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Identification of Proteins Overexpressed in Papillary Thyroid Tumors

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Abstract—A modified method of proteome comparative analysis based on preliminary removal of cell structural proteins by extraction using salt buffer and subsequent separation of extracts by two-dimensional gel electrophoresis was developed. Identification of differentially expressed proteins by mass spectrometry has revealed three proteins with noticeably increased level of synthesis in most samples of papillary thyroid tumors compared to normal tissues. An increase in ubiquitin content was found for the first time. Oncomarker search efficiencies by two-dimensional gel electrophoresis and bioinformatic search were compared.

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Key words: differential proteomics, two-dimensional gel electrophoresis, thyroid cancer, protein oncomarkers

Thyroid cancer (TC) is the most widespread tumor of the endocrine system [1]. Epidemiological investigations of recent decades have shown a clear tendency to growth in the number of TC patients in many regions of the world [2]. According to the World Health Organization, during the last decade the number of TC cases in the world has doubled [3]. In Russia every year over 8000 cases of primary thyroid cancer are registered [4]. Ecological problems are considered as the main reason for this growth, in particular, increased level of radiation. Thus, in five years after the accident at the Chernobyl nuclear power station an unprecedented growth of TC cases among children and teenagers living at contaminated territories was registered [3].

Thyroid tumors are revealed in diverse clinical variants from microfoci (i.e. slowly progressing highly differentiated latent forms of papillary and follicular cancer) to extremely aggressive course in the case of anaplastic TC [5]. According to the theory of "multiple distortions", thyroid cancer develops due to a series of mutations resulting in activation of oncogenes and inactivation of tumor suppressor genes [6, 7]. It became obvious during recent years that anomalous activation of the RAS-RAF-MEK signaling pathway is one of main factors responsible for the development of TC. This cascade is activated in 70% of cases of papillary TC form, and the most frequent mutations are in BRAF protein (45%) [6]. Such mutations in one gene allele can be inherited, but much more often they evolve sporadically in both alleles in somatic cells [2, 8]. Accounting for significant growth of TC cases in young people, prospective diagnostics of tumors in high-risk groups during regular dispensary examinations becomes very important.

About 80% of all primary malignant thyroid tumors are papillary cancer. Papillary cancer variants consisting of cylindrical, columnar, and oxyphilic cells (Hurthle cells) are characterized by aggressive growth and high mortality, and, therefore, require early surgical intervention [2, 5]. The "gold standard" of papillary tumor diagnostics is the fine-needle biopsy under clinical ultrasound

Abbreviations: EST, expressed mRNA sequences; IHCh, immunohistochemistry; MALDI-TOF mass spectrometry, time-of-flight mass spectrometry with laser desorption/deionization; TC, thyroid cancer.

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control [2, 3]. Since the information content of biopsy analysis in the case of thyroid cancer is 50-90% [2, 5], the search for more efficient and sensitive methods of molecular diagnostics is required for this type of tumors.

Proteome comparative analysis can be used to identify protein oncomarkers whose levels of synthesis in normal and tumor cells differ most noticeably and often. Antibodies to secretory proteins of this type enable creation of reliable and sensitive immunological tests for serum diagnostics, prognosis, and postoperative monitoring of tumors. *In vivo* profile proteomics is widely used for searching for potential protein tumor markers. This method is based on comparison of the level of protein synthesis in normal and tumor tissues by two-dimensional gel electrophoresis, revealing proteins with most reliable differences in their content in normal and tumor tissues, and identification of these proteins by mass spectroscopy [9, 10]. The available data on thyroid cancer profile proteomics are summarized in the table.

The use of standard methods of differential proteomics for searching for protein oncomarkers in total extracts is complicated by the existence of major fractions of structural and cellular cytoskeleton proteins whose concentrations exceed by 10^5-10^{12} times those of most soluble proteins [11]. We have used preliminary extraction of proteins soluble under physiological conditions before proteomic analysis in order to remove most abundant cellular proteins. Comparison of two-dimensional gels revealed 10 protein spots with consistently increased content in papillary thyroid tumors compared to norm.

MATERIALS AND METHODS

Extraction of proteins. After resection, clinical samples were quickly frozen in liquid nitrogen and stored at −80°C. Six pairs of samples of papillary thyroid contralateral lobe primary tumors and normal tissues at the distance of 1-2 cm from the tumor were used in this work. The absence of tumor cells in normal samples was confirmed by immunohistochemical analysis.

