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(54) **METHODE DE DIAGNOSTIC PRECOCE DE CARCINOMES**
(54) **METHOD FOR EARLY DIAGNOSIS OF CARCINOMAS**

(57) L'invention concerne une méthode de diagnostic précoce de carcinomes et de leurs stades préalables, qui comprend la détermination de la surexpression d'une protéine régulatrice de cycle cellulaire dans un échantillon organique. L'invention concerne en outre un coffret approprié à cet effet.

(57) The present invention relates to a method for early diagnosis of carcinomas and the precursors thereof, comprising determination of the over-expression of a cell cycle regulator protein in an organic sample. The invention also relates to a kit that can be used for said method.

A Method for the Early Diagnosis of Carcinomas

The present invention relates to a method for the early diagnosis of carcinomas as well as their preliminary stages, particularly carcinomas of the upper respiratory tract or the anogenital tract.

Preventive programs have been offered for the most differing carcinomas since the middle of the 50ies. Regarding the cervical carcinoma they are based mainly on the morphological and cytological examination of cytosmears of the cervix uteri, what is called the Pap test, which is made on the basis of gynecological routine examinations at regular intervals in women from the 20th year on. By means of the morphology of the cells, the smears are divided into various intensity degrees of dysplastic cellular changes. According to Pap I-V, these intensity degrees are referred to as normal, mild dysplasia, fairly serious dysplasia, serious dysplasia and invasive carcinoma, respectively. If the Pap test leads to a striking result, a small biopsy will be taken and subjected to a histopathologic examination, by which the kind and intensity of the dysplasia are determined and classified as cervical intraepithelial neoplasia (CINI-III).

In spite of all preventive programs the cervical carcinoma which leads to 400,000 new cases per year is the most frequent carcinoma but one in women. This is *inter alia* due to the fact that up to 30 % of the results of the Pap test are false-negative.

Therefore, it is the object of the present invention to provide a method by which cervical carcinomas can be diagnosed early and reliably. In addition, a differentiation should be possible by this method with respect to benign inflammatory or metaplastic changes of dysplastic preneoplasias.

According to the invention this is achieved by the subject matters defined in the claims.

The present invention is based on the applicant's insights that cell cycle regulatory proteins are overexpressed in many carcinomas, e.g. carcinomas of the upper respiratory tract or anogenital carcinomas, particularly cervical carcinoma, and preliminary stages of these carcinomas, respectively. Examples of the cell cycle regulatory proteins are cyclins. Cyclin-dependent kinases which regulate the cyclins are to be mentioned particularly. Cyclin-dependent kinase inhibitors which, in turn, regulate the cyclin-dependent kinases, are to be mentioned even more particularly. Examples of the cyclin-dependent kinase inhibitors are the proteins p14, p15, p16, p19, p21 and p27. The applicant has found out that the intensity of cell cycle regulatory protein overexpression correlates with the degree of cell dysplasia.

According to the invention the applicant's insights are used for a method for the early diagnosis of carcinomas and their preliminary stages, which comprises determining the overexpression of cell cycle proteins in a body sample.

The expression "carcinomas and their preliminary stages" comprises carcinomas of any kind and origin and preliminary stages thereof, respectively. For example, they may be carcinomas of the upper respiratory tract or anogenital carcinomas, particularly the cervical carcinoma. In connection with the latter, its preliminary stages, e.g. cervical intraepithelial neoplasias (CINI-III), carcinomas *in situ* (CIS), etc., have to be mentioned particularly.

The expression "cell cycle regulatory proteins" comprises cell cycle regulatory proteins of any kind and origin. For example, the proteins may be cyclins. In particular, they may be cyclin-dependent kinases which regulate the cyclins. Examples of the cyclin-dependent kinases are the proteins cdk4 and cdk6. More particularly, they may be cyclin-

dependent kinase inhibitors which, in turn, regulate the cyclin-dependent kinases. Examples of cyclin-dependent kinase inhibitors are the proteins p14, p15, p16, p18, p19, p21 and p27, with p16 being preferred.

The expression "body sample" comprises any body samples in which cell cycle regulatory proteins can be detected. Examples of such body samples are blood, smears, sputum, urine, stool, liquor, bile, gastrointestinal secretions, lymph, bone marrow, organ punctates or aspirates and biopsies. In particular, smears and biopsies are indicated when the detection of anogenital carcinomas, e.g. cervical carcinomas, is concerned.

