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Research Article

Immunochemical Diagnosis of Cancer. Prototyping

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Abstract

Early detection of malignant neoplasms to date is a serious problem. A prototype of the method of immunochemical detection of different types of solid cancers (primary and recurrent) in the early stages was developed. According to the initial hypothesis, sera of patients with malignant tumors of different localization and histological nature contain different sets of autoantibodies (auto-Ab) of IgG class to many cancer-associated antigens. The different content of such auto-Ab determines the difference in serum immunoreactivity profiles of cancer patients and patients with non-malignant chronic inflammatory diseases. Confirmation of this hypothesis opens up prospects for the creation of simple and cheap laboratory methods of mass preventive examination of the population for the early detection of cancer. Received confirmation of the principle of justice proposed hypotheses. Even with non-optimal sets of test antigens, it is possible to achieve a sensitivity of 71% and specificity of 68% in the differentiation of blood serum of cancer (cancers of the lung, stomach, ovarian, prostate) and cancer patients (chronic inflammatory diseases of the lung, stomach, ovary, prostatic gland) and nearly 90% in the differentiation of healthy individuals from cancer patients.

Keywords: Oncology; Solid Tumors; Laboratory Diagnosis; Enzyme-Linked Immunosorbent Assay; Autoantibodies

Introduction

One of the most important tasks of practical Oncology is early detection and diagnosis of actively growing malignant tumors at the earliest stages of the disease. The solution of this problem should reverse the progressive growth of malignant diseases which does observed at least during the last half century. Noting that risk reduction has the potential to prevent around half of all cancers [Seventieth World Health Assembly | WHA70.12 | Agenda item 15.6 | 31 May 2017]. It can be assumed that immunochemical methods can play a decisive role here [1].

Serological studies have long been routine methods of laboratory diagnosis. To increase the titers of specific antibodies (Ab) to antigens, for example, HIV-1, Chlamidia trachomatis and other viruses or bacteria, suggests an increase in the content of certain microbes in the body. Parenteral administration of its own antigens, for example, human chorionic gonadotropin (HCG), leads to an increase in serum content of Ab to HCG, despite the fact that it is not "Alien", and "Self" [2]. Increasing the body's production of its own antigens also causes an increase in the synthesis of respective Ab. For example, increased expression of insulin receptors, long before the development of diabetes mellitus type 2, accompanied by an increase in antibody to the insulin receptor [3], and increased synthesis of the regulator of apoptosis p53 is accompanied by the rise of production of antibody to this protein [4]. These and similar examples illustrate an important property of the immune system: the immune reflection, i.e. the ability to react to quantitative changes of ANY antigens in the human body by increasing the synthesis

of the corresponding Ab, regardless of whether the antigens are "Self" or "Alien". At the heart of this phenomenon is the basic (archetypal) function of the immune system – its participation in the clearance of the body from the excess of any molecules that can disrupt homeostasis – antigens of pathogenic microbes, or own molecules, synthesized in abnormal quantities or coming in excess from the dying cells [4].

Both phenomena are typical for malignancy: abnormal expression of a number of tumor associated antigens (TAAG), for example, p53, CEA, AFP, etc., and activation of apoptosis/necrosis with extracellular release of excess antigens of tumor cells. All this induces an increased synthesis of the corresponding Abs, repeatedly confirmed by experimental observations [Belousov., *et al*. 2008]. It is natural to conclude that the immune system" sees" a growing tumor [5], although it does not destroy it. The amplification of the immunochemical signal is characteristic: 100-1000 or more Abs molecules can be synthesized for each TAAG molecule. Therefore, methods based on the identification of Abs to TAAG are many times more sensitive than methods based on the identification of the actual TAAG currently used.

The phenomenon of tumor-associated "shifts" in serum Abs formulations is tempting to be used for diagnostic purposes. Selection of adequate sets of antigens will allow develop diagnostic test systems. With the help of which it will be possible to identify changes typical for neoplastic processes. This can be one of the most effective tools for mass diagnostic examinations to detect cancer in the early stages of development, as well as to confirm (or reject) the malignant nature of the formations identified, for example, with the help of MRI or ultrasound without biopsy and histological studies.

