TURUN YLIOPISTON JULKAISUJA
ANNALES UNIVERSITATIS TURKUENSIS

SARJA - SER. D OSA - TOM. 924

MEDICA - ODONTOLOGICA

THE APPLICATION OF cDNA AND TISSUE MICROARRAY METHODS IN THE STUDY OF HUMAN CARCINOMAS

by

Kati Talvinen

TURUN YLIOPISTO
UNIVERSITY OF TURKU
Turku 2010

From the Department of Pathology, University of Turku, Turku, Finland

Supervised by:
Docent Pauliina Kronqvist, M.D., Ph.D.
Department of Pathology
University of Turku
Turku, Finland

Jari Sundström, M.D., Ph.D.
Department of Pathology
University of Turku
Turku, Finland

Reviewed by:
Professor Veli-Matti Kosma, M.D., Ph.D.
Department of Pathology and Forensic Medicine
Institute of Clinical Medicine
School of Medicine
Faculty of Health Sciences
University of Eastern Finland
Kuopio, Finland

Annika Brandt, Ph.D.
Faculty of Life Sciences and Business
Turku University of Applied Sciences
Turku, Finland

Opponent:
Docent Anita Naukkarinen, Ph.D.
Department of Pathology and Forensic Medicine
University of Eastern Finland
Kuopio, Finland

ISBN 978-951-29-4415-6 (PRINT)
ISBN 978-951-29-4416-3 (PDF)
ISSN 0355-9483
Painosalama Oy – Turku, Finland 2010

To my family

ABSTRACT

Talvinen Kati

The application of cDNA and tissue microarray methods in the study of human carcinomas

Department of Pathology, University of Turku, Turku, Finland

Annales Universitatis Turkuensis 2010

Currently, numerous high-throughput technologies are available for the study of human carcinomas. In literature, many variations of these techniques have been described. The common denominator for these methodologies is the high amount of data obtained in a single experiment, in a short time period, and at a fairly low cost. However, these methods have also been described with several problems and limitations.

The purpose of this study was to test the applicability of two selected high-throughput methods, cDNA and tissue microarrays (TMA), in cancer research. Two common human malignancies, breast and colorectal cancer, were used as examples. This thesis aims to present some practical considerations that need to be addressed when applying these techniques.

cDNA microarrays were applied to screen aberrant gene expression in breast and colon cancers. Immunohistochemistry was used to validate the results and to evaluate the association of selected novel tumour markers with the outcome of the patients. The type of histological material used in immunohistochemistry was evaluated especially considering the applicability of whole tissue sections and different types of TMAs. Special attention was put on the methodological details in the cDNA microarray and TMA experiments.

In conclusion, many potential tumour markers were identified in the cDNA microarray analyses. Immunohistochemistry could be applied to validate the observed gene expression changes of selected markers and to associate their expression change with patient outcome. In the current experiments, both TMAs and whole tissue sections could be used for this purpose. This study showed for the first time that securin and p120 catenin protein expression predict breast cancer outcome and the immunopositivity of carbonic anhydrase IX associates with the outcome of rectal cancer. The predictive value of these proteins was statistically evident also in multivariate analyses with up to a 13.1-fold risk for cancer specific death in a specific subgroup of patients.

Keywords: cDNA microarray, tissue microarray, breast cancer, colorectal cancer, securin, p120 catenin, carbonic anhydrase IX

TIIVISTELMÄ

Kati Talvinen
cDNA- ja kudossirumenetelmien soveltaminen syövän tutkimuksessa
Patologian oppiaine, Turun yliopisto, Turku
Annales Universitatis Turkuensis 2010

Tällä hetkellä lukuisia suurenkapasiteetin menetelmiä käytetään syöpätutkimuksessa. Näistä tekniikoista on esitetty kirjallisuudessa useita muunnelmia. Menetelmille on yhteistä kerralla nopeasti ja suhteellisen alhaisilla kustannuksilla saatu suuri tietomäärä. Näihin menetelmiin kuitenkin liittyy myös useita ongelmia ja rajoituksia.

Tämän työn tarkoitus oli testata kahden valikoidun suurenkapasiteetin menetelmän, cDNA- ja kudossirujen, soveltuvuutta syöpätutkimukseen. Esimerkkinä käytettiin kahta tavallista ihmisen pahanlaatuista kasvainta, rintasyöpää sekä paksu- ja peräsuolisyöpää. Tämän yhteenvedon tarkoituksena on esitellä joitakin käytännön näkökohtia, jotka on huomioitava kyseisiä tekniikoita sovellettaessa.

cDNA-siruja käytettiin rintasyövän ja paksusuolisyövän poikkeavan geenien ilmentymisen seulomiseen. Immunohistokemiallista tutkimusta käytettiin tulosten vahvistamisessa sekä selvitettäessä valikoitujen uusien kudosmerkkiaineiden ja syöpäpotilaiden eloonjäämisen välistä yhteyttä. Tällöin arvioitiin etenkin erilaisten histologisten aineistojen, kuten suurten kudosleikkeiden ja erilaisten kudossirujen, käyttökelpoisuutta. Työssä pyrittiin erityisesti huomioimaan cDNA- ja kudossirutekniikoihin liittyviä teknisiä yksityiskohtia.

Tässä työssä tunnistettiin cDNA-sirututkimuksen avulla useita mahdollisia syöpään liittyviä kudosmerkkiaineita. Immunohistokemiallisia tutkimuksia käyttäen pystyttiin varmistamaan valikoitujen geenien ilmentymisessä havaitut muutokset, ja lisäksi osoittamaan näiden muutosten yhteys potilaiden eloonjäämiseen. Sekä kudossiruja että kokonaisia kudosleikkeitä voitiin soveltaa immunohistokemiallisiin tutkimuksiin. Tässä työssä osoitettiin ensimmäistä kertaa, että securin- ja p120 catenin proteiini-ilmentymät ennustavat rintasyövän ja hiilihappoanhydraasi IX peräsuolisyövän eloonjäämistä. Näiden proteiinien ennusteellinen arvo oli tilastollisesti ilmeinen myös monimuuttuja-analyyseissä, joissa osoitettiin korkeimmillaan 13.1-kertainen syöpäkuoleman riski tietyssä potilasryhmässä.

Avaintermit: cDNA mikrosiru, kudosmikrosiru, rintasyöpä, paksu- ja peräsuolisyöpä, securin, p120 catenin, hiilihappoanhydraasi IX

TABLE OF CONTENTS

ABBREVIATIONS **8**

LIST OF ORIGINAL PUBLICATIONS **10**

1. INTRODUCTION **11**

2. REVIEW OF THE LITERATURE **13**

2.1. Review of the cDNA microarray method 13
2.1.1. Background of the cDNA microarray method 13
2.1.2. Principles of the cDNA microarray method 14
2.1.2.1. Constructing cDNA microarrays 14
2.1.2.2. Applying cDNA microarrays 15
2.1.3. Evaluation of the cDNA microarray method 16
2.1.3.1. Validation of cDNA microarrays 16
2.1.3.2. Advantages of cDNA microarrays 18
2.1.3.3. Limitations of cDNA microarrays 19
2.2. Review of the tissue microarray (TMA) method 22
2.2.1. Background of the TMA method 22
2.2.2. Principles of the TMA method 23
2.2.2.1. Constructing TMAs 23
2.2.2.2. Applying TMAs 23
2.2.3. Evaluation of the TMA method 25
2.2.3.1. Validation of TMAs 25
2.2.3.2. Advantages of TMAs 25
2.2.3.3. Limitations of TMAs 26
2.3. Examples of cDNA microarrays and TMAs in breast and colorectal carcinomas 28
2.3.1. Background of breast carcinoma 29
2.3.2. Background of colorectal carcinoma 29
2.3.3. Examples of cDNA microarrays in breast carcinoma research 29
2.3.4. Examples of cDNA microarrays in colorectal carcinoma research 30
2.3.5. Examples of TMAs in breast carcinoma research 31
2.3.6. Examples of TMAs in colorectal carcinoma research 31
2.4. Review of securin, carbonic anhydrase IX and p120 catenin 32
2.4.1. Review of securin 32
2.4.1.1. Introduction 32
2.4.1.2. Cellular functions 33
2.4.1.3. Examples of expression in carcinoma 34