The expression "determining the overexpression of cell cycle regulatory proteins" comprises any methods which are suited for detecting the expression of cell cycle regulatory proteins or their encoding mRNAs and an amplification of the corresponding genes, respectively. In order to determine an overexpression it is an obvious thing to compare the body sample to be examined with a corresponding body sample which originates from a healthy person. Such a sample can be present in standardized form. The (over)expression of cell cycle regulatory proteins can be detected on a nucleic acid level and protein level, respectively. Regarding the detection on a protein level, it is possible to use e.g. antibodies which are directed against cell cycle regulatory proteins. These antibodies can be used in the most varying methods such as Western blot, ELISA or immunoprecipitation. It may be favorable for the antibodies to be fixed on solid carriers such as test strips or latex particles.

By means of the present invention it is possible to diagnose carcinomas early, i.e. in their preliminary stages.

A further subject matter of the present invention relates to a kit for carrying out a method according to the invention. Such a kit comprises:

(a) a reagent for detecting the expression of a cell cycle regulatory protein, e.g. an antibody directed against such a protein or a nucleic acid coding for such a protein and parts thereof, respectively,

(b) conventional auxiliary agents, such as buffers, carriers, markers, etc., and optionally

(c) an agent for control reactions, e.g. a cell cycle regulatory protein, a nucleic acid coding for such a protein and parts thereof, respectively, or a preparation of cells e.g. a tissue section or cells fixed on a slide.

The above statements apply correspondingly to the individual components of the kit. Furthermore, one or several representatives of the individual components may be present.

By means of the present invention it is possible to diagnose carcinomas early. In particular, preliminary stages of carcinomas can be detected early. It must also be emphasized that it is possible to make a differentiation with respect to benign inflammatory or metaplastic changes of dysplastic preneoplasias. Another characteristic is that the results obtained by a method according to the invention are not subject to a subjective evaluation, so that e.g. the false-negative results and false-positive results, respectively, of a Pap test or of histological preparations can be avoided. In addition, the present invention distinguishes itself by rapid and simple handling, so that it can be used for extensive screening measures, particularly also in third-world countries. Thus, the present invention represents an important contribution to today's diagnostics of cancerous diseases.

Brief description of the drawing.

Figure 1 shows the detection of the cdk4 overexpression in HPV16-transformed cervical carcinoma cells CaSki. The indications 4 h, 8 h, 12 h, 24 h refer to the

times of cell extract removal. The indication co stands for control while arr indicates the addition of the serum.

Figure 2 shows the detection of the overexpression of cdk6 and p19 in HPV16-transformed NIH3T3 cells. The indication co stands for control.

The invention is explained by the following examples.

Example 1: Detection of the overexpression of p16 in biopsies of the cervix uteri

(A) Paraffin sections having a thickness of 3 to 5 μm are produced from 20 biopsies of the cervix uteri, which comprise all degrees of the dysplastic progression from normal tissue (n=2) via CIN I (n=4), II (n=4), III (n=5) lesions to the invasive carcinoma (n=5). They are deparaffinized in xylene for 2 x 10 min. and rehydrogenated using ethanol. The antigens are demasked in 10 mM citrate buffer (pH 6.0) in an autoclave at 110°C for 10 min. Thereafter, the endogenous peroxidases are inactivated using 0.25 % H_2O_2 in PBS. Following the blocking of unspecific binding sites with horse serum (Vectastain ABC detection kit, Vector Laboratories, Burlingame, California, U.S.A.) at room temperature for 20 minutes, the sections are incubated with a p16-specific monoclonal antibody (Neomarkers, Fremont, California, U.S.A.) in the presence of 3 % fetal calf serum at room temperature for 45 min. For the detection of the p16-antibody binding, a biotinylated secondary antibody (horse anti-mouse IgG, Vectastain kit, see above) is then added for 30 minutes. Thereafter, the bound secondary antibody is detected by means of the reagents and in accordance with the Vectastain kit instructions and a core counterstain is carried out using Mayer's hemalum solution.

It shows that an overexpression of p16 exists in dysplasia cells. It also shows that the intensity of p16 overexpression correlates with the degree of cell dysplasia.

(B) In addition, paraffin sections are prepared from 78 biopsies of the cervix uteri. The biopsies relate to normal tissue (n = 12), dysplastic lesions of stages CIN I (n = 15), II (n = 14) and III (n = 18) as well as invasive carcinomas (n = 19). The paraffin sections are treated as described in (A). The data indicated in Table 1 are obtained.

