Title: USE OF PROTEIN UBC13 AS A MARKER FOR BREAST CANCER

Abstract: The present invention relates to the diagnosis of breast cancer. It discloses the use of ubiquitin-conjugating enzyme E2N (UBC13) in the diagnosis of breast cancer. It relates to a method for diagnosis of breast cancer from a liquid sample, derived from an individual by measuring UBC13 in said sample. Measurement of UBC13 can, e.g., be used in the early detection or diagnosis of breast cancer.
Use of protein UBC13 as a marker for breast cancer

The present invention relates to the diagnosis of breast cancer. It discloses the use of ubiquitin-conjugating enzyme E2N (= UBC13, and also known as UBCN) in the diagnosis of breast cancer. Furthermore, it especially relates to a method for diagnosis of breast cancer from a liquid sample, derived from an individual by measuring UBC13 in said sample. Measurement of UBC13 can, e.g., be used in the early detection or diagnosis of breast cancer.

Cancer remains a major public health challenge despite progress in detection and therapy. Amongst the various types of cancer, breast cancer (= BC) is one of the most frequent cancers among women in the Western world.

The earlier cancer can be detected/diagnosed, the better is the overall survival rate. This is especially true for BC. The prognosis in advanced stages of tumor is poor. More than one third of the patients will die from progressive disease within five years after diagnosis, corresponding to a survival rate of about 40% for five years. Current treatment is only curing a fraction of the patients and clearly has the best effect on those patients diagnosed in an early stage of disease.

With regard to BC as a public health problem, it is essential that more effective screening and preventative measures for breast cancer will be developed.

The earliest detection procedures available at present for breast cancer involve using clinical breast examination and mammography. However, significant tumor size must typically exist before a tumor is palpable or can be detected by a mammogram. The density of the breast tissue and the age are important predictors of the accuracy of screening mammography. The sensitivity ranges from 63% in women with extremely dense breasts to 87% in women with almost entirely fatty breasts. The sensitivity increases with age from 69% in women of about 40 years of age to 83% in women 80 years and older (Carney, P.A., et al., Ann. Intern. Med. 138 (3) (2003) 168-175). Only 20 – 25 % of mammographically detected abnormalities that are biopsied prove to be malignant. The visualization of precancerous and cancerous lesions represents the best approach to early detection, but mammography is an expensive test that requires great care and expertise both

In the recent years a tremendous amount of so-called breast specific or even so-called breast cancer specific genes has been reported. The vast majority of the corresponding research papers or patent applications are based on data obtained by analysis of RNA expression patterns in breast (cancer) tissue versus a different tissue or an adjacent normal tissue, respectively. Such approaches may be summarized as differential mRNA display techniques.

As an example for data available from mRNA-display techniques, WO 00/60076 shall be mentioned and discussed. This application describes and claims more than two hundred isolated polynucleotides and the corresponding polypeptides as such, as well as their use in the detection of BC. However, it is general knowledge that differences on the level of mRNA are not mirrored by the level of the corresponding proteins. A protein encoded by a rare mRNA may be found in very high amounts and a protein encoded by an abundant mRNA may nonetheless be hard to detect and find at all (Chen, G., et al., Molecular and Cellular Proteomics, 1.4 (2002) 304-313). This lack of correlation between mRNA-level and protein level is due to reasons like mRNA stability, efficiency of translation, stability of the protein, etc.

There also are recent approaches investigating the differences in protein patterns between different tissues or between healthy and diseased tissue in order to identify candidate marker molecules which might be used in the diagnosis of BC. Wulfkuhle et al. Cancer Research 62 (2002) 6740-6749 have identified fifty-seven proteins which were differentially expressed between BC tissue and adjacent normal tissue. No data from liquid samples obtained from an individual are reported.

WO 02/23200 reports about twelve breast cancer-associated spots as found by surface-enhanced laser desorption and ionization (SELDI). These spots are seen more frequently in sera obtained from patients with BC as compared to sera obtained from healthy controls. However, the identity of the molecule(s) comprised in such spot, e.g. their sequence, is not known.

Nipple aspirate fluid (NAF) has been used for many years as a potential non-invasive method to identify breast cancer-specific markers. Kuerer et al. compared
bilateral matched pair nipple aspirate fluids from women with unilateral invasive breast carcinoma by 2D gel electrophoresis (Kuerer, H.M., et al., Cancer 95 (2002) 2276-2282). 30 to 202 different protein spots were detected in the NAF of breasts suffering from breast carcinoma and not in the matched NAF of the healthy breasts. These spots were detected by a gel image analysis. But the identity of the protein spots is not known.

Despite the large and ever growing list of candidate protein markers in the field of BC, to date clinical/diagnostic utility of these molecules is not known. In order to be of clinical utility a new diagnostic marker as a single marker should be at least as good as the best single marker known in the art. Or, a new marker should lead to a progress in diagnostic sensitivity and/or specificity either if used alone or in combination with one or more other markers, respectively. The diagnostic sensitivity and/or specificity of a test is best assessed by its receiver-operating characteristics, which will be described in detail below.

At present, only diagnostic blood tests based on the detection of cancer antigen 15-3 (CA 15-3), a tumor-associated mucin, and carcinoembryonic antigen (CEA), a tumor associated glycoprotein, are available to assist diagnosis in the field of BC. CA 15-3 is usually increased in patients with advanced breast cancer. CA 15-3 levels are rarely elevated in women with early stage breast cancer (Duffy, M.J., Critical Reviews in Clinical Laboratory Sciences 38 (2001) 225-262). Cancers of the ovary, lung and prostate may also raise CA 15-3 levels. Elevated levels of CA 15-3 may be associated with non-cancerous conditions, such as benign breast or ovary disease, endometriosis, pelvic inflammatory disease, and hepatitis. Pregnancy and lactation can also cause CA 15-3 levels to raise (National Cancer Institute, Cancer Facts, Fact Sheet 5.18 (1998) 1-5). The primary use of CEA is in monitoring colon cancer, especially when the disease has metastasized. However, a variety of cancers can produce elevated levels of CEA, including breast cancer.