2.4.2. Review of carbonic anhydrase IX 35
2.4.2.1. Introduction 35
2.4.2.2. Cellular functions 37
2.4.2.3. Examples of expression in carcinoma 37
2.4.3. Review of p120 catenin 38
2.4.3.1. Introduction 38
2.4.3.2. Cellular functions 39
2.4.3.3. Examples of expression in carcinoma 40

3. AIMS OF THE STUDY 41

4. MATERIALS AND METHODS 42

4.1. Patient and tissue material 42
4.2. The cDNA microarray method 44
4.3. Immunohistochemistry 47
4.4. Western analysis 51

5. RESULTS 52

5.1. cDNA microarray 52
5.2. Immunohistochemistry 55
5.3. Western analysis 57
5.4. Correlation with patient outcome 57

6. DISCUSSION 59

6.1. Aberrant expression of the selected genes and proteins 59
6.2. Experiences on cDNA microarray 60
6.3. Immunohistochemistry and western analysis 62
6.4. Experiences on TMAs 64

7. CONCLUSIONS 67

8. ACKNOWLEDGEMENTS 68

9. REFERENCES 70

10. ORIGINAL PUBLICATIONS 89

ABBREVIATIONS

AGE	agarose gel electrophoresis
BC	breast cancer
BLAST	basic local alignment search tool
BRCA	breast cancer susceptibility protein
BTC	Turku Centre for Biotechnology
CA	carbonic anhydrase
CCD	charge-coupled device
cDNA	complementary deoxyribonucleic acid
CRC	colorectal cancer
cRNA	complementary ribonucleic acid
CTNND	catenin (cadherin-associated protein), delta
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
DIG	digoxigenin
DNA	deoxyribonucleic acid
dTTP	deoxythymidine triphosphate
dUTP	deoxyuridine triphosphate
Cy	cyanine
EST	expressed sequence tag
FFPE	formalin fixed paraffin embedded
HIER	heat induced epitope retrieval
ICC	intraclass correlation coefficient
IHC	immunohistochemistry
ISH	in situ hybridization
κ	kappa
kb	kilobase
kDa	kilodalton
κ_w	weighted kappa
LOWESS	locally weighted scatterplot smoothing
MAQC	microarray quality control

MGED	microarray gene expression data group
MIAME	minimal information about a microarray experiment
mRNA	messenger ribonucleic acid
MS	mass spectrometry
NCBI	National Center for Biotechnology Information
ND	not determined
NGS	next generation sequencing
p120(ctn)	p120 catenin
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PTTG	pituitary tumor transforming gene
RefSeq	reference sequence
RNA	ribonucleic acid
RPA	ribonuclease protection assay
RT	room temperature
RT PCR	reverse transcription polymerase chain reaction
s.d.	standard deviation
SDS	sodium dodecyl sulphate
TMA	tissue microarray
tRNA	transfer ribonucleic acid
TSA	tyramide signal amplification

LIST OF ORIGINAL PUBLICATIONS

This study is based on the following original communications and unpublished results. The publications are referred to in the text by roman numerals (I – V).

I Talvinen K, Tuikkala J, Grönroos J, Huhtinen H, Kronqvist P, Aittokallio T, Nevalainen O, Hiekkanen H, Nevalainen T, Sundström J. Biochemical and clinical approaches in evaluating the prognosis of colon cancer. Anticancer Res 26:4745-4751, 2006.

II Talvinen K, Tuikkala J, Nevalainen O, Rantanen A, Hirsimäki P, Sundström J, Kronqvist P. Proliferation marker securin identifies favourable outcome in invasive ductal breast cancer. Br J Cancer 99:335-340, 2008.

III Korkeila E, Talvinen K, Jaakkola PM, Minn H, Syrjänen K, Sundström J, Pyrhönen S. Expression of carbonic anhydrase IX suggests poor outcome in rectal cancer. Br J Cancer 100:874-880, 2009.

IV Talvinen K, Karra H, Hurme S, Nykänen M, Nieminen A, Anttinen J, Kuopio T, Kronqvist P. Securin promotes the identification of favourable outcome in invasive breast cancer. Br J Cancer 101:1005-1010, 2009.

V Talvinen K, Tuikkala J, Nykänen M, Nieminen A, Anttinen J, Nevalainen OS, Hurme S, Kuopio T, Kronqvist P. Altered expression of p120catenin predicts poor outcome in invasive breast cancer. J Cancer Res Clin Oncol 136:1377-1387, 2010.

1. INTRODUCTION

During the last few years, high-throughput methods of molecular biology have increasingly been applied to meet different research goals. So far, numerous methods and methodological variations have been used for this purpose. These techniques are especially valuable in the study of malignant diseases where the treatment of each individual patient should be optimized according to the risk of cancer death. The advantages of these methods are to achieve high amounts of information on the molecular pathological events at the time and without necessarily requiring a specially defined primary hypothesis. (Sara et al., 2010, Morozova et al., 2009)

High-throughput methods have substantially enhanced our understanding of the biology of cancer. For example, the development of breast cancer is no longer considered a single multi-step process towards invasive disease and metastasis but, instead, heterogeneous in nature and advancing via divergent pathways characterized by a complex series of genetic events (Simpson et al., 2005, Geyer et al., 2009). As another example, some newly identified cell biological markers and gene expression signatures are under study for evaluating the prognosis of colorectal carcinomas (Markowitz et al., 2009, Walther et al., 2009). It has been suggested that with time, gene expression signatures will become a powerful approach in cancer prognostication and clinical practice (Abdullah-Sayani et al., 2006, Geyer et al., 2009).

This thesis concentrates on two selected novel methods of molecular pathology, cDNA (complementary deoxyribonucleic acid) microarrays and tissue microarrays (TMA). The scientific community has accepted DNA microarrays with great enthusiasm as indicated by a huge number of PubMed citations since the late 1990's (Loring, 2006). Despite several clear benefits provided by DNA and tissue microarrays, there has also been criticism and limitations related to these methods. Results obtained applying DNA microarrays have been accused of high variation because of the versatile approaches and analytical tools used in the experiments (Bammler et al., 2005). A difficulty in both of these methods is heterogeneity, which concerns both tissue material and expression. As a consequence of tissue heterogeneity, the specimen may not accurately represent the whole lesion and/or may contain a variety of cell types. Heterogeneity of expression may result in focal or uneven patterns. (Tumor Analysis Best Practices Working Group, 2004, Hoos et al., 2001, Gillett et al., 2000) In the case of TMA, it is not uncommon that the specimen is totally devoid of the cells under study. As a technical problem, detached cores may cause the loss of data points in TMAs. (Emerson et al., 2009, Camp et al., 2002)

In this thesis, the applications of cDNA and tissue microarray methods in screening novel tumour markers of potential value in assessing patient outcome are tested and evaluated. Special emphasis is put on advantages and possible limitations in these methodologies. The focus is on practical considerations of the principles and technical details in the applications of these methods. The thesis is based on original papers, where the application of cDNA and tissue microarrays is tested for evaluating the outcome of common types of human malignancies, breast and colorectal carcinomas.