Table 1

		p 16 expression intensity			
histology	n=	-	+	++	+++
normal	12	9	3		
CIN I	15	10	3	2	
CIN II	14	1	4	9	
CIN III	18			9	9
CxCa	19			1	18
total	78	20	10	21	27

It follows from the data of Table 1 that p16 is overexpressed in cells of dysplasias and invasive carcinomas, the overexpression increasing with the degree of dysplasia towards the invasive carcinoma.

(C) Moreover, paraffin sections from 180 biopsies of the cervix uteri are treated as described in (A). In addition, the percentage cell number is determined

which reacts with the above-mentioned p16-specific monoclonal antibody. A distinction is also made between HPV-positive and HPV-negative dysplasias and invasive carcinomas, respectively. The data indicated in Table 2 are obtained.

Table 2

Percentage of cells overexpressing p16

	n	average percentage ± standard deviation
CIN I	32	54.9 ± 24.0
HPV-negative	17	54.0 ± 27.2
HPV-positive	15	55.9 ± 21.0
CIN II	32	70.8 ± 18.9
HPV-negative	14	76.0 ± 15.8
HPV-positive	18	66.8 ± 20.5
CIN III	60	92.4 ± 10.2
HPV-negative	9	94.4 ± 7.5
HPV-positive	51	92.1 ± 10.7
Invasive carcinoma	58	97.8 ± 5.2
HPV-negative	5	96.4 ± 8.1
HPV-positive	53	97.9 ± 4.9

The data of Table 2 disclose that p16 is overexpressed in both HPV-positive cells and HPV-negative cells of dysplasias and invasive carcinomas. This result is confirmed by controls with normal tissue. The data also show that the percentage of cells reacting with p16 increases with the degree of dysplasia towards the invasive carcinoma.

Example 2: Detection of the overexpression of cell cycle regulatory proteins in HPV-transformed cells

(A) Cervical carcinoma cells CaSki which are transformed with HPV16 are cultured in the absence of serum for 72 h. Following the addition of serum, cell extracts are collected at various times, subjected to SDS-PAGE and transferred to PVDF membranes (Du Pont). The expression of cdk4 is determined using polyclonal antiserum (1 : 1000) from Santa Cruz. Furthermore, the expression of HPV16 - E7 protein is determined with a monoclonal antibody against HPV16 - E7 (1 : 50) from Triton. The individual immune responses are detected via peroxidase-linked second antibodies and a chemiluminescence detection system (NEN, Du Pont).

It turns out that cdk4 is overexpressed (cf. figure 1).

(B) NIH3T3 cells are transformed with HPV16 so as to obtain an expression of HPV16-E7 protein. Cell extracts of the transformed cells are obtained and treated as described in (A). For detecting the expression of cdk6 and p19, respectively, polyclonal antisera (1: 1000) from Santa Cruz are used. As far as the detection of the expression of HPV16-E7 protein and the detection of the individual immune responses are concerned, reference is made to the above statements under item (A).

It turns out that cdk6 and p19 are overexpressed (cf. figure 2).

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Claims

1. Method for the early diagnosis of carcinomas and their precursors, comprising determining the overexpression of a cell cycle regulatory protein in a body sample, the cell cycle regulatory protein being a cyclin-dependent kinase inhibitor.

2. The method according to claim 1, wherein the carcinomas are those of the upper respiratory tract.

3. The method according to claim 1, wherein the carcinomas are anogenital carcinomas.

4. The method according to claim 3, wherein the anogenital carcinoma is a cervical carcinoma.

5. The method according to any of claims 1 to 4, wherein the cyclin-dependent kinase inhibitor is a protein p14, p15, p16, p18, p19, p21 or p27.

6. The method according to any of claims 1 to 5, wherein the body sample is blood, smears, sputum, urine, stool, liquor, bile, bone marrow, gastrointestinal secretions, organ punctates, biopsin and/or lymph.

7. The method according to any of claims 1 to 6, wherein the overexpression is determined on the nucleic acid level.

8. The method according to any of claims 1 to 6, wherein the overexpression is determined on the protein level.

9. The method according to claim 8, wherein an antibody directed against a cell cycle regulatory protein is used for the determination.

10. A kit for carrying out the method according to any of claims 1 to 9, comprising:

(a) a reagent for detecting the expression of a cell cycle regulatory protein,
(b) conventional auxiliary agents, such as buffers, carriers, markers, etc., and optionally
(c) means for control reactions.