Due to the lack of organ and tumor specificity, neither measurement of CA 15-3 nor measurement of CEA are recommended for screening of BC. These tumor markers are helpful diagnostic tools in follow-up care of BC patients (Untch, M., et al., J. Lab. Med. 25 (2001) 343-352).
Whole blood, serum, plasma, or nipple aspirate fluid are the most widely used sources of sample in clinical routine. The identification of an early BC tumor marker that would allow reliable cancer detection or provide early prognostic information could lead to a diagnostic assay that would greatly aid in the diagnosis and in the management of this disease. Therefore, an urgent clinical need exists to improve the diagnosis of BC from blood. It is especially important to improve the early diagnosis of BC, since for patients diagnosed early, chances of survival are much higher as compared to those diagnosed at a progressed stage of disease.

It was the task of the present invention to investigate whether a new marker can be identified which may aid in BC diagnosis.

Surprisingly, it has been found that use of the marker UBC13 can at least partially overcome the problems known from the state of the art.

The present invention therefore relates to a method for the diagnosis of breast cancer comprising the steps of a) providing a liquid sample obtained from an individual, b) contacting said sample with a specific binding agent for UBC13 under conditions appropriate for formation of a complex between said binding agent and UBC13, and c) correlating the amount of complex formed in (b) to the diagnosis of breast cancer.

Another preferred embodiment of the invention is a method for the diagnosis of breast cancer comprising the steps of a) contacting a liquid sample obtained from an individual with a specific binding agent for UBC13 under conditions appropriate for formation of a complex between said binding agent and UBC13, and b) correlating the amount of complex formed in (a) to the diagnosis of breast cancer.

As the skilled artisan will appreciate, any such diagnosis is made in vitro. The patient sample is discarded afterwards. The patient sample is merely used for the in vitro diagnostic method of the invention and the material of the patient sample is not transferred back into the patient's body. Typically, the sample is a liquid sample.
The ubiquitin-conjugating enzyme E2N (UBC13 or UBCN; gene name UBE2N; Swiss-PROT: Q16781) is characterized by the sequence given in SEQ ID NO:1. This sequence translates to a calculated molecular weight of 17138 Da and to an isoelectric point at pH 6.54.

In 1996 Yamaguchi et al. cloned the cDNA encoding UBE2N, a human ubiquitin-conjugating enzyme, from an epidermoid carcinoma KB cDNA library. Northern blot analysis detected a major 1.4- and a minor 2.4- kb transcript in all tissues examined with the highest expression in heart, skeletal muscle, and testis (Yamaguchi, T., et al., J. Biochem. 120 (1996) 494-497).

Ubiquitin can be covalently attached to target proteins, leading to their ubiquitination. Many ubiquitinated proteins degraded by the proteasome. Ubiquitination-mediated degradation and change in activity regulate many molecules of the cell death machinery, such as p53, caspases, and Bcl-2 family members. It has been demonstrated that the degradation of the IAP (inhibitor of apoptosis) proteins is required for apoptosis to occur in some systems, indicating apoptosis proceeds by activating death pathways as well as eliminating “roadblocks” through ubiquitination (Yang, Y., Yu, X., The FASEB Journal 17 (2003) 790-799).

Selective protein degradation by the ubiquitin-proteasome pathway has emerged as a powerful regulatory mechanism in a wide variety of cellular processes. Ubiquitin conjugation requires the sequential activity of three enzymes or protein complexes called the ubiquitin-activity enzyme (E1), the ubiquitin-conjugating enzyme (E2), and the ubiquitin-protein ligase (E3). The specific selection of target proteins is accomplished by the E2 and E3 proteins (Del Pozo, J.C., Estelle, M., Plant Molecular Biology 44 (2000) 123-128).

E2 proteins have a highly conserved core domain of approximately 150 amino acids which contains the active-site cysteine (Haldeman M.T., et al., Biochemistry 36 (1997) 10526-10537).

The E2 enzyme, UBC13, and the E2 enzyme variants, Uevs, form stable, high affinity complexes for the assembly of Lys63-linked ubiquitin chains. This process is involved in error-free DNA postreplication repairs and other cellular processes (Ashley, C., et al., Gene 285 (2002) 183-191).
As obvious to the skilled artisan, the present invention shall not be construed to be limited to the full-length protein UBC13 of SEQ ID NO:1. Physiological or artificial fragments of UBC13, secondary modifications of UBC13, as well as allelic variants of UBC13 are also encompassed by the present invention. Artificial fragments preferably encompass a peptide produced synthetically or by recombinant techniques, which at least comprises one epitope of diagnostic interest consisting of at least 6 contiguous amino acids as derived from the sequence disclosed in SEQ ID NO:1. Such fragment may advantageously be used for generation of antibodies or as a standard in an immunoassay. More preferred the artificial fragment comprises at least two epitopes of interest appropriate for setting up a sandwich immunoassay.

In preferred embodiments, the novel marker UBC13 may be used for monitoring as well as for screening purposes.

When used in patient monitoring the diagnostic method according to the present invention may help to assess tumor load, efficacy of treatment and tumor recurrence in the follow-up of patients. Increased levels of UBC13 are directly correlated to tumor burden. After chemotherapy a short term (few hours to 14 days) increase in UBC13 may serve as an indicator of tumor cell death. In the follow-up of patients (from 3 months to 10 years) an increase of UBC13 can be used as an indicator for tumor recurrence.

In a preferred embodiment the diagnostic method according to the present invention is used for screening purposes. I.e., it is used to assess subjects without a prior diagnosis of BC by measuring the level of UBC13 and correlating the level measured to the presence or absence of BC.

The staging of cancer is the classification of the disease in terms of extent, progression, and severity. It groups cancer patients so that generalizations can be made about prognosis and the choice of therapy.

Today, the TNM system is the most widely used classification of the anatomical extent of cancer. It represents an internationally accepted, uniform staging system. There are three basic variables: T (the extent of the primary tumor), N (the status of regional lymph nodes) and M (the presence or absence of distant metastases). The TNM criteria are published by the UICC (International Union Against Cancer)

What is especially important is, that early diagnosis of BC translates to a much better prognosis. Therefore, best prognosis have those patients as early as in stage T\textsubscript{is}, N0, M0 or T1-3; N0; M0, if treated properly have a more than 90% chance of survival 5 years after diagnosis as compared to a 5-years survival rate of only 18% for patients diagnosed when distant metastases are already present.