2. REVIEW OF THE LITERATURE

2.1. REVIEW OF THE cDNA MICROARRAY METHOD

2.1.1. Background of the cDNA microarray method

Basically, there are two types of DNA microarrays, oligonucleotide and cDNA microarrays. The former can be further divided into short and long oligonucleotide microarrays. (Barrett and Kawasaki, 2003) DNA microarray refers to a methodology which utilizes small areas of solid support material, such as glass, plastic or silicon to hold thousands of individual ”spots” which are composed of specific DNA sequences in precisely arrayed series (Abdullah-Sayani et al., 2006). DNA microarrays are widely used in monitoring gene expression changes in order to detect variations in abundances of specific messenger ribonucleic acid (mRNA) species in a large set of genes in a single experiment (Bier et al., 2008, Barrett and Kawasaki, 2003). Among all DNA microarrays, the special focus of this thesis is on gene expression profiling using slide-based cDNA microarrays. However, cDNA and oligonucleotide microarrays are variant platforms of the same technology, thus sharing many of the following details.

The methodology of DNA microarray as it is now understood has evolved along with robotic devices used for arraying (Jordan, 2002). However, dot blot hybridization applying radioactive labels and cDNA spotted on nitrocellulose filters have been applied in semiquantitative expression analysis already in the seventies (Kafatos et al., 1979). The approach has developed over the years through expression profiling using membrane based cDNA macroarrays to actual membrane microarrays with more miniaturized size (Lennon and Lehrach, 1991, Nguyen et al., 1995, Chen et al., 1998, Bertucci et al., 1999). A small array spotted on a glass microscope slide containing a small number of amplified cDNAs was first published in 1995 by Schena et al. using *Arabidopsis thaliana* as a model organism. The next year the same authors applied the method on human samples with over one thousand probes (Schena et al., 1996). In 1996, DeRisi and co-workers also published a corresponding experiment on gene expression in human melanoma cell line (DeRisi et al., 1996). At present, robotic printers can array onto a single slide over 50 000 spots, which exceeds the amount of genes in the whole human genome (Barrett and Kawasaki, 2003). At the moment, nitrocellulose and nylon filters as support material have been almost abandoned, and also the use of glass slides is rapidly decreasing. These platforms have been substituted by oligonucleotide microarrays. (Abdullah-Sayani et al., 2006) Presently, it is expected that next generation sequencing (NGS) approaches may displace DNA microarrays in the study of transcriptomes (Shendure, 2008).

In medicine, gene expression profiling is used to identify differently expressed genes in predefined types of specimen, to discover new disease subsets, and develop prognostic models with the idea that the molecular level can mirror the biological differences reflected in morphological changes and clinical signs (Simon et al., 2002). Special interest has been focused on gene expression profiles in cancers where the method has been extensively applied for identification of gene expression markers for disease classification (Khan et al., 2001), development, progression and survival (Eschrich et al., 2005), drug sensitivity (Zembutsu et al., 2002), and detection of an unknown primary tumour (Tothill et al., 2005).

2.1.2. Principles of the cDNA microarray method

2.1.2.1. Constructing cDNA microarrays

According to the nomenclature recommended by Phimister (1999), the term "probe" is used in this thesis to describe the sequence immobilized on the solid support, whereas the term "target" means the free nucleic acid sample. The most conventional cDNA microarrays contain probes typically produced by polymerase chain reaction (PCR) amplification with primers annealing to vector portions of cDNA or expressed sequence tag (EST) library clones (Schena et al., 1995, Yue et al., 2001, Deyholos and Galbraith 2001). Also gene specific amplification primers (Deyholos and Galbraith, 2001) or recombinant plasmids purified from the library can be arrayed (Wang W. et al., 2001). PCR-produced amplicons are purified and checked for both quality and quantity prior to spotting. The quality control is performed visually by inspecting the results from agarose gel electrophoresis (AGE) of amplicons for the presence, single number, and correct size of the amplicon. (Taylor et al., 2001, Yue et al., 2001) In quantitation, e.g. AGE, measurements of absorbance at 260 nm or measurements using DNA sensitive dyes can be used (Deyholos and Galbraith 2001, Yue et al., 2001, Bowtell and Sambrook (Eds.), 2003, p. 27). Amplified probes are arrayed by robotic device (Bier et al., 2008). The quality of the newly spotted array, indicated by the amount of DNA and its homogeneity across the slide, can be checked by, for example, staining with fluorescent DNA dyes or hybridization with labelled oligonucleotide complementary to the vector sequence present in spotted probes (DeRisi et al., 1996, Yue et al., 2001, http://las.perkinelmer.com/content/technicalinfo/tch_scanarraymicroarrayspotqc.pdf 25.9.2009). The presence or absence of the spots, as well as their size and the shape, can be evaluated utilizing salt crystals originating from spotting buffer and visualized by laser scanning at 633 nm (Bier et al., 2008, http://las.perkinelmer.com/content/technicalinfo/tch_scanarraymicroarrayspotqc.pdf 25.9.2009). Each probe on the microarray represents one gene and the length of the probes spotted on the cDNA microarray varies from hundreds of bases to several kilobases in length (Barrett and Kawasaki, 2003).

2.1.2.2. Applying cDNA microarrays

For a cDNA microarray experiment, either total or messenger RNA is isolated from a tissue or cell line under study (Yu et al., 2002, Schena et al., 1996). RNA amplification prior to labelling may be necessary if the available starting material is limited, for example, in the case of microdissected samples (Luo et al., 1999, van Gelder et al., 1990). Amplification of mRNA may induce bias in the transcriptome but, according to the literature, bias can be controlled with properly optimized protocol and pooling of replicate reactions can prevent random transcript drop-out during the priming of amplification (Chuaqui et al., 2002). As an alternative, some reports recommend the pooling of several corresponding samples to increase the amount of starting material (Simon et al., 2002).

Purified RNA is labelled utilizing cDNA synthesis (Schena et al., 1995) or *in vitro* transcription (Frederiksen et al., 2003). Among the several possible alternatives for labels, fluorescent dyes, especially cyanine dyes (Cy5 and Cy3), are the most common choice but radioactive and enzymatic labels have been used as well (Bier et al., 2008). In cDNA synthesis, either oligo(dT) (Schena et al., 1995) or random oligomers (Yue et al., 2001) can be used as a primer. Labelling can occur via direct or indirect procedures where the direct protocol is based on nucleotides directly conjugated to labels (Schena et al., 1995) and the indirect protocol to either amino-allyl nucleotide analogs (Yu et al., 2002), 3DNA (Stears et al., 2000) or the tyramide signal amplification (TSA) system (Yoshida et al., 2002). In the case of the amino-allyl procedure, special fluorescent dyes are conjugated to nucleotide analogs after their incorporation in the nascent cDNA or cRNA (Manduchi et al., 2002, Frederiksen et al., 2003). In the 3DNA system, tailed primers are used in cDNA synthesis, which after microarray hybridization capture a special reagent dendimer containing hundreds of fluorescent molecules (Stears et al., 2000). As a special advantage, 3DNA technology allows expression profiling with a low background signal using only a limited input of the original RNA sample (Stears et al. 2000, Yu et al., 2002). As another option for signal enhancement, test and reference targets can be differently labelled during cDNA synthesis with, for example, biotin, fluorescein or dinitrophenyl (TSA system). After hybridization, they are sequentially detected with streptavidin or antibodies conjugated with horseradishperoxidase and different fluorescent reporter molecules conjugated with tyramide. Horseradishperoxidase activates tyramides, which can then covalently bind to the microarray surface at the location of their formation. (Huddleston et al., 2005, Ishibashi et al., 2002, Andras et al., 2001)