Identification of proteins exhibiting increased content in papillary thyroid cancer based on results of this work, analysis of literature sources, and bioinformatics search

Protein	Source	Method of protein/mRNA synthesis determination	Level of protein synthesis	
			dbEST	Oncomine
ANXA1	[20], this work	2D, WB	+	+
ANXA2	[21], this work	2D, MC	+/–	+
UBB	this work	2D	+	+
LGALS3	[22, 27]	IHCh	–	–/+
MUC1	[23]	IHCh	n.d.	–/+
EPCAM	[24]	IHCh	–	+
S100A4	[25]	IHCh	n.d.	+
CITED1	[26, 27]	IHCh, MC, RT-PCR	+/–	+
SFTPB	[27]	IHCh, MC	+/–	+
FN1	[26, 27]	IHCh, MC, RT-PCR	+	+
KRT19	[26, 27]	IHCh, MC	–	+
DPP4	[27]	IHCh, MC	n.d.	+
MET	[27, 28]	IHCh, MC, RT-PCR	+	+
SERPINA1	[27, 28]	IHCh, MC, WB	+/–	+
LAMB1	[29]	IHCh	n.d.	+
MDK	[26, 27]	IHCh, MC	n.d.	–/+
TIMP1	[27]	IHCh, MC	–	+
TIMP2	[27]	IHCh	–	+
MMP2	[27]	IHCh	+	–
MMP9	[27]	IHCh	n.d.	–/+

Note: n.d., no data available; + and –, protein synthesis levels in thyroid cancer are increased or decreased, respectively, compared to norm; +/– and –/+, data are contradictory, the level of protein synthesis is most likely increased or decreased, respectively; IHCh, immunohistochemistry; WB, Western blot analysis; RT-PCR, PCR with reverse transcription; MC, microchip technology.

Tissues were homogenized using a Sartorius Mikro-Dismembrator U ball mill (Germany) at 7200 rpm for 60 sec with cooling by liquid nitrogen. Proteins were extracted by salt buffer (0.12 M NaCl, 20 mM KCl, 20 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM EDTA). After centrifugation at 16,000*g* for 5 min at 4°C, soluble proteins of the supernatant were precipitated by five volumes of acetone for 16 h at 4°C and collected by centrifugation (16,000*g*, 10 min, 4°C). The precipitate was air dried and dissolved in application buffer according to O'Farrell [12] for subsequent two-dimensional electrophoresis. Protein concentration was determined according to Bradford [13].

Analysis of extracts by two-dimensional gel electrophoresis. Electrophoresis was carried out by the classic O'Farrell method [12] with some modifications. In all experiments ~100 μg total protein was applied per tube, and isoelectrofocusing was carried out towards the acidic buffer (300 V, 16 h). Gel columns were removed from the tubes, washed for 40 min in buffer system according to Laemmli [14], and frozen at −80°C. Separation in the second direction was carried out in gradient polyacrylamide gel (10-22%) at 25 mA for 16 h. After electrophoresis gels were stained using silver salts [15]. Protein spots were scanned on an Epson Perfection V700 Photo device (Epson, Japan) making it possible to obtain images of transparent objects in the visible region with resolution of 4800 dpi.

Identification of protein spots. For dye removal, gel pieces of ~1 × 1 mm were washed twice in 100 μl of 0.1 M NH_4HCO_3 solution in 40% acetonitrile for 30 min at 37°C. The solution was removed and the gel was dehydrated by addition of 100 μl acetonitrile. After removal of the acetonitrile and drying of the gel piece, 3 μl (15 μg/ml) of modified trypsin solution (Promega, USA) in 0.05 M NH_4HCO_3 was added. The samples were incubated for 12 h at 37°C and 7 μl of 0.5% trifluoroacetic acid (TFA) in aqueous acetonitrile solution (10%) was added and thoroughly mixed. The gel-bathing solution (2 μl) was mixed with 0.3 μl of 2,5-dihydrobenzoic acid solution (Aldrich, USA) at concentration of 20 mg/ml in 20% aqueous acetonitrile with addition of 0.5% TFA. The mixture was air-dried.

Mass spectrometry of the gel-bathing solutions was performed on an Ultraflex II tandem MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Germany) equipped with a UV laser (Nd) in the positive ion regime using a reflectron (700-4500 Da). Total accelerating potential in reflectron mode was 25 kV. Fragmentation spectra of separate peptides were obtained in tandem mode (Lift). Accelerating potential of parental ions was 7 kV. The measured mass accuracy after additional calibration by trypsin autolysis peaks was at least 0.005%, while accuracy of measured fragment mass values was 1 Da. Mass spectra were processed using the FlexAnalysis 2.0 program (Bruker Daltonics). Proteins were identified by peptide mass fingerprint using the Mascot (Matrix science, http://matrixscience.com/) program and NCBI database of human proteins accounting for oxidation of methionine by air oxygen and modification of cysteine by acrylamide. Protein identification was considered as reliable with the integral parameter value >71 ($p < 0.05$). If protein identification did not meet criteria of reliability, fragmentation spectra of separate peptides were obtained and searched by MS + MS/MS using the BioTools v.3 program (Bruker Daltonics). Protein identification was considered as reliable if the integral parameter level exceeded the threshold.