In the sense of the present invention early diagnosis of BC refers to a diagnosis at a pre-cancerous state (DCIS) or at a tumor stage where no metastases at all (neither proximal nor distal), i.e., T\textsubscript{is}, N0, M0 or T1-4; N0; M0 are present. T\textsubscript{is} denotes carcinoma \textit{in situ}.

In a preferred embodiment UBC13 is used to diagnose BC in a non-metastatic stage, i.e., that diagnosis is made at stage T\textsubscript{is}, N0, M0 or T1-3; N0; M0 (\(=T\textsubscript{is}-3; N0; M0\)).

The diagnostic method according to the present invention is based on a liquid sample which is derived from an individual. Unlike to methods known from the art UBC13 is specifically measured from this liquid sample by use of a specific binding agent.

A specific binding agent is, e.g., a receptor for UBC13, a lectin binding to UBC13 or an antibody to UBC13. A specific binding agent has at least an affinity of \(10^7\) l/mol for its corresponding target molecule. The specific binding agent preferably has an affinity of \(10^8\) l/mol or even more preferably of \(10^9\) l/mol for its target molecule. As the skilled artisan will appreciate the term specific is used to indicate that other biomolecules present in the sample do not significantly bind to with the binding agent specific for UBC13. Preferably, the level of binding to a biomolecule other than the target molecule results in a binding affinity which is only 10%, more preferably only 5% of the affinity of the target molecule or less. A most preferred specific binding agent will fulfill both the above minimum criteria for affinity as well as for specificity.
A specific binding agent preferably is an antibody reactive with UBC13. The term antibody refers to a polyclonal antibody, a monoclonal antibody, fragments of such antibodies, as well as to genetic constructs comprising the binding domain of an antibody. Any antibody fragment retaining the above criteria of a specific binding agent can also be used.

Antibodies are generated by state of the art procedures, e.g., as described in Tijssen (Tijssen, P., Practice and theory of enzyme immunoassays 11 (1990) the whole book, especially pages 43-78; Elsevier, Amsterdam). In addition, the skilled artisan is well aware of methods based on immunosorbents that can be used for the specific isolation of antibodies. By these means the quality of polyclonal antibodies and hence their performance in immunoassays can be enhanced. (Tijssen, P., supra, pages 108-115).

For the achievements as disclosed in the present invention monoclonal and polyclonal antibodies have been used. Polyclonal antibodies have been raised in rabbits. However, clearly also polyclonal antibodies from different species, e.g. rats or guinea pigs can also be used. Monoclonal antibodies have been produced using spleen cells from immunized mice. Since monoclonal antibodies can be produced in any amount required with constant properties, they represent ideal tools in development of an assay for clinical routine. The generation and use of monoclonal antibodies to UBC13 in a method according to the present invention is yet another preferred embodiment.

As the skilled artisan will appreciate now, that UBC13 has been identified as a marker which is useful in the diagnosis of BC, alternative ways may be used to reach a result comparable to the achievements of the present invention. For example, alternative strategies to generate antibodies may be used. Such strategies comprise amongst others the use of synthetic peptides, representing an epitope of UBC13 for immunization. Preferably, a synthetic peptide comprises a subsequence of SEQ ID NO:1 which is specific for UBC13, i.e., which has a comparatively low homology to other/related polypeptides. It is preferred that the synthetic peptide comprises a contiguous subsequence consisting of 5 to 25 amino acid residues of SEQ ID NO:1. More preferred, the peptide comprises a contiguous subsequence consisting of 10 to 15 amino acid residues of SEQ ID NO:1.
Alternatively, DNA immunization also known as DNA vaccination may be used.

For measurement the liquid sample obtained from an individual is incubated with the specific binding agent for UBC13 under conditions appropriate for formation of a binding agent UBC13-complex. Such conditions need not be specified, since the skilled artisan without any inventive effort can easily identify such appropriate incubation conditions.

As a final step according to the method disclosed in the present invention the amount of complex is measured and correlated to the diagnosis of BC. As the skilled artisan will appreciate there are numerous methods to measure the amount of specific binding agent UBC13-complex all described in detail in relevant textbooks (cf., e.g., Tijssen P., supra, or Diamandis et al., eds. (1996) Immunoassay, Academic Press, Boston).

Preferably UBC13 is detected in a sandwich type assay format. In such assay a first specific binding agent is used to capture UBC13 on the one side and a second specific binding agent, which is labeled to be directly or indirectly detectable is used on the other side.

As mentioned above, it has surprisingly been found that UBC13 can be measured from a liquid sample obtained from an individual sample. No tissue and no biopsy sample is required to apply the marker UBC13 in the diagnosis of BC.

In a preferred embodiment the method according to the present invention is practiced with serum as liquid sample material.

In a further preferred embodiment the method according to the present invention is practiced with plasma as liquid sample material.

In a further preferred embodiment the method according to the present invention is practiced with whole blood as liquid sample material.

In a further preferred embodiment the method according to the present invention is practiced with nipple aspirate fluid as liquid sample material.
Whereas application of routine proteomics methods to tissue samples, leads to the identification of many potential marker candidates for the tissue selected, the inventors of the present invention have surprisingly been able to detect UBC13 in a bodily fluid sample. Even more surprising they have been able to demonstrate that the presence of UBC13 in such liquid sample obtained from an individual can be correlated to the diagnosis of breast cancer.

Antibodies to UBC13 with great advantage can be used in established procedures, e.g., to detect breast cancer cells in situ, in biopsies, or in immunohistological procedures.

Preferably, an antibody to UBC13 is used in a qualitative (UBC13 present or absent) or quantitative (UBC13 amount is determined) immunoassay.

Measuring the level of protein UBC13 has proven very advantageous in the field of BC. Therefore, in a further preferred embodiment, the present invention relates to use of protein UBC13 as a marker molecule in the diagnosis of breast cancer from a liquid sample obtained from an individual.

The term marker molecule is used to indicate that an increased level of the analyte UBC13 as measured from a bodily fluid of an individual marks the presence of BC.

It is especially preferred to use the novel marker UBC13 in the early diagnosis of breast cancer.