Commonly, two differently labelled targets are generated for cDNA microarrays – one from the sample under study and the other from the chosen reference material. The labelled targets are mixed and allowed to hybridize to the microarray probes. (Churchill, 2002, Ramaswamy and Golub, 2002, Simon et al., 2002) Special attention should be placed on choosing reference material, since it must be abundantly available,

homogeneous, and stable over time (Churchill, 2002), but not necessarily biologically relevant (Simon et al., 2002). According to Simon and co-workers (2002), the practicable reference sample should express from the array as many genes as possible, but not at such a high level as to saturate the detection system. Also, a paired non-diseased sample from the same tissue of a patient can be used as reference material, if substantial differences in the gene expression of non-diseased tissues between the patients can be expected (Simon et al., 2002). In the literature, many report on non-standardized reference material, consisting of, for example, pooled RNA from different cell lines, tumour samples or non-diseased tissues (Abdullah-Sayani et al., 2006). To improve the quality of microarray studies, the Microarray Quality Control (MAQC) Project, initiated by the US Food and Drug Administration has worked to provide established standards and quality measures (http://www.fda.gov/ScienceResearch/BioinformaticsTools/MicroarrayQualityControlProject/default.htm 18.9.2009). The MAQC Project has especially concentrated on oligonucleotide microarrays, but provided also for cDNA microarrays, well-characterized reference materials and data sets, as well as valuable information to aid in quality control actions and to facilitate intra- and inter-laboratory reproducibility (MAQC Consortium, 2006, Simon et al., 2002).

After target hybridization, the amount of bound fluorescence is measured utilizing a laser scanner or CCD (charge-coupled device) camera-based system (Deyholos and Galbraith, 2001). There are many alternative software products used in image acquisition with distinct properties to specify, for example, spot or signals from the background (Mello-Coelho and Hess, 2005). The signal strengths from the two labels are proportional to the amounts of corresponding mRNA in studied and reference samples. The ratio of the signal from the studied to reference sample is used as a measure of the difference in gene expression (Schena et al., 1996).

2.1.3. Evaluation of the cDNA microarray method

2.1.3.1. Validation of cDNA microarrays

The several sources of variation throughout the whole cDNA microarray experiment can be evaluated and partly managed with the help of various types of controls. Utilizing these, dynamic range, sensitivity and specificity of the hybridization can be estimated. (Yue et al., 2001, DeRisi et al., 1996, Churchill, 2002) Each gene can be included in the cDNA microarray as multiple spots representing different clones of a gene with distinctive sequences to evaluate hybridization specificity, RNA integrity and efficiency of cDNA synthesis (Dietel and Sers, 2006, Bowtell and Sambrook (Eds.), 2003, pp. 42 – 44).

With the help of negative controls, the degree of nonspecific hybridization can be assessed and they can be applied for determining threshold signals defining the presence of mRNA in a sample. Negative controls can be prepared using, for example, either poly(dA), non-coding genomic sequences, printing buffer devoid of DNA, or genes, which are evolutionarily distant from the studied species but with approximately the same cytosine-guanine-content. (Bowtell and Sambrook (Eds.), 2003, pp. 42 – 44, Schena et al., 1996, Manduchi et al., 2002, Deyholos and Galbraith, 2001) Evolutionary distant DNA species can be used also to prepare so-called spiked controls, which are both spotted on the array and used as templates to synthesize RNA targets to be added into studied and reference samples before labelling (Ishibashi et al., 2002, Schena et al., 1996). With spiked controls, equivalent labelling of test and reference samples can be verified (Bowtell and Sambrook (Eds.), 2003, pp. 42 – 44, DeRisi et al., 1996).

Positive controls are most commonly prepared combining equal volumes of all the probe elements to be spotted and printed along with single specific probes on the array. Optimally, test and reference samples should produce expression ratios close to equal. Printing can also be performed in a dilution series. The signal strength obtained from hybridizations of these series should be proportional to the amount of DNA in a wide range of concentrations. (Bowtell and Sambrook (Eds.), 2003, pp. 42 – 44, Yue et al., 2001) Among several possible normalization schemes, also spiked controls or genes with no expected expression change, such as housekeeping genes, have been suggested for use to eliminate systematic variation (Ishibashi et al., 2002, Kitahara et al., 2001, Mello-Coelho and Hess, 2005).

It has been recommended that cDNA microarray experiments, especially in exploratory study designs, should be confirmed by conventional methods, such as reverse transcription polymerase chain reaction (RT PCR) (Simon et al., 2002, Bucca et al., 2004, Sherlock, 2005). As yet, the methodological practise is, however, still not established, and also cost and availability of the reagents, as well as labour-intensiveness, may direct the selection of validation methodology. It has been suggested that also *in silico* procedure could be used in validation. That means comparing the results to available literature without further laboratory experimentation. (Chuaqui et al., 2002) The validation can include either the same set of samples as used in the original microarray experiment or an independent one (Chuaqui et al., 2002, Hewitt, 2006). The use of an independent set of samples has been suggested as being especially beneficial in prognostic study designs (Manning et al., 2007), where validation data confirms the applicability of the results (Chuaqui et al., 2002).

Based on the literature, quantitative RT PCR appears to be the most popular method for validation of cDNA microarray experiments, but also Northern and in situ hybridization (ISH), as well as ribonuclease protection assay (RPA) have been used. RT PCR is a rapid,

rather inexpensive and practical technique since it requires only a minimal amount of starting template. (Chuaqui et al., 2002) The special advantage of Northern hybridization is that the size of the detected transcripts can be determined, revealing the specificity and possible variations in mRNA splicing (Senft and LeVine, 2005). The advantage of ISH is the possibility to evaluate gene expression in relation to histology (Hewitt, 2006). As compared to Northern hybridization, RPA may allow for more sensitive detection of transcripts, but with the cost of losing information on transcript size (Qu and Boutjdir, 2007).

Validation of cDNA microarray results is commonly analysed at the protein level, applying immunoblots or immunohistochemistry (IHC) (Chuaqui et al., 2002), but with the disadvantage that the mRNA expression level does not always reflect the amount of protein (Hewitt, 2006). Obviously, gene expression profile obtained from a microarray reflects only those cellular changes that affect mRNA synthesis (Abdullah-Sayani et al., 2006), so that biologically crucial post-translational modifications and protein-protein interactions can not be recognized with cDNA microarray technology (Hewitt, 2006, Dietel and Sers, 2006). In patient care, the protein level is considered to be the most relevant, since the vast majority of measurements in clinical routine concern proteins (Hewitt, 2006). Presently, immunohistochemical detection performed on tissue microarrays is a powerful method for the validation of DNA microarray results (Hewitt, 2006, Chuaqui et al., 2002).