Materials and Methods

Patients

Immunoreactivity of blood serum samples of patients with histologically confirmed cancers of different localization was investigated (Group-1; n = 38; Table 1) and patients with chronic non-cancer diseases of the same organs (Group-2; n = 40; Tab. 2).

No	Affected organ	Number of patients
1	Ovary	9
2	Prostate	15
3	Lung	7
4	Stomach	7
Total: n = 38 (m =25, f = 13, mean age 64)		

Table 1: Oncology patients (Group-1).

No	Affected organ	Number of patients
1	Ovary	10
2	Prostate	10
3	Lung	10
4	Stomach	10
Total: n = 40 (m = 16, f = 24, mean age 59)		

Table 2: Patients with chronic non-cancer diseases (Group-2).

Serum samples were provided by the Oncology Center of the Republic of Bashkortostan, Ufa. The samples were heated for 30 minutes at 56°C, after which they were frozen and stored until the study at a temperature of-20°C for no more than 6 months.

Immunochemical methods

With the help of solid-phase ELISA on 96-well polystyrene plates NUNC MaxiSorp (Denmark) determined immunoreactivity profiles of serum samples, depending on the content of auto-Abs to TAAG, as described above [3]. Synthetic fragments (peptides-epitopes) proteins were adsorbed into the wells of the plates. Synthesis of peptide fragments of TAAG was made in the branch of the Institute of Bioorganic chemistry RAS (Pushchino, Moscow Region, Russia).

Homep	TAAG (proteins)	The code of TAAG proteins fragments
1	NOTCH3	N15Y
2	Clusterin	Q15Y
3	Jagged-1	Abu20D
4	NOTCH-1	K14A
5	DNA-topoisomerase 2-alpha	K14K
6	The same TAAG, but other epitope	K14KC
7	MYC proto-oncogene	A15S
8	The same TAAG, but other epitope	A15SC
9	MAGE Family Member A3	F16E
10	The same TAAG, but other epitope	F16EC
11	Kita-kyushu lung cancer antigen 1	L15T
12	The same TAAG, but other epitope	L15TC
13	p53	E25L
14	Myb transcriptional activator	N15S
15	p90	KEE
16	Human Epidermal growth factor Receptor 2	QVV

Table 3: Components of the test system.

Information about the corresponding proteins (TAAG) can be found in: http://www.uniprot.org/uniprot/

Data calculation

The averaged individual immune reactivity of analyzed serum samples with 16 used antigens and normalized immune reactivity of serum autoantibodies (auto-Abs) with each of the antigens (serum profiles) was calculated as described [3], with using a special PC software.

1. The arithmetic mean values of the optical density values in the reaction with each antigen for the control serum and for the samples of the analyzed blood serum were calculated.
2. The average individual immunoreactivity of each analyzed blood serum sample with all used antigens was calculated according to the formula:

$$IR(avg) = \left(\frac{OD(AG1) \times 100}{OD(k1)} - 100 + \frac{OD(AG2) \times 100}{OD(k2)} - 100 + \ldots\ldots + \frac{OD(AG16) \times 100}{OD(k16)} - 100\right) : 16$$

Explanation

- IR (avg) - the average individual immunoreactivity of the serum of a particular patient, expressed as a percentage of the mean population (control) values.
- OD (AG1, AG2…AG16) - the value of the optical density of the analyzed blood serum in the wells with the used antigens (AG1, AG2…AG16).
- OD (k1, k2 …k16) - the value of the optical density of the control blood serum in the wells with the same antigens (AG1, AG2…AG16).