The use of protein UBC13 itself, represents a significant progress to the challenging field of BC diagnosis. Combining measurements of UBC13 with other known markers, e.g. CA 15-3 and CEA, or with other markers of BC presently known or yet to be discovered, leads to further improvements. Therefore in a further preferred embodiment the present invention relates to the use of UBC13 as a marker molecule for breast cancer in combination with one or more marker molecules for breast cancer in the diagnosis of breast cancer from a liquid sample obtained from an individual. In this regard, the expression “one or more” denotes 1 to 10, preferably 1 to 5, more preferred 3. Preferred selected other BC markers with which the measurement of UBC13 may be combined are CEA and CA 15-3. Most preferred, UBC13 is used as part of a marker panel at least comprising UBC13 and
CA 15-3. Thus, a further preferred embodiment of the present invention is the use of the protein UBC13 as a marker molecule for breast cancer in combination with one or more marker molecules for breast cancer in the diagnosis of breast cancer from a liquid sample obtained from an individual, whereby the at least one other marker molecule is CA 15-3.

Preferably, the inventive method is used with samples of patients suspected of suffering from breast cancer. An individual suspected of suffering from breast cancer is an individual for which other types of cancers have been excluded. Other cancers include but are not limited to cancers of the colon, lung, stomach, ovary, and prostate. A preferred embodiment of the invention is therefore a method for the diagnosis of breast cancer comprising the steps of a) providing a liquid sample obtained from an individual suspected of suffering from breast cancer, b) contacting said sample with a specific binding agent for UBC13 under conditions appropriate for formation of a complex between said binding agent and UBC13, and c) correlating the amount of complex formed in (b) to the diagnosis of breast cancer.

Diagnostic reagents in the field of specific binding assays, like immunoassays, usually are best provided in the form of a kit, which comprises the specific binding agent and the auxiliary reagents required to perform the assay. The present invention therefore also relates to an immunological kit comprising at least one specific binding agent for UBC13 and auxiliary reagents for measurement of UBC13.

Accuracy of a test is best described by its receiver-operating characteristics (ROC) (see especially Zweig, M. H., and Campbell, G., Clin. Chem. 39 (1993) 561-577). The ROC graph is a plot of all of the sensitivity/specificity pairs resulting from continuously varying the decision thresh-hold over the entire range of data observed.

The clinical performance of a laboratory test depends on its diagnostic accuracy, or the ability to correctly classify subjects into clinically relevant subgroups. Diagnostic accuracy measures the test's ability to correctly distinguish two different conditions of the subjects investigated. Such conditions are for example health and disease or benign versus malignant disease.
In each case, the ROC plot depicts the overlap between the two distributions by plotting the sensitivity versus 1 - specificity for the complete range of decision thresholds. On the y-axis is sensitivity, or the true-positive fraction [defined as (number of true-positive test results) / (number of true-positive + number of false-negative test results)]. This has also been referred to as positivity in the presence of a disease or condition. It is calculated solely from the affected subgroup. On the x-axis is the false-positive fraction, or 1 - specificity [defined as (number of false-positive results) / (number of true-negative + number of false-positive results)]. It is an index of specificity and is calculated entirely from the unaffected subgroup. Because the true- and false-positive fractions are calculated entirely separately, by using the test results from two different subgroups, the ROC plot is independent of the prevalence of disease in the sample. Each point on the ROC plot represents a sensitivity/-specificity pair corresponding to a particular decision threshold. A test with perfect discrimination (no overlap in the two distributions of results) has an ROC plot that passes through the upper left corner, where the true-positive fraction is 1.0, or 100% (perfect sensitivity), and the false-positive fraction is 0 (perfect specificity). The theoretical plot for a test with no discrimination (identical distributions of results for the two groups) is a 45° diagonal line from the lower left corner to the upper right corner. Most plots fall in between these two extremes. (If the ROC plot falls completely below the 45° diagonal, this is easily remedied by reversing the criterion for "positivity" from "greater than" to "less than" or vice versa.) Qualitatively, the closer the plot is to the upper left corner, the higher the overall accuracy of the test.

One convenient goal to quantify the diagnostic accuracy of a laboratory test is to express its performance by a single number. The most common global measure is the area under the ROC plot. By convention, this area is always ≥ 0.5 (if it is not, one can reverse the decision rule to make it so). Values range between 1.0 (perfect separation of the test values of the two groups) and 0.5 (no apparent distributional difference between the two groups of test values). The area does not depend only on a particular portion of the plot such as the point closest to the diagonal or the sensitivity at 90% specificity, but on the entire plot. This is a quantitative, descriptive expression of how close the ROC plot is to the perfect one (area = 1.0).

Clinical utility of the novel marker UBC13 has been assessed in comparison to and in combination with the established marker CA 15-3 using a receiver operator
curve analysis (ROC; Zweig, M. H., and Campbell, G., Clin. Chem. 39 (1993) 561-577). This analysis has been based on well-defined patient cohorts consisting of 50 samples each from patients with invasive ductal or lobular carcinoma in T1-3; N0; M0, more progressed tumor, i.e., T4 and/or various severity of metastasis (N+ and/or M+), medullary, papillary, mucinous and tubular carcinoma, ductal carcinoma in situ, and healthy controls, respectively.

The following examples, references, sequence listing and figure are provided to aid the understanding of the present invention, the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

**Description of the Figure**

Figure 1  Figure 1 shows a typical example of a 2D-gel, loaded with a tumor sample (left side), and a gel, loaded with a matched control sample (right side). The circle in the enlarged section of these gels indicates the position for the protein UBC13. Using the same method this protein has not been detected in healthy tissue.

**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABTS</td>
<td>2,2'-Azino-di- [3-ethylbenzthiazoline sulfonate (6)] diammonium salt</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CHAPS</td>
<td>(3-[(3-Cholamidopropyl)-dimethylammonio]- 1-propane-sulfonate)</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diamine tetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>IAA</td>
<td>iodacetamid</td>
</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
</tr>
</tbody>
</table>
Example 1
Identification of UBC13 as a potential breast cancer marker

Sources of tissue

In order to identify tumor-specific proteins as potential diagnostic markers for breast cancer, analysis of two different kinds of tissue is performed using proteomics methods.