2.1.3.2. Advantages of cDNA microarrays

The greatest advantage attained by DNA microarrays with a miniaturized format is that the expression changes of thousands of genes, even the whole genome, can be explored in a single experiment (Barrett and Kawasaki 2003, Yue et al., 2001). In comparison with the more conventional methods, such as Northern hybridization or RPA, cDNA microarrays allow for exploring a considerably larger number of genes at a time. With RT PCR, at best, no more than a couple of hundred genes may be quantified (Bucca et al., 2004, Medeiros et al., 2007), and it has been estimated that this is not enough to understand gene expression changes for analysing and classifying complicated diseases, such as cancer (Dietel and Sers, 2006). cDNA microarrays also have some special advantages over the oligonucleotide microarrays. The precise sequence information of all genes is not required prior to the construction of the array (Schena et al., 1996). This is not a critical issue in the research of human tissues, since the completion of the Human Genome Project has made sequences of all human genes available in databases (Abdullah-Sayani et al., 2006). Instead, when studying less characterized species, this feature may be invaluable (Deyholos and Galbraith, 2001). Technically, as compared to the membrane based approach, glass slides offer the advantage of small hybridization volumes, high array densities, and the use of fluorescent labels (Schena et al., 1996).

In addition, the two-colour hybridization scheme of slide-based cDNA microarray experiments minimizes technical variation (Schena et al., 1995). At present, also the reasonable price of microarray experiments makes their use accessible to researchers (Abdullah-Sayani et al., 2006).

2.1.3.3. Limitations of cDNA microarrays

The quality of RNA is an important issue in microarray experiments (Russo et al., 2003). In present practise, frozen tissue is indispensable for microarray analysis (Abdullah-Sayani et al., 2006). Still, tissue material in the great majority of pathology laboratories is stored as fixed and paraffin embedded tissue blocks producing for microarray experiments less intact RNA (Chung et al., 2006), with compromised quality due to long-term archival storage (Cronin et al., 2004). Also, the freezing of the samples should be performed in a standardized fashion (Abdullah-Sayani et al., 2006), since even subtle variations in tissue handling may affect measured gene expression levels (Simon et al., 2002). The time between surgical removal and the freezing of tissue is critical to the gene expression profiles, because of fragility of RNA molecules or possible actual gene expression changes caused by tissue manipulation. Optimally, the tissue sample should be transported on ice (Micke et al., 2006), frozen within twenty to thirty minutes after surgical removal, and stored at -80 °C or below (Huang et al., 2001, Ramaswamy and Golub, 2002). The size of the stored tissue specimen should be kept small, approximately 0.1 cm^3 in volume, and placed in airtight containers with fragments of ice to prevent the sample from drying out (Medeiros et al., 2007, Tumor Analysis Best Practices Working Group, 2004). According to some researchers, a commercial reagent called RNAlater (Applied Biosystems/Ambion, Austin, TX, U.S.A.) may preserve RNA quality for expression profiling as efficiently as snap-freezing (Chowdary et al., 2006, Grotzer et al., 2000). For DNA microarray methodology, there are no general recommendations available for RNA extraction methods or the long-term storage of isolated RNA (Medeiros et al., 2007). The RNA extraction step is known as a source of variation in microarray experiments (Simon et al., 2002), and the severity of the influence of RNA degradation depends on study design. Those experiments where cDNA synthesis has been performed using oligo(dT) have been suggested to be more sensitive to degradation (Medeiros et al., 2007). RNA degradation may not be appreciable in standard ethidium bromide stained gels, although the gene expression profile is significantly altered (Huang et al., 2001).

Especially in cancer research, heterogeneity of the lesion may cause the tissue sample to contain both diseased and normal cell types (Abdullah-Sayani et al., 2006). This feature may be a major confounding variable in many microarray experiments (Tumor Analysis Best Practices Working Group, 2004). For this purpose, Medeiros et al. (2007) have urged defining a minimum volume and percentage of tumour tissue of samples for DNA microarray experiments. Another remedy for the problem could be the use of isolated

pure cell populations by, for example, laser capture microdissection (Tumor Analysis Best Practices Working Group, 2004).

According to Bammler et al. (2005), the type of platform applied is among the important sources of variation in DNA microarray experiments. In the literature, conflicting results of gene expression changes have been presented when cDNA and oligonucleotide microarrays have been compared (Kuo et al., 2002, Tan et al., 2003). There are, however, also several reports suggesting relatively concordant results between the platforms (Yauk et al., 2004, Irizarry et al., 2005, Larkin et al., 2005). There is no simple answer explaining the reason for divergent observations obtained from different comparative studies (Petersen et al., 2005), although the role of measuring different splice variants of the same gene with different platforms has been speculated (Sherlock, 2005). Cross-platform consistency may be improved if the probes on the compared arrays represent the same sequence (Mecham et al., 2004). The explanation may also be in the different RNA preparations and protocols used in the comparisons (Kuo et al., 2002). According to the literature, better concordance may be achieved by evaluating direction instead of magnitude of expression change, by using standardized methods throughout the experiment and by surveying deregulated functional groups instead of single deregulated genes (Petersen et al., 2005, Bammler et al., 2005, Tan et al., 2003).

In a fairly early phase of the development of the methodology, it became evident that data obtained from cDNA microarray experiments may not be reproducible or comparable to similar studies conducted by other, or even by the same laboratory (Barrett and Kawasaki, 2003). One explanation for this may have been that publications on cDNA microarray experiments often lack the vital technical details necessary for repeating the study (Nature 419, p. 323, 2002). Recently, an international co-operative project, the Microarray Gene Expression Data Group (MGED), has been established among specialists of biology, information technology and biostatistics, to promote the integration of data (Brazma et al., 2000, http://www.mged.org/ 17.9.2009). MGED has proposed guidelines called MIAME (Minimal Information About a Microarray Experiment) for publication of DNA microarray data (Brazma et al., 2001). Presently, MIAME 2.0 guidelines (http://www.mged.org/Workgroups/MIAME/miame.html 17.9.2009), user-friendly spreadsheet-based format (Rayner et al., 2006), and vocabulary accordant with MGED Ontology (Whetzel et al., 2006), are available for microarray data publication and database deposition. In practice, MIAME provides criteria for submission of DNA microarray data for publication for unambiguous interpretation of the data. This information should include details on the experimental design, array design, description of probes, and sample annotation with associated key experimental variables. In addition, experimental protocols, measurements and data processing should be detailed if they differ from the standard. Both raw and final processed data obtained from an experiment should be stored in a public repository. (Brazma et al., 2001, Stoeckert et al., 2002, http://www.mged.org/Workgroups/

MIAME/miame_2.0.html 17.9.2009). At present, there are at least fifty journals following the MIAME rules in publication (http://www.mged.org/Workgroups/MIAME/journals.html 17.9.2009).

For many years, analysis of microarrays suffered from lack of sophisticated software and bioinformatics tools, and of poorly annotated EST databases (Jordan, 2002, Loring, 2006). At present, there are several bioinformatics tools available and under development in the internet (Mello-Coelho and Hess, 2005, http://www.bioconductor.org/ 22.10.2009). The chosen analytical approaches greatly influence the accuracy and reliability of the results (Abdullah-Sayani et al., 2006) but, presently, there is no consensus on their applicability (Chuaqui et al., 2002, Desai et al., 2002).