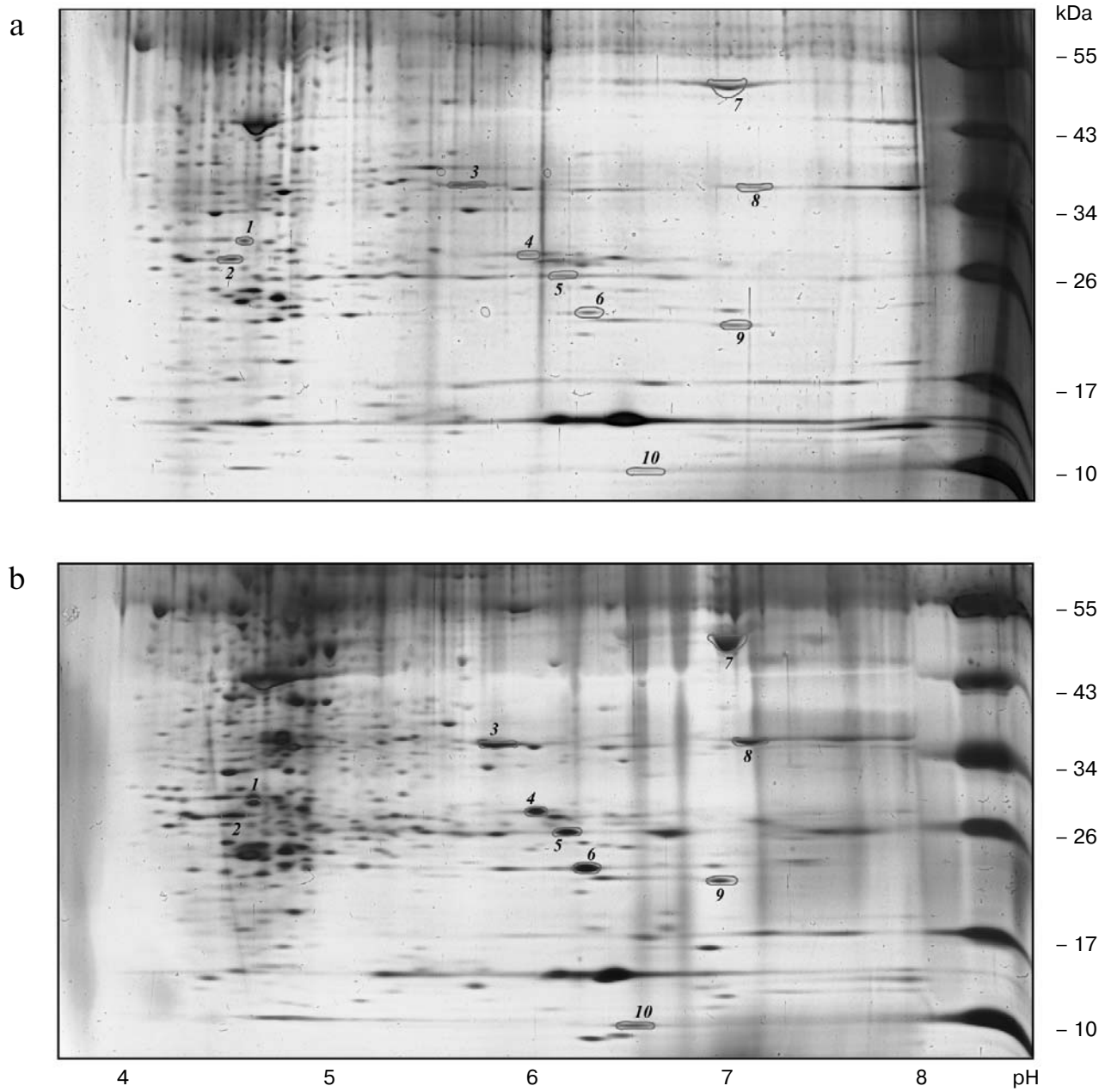
Bioinformatic search. Potential protein oncomarkers with altered synthesis level in papillary thyroid tumors were searched using the dbEST database (dbEST, http://www.ncbi.nlm.nih.gov/sites/entrez?db=nucest) as described earlier [16] and in the Oncomine database (https://www.oncomine.org).