In total, tissue specimen from 14 patients suffering from breast cancer are analyzed. From each patient two different tissue types are collected from therapeutic resections: Tumor tissue (> 80% tumor) (T), and adjacent healthy tissue (N). The latter tissue type serves as matched healthy control sample. Tissues are immediately snap frozen after resection and stored at −80°C before processing. Tumors are diagnosed by histopathological criteria.

Tissue preparation

0.8-1.2 g of frozen tissue are put into a mortar and completely frozen by liquid nitrogen. The tissue is pulverized in the mortar, dissolved in the 10-fold volume (w/v) of lysis buffer (40 mM Na-citrate, 5 mM MgCl₂, 1% Genapol X-080, 0.02% Na-azide, Complete® EDTA-free [Roche Diagnostics GmbH, Mannheim,
Germany, Cat. No. 1 873 580] and subsequently homogenized in a Wheaton® glass homogenizer (20 x loose fitting, 20 x tight fitting). 3 ml of the homogenate are subjected to a sucrose-density centrifugation (10-60% sucrose) for 1 h at 4,500 x g. After this centrifugation step three fractions are obtained. The fraction on top of the gradient contains the soluble proteins and is used for further analysis.

**Immobilization of monoclonal antibody anti-human albumin on CNBr-activated Sepharose 4B**

Freeze-dried CNBr-activated Sepharose 4B (Amersham Biosciences, 17-0430-01) is reswollen and washed according to the instructions of the manufacturer. Monoclonal antibody directed against human albumin is dissolved in 0.1 M NaHCO₃, pH 8.3, 0.5 M NaCl, 10 mg/ml. 1 ml antibody solution is mixed with 1 ml reswollen CNBr-activated Sepharose 4B. The reaction time is 1 h. Blocking of the remaining active groups and washing of the gel is carried out according to the instructions of the manufacturer.

**Depletion of serum albumin**

7 ml anti-albumin gel is equilibrated in lysis buffer without Genapol X-080. 7 ml of the upper fraction of the sucrose-density centrifugation (see above, tissue preparation) are applied onto the column and washed through with lysis buffer without Genapol X-080. The combined effluent is used for the isoelectric focussing experiments.

**Isoelectric focussing (IEF) and SDS-PAGE**

For IEF, 3 ml of the HSA-depleted tissue preparation are mixed with 12 ml sample buffer (7 M urea, 2 M thiourea, 2% CHAPS, 0.4% IPG buffer pH 4-7, 0.5% DTT) and incubated for 1 h. The samples are concentrated in an Amicon® Ultra-15 device (Millipore GmbH, Schwalbach, Germany) and the protein concentration is determined using the Bio-Rad® protein assay (Cat.No. 500-0006; Bio-Rad Laboratories GmbH, München, Germany) following the instructions of the supplier's manual. To a volume corresponding to 1.5 mg of protein sample buffer is added to a final volume of 350 µl. This solution is used to rehydrate IPG strips pH 4-7 (Amersham Biosciences, Freiburg, Germany) overnight. The IEF is
performed using the following gradient protocol: (1.) 1 minute to 500 V; (2.) 2 h to 3500 V; (3.) 22 h at constant 3500 V giving rise to 82 kVh. After IEF, strips are stored at –80°C or directly used for SDS-PAGE.

Prior to SDS-PAGE the strips are incubated in equilibration buffer (6 M urea, 50 mM Tris/HCl, pH 8.8, 30% glycerol, 2% SDS), for reduction DTT (15 min, + 50 mg DTT/10 ml), and for alkylation IAA (15 min, + 235 mg iodoacetamide/10 ml) is added. The strips are put on 12.5% polyacrylamide gels and subjected to electrophoresis at 1 W/gel and thereafter 1 h at 17 W/gel. Subsequently, the gels are fixed (50% methanol, 10% acetate) and stained overnight with Novex™ Colloidal Blue Staining Kit (Invitrogen, Karlsruhe, Germany, Cat No. LC6025, 45-7101)

**Detection of UBC13 as a potential marker for breast cancer**

Each patient is analyzed separately by image analysis with the ProteomeWeaver® software (Definiens AG, Germany, München). In addition, all spots of the gel are excised by a picking robot and the proteins present in the spots are identified by MALDI-TOF mass spectrometry (Ultraflex™ Tof/Tof, Bruker Daltonik GmbH, Bremen, Germany). For each patient, 4 gels from the tumor sample are compared with 4 gels each from adjacent tissue and analyzed for distinctive spots corresponding to differentially expressed proteins. By this means, protein UBC13 is found to be specifically expressed or strongly overexpressed in tumor tissue and not detectable in healthy control tissue. It therefore – amongst many other proteins – qualifies as a candidate marker for use in the diagnosis of breast cancer.

**Example 2**

**Generation of antibodies to the breast cancer marker protein UBC13**

Polyclonal antibody to the breast cancer marker protein UBC13 is generated for further use of the antibody in the measurement of serum and plasma and blood levels of UBC13 by immunodetection assays, e.g. Western Blotting and ELISA

**Recombinant protein expression and purification**

In order to generate antibodies to UBC13, recombinant expression of the protein is performed for obtaining immunogens. The expression is done applying a
combination of the RTS 100 expression system and E. coli. In a first step, the DNA sequence is analyzed and recommendations for high yield cDNA silent mutational variants and respective PCR-primer sequences are obtained using the "ProteoExpert RTS E.coli HY" system. This is a commercial web-based service (www.proteoexpert.com). Using the recommended primer pairs, the "RTS 100 E. coli Linear Template Generation Set, His-tag" (Roche Diagnostics GmbH, Mannheim, Germany, Cat.No. 3186237) system to generate linear PCR templates from the cDNA for in-vitro transcription and expression of the nucleotide sequence coding for the UBC13 protein is used. For Western-blot detection and later purification, the expressed protein contains a His-tag. The best expressing variant is identified. All steps from PCR to expression and detection are carried out according to the instructions of the manufacturer. The respective PCR product, containing all necessary T7 regulatory regions (promoter, ribosomal binding site and T7 terminator) is cloned into the pBAD TOPO® vector (Invitrogen, Karlsruhe, Germany, Cat. No. K 4300/01) following the manufacturer's instructions. For expression using the T7 regulatory sequences, the construct is transformed into E. coli BL21 (DE3) (Studier, F.W., et al., Methods Enzymol. 185 (1990) 60-89) and the transformed bacteria are cultivated in a 1 l batch for protein expression.