In the literature, some limitations have been related specifically to the cDNA microarray platform, but not to oligonucleotide microarrays. Firstly, original cDNA libraries used for microarray construction, are known to contain cross-contaminated and incorrect clones, and false annotation information concerning, for example, the size of the products of amplified cDNA. Also, they may not yield sufficient amounts of product to construct a microarray. Since it is a multi-step process to reach from the original library to a finished cDNA microarray, there are many sources for errors also. As a consequence, from almost 20% to 40% error rates, as compared to original annotation, have been reported. Sequencing of the original cDNA libraries, or amplified probes, helps to minimize the amount of false results. Sequence verification is, however, a very laborious, costly, and time-consuming process. Besides sequencing, AGE of clones prepared from bacterial colonies, or of amplified PCR products, is an alternate way to get some insight into the fidelity of the cDNA library. (Halgren et al., 2001, Taylor et al., 2001)

Non-specific hybridization and cross-hybridization are other problems which have been related to cDNA microarrays in the literature. Chuaqui et al. (2002) and Emmert-Buck et al. (2000) have pointed out that cDNA microarrays may be susceptible to non-specific background signals due to the hybridization of the target via repetitive elements, poly(A) tails or common motifs, for example, sequence homology among gene-family members. It has also been suggested that cDNA microarrays suffer from special technical problems, such as heavy background, air bubbles trapped under the coverslips during hybridization, scratches on the surfaces of the arrays, and variations in the size and shape of the probe spots (Simon et al., 2002).

All in all, the sources of variation in DNA microarray experiments are best managed with rigorously and carefully designed (Dietel and Sers, 2006) standardized procedures (Bammler et al., 2005), and close cooperation between specialists of different backgrounds, such as surgeons, pathologists, molecular biologists and bioinformaticists (Abdullah-Sayani et al., 2006).

2.2. REVIEW OF THE TISSUE MICROARRAY (TMA) METHOD

2.2.1. Background of the TMA method

Multitissue array is a high-throughput methodology producing for histopathological analysis tissue blocks constructed from multiple samples arranged in either tissue rods or cores (Eguíluz et al., 2006). These blocks can contain from approximately a hundred up to a thousand separate tissue specimens in a limited area (Kononen et al., 1998). The technique was originally introduced in 1986 by H. Battifora, as "the multitumor (sausage) tissue block", which contained over a hundred rod-shaped tissue samples wrapped in sheets of small intestine and embedded in a normal-sized paraffin block. In 1990, Battifora and Mehta described an improved version, "the checkerboard tissue block", where the origin of each sample could be identified in the even distribution of tissues. During the last two decades many more slightly modified variations of the construction techniques have been described for both rod-shaped blocks (Miller and Groothuis, 1991, Press et al., 1994), and tissue-core blocks (Wan et al., 1987, Kraaz et al., 1988, Kononen et al., 1998, Pan et al., 2004, Pires et al., 2006). Eventually, tissue-core technologies using 16-gauge needles (Wan et al., 1987) and skin punch biopsy instruments (Kraaz et al., 1988), were substituted by custom-built devices enabling the rapid and reproducible construction of multitissue blocks, called "tissue microarrays" (Kononen et al., 1998). Currently, many commercial manual and automated microarrayer instruments are available for the diverse needs of laboratories (e.g. Beecher Instruments, Sun Prairie, WI, USA; Veridiam, Poway, CA, USA). Meanwhile, TMAs have become a widely applied tool in modern pathology research (Camp et al., 2008, Giltnane and Rimm, 2004).

In the literature, TMAs have been applied to cancer research with varying goals (Kallioniemi et al., 2001), with the spectrum of applications including, for example, the correlation of single or multiple protein markers to disease aggressiveness (Salvucci et al., 2006), progression (Yang et al. 2006) or patient outcome (Torhorst et al., 2001), and therapy or drug response (Umar et al., 2009, Rocchi et al., 2004). While Hao et al. (2004), made comparisons between paired primary tumour and metastasis, Couvelard et al. (2009), studied expression heterogeneity within metastasis and between metastases, and Nishizuka et al. (2003), applied TMA for differential diagnosis of cancers. Furthermore, the TMA technique has also been used for tumour classifications (Callagy et al., 2003, Makretsov et al., 2004), and antibody validation for therapeutic decision-making (Cheang et al., 2006, Camp et al., 2008). Multitumour TMAs – sometimes called "prevalence TMAs" – have been introduced, for example, in prevalence studies of the abundance of a certain protein or gene amplification (van de Rijn and Gilks, 2004, Yang

et al., 2007, Andersen et al., 2002). The TMA technique has turned out to be useful also as an IHC quality control tool (Mengel et al., 2002, de Jong et al., 2009).

2.2.2. Principles of the TMA method

2.2.2.1. Constructing TMAs

When constructing a TMA, tissue areas of interest are first selected by a histopathology expert from routine stained whole tissue sections. The selection of the areas for TMA cores must be done from the previous section of the paraffin block. One or several representative areas are marked on the slide and the corresponding area is identified on the original tissue block. (Hewitt, 2004) Cylindrical tissue cores from the chosen area on the block are then punched using a hollow needle, either manually or with a tissue-arraying instrument (Bowtell and Sambrook (Eds.), 2003, pp. 603 – 607). When constructing the TMA, the punched tissue cores are mounted into pre-made holes of a recipient block in grid-pattern with precise coordinates (Kononen et al., 1998). For easier microscopic examination, the tissue cores may be arrayed into subsections (Simon and Sauter, 2002). While constructing the TMA, special consideration should be put on the type, quality and heating of paraffin wax, size and distance of cores to each other and to the surface of the cassette, handling of the blocks, and direction of sectioning in order to avoid the TMA breaking in sectioning (Dennis et al., 2003, Eguíluz et al., 2006).

2.2.2.2. Applying TMAs

In general, any type of tissue – normal or diseased – can be arrayed, but in constructing a TMA several considerations are necessary. TMAs are not optimal for evaluating distant or scarce structures, such as glomeruli of the kidney or portal tracts of liver, since they are not likely to be sufficiently represented in small tissue cores. Similarly, it is not recommendable to apply TMA methodology for the study of small lesions or intricate disease classifications, such as in situ tumour lesions. Technically, hard or fragile tissues, such as bone or cartilage may be challenging to array. (Hewitt, 2004) Currently, the most commonly applied form of TMA consists of hundreds of formalin fixed paraffin embedded (FFPE) cores from large tissues (Takikita et al., 2007) but, according to the literature, also cell lines (Li et al., 2005, Montgomery et al., 2005) and tissue core needle biopsies (Datta et al., 2005) have been arrayed in similar fashion.

In punching the blocks, the idea is to get adequate and acceptable representation of tissue with the minimal number and diameter of cores. Cores smaller than 0.6 mm in diameter are not likely to fulfil the requirement of representation, whereas cores larger than 3 mm are contrary to the sense of tissue arraying in the first place. (Bowtell and Sambrook (Eds.), 2003, pp. 603 – 607) With a tissue core height of 3 – 4 mm, approximately 200 –

300 sections can be obtained from each TMA block (Shergill et al., 2004, Eguíluz et al., 2006). Using equally thick donor blocks, the uniformity of sections throughout the TMA can be ensured (Henshall, 2003). If the donor tissues are thin, several cores from the same block can be placed on top of each other to increase the height of the TMA (Hoos and Cordon-Cardo, 2001).

As in standard histopathology practise, sections of TMA are cut with microtome, floated in a water bath, and collected on positively charged slides or treated with the tape transfer technique (Quraishi et al., 2007). The adhesive tape system has been reported to work particularly well for high-density TMA slides, minimizing tissue loss and section stretching (Henshall, 2003, Hewitt, 2004), and preventing loss of antigenicity because of the oxidation of tissue sections during the water bath (Quraishi et al., 2007). While cutting multiple sections from a TMA, it is advisable to check the quality and correct number of tissue cores with a routine staining once every 50 slides (Hewitt, 2004). Furthermore, sections of TMAs are readily applicable with the same methods as standard whole sections, such as IHC and ISH (Kallioniemi et al., 2001). The great majority of reports deal with the use of FFPE TMAs with IHC, while fewer studies have reported RNA ISH (Shergill et al., 2004, Camp et al., 2008). Also DNA ISH, PCR ISH, and immunocytochemistry techniques could be used along with TMAs (Bowtell and Sambrook (Eds.), 2003, pp. 603 – 607).