The immunoreactivity profile of the analyzed blood serum sample with each of the antigens used as a percentage of the average individual immunoreactivity of the serum of a of each patient was calculated by the formula:

$$Dev\ AG1 = \left(\frac{OD(AG1) * 100}{OD\ (k1)}\right) - 100 - IR(avg)$$

$$Dev\ AG2 = \left(\frac{OD(AG2) * 100}{OD\ (k2)}\right) - 100 - IR(avg)$$

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$$Dev\ AG16 = \left(\frac{OD(AG16) * 100}{OD\ (k16)}\right) - 100 - IR(avg)$$

Explanation

- Dev AG1, AG2 … AG16 – deviation of the immune reactivity of the analyzed serum with each of used antigen (AG1, AG2…. AG16) in per cents from the average normalized level of reactivity this serum.
- OD(AG1, AG2…. AG16) – optical density of reaction of blood serum sample with antigens AG1, AG2, … AG16.
- OD(k1, k2…. k16) – optical density of the reaction of control serum with antigens AG1, AG2, … AG16.
- IR(avg) – the average individual immunoreactivity of the serum of a particular patient, expressed as a percentage of the average population values obtained by the method of ELI-Viscero-Test.

Analysis of features of serum immunoreactivity profiles

The following statistical approach was used for the differential diagnosis of serum samples of cancer patients and control persons with chronic inflammatory diseases without signs of malignancy:

1. Cancer patients were indicated by the index “unit” (1); control patients were indicated by the index “zero” (0). This new variable was called the cancer index (CRI).
2. Analysis of the correlations between the levels of auto-Abs to TAAG and CRI has shown that the reaction of many auto-Abs were correlated among themselves and that information on the immunoreactivity of any one auto-Abs was insufficient for differential diagnosis.
3. To analyze the data with using CRI and hence differential diagnosis, we used a step-by-step regression analysis method [6]. This method made it possible to distinguish 2-4 auto-Abs to TAAG taking into account their correlation with each other and, at the same time, the most closely related to the development of cancer and to construct a regression formula for estimating the value of CRI. Step-by-step regression was performed with the inclusion of variables $p < 0.05$ and exclusion of variables with $p > 0.10$.
4. In a number of experiments, the set of the most informative auto-Abs gave unstable results that could change with minor changes in the data (for example, when 2-3 cancer patients or control patients were excluded from the analysis). To increase the stability (robustness) of the obtained results, the “resampling” method was used [7]: data for 10% of patients and the control group were repeatedly randomly excluded from the entire sampling, and step-by-step regression was performed for a reduced sampling. As a result of these calculations, those auto-ABS were selected that fell into the Formula more often than others. These auto-Abs were considered as stable markers of the presence of cancer.

Results

Based on data on immunoreactivity serum samples from patients of Group-1 and Group-2 with TAAG fragments (Table. 3) the coefficients of the formula for calculating the CRI were evaluated. As a result, a linear regression equation of the following form was obtained:

CRI = 0.51 – 0.0127* K14A – 0.0118* K14KC – 0.0143* Q15Y

(K14A, K14KC and Q15Y – fragments of TAAG most suitable for samples differentiating; Table 3).

The correlation coefficient between the calculated CRI calculated by the above formula and the initial values of 0 and 1 (for non-malignant diseases, and for cancer patients, accordingly) was equal to R = 0.45, which is characterized as a weak relationship on a Chaddock' scale.

The calculated value of CRI took arbitrary fractional values, but not 1 or 0. To use these values for diagnosis, it was necessary to round the calculated values as follows: if the calculated CRI was greater than 0.5, then the patient was assigned a sign of the presence of cancer (CRI =1), if the calculated CRI was less than or equal to 0.5, then the patient was assigned a sign of the presence of chronic non-malignant disease (CRI =0).

Comparison of the rounded values of the calculated CRI with the initial values made it possible to propose a laboratory method of differentiation of blood serum samples of cancer and non-cancer patients according to the characteristic profiles of immunoreactivity, having a sensitivity of 71% and specificity of 68%.