Purification of His-UBC13 fusion protein is done following standard procedures on a Ni-chelate column. Briefly, 1 l of bacteria culture containing the expression vector for the His-UBC13 fusion protein is pelleted by centrifugation. The cell pellet is resuspended in lysis buffer, containing phosphate, pH 8.0, 7 M guanidium chloride, imidazole and thioglycerol, followed by homogenization using a Ultra-Turrax®. Insoluble material is pelleted by high speed centrifugation and the supernatant is applied to a Ni-chelate chromatographic column. The column is washed with several bed volumes of lysis buffer followed by washes with buffer, containing phosphate, pH 8.0 and urea. Finally, bound antigen is eluted using a phosphate buffer containing SDS under acid conditions.

**Synthesis of hemocyanin-peptide-conjugates for the generation of antibodies**

Synthesis is carried out using heterobifunctional chemistry (maleimide/SH-chemistry). Selected cysteine containing UBC13-peptides are coupled to 3-maleimidoheanoyl-N-hydroxysuccinimidester (MHS) activated hemocyanin from Concholepas concholepas (Sigma, B-8556).
Hemocyanin is brought to 10 mg/ml in 100 mM NaH₂PO₄/NaOH, pH 7.2. Per ml hemocyanin 100 μl MHS (12.3 mg in DMSO) are added and incubated for 1 h. The sample is dialyzed over night against 100 mM NaH₂PO₄/NaOH, pH 6.5 and adjusted to 6 mg/ml with dialysis buffer. A selected cysteine containing UBC13-peptide was dissolved in DMSO (5 mg/ml for a peptide of 1500 Dalton). Per ml MHS-activated hemocyanin (6 mg/ml) 20 μl of 100 mM EDTA, pH 7.0 and 100 μl of the selected cysteine containing UBC13-peptide are added. After 1 h the remaining maleimide groups are blocked by the addition of 10 μl 0.5 M cysteine/HCl per ml reaction mixture. This preparation is used for immunization without further purification.

Production of monoclonal antibodies against UBC13

a) Immunization of mice

12 week old A/J mice are initially immunized intraperitoneally with 100 μg UBC13 or hemocyanin-peptide-conjugate (see above). This is followed after 6 weeks by two further intraperitoneal immunizations at monthly intervals. In this process each mouse is administered 100 μg UBC13 or hemocyanin-peptide-conjugate adsorbed to aluminium hydroxide and 10⁹ germs of Bordetella pertussis. Subsequently the last two immunizations are carried out intravenously on the 3rd and 2nd day before fusion using 100 μg UBC13 or hemocyanin-peptide-conjugate in PBS buffer for each.

b) Fusion and cloning

Spleen cells of the mice immunized according to a) are fused with myeloma cells according to Galfre, G., and Milstein, C., Methods in Enzymology 73 (1981) 3-46. In this process ca. 1x10⁸ spleen cells of the immunized mouse are mixed with 2x10⁷ myeloma cells (P3X63-Ag8-653, ATCC CRL1580) and centrifuged (10 min at 300 x g and 4°C.). The cells are then washed once with RPMI 1640 medium without foetal calf serum (FCS) and centrifuged again at 400 x g in a 50 ml conical tube. The supernatant is discarded, the cell sediment is gently loosened by tapping, 1 ml PEG (molecular weight 4000, Merck, Darmstadt) is added and mixed by pipetting. After 1 min in a water-bath at 37°C., 5 ml RPMI 1640 without FCS is added drop-wise at room temperature within a period of 4-5 min. Afterwards 5 ml RPMI 1640
containing 10% FCS is added drop-wise within ca. 1 min, mixed thoroughly, filled to 50 ml with medium (RPMI 1640+10% FCS) and subsequently centrifuged for 10 min at 400 x g and 4°C. The sedimented cells are taken up in RPMI 1640 medium containing 10% FCS and sown in hypoxanthine-azaserine selection medium (100 mmol/l hypoxanthine, 1 µg/ml azaserine in RPMI 1640+10% FCS). Interleukin 6 at 100 U/ml is added to the medium as a growth factor.

After ca. 10 days the primary cultures are tested for specific antibody. UBC13-positive primary cultures are cloned in 96-well cell culture plates by means of a fluorescence activated cell sorter. In this process again interleukin 6 at 100 U/ml is added to the medium as a growth additive.

c) Immunoglobulin isolation from the cell culture supernatants

The hybridoma cells obtained are sown at a density of 1x10^5 cells per ml in RPMI 1640 medium containing 10% FCS and proliferated for 7 days in a fermenter (Thermodux Co., Wertheim/Main, Model MCS-104XL, Order No. 144-050). On average concentrations of 100 µg monoclonal antibody per ml are obtained in the culture supernatant. Purification of this antibody from the culture supernatant is carried out by conventional methods in protein chemistry (e.g. according to Bruck, C., et al., Methods in Enzymology 121 (1986) 587-695).

Generation of polyclonal antibodies

a) Immunization

For immunization, a fresh emulsion of the protein solution (100 µg/ml UBC13 or hemocyanin-peptide-conjugate) and complete Freund's adjuvant at the ratio of 1:1 is prepared. Each rabbit is immunized with 1 ml of the emulsion at days 1, 7, 14 and 30, 60 and 90. Blood is drawn and resulting anti-UBC13 serum used for further experiments as described in Examples 3 and 4.
b) Purification of IgG (immunoglobulin G) from rabbit serum by sequential precipitation with caprylic acid and ammonium sulfate

One volume of rabbit serum is diluted with 4 volumes of acetate buffer (60 mM, pH 4.0). The pH is adjusted to 4.5 with 2 M Tris-base. Caprylic acid (25 μl/ml of diluted sample) is added drop-wise under vigorous stirring. After 30 min the sample is centrifuged (13,000 x g, 30 min, 4°C), the pellet discarded and the supernatant collected. The pH of the supernatant is adjusted to 7.5 by the addition of 2 M Tris-base and filtered (0.2 μm).

The immunoglobulin in the supernatant is precipitated under vigorous stirring by the drop-wise addition of a 4 M ammonium sulfate solution to a final concentration of 2 M. The precipitated immunoglobulins are collected by centrifugation (8,000 x g, 15 min, 4°C).