Automated quantification is a suggested solution to deal with the huge amount of tissue samples stained in a TMA, and the eventual time restraints (Simon and Sauter, 2002) and accuracy demands of the histological evaluation (Camp et al., 2002). TMA with an automated computerized evaluation provides a feasible platform for rapid continuous scale quantification, and helps to fix the problem of subjectivity (Camp et al., 2008). The TMA technique is especially well suited for automated quantification, because the small pre-selected tissue cores can be expected to represent a cell population with minimal heterogeneity (Simon and Sauter, 2002, Rubin et al., 2002). Presently, there are several software solutions commercially available for this purpose (Aguilar-Mahecha et al., 2006, Giltnane and Rimm, 2004). Automated quantification is expected to speed up the interpretation and improve the accuracy, reproducibility, and prognostic significance of TMAs (Camp et al., 2002, Wang S. et al., 2001). Automated quantitative analysis has also been established for ISH (Jubb et al., 2003).

It has been suggested that linking automated quantitative scores of TMA to patient data may lead to true high-throughput data processing (Giltnane and Rimm, 2004). In this attempt, information management systems and databases archiving TMA data together with histological and clinical data have been introduced (Henshall, 2003, Manley et al., 2001). In the future, production of large-scale TMAs may even be centralized for collaborative use so that standardized studies for biomarkers can be run simultaneously

in several laboratories. This would ultimately constitute a huge depository of knowledge, disseminating valuable data to the whole research community. (Moch et al., 2001) Despite possible future promises, automated quantification is still relatively early in its development and programs are struggling with several problems, such as the identification of background staining (Aguilar-Mahecha et al., 2006, Takikita et al., 2007). The instrumentation needed is rather expensive and requires extensive training to use (Hewitt, 2004). Many bioinformatics tools are also not optimal for analysis of quantitative TMA data, since they were originally developed for analysing relational values of gene expression arrays (Giltnane and Rimm, 2004). Thus far, the few unsupervised clustering algorithms used in breast cancer (Callagy et al., 2003, Makretsov et al., 2004) should be applied with caution (Hewitt, 2006).

2.2.3. Evaluation of the TMA method

2.2.3.1. Validation of TMAs

In order to perform reliable TMA experiments, the donor tissue should be standardized for the protocols of tissue processing with a choice of fixatives and paraffins, especially when the specimens originate from different institutions (Hewitt, 2004, Chiriboga, et al., 2004). Also, the influence of tissue storing should be considered by ruling out any systemic association between the type of staining and the age of the archival tissue in the TMA (Hewitt, 2004, Camp et al., 2008, Hecht et al., 2008). According to custom practise, TMA blocks are stored at room temperature in a dry environment (Hewitt, 2004).

The optimal design for TMA takes into consideration the maximal surface area that can be uniformly stained. Distributing the control tissue specimen, such as cores of normal tissue or mixed cell lines, on the TMA serves as verification of even staining across the entire array. Placing control tissue cores as a frame around the array minimizes the loss of study material during sectioning, and protects it from artificial staining at the section borders, both phenomena sometimes seen in the periphery of the TMA. (Hewitt, 2004, Hoos and Cordon-Cardo, 2001) For the use of the biotin-avidin-based detection system, inclusion of endogenous-biotin containing kidney and liver tissues on the TMA helps to identify false-positive background caused by the failure of biotin blocking (Hewitt, 2004).

2.2.3.2. Advantages of TMAs

TMAs have many benefits over the traditional whole tissue sections. The most obvious of them is its effectiveness over time, reagents, laboratory capacity, and storage. Also, the saving of scarce tissue material is often important. (Camp et al., 2008, Eguíluz et al., 2006, Sapino et al., 2006) TMAs are achievable from archival tissue specimens to

produce large materials with long-term follow-up providing adequate statistical power to test the clinical relevance of numerous potential new biomarkers (Kallioniemi et al., 2001, Kononen et al., 1998, Voduc et al., 2008). The initial cost of the production of a TMA will be substantially reduced along with continuing experiments (Mucci et al., 2000). The cost-effectiveness of TMAs has been demonstrated also in routine clinical use (Sapino et al., 2006) but the technique may require specially trained and dedicated laboratory personnel (Camp et al., 2008, Sapino et al., 2006, Simon and Sauter, 2002).

TMAs may improve the consistency of experiments in providing uniform conditions for antigen retrieval, temperature, washing and staining conditions, and antibody dilution over the whole section (Camp et al., 2008, Eguíluz et al., 2006). It has also been pointed out that the use of TMAs may improve reproducibility of results by minimizing subjectivity in selecting the area for histological evaluation (Kyndi et al., 2008, Torhorst et al., 2001, Voduc et al., 2008). Interassay variations between separate TMA analyses may be managed by incorporating internal controls from cell lines, or either normal or diseased tissue specimens (Simon and Sauter, 2002, García et al., 2003, Fergenbaum et al., 2004). Different concentrations of peptides attached to slides may be used for quantitative controls (Vani et al., 2008). Small-scale TMAs are generally applied as quality control tools on traditional whole section slides of routine pathology (Packeisen et al., 2002), and in the quality assurance of new aliquots of antibodies (van de Rijn and Gilks, 2004) in a single laboratory or between several institutions (Camp et al., 2008).

2.2.3.3. Limitations of TMAs

One of the most debatable issues in TMAs is tissue heterogeneity i.e. how well a tissue core can represent a larger lesion or a whole tumour. This is dependent on the type of tissue, so that while homogeneous tissues may be accurately represented with a single core, several or larger cores are needed for evaluating heterogeneous tissues, such as tumours. (Bowtell and Sambrook (Eds.), 2003, pp. 603 – 607, Camp et al., 2000, Rubin et al., 2002, García et al., 2003, Hoos and Cordon-Cardo, 2001) Some researchers have suggested that moderately complex tissues can be covered by two or three cores taken far apart (Bowtell and Sambrook (Eds.), 2003, pp. 603 – 607, Camp et al., 2000).

As to expression heterogeneity, several reports have highlighted the relevance of individual validation of each antigen for TMAs (Fons et al., 2007, Gillett et al., 2000), because comparative studies recommend arraying tissue cores in double, triple or quadruple (Camp et al., 2000, Hoos et al., 2001, Fernebro et al., 2002). In some studies, however, no, or only marginal, improvement in the agreement between the TMA and whole tissue section has been achieved using multiple instead of a single core (Gillett et al., 2000, Hecht et al., 2008, Kyndi et al., 2008).

Some particular molecular markers may not be optimal for analysis in TMAs, because of focal or heterogeneous expression patterns in "hot-spots" (Gillett et al., 2000), or at the leading edge of the tumour (Brooks and Leathem, 1995, Cardillo et al., 1997). This applies, for example, to hypoxia (van Diest et al., 2005) and cell proliferation markers (Beliën et al., 1999, Salminen et al., 2005). In addition, some markers may be expressed in different subcellular locations at the edge and the centre of the tumour (Brabletz et al., 2005). It has been suggested that cores for TMAs are taken from distinct areas far apart (Iakovlev et al., 2007), such as both centre and the edge of the tumour (Camp et al., 2000). Also, the tumour area selected for the TMA should reflect the requirements of the studied phenomenon or biomarker, for example, the proliferation of breast cancer should be evaluated at the periphery of the tumour (Packeisen et al., 2003). Peripheral cores are often recommended also because the central region of the tumour may show inadequate tissue fixation, possibly hampering the detection of biomarkers sensitive to underfixation (Rubin et al., 2001, Walker, 2008).