11. The kit according to claim 10, wherein the reagent is an antibody directed against a cell cycle regulatory protein.

12. The kit according to claim 10, wherein the reagent is a nucleic acid coding for a cell cycle regulatory protein or parts thereof.

13. The kit according to any of claims 10 to 12, wherein the means is a cell cycle regulatory protein or a nucleic acid coding for it or parts thereof.

14. The kit according to any of claims 10 to 12, wherein the means is a cell preparation or cells fixed on a slide.

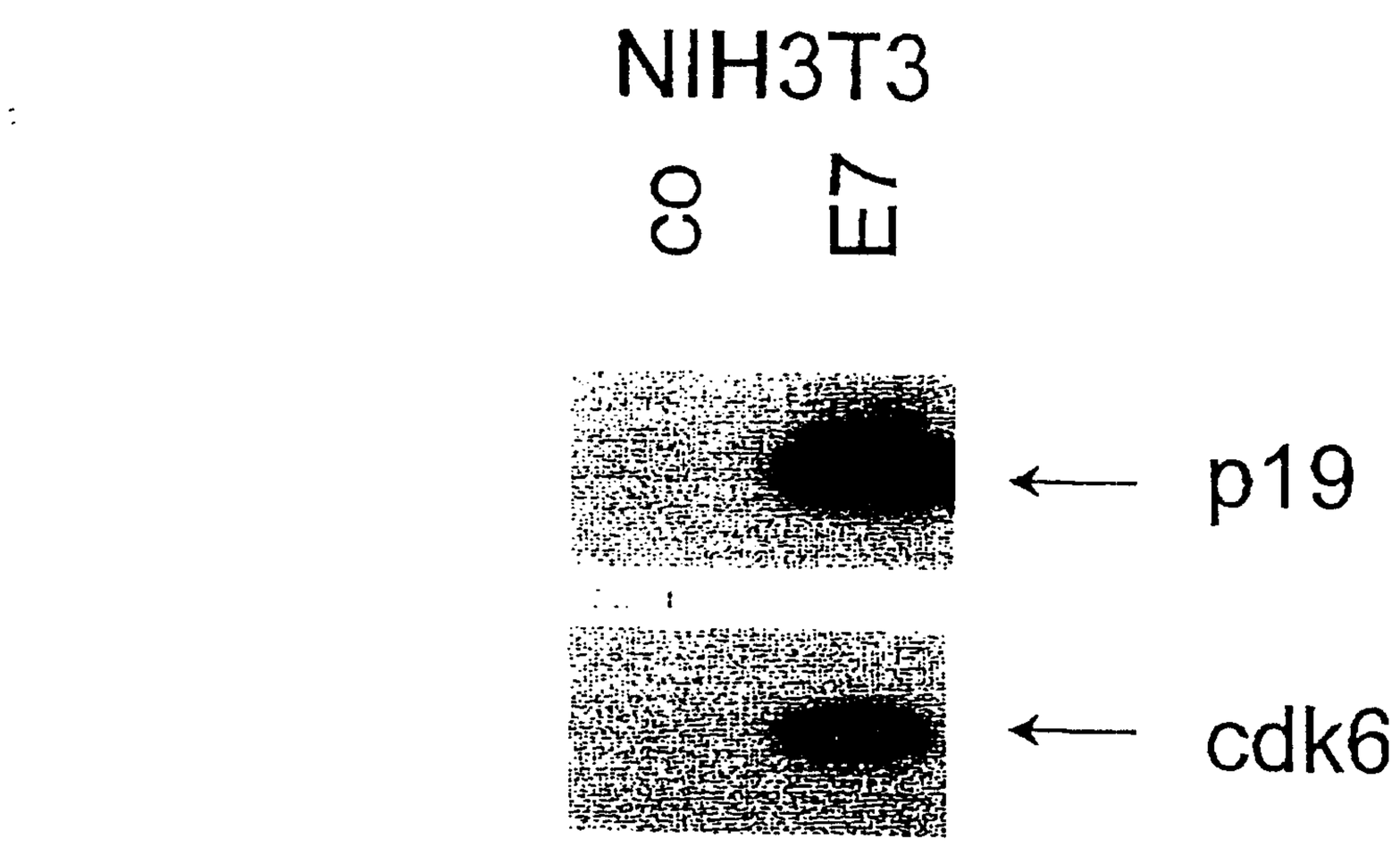


Fig. 1



Fig. 2