The supernatant is discarded. The pellet is dissolved in 10 mM NaH₂PO₄/NaOH, pH 7.5, 30 mM NaCl and exhaustively dialyzed. The dialysate is centrifuged (13,000 x g, 15 min, 4°C) and filtered (0.2 μm).

**Biotinylation of polyclonal rabbit IgG**

Polyclonal rabbit IgG is brought to 10 mg/ml in 10 mM NaH₂PO₄/NaOH, pH 7.5, 30 mM NaCl. Per ml IgG solution 50 μl Biotin-N-hydroxysuccinimide (3.6 mg/ml in DMSO) are added. After 30 min at room temperature, the sample is chromatographed on Superdex 200 (10 mM NaH₂PO₄/NaOH, pH 7.5, 30 mM NaCl). The fraction containing biotinylated IgG are collected. Monoclonal antibodies are biotinylated according to the same procedure.

**Digoxigenylation of polyclonal rabbit IgG**

Polyclonal rabbit IgG is brought to 10 mg/ml in 10 mM NaH₂PO₄/NaOH, 30 mM NaCl, pH 7.5. Per ml IgG solution 50 μl digoxigenin-3-O-methylcarbonyl-e-aminocaproic acid-N-hydroxysuccinimide ester (Roche Diagnostics, Mannheim, Germany, Cat. No. 1 333 054) (3.8 mg/ml in DMSO) are added. After 30 min at room temperature, the sample is chromatographed on Superdex® 200 (10 mM NaH₂PO₄/NaOH, pH 7.5, 30 mM NaCl). The fractions containing digoxigenylated
IgG are collected. Monoclonal antibodies are labeled with digoxigenin according to the same procedure.

**Example 3**

Western blot for the detection of UBC13 in human serum and plasma samples.

SDS-PAGE and Western Bloting are carried out using reagents and equipment of Invitrogen, Karlsruhe, Germany. Human plasma samples are diluted 1:20 in reducing NuPAGE® (Invitrogen) LDS sample buffer and heated for 5 min at 95°C. 10 µl aliquots are run on 4-12 % NuPAGE® gels (Bis-Tris) in the MES running buffer system. The gel-separated protein mixture is blotted onto nitrocellulose membranes using the Invitrogen XCell II™ Blot Module (Invitrogen) and the NuPAGE® transfer buffer system. The membranes are washed 3 times in PBS/0.05 % Tween-20 and blocked with SuperBlock Blocking Buffer (Pierce Biotechnology, Inc., Rockford, IL, USA). The biotinylated primary antibody is diluted in SuperBlock Blocking Buffer (0.01-0.2 µg/ml) and incubated with the membrane for 1h. The membranes are washed 3 times in PBS/0.05 % Tween-20. The specifically bound biotinylated primary antibody is labeled with a streptavidin-HRP-conjugate (20 mU/ASTS/ml in SuperBlock Blocking Buffer). After incubation for 1 h, the membranes are washed 3 times in PBS/0.05 % Tween-20. The bound streptavidin-HRP-conjugate is detected using a chemiluminescent substrate (SuperSignal West Femto Substrate, Pierce Biotechnology, Inc., Rockford, IL, USA) and autoradiographic film. Exposure times varies from 10 min to over night.

**Example 4**

ELISA for the measurement of UBC13 in human serum and plasma samples.

For detection of UBC13 in human serum or plasma, a sandwich ELISA is developed. For capture and detection of the antigen, aliquots of the anti-UBC13 polyclonal antibody (see Example 2) are conjugated with biotin and digoxygenin, respectively.

Streptavidin-coated 96-well microtiter plates are incubated with 100 µl biotinylated anti-UBC13 polyclonal antibody for 60 min at 10 µg/ml in 10 mM phosphate, pH 7.4, 1% BSA, 0.9% NaCl and 0.1% Tween-20. After incubation, plates are washed three times with 0.9% NaCl, 0.1% Tween-20. Wells are then incubated for
2 h with either a serial dilution of the recombinant protein (see Example 2) as standard antigen or with diluted plasma samples from patients. After binding of UBC13, plates are washed three times with 0.9% NaCl, 0.1% Tween-20. For specific detection of bound UBC13, wells are incubated with 100 μl of digoxygenylated anti-UBC13 polyclonal antibody for 60 min at 10 μg/ml in 10 mM phosphate, pH 7.4, 1% BSA, 0.9% NaCl and 0.1% Tween-20. Thereafter, plates are washed three times to remove unbound antibody. In a next step, wells are incubated with 20 mU/ml anti-digoxigenin-POD conjugates (Roche Diagnostics GmbH, Mannheim, Germany, Catalog No. 1633716) for 60 min in 10 mM phosphate, pH 7.4, 1% BSA, 0.9% NaCl and 0.1% Tween-20. Plates are subsequently washed three times with the same buffer. For detection of antigen-antibody complexes, wells are incubated with 100 μl ABTS solution (Roche Diagnostics GmbH, Mannheim, Germany, Catalog No. 11685767) and OD is measured after 30-60 min at 405 nm with an ELISA reader.

Example 5
ROC analysis to assess clinical utility in terms of diagnostic accuracy.

Accuracy is assessed by analyzing individual liquid samples obtained from well-characterized patient cohorts, i.e., 50 patients having undergone mammography and found to be free of BC, 50 patients each diagnosed and staged as invasive ductal and invasive lobular T1-3, N0, M0 of BC, 50 patients diagnosed with progressed BC, having at least tumor infiltration in at least one proximal lymph node or more severe forms of metastasis, 50 patients each diagnosed with medullary, mucinous, tubular, or papillary breast carcinoma, and 50 patients diagnosed with DCIS, respectively. CA 15-3 as measured by a commercially available assay (Roche Diagnostics, CA 15-3-assay (Cat. No. 0 304 5838 for Elecsys® Systems immunoassay analyzer) and UBC13 measured as described above have been quantified in a serum obtained from each of these individuals. ROC-analysis is performed according to Zweig, M. H., and Campbell, supra. Discriminatory power for differentiating patients in the group T_1-, N0, M0 from healthy individuals for the combination of UBC13 with the established marker CA 15-3 is calculated by regularized discriminant analysis (Friedman, J. H., Regularized Discriminant Analysis, Journal of the American Statistical Association 84 (1989) 165-175).
Preliminary data indicate that UBC13 may also be very helpful in the follow-up of patients after surgery.
List of References

