 2001. – Т.1059. - № 4.- С.115-118.
2. Комаревцева Е.В. Апоптоз и узловые формы заболеваний щитовидной железы //Патофизиология и современная медицина. – 2004.- С. 207-209.
3. Черенько С.М. Диференційна діагностика вузлових утворень щитовидної залози // Лікарська справа. - 1998. - № 6. - С. 43-47.
4. Andrikoula M., Tsatsoulis A. The role of FAS-mediated apoptosis in thyroid desiase // Eur. J. Endocrin.- 2001.- Vol. 144.-P. 561-568.
5. Derwahl M. Molecular aspects of the pathogenesis of nodular goiters, thyroid nodules and adenomas // Exp. Clin. Endocrinol. Diabetes.- 1996.- Vol. 104, (Suppl 4). – P.32–35.
6. Knudsen N., Lauberg P., Perrid H. Risk factors for goiter and thyroid nodules// Thyroid.- 2002.- Vol.12. - № 10.- P.879-888.
7. Kotani T., Aratake Y., Ohtaki S. Apoptosis in Hashimoto's thyroiditis //Rinsho Byori.-1997.-Vol.45. - № 11.-P.1038-1047.
8. Lansford C.D., Teknos T.N. Evaluation of the Thyroid nodule //Cancer Control.-2006.-Vol.13. - № 32.- P.89-98.
9. Messmer U.K., Verena A.B. Basic fibroblast growth factor selectively enhances TNF – induced apoptotic cell death in glomerular endotelial cells //Biochem. I. – 1996. – Vol. 319. – P. 299-305.
10. Mezosi E., Yamazaki H., Bretz J.D. Aberrant Apoptosis in Thyroid Epithelial Cells from Goiter Nodules // J. Clin. Endocrinology & Metabolism.- 2002.- Vol. 87. - №. 9.- P. 4264-4272.

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