Discussion

It is known that malignant cells do not remain without attention of the tumor-bearing person's immune system [1,5,8]. However, TAAGs are not considered by the immune system as something alien (hazard) and initiate only indicative, but not destructive immune response in the form of increased production of antibody to oncofetal proteins (AFP, CEA) and some other (e.g., p53) [2]. These antigens are abundantly produced by the fetus and in small amounts by the adult organism. During malignization is observed only multiplying activation of their expression. If the primary cause of cancer were mutations of oncogenes, tumor cells would produce qualitatively new TAAGs. In this case, the apparent insufficiency of anti-cancer activity of the immune system would be difficult to explain. Thus, verified information indicates that critical changes in the antigenic composition of the growing malignant tumor are, apparently, quantitative. These changes are accompanied by secondary (also quantitative) shifts in the production of auto-Abs corresponding antigenic specificity. Therefore, during the formation and growth of malignant tumors, the difference in immunoreactivity is reduced only to changes in the relative serum content of many natural auto-Abs present in the norm. This leads to changes in serum immunoreactivity profiles associated with many auto-Abs. It is possible that as a result of mutagenesis or post-transcription "errors" in the body can regularly occur malignant cells expressing qualitatively new antigens. However, such cells are quickly and effectively eliminated and do not give rise to malignant growth.

It is known that malignancy is characterized by a gradual increase in the proportion of less Mature cells (the phenomenon of embryonality) [9] that carry less and less features of the tissue-progenitor (the phenomenon of convergence of tumor characteristics; in this case, tumor cells originating from different tissues acquire similarity between themselves in the antigenic composition. The latter leads to the principal possibility of creating universal screening "onco-tests" suitable for the identification of persons at risk for the development of malignant tumors of different types and also gives hope for the future development of unified therapeutic vaccines suitable for the prevention and treatment of different types of tumors. Taking into account the fact that currently used tests for "oncomarkers", over the years of application showed low specificity and sensitivity [5], the need for new laboratory methods of cancer diagnosis is very high.

The approach used in our work revealed differences in the profiles of serum immunoreactivity in individuals belonging to the group of chronic inflammatory diseases of the lungs, kidneys, ovaries, prostate and patients with cancers of the same organs. As a result, by the peculiarities of serum immunoreactivity it was possible to differentiate persons with chronic inflammatory diseases and cancers of the same organs with sensitivity and specificity of about 71% and 68%, respectively. I.e. the principal possibility of creation of simple laboratory immunochemical methods of detection of oncological diseases at mass examinations was experimentally demonstrated. Earlier we showed the possibility of distinguishing sera of healthy individuals and cancer patients with sensitivity and specificity above 90% using similar approaches [1]. However, for a long time it was not possible to achieve differentiation between sera of cancer patients and patients with chronic inflammatory diseases. To achieve today's (reliable) sensitivity and specificity, we

had to carry out an empirical search of many variants of sets from combinations of dozens of different TAAG and their fragments, which additionally took 8 years of experimental work (after the development of the method of immunochemical differentiation of serum samples of clinically healthy individuals and cancer patients). This fact is clearly interesting: why sets of serum at in clinically healthy individuals and cancer patients differ significantly, but in cancer patients and patients with chronic non-malignant diseases differences are much weaker? Should we assume that any chronic inflammation carries the "potency of malignancy"? Is this not an indirect confirmation of the idea expressed by Harold Dvorak in his famous article "Tumors: wounds that do not heal" [10] about inflammation as a precursor of malignancy? [11-14].

Conclusion

As noted, similar in content work is carried out in many laboratories around the world. However, judging by the available publications, so far none of the developments has not been brought to the stage of practical use. Probably difficulties in differentiation between serum immunoreactivity from patients with chronic non-malignant inflammatory diseases and cancer patients are the main "stumbling block" in trying to create immunochemical methods for the detection of malignant tumors. We hope that the continuation of our research (experimental study of variants of multicomponent test systems based on combinations of many TAAG) will achieve sensitivity and specificity in the differentiation of serum samples of cancer patients at least 85-90%. That, in turn, will allow to start the practical implementation of appropriate methods.

Authors' Contributions

All authors are equally contributed.

Conflict of Interest

The authors declare that they have no conflicting interests.

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Volume 3 Issue 7 July 2019