Del Pozo, J.C., Estelle, M., Plant Molecular Biology 44 (2000) 123-128
Duffy, M.J., Critical Reviews in Clinical Laboratory Sciences 38 (2001) 225-262
Kuerer, H.M., et al., Cancer 95 (2002) 2276-2282
National Cancer Institute, Cancer Facts, Fact Sheet 5.18 (1998) 1-5
Tijssen, P., Practice and theory of enzyme immunoassays 11 (1990) the whole book,
especially pages 43-78; Elsevier, Amsterdam
UICC (International Union Against Cancer), Sobin, L.H., Wittekind, Ch. (eds),
TNM Classification of Malignant Tumours, fifth edition, 1997
WHO, Screening for Breast Cancer, May 10, 2002
WO 00/60076
WO 02/23200
Yamaguchi, T., et al., J. Biochem. 120 (1996) 494-497
Patent Claims

1. A method for the diagnosis of breast cancer comprising the steps of
   a) providing a liquid sample obtained from an individual,
   b) contacting said sample with a specific binding agent for ubiquitin-conjugating enzyme E2N (UBC13) under conditions appropriate for formation of a complex between said binding agent and UBC13, and
   c) correlating the amount of complex formed in (b) to the diagnosis of breast cancer.

2. The method according to claim 1, further characterized in that said sample is serum.

3. The method according to claim 1, further characterized in that said sample is plasma.

4. The method according to claim 1, further characterized in that said sample is whole blood.

5. The method according to claim 1, further characterized in that said sample is nipple aspirate fluid.

6. Use of protein UBC13 as a marker molecule in the diagnosis of breast cancer from a liquid sample obtained from an individual.

7. Use of protein UBC13 as a marker molecule in the early diagnosis of breast cancer from a liquid sample obtained from an individual.

8. Use according to claim 7, wherein the early diagnosis is made with a sample derived from breast cancer patients in stage T1: N0; M0.

9. Use of protein UBC13 as a marker molecule for breast cancer in combination with one or more marker molecules for breast cancer in the diagnosis of breast cancer from a liquid sample obtained from an individual.
10. Use according to claim 9, wherein the at least one other marker molecule is CA 15-3.

11. An immunological kit comprising at least one specific binding agent for UBC13 and auxiliary reagents for measurement of UBC13.
Fig. 1

Tumor sample

matched control sample

UBC13

magnification of clipping
SEQUENCE LISTING

<110> Roche Diagnostics GmbH
       P. Hoffmann-La Roche AG

<120> Use of UBC13 as a marker for breast cancer

<130> 22242

<150> EP 03023506.3
<151> 2003-10-15

<160> 1

<170> PatentIn version 3.2

<210> 1
<211> 152
<212> PRT
<213> Homo sapiens

<220>
<221> MISC_FEATURE
<223> ubiquitin-conjugating enzyme E2N (UBC13)

<400> 1

Met Ala Gly Leu Pro Arg Arg Ile Ile Lys Glu Thr Gln Arg Leu Leu
 1     5  10  15

Ala Glu Pro Val Pro Gly Ile Lys Ala Glu Pro Asp Glu Ser Asn Ala
 20  25  30

Arg Tyr Phe His Val Val Ile Ala Gly Pro Gln Asp Ser Pro Phe Glu
 35  40  45

Gly Gly Thr Phe Lys Leu Glu Leu Phe Leu Pro Glu Glu Tyr Pro Met
 50  55  60

Ala Ala Pro Lys Val Arg Phe Met Thr Lys Ile Tyr His Pro Asn Val
 65  70  75  80

Asp Lys Leu Gly Arg Ile Cys Leu Asp Ile Leu Lys Asp Lys Trp Ser
 85  90  95

Pro Ala Leu Gln Ile Arg Thr Val Leu Leu Ser Ile Gln Ala Leu Leu
100 105 110
Ser Ala Pro Asn Pro Asp Asp Pro Leu Ala Asn Asp Val Ala Glu Gln
115 120 125

Trp Lys Thr Asn Glu Ala Gln Ala Ile Glu Thr Ala Arg Ala Trp Thr
130 135 140

Arg Leu Tyr Ala Met Asn Asn Ile
145 150
# INTERNATIONAL SEARCH REPORT

## A. CLASSIFICATION OF SUBJECT MATTER

| IPC   | GOIN33/574 |

According to International Patent Classification (IPC) or to both national classification and IPC.

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

| IPC   | GOIN |

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched.

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

- EPO-Internal
- BIOSIS
- WPI Data

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>column 2, paragraph 3 abstract</td>
<td>1-10</td>
</tr>
</tbody>
</table>

X: Further documents are listed in the continuation of box C. X: Patent family members are listed in annex.

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>column 2, paragraph 3 abstract</td>
<td>1-10</td>
</tr>
</tbody>
</table>

X: Further documents are listed in the continuation of box C. X: Patent family members are listed in annex.

**A** document defining the general state of the art which is not considered to be of particular relevance

**E** earlier document but published on or after the international filing date

**I** document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

**O** document relating to an oral disclosure, use, exhibition or other means

**F** document published prior to the international filing date but later than the priority date claimed

**T** later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

**X** document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

**Y** document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

**A** document member of the same patent family

Date of the actual completion of the international search: 11 January 2005

Date of mailing of the international search report: 08/02/2005

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentnieuwe 2 NL-2280 HV Rijswijk

Tel. (+31-70) 340-3540, Tx. 31 651 epos nl, Fax (+31-70) 340-3516

Authorized officer: Bigot-Maucher, C
<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevance to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>abstract; figure 7</td>
<td>1-10</td>
</tr>
<tr>
<td>Patent document cited in search report</td>
<td>Publication date</td>
<td>Patent family member(s)</td>
</tr>
<tr>
<td>---------------------------------------</td>
<td>-----------------</td>
<td>-------------------------</td>
</tr>
<tr>
<td>US 2003073097 AI</td>
<td>17-04-2003</td>
<td>NONE</td>
</tr>
</tbody>
</table>