RESULTS AND DISCUSSION

Most works on proteomics use total extract of cell proteins. In this work soluble proteins of analyzed samples were fractionated in advance by extraction with salt buffer. Then the extracts of primary papillary thyroid tumors and normal tissues were separated by two-dimensional gel electrophoresis. As a result, there was significant enrichment of soluble proteins that most noticeably penetrate in the blood circulation, if secreted by tumors.

Gels obtained after separation of proteins from extracts of normal and tumor thyroid tissues are shown in the figure. Comparison of six pairs of gels containing ~650 protein spots per gel revealed 10 spots with increased content in tumor tissue compared to the norm in at least four samples. Mass spectrometric analysis of the protein spots reliably identified three proteins – ANXA1, ANXA2, and UBB. Increased level of synthesis of first two proteins in papillary thyroid tumors was described earlier [10], whereas we have found the increase in UBB protein synthesis for the first time. None of the identified proteins is secretory, and therefore cannot be used for serum diagnostics of thyroid cancer. However, these proteins are potential prognostic oncomarkers. Estimation of their prognostic potential will require additional investigations using immunological methods.

Integral parameters obtained by mass spectrometric analysis of proteins in the other seven spots did not follow the established criterion of reliability. Therefore, in these cases final identification of potential oncomarkers of TC requires additional comparative investigations of normal and tumor samples (e.g. by Western blotting). In our opinion, proteins PRDX1, TPI1, and ENO1 (integral parameters are −69, −60, and −47, respectively) are the most promising candidates for final identification. Rather high values of integral parameter are characteristic of the



Two-dimensional electrophoregrams of proteins obtained by salt extraction from normal (a) and tumor (b) tissues: *1*) FKBP7; *2*) FAM109A; *3*) ANXA1; *4*) RND3; *5*) TPI1; *6*) PRDX1; *7*) ENO1; *8*) ANXA2; *9*) CD3D; *10*) UBB

first two proteins, while the increase in their expression was registered in a significant number of tumors (although not in TC) [17, 18]. An increase in ENO1 synthesis was found in malignant thyroid oncocytomas [19].

Comparative estimation of the efficiency of identification of proteins overexpressed in TC by methods of protein analysis and bioinformatic search in the dbEST database using a selected collection of 20 proteins has shown that the data coincide only in 25% of cases (table). The data obtained by protein analysis are absent in the dbEST database for 30% of cases, do not coincide with the results of protein analysis for 25% of cases, and the information is contradictory in 20% of cases. For the Oncomine database the results coincide in 75% of cases (in 20% of cases data are contradictory and in 5% of cases do not coincide). Thus, data of the Oncomine database much better match the results of protein analysis than those of dbEST, which can be explained by insufficient number of clones that were sequenced upon creation of the dbEST databases.

Existing differences between results of the Oncomine database and protein expression results can be explained

as follows. First, in two of five cases (MUC1 and MMP9 proteins) analysis of protein expression was carried out only in one sample and cannot be considered as final; second, the differences in case of LGALS3 and MDK proteins, obtained in two independent laboratories, can be explained by inhibition of synthesis of microRNAs, suppressing protein expression at the translational level [30]; third, in one case (MMP2) the result of protein analysis is confirmed by dbEST, and hence the result of search of the Oncomine database is most likely an artifact.