Previous studies have also attempted to deal with the challenge of tissue and expression heterogeneity by increasing the diameter of the punched core in TMAs (Hewitt, 2004). A large tumour of several centimeters in diameter is hardly more accurately represented by, for example, a tissue core 2 mm in diameter (3 mm^2), than one 0.6 mm in diameter (0.27 mm^2) (Kallioniemi et al., 2001, Packeisen et al., 2003). Although sampling of multiple cores may better deal with tissue heterogeneity, a larger core may still provide more tissue material for a better microscopic evaluation of tissue architecture and histology (Hewitt, 2004).

Loss of data points is a common problem in many TMA studies (Boone et al., 2008, Cheang et al., 2006, Fernebro et al., 2002, Gillett et al., 2000). Reported rates of non-informative tissue cores vary extensively (Hoos and Cordon-Cardo, 2001), for example, from 4% (Jourdan et al., 2003) to even 30 % (Torhorst et al., 2001) lost tissue cores in a TMA. Some researchers suggest that the loss of tissue material in processing TMAs may be dependent on the diameter of the tissue core (Henshall, 2003, Kramer et al., 2007), and on the fixation time of the donor tissue (Hoos and Cordon-Cardo, 2001). Previous reports have also considered the influence of the location of the core in the TMA, and the type of tissue studied for tissue loss in TMAs, but the results are contradictory (Hoos and Cordon-Cardo, 2001, Henshall, 2003, Mucci et al., 2000).

Although many proteins retain their antigenicity in FFPE blocks for more than sixty years (Camp et al, 2000), it has been shown that antigenicity changes rapidly in pre-cut TMA slides stored at room temperature in air. DiVito et al. (2004) have reported that already six days of storage results in a statistically significant difference in detectable antigen quantity as compared to fresh cut sections. For this reason, it may not be advisable to prepare extra sections in advance (Camp et al., 2008). On the other hand, the tissue

block has to be refaced every time when remounted on a microtome, which can result in exhaustion of tissue cores or tumour tissue within the cores (Fergenbaum et al., 2004). The severity of the observed loss depends on the antigen studied, so that membranous antigens seem to be more susceptible to the effects of storage time and temperature (DiVito et al., 2004, Fergenbaum et al., 2004). According to Mirlacher et al. (2004), however, some of the observed clinicopathological associations with patient survival are retained, in spite of a decrease in staining intensity during the storage of slides. It has been proposed that re-coating the slides by dipping them in melted paraffin and storing them in a nitrogen desiccator may serve as a remedy for antigen deterioration on pre-cut sections (DiVito et al., 2004, Su et al., 2006). Hewitt (2004) has suggested that humidity and oxygen are more important factors than temperature in the storage of pre-cut paraffin sections.

In summary, no standard for TMA construction has been achieved thus far. Also, there is presently no consensus on whether TMA is a suitable platform for the analysis of heterogeneously expressed proteins or not (Hoos et al., 2001, Fernebro et al., 2002, García et al., 2003, Linderoth et al., 2007, Merseburger et al., 2003). Instead, the real question is if clinical correlations can be reproduced in TMAs (Simon and Sauter, 2002). Many studies have confirmed that if the TMA comprises a large enough patient cohort, even one 0.6 mm core may be sufficient for statistical significance in clinical correlations (Cheang et al., 2006, Torhorst et al., 2001, Nocito et al., 2001). Consequently, the TMA is repeatedly suggested as a powerful tool when examining large populations in a research context, but not in individual cases, which hampers its use as a tool for patient management (Kallioniemi et al., 2001, Nocito et al., 2001, Gulmann et al., 2006).

2.3. EXAMPLES OF cDNA MICROARRAYS AND TMAs IN BREAST AND COLORECTAL CARCINOMAS

Over the years, cDNA microarrays have been used to study numerous malignant diseases (Russo et al., 2003) but with only limited consensus concerning the numerous inter-experimental variations and interpretation of the results (Abdullah-Sayani et al., 2006). Recently, new gene expression patterns have been discovered at a rapid pace, using modern high-throughput technology, such as cDNA microarrays or proteomic screening approaches (Sallinen et al., 2000, Hudelist et al., 2004, Umar et al., 2009). Simultaneously, a growing demand has arisen to validate the plethora of identified gene expression changes in respect to histology in a large series of samples (Bowtell and Sambrook (Eds.), 2003, pp. 603 – 607). The development of the TMA technique has provided a convenient platform for this task (Hewitt, 2006, Moch et al., 1999, Bubendorf et al., 1999).

2.3.1. Background of breast carcinoma

Invasive breast cancer (BC) is the most common malignancy among women in Finland, accounting for up to 31.4% of all female carcinomas. The yearly incidence is presently 4 313 and constantly increasing. (Finnish Cancer Registry, 2008) BC is a multifactorial hormone-dependent malignancy related to Western reproductive factors and lifestyle, but also hereditary forms of BC occur (Tavassoli and Devilee (Eds.), 2003, pp. 13 – 14). BC may manifest as a palpable breast lump but, presently, a substantial part of BCs are symptomless (Tavassoli and Devilee (Eds.), 2003, p. 17). Mammographic screening has been practised in Southwestern Finland since 1987, and resulted in a significant decrease in tumour size and disease progression at the time of diagnosis, and improved disease outcome (Finnish Cancer Registry, 2008). The corner stone of BC detection is the so-called triple diagnostics, i.e. the combination of clinical, imaging, and histological examinations.

2.3.2. Background of colorectal carcinoma

Colorectal cancer (CRC) is one of the most common malignant tumours in Finland. There are 2 704 new cases per year (Finnish Cancer Registry, 2008), and the incidence is rising. The risk factors for CRC are not exactly known, but hereditary factors and dietary habits with a high consumption of animal fat and a low content of vegetable fibre have been linked to an increased risk of the disease (Wei et al. 2004). The symptoms of CRC include abdominal complaints, anaemia, and blood in stools (Kent et al., 2009). The best way to diagnose the CRC is with a colonoscopy, which also allows for a histological diagnosis. Radical surgery is the cornerstone of the treatment (Wilkinson and Scott-Conner 2008). Chemotherapy and/or radiotherapy are used as an adjuvant treatment for high-risk patients in order to reduce the risk of a relapse, or as a palliative treatment for patients with disseminated cancer (Wolpin and Mayer 2008). The survival of CRC depends on the stage of the disease.

2.3.3. Examples of cDNA microarrays in breast carcinoma research

Soon after Schena et al. (1995) introduced glass slide-based cDNA microarrays, Perou et al. (1999) used the method to classify breast tumours on the basis of gene expression profiles. The paper indicated that, in spite of the known heterogeneous nature with mixed cell populations, breast carcinomas could be classified using gene expression profiling and the expression changes could further be validated by IHC in archival tumour samples. In their later work, Perou et al. (2000) were able to associate sets of co-expressed genes to physiological features, for example, to proliferation rates as measured by the mitotic index.

According to present understanding, most breast carcinomas are sporadic and induced by the accumulation of various mutations or alterations in gene expression (Desai et al., 2002). On the other hand, in the hereditary form of breast cancer, the risk to develop the disease has been associated with mutations in BRCA1 and BRCA2 genes, for example (Tavassoli and Devilee (