REFERENCES

1. Kenneth, B. (2000) in *Thyroid Diseases* (Brawerman, L. I., ed.) [Russian translation], Meditsina, Moscow, pp. 313-320.
2. Sigematsu, I. (1996) *Diagnostics of Thyroid Diseases* [Russian translation], Sasakava Foundation for Help in Public Health Development, Tokyo.
3. Romanchishin, A. F., and Bogaturiya, G. O. (2004) in *Surgical Endocrinology* (Kalinin, A. P., ed.) [in Russian], Piter, St. Petersburg, pp. 164-172.
4. Romanchishin, A. F., Kolosyuk, V. A., and Bogaturiya, G. O. (2003) *Thyroid Cancer* [in Russian], Welcome, St. Petersburg.
5. Rumyantsev, P. O., and Il'in, A. A. (2009) *Thyroid Cancer* [in Russian], GEOTAR, Moscow.
6. Dolgov, V. V., and Shabalova, I. P. (2002) *Laboratory Diagnostics of Thyroid Diseases* [in Russian], Triada, Moscow.
7. Shapiro, N. A., and Kamneva, T. N. (2003) *Cytological Diagnostics of Thyroid Diseases* [in Russian], ReproCenter, Moscow.
8. Chissov, V. I., and Daryalova, S. L. (2009) *Oncology* [in Russian], GEOTAR-Media, Moscow.
9. Belfiore, A., and la Rosa, G. L. (2002) *Endocrinol. Metab. Clin. North*, **30**, 361-400.
10. Hillebrandt, S., and Streffer, C. (1995) *Cancer Res.*, **55**, 5617-5620.
11. Kimura, E. T., Nikiforova, M. N., Zhu, Z., Knauf, J. A., Nikiforov, Y. E., and Fagin, J. A. (2003) *Cancer Res.*, **63**, 1454-1457.
12. O'Farrell, P. H. (1975) *J. Biol. Chem.*, **250**, 4007-4021.
13. Bradford, M. M. (1976) *Anal. Biochem.*, **72**, 248-254.
14. Laemmli, U. K. (1970) *Nature*, **227**, 680-685.
15. Mortz, E., Krogh, T., Vorum, H., and Gorg, A. (2001) *Proteomics*, **1**, 1359-1363.
16. Bukurova, Yu. A., et al. (2010) *Mol. Biol.*, in press.
17. Rho, J. H., Qin, S., Wang, J. Y., and Roehrl, M. H. (2008) *J. Proteome Res.*, **7**, 2959-2972.
18. Hamaguchi, T., Iizuka, N., Tsunedomi, R., Hamamoto, Y., Miyamoto, T., Iida, M., Tokuhisa, Y., Sakamoto, K., Takashima, M., Tamesa, T., and Oka, M. (2008) *Int. J. Oncol.*, **33**, 725-731.
19. Baris, O., Savagner, F., Nasser, V., Loriod, B., Granjeaud, S., Guyetant, S., Franc, B., Rodien, P., Rohmer, V., Bertucci, F., Birnbaum, D., Malthiery, Y., Reynier, P., and Houlgatte, R. J. (2004) *Clin. Endocrinol. Metab.*, **89**, 994-1005.
20. Petrella, A., Festa, M., Ercolino, S. F., Zerilli, M., Stassi, G., Solito, E., and Parente, L. (2006) *Cancer Biol. Ther.*, **5**, 643-647.
21. Delys, L., Detours, V., Franc, B., Thomas, G., Bogdanova, T., Tronko, M., Libert, F., Dumont, J. E., and Maenhaut, C. (2007) *Oncogene*, **26**, 7894-7903.
22. Turkoz, H. K., Oksuz, H., Yurdakul, Z., and Ozcan, D. (2008) *Endocr. Pathol.*, **19**, 92-96.
23. Abrosimov, A., Saenko, V., Meirmanov, S., Nakashima, M., Rogounovitch, T., Shkurko, O., Lushnikov, E., Mitsutake, N., Namba, H., and Yamashita, S. (2007) *Endocr. Pathol.*, **18**, 68-75.
24. Ensinger, C., Kremser, R., Prommegger, R., Spizzo, G., and Schmid, K. W. (2006) *J. Immunother.*, **29**, 569-573.
25. Min, H. S., Choe, G., Kim, S. W., Park, Y. J., Park, J., Youn, Y. K., Park, S. H., Cho, B. Y., and Park, S. Y. (2008) *Mod. Pathol.*, **21**, 748-755.
26. Prasad, M. L., Pellegata, N. S., Huang, Y., Nagaraja, H. N., de la Chapelle, A., and Kloos, R. T. (2005) *Mod. Pathol.*, **18**, 48-57.
27. Huang, Y., Prasad, M., Lemon, W. J., Hampel, H., Wright, F. A., Kornacker, K., LiVolsi, V., Frankel, W., Kloos, R. T., Eng, C., Pellegata, N. S., and de la Chapelle, A. (2001) *Proc. Natl. Acad. Sci. USA*, **98**, 15044-15049.
28. Jarzab, B., Wiench, M., Fujarewicz, K., Simek, K., Jarzab, M., Oczko-Wojciechowska, M., Wloch, J., Czarniecka, A., Chmielik, E., Lange, D., Pawlaczek, A., Szpak, S., Gubala, E., and Swierniak, A. (2005) *Cancer Res.*, **65**, 1587-1597.
29. Maatta, M., Virtanen, I., Burgeson, R., and Autio-Harmainen, H. (2001) *J. Histochem. Cytochem.*, **49**, 711-726.
30. Bartel, D. P. (2004) *Cell*, **116**, 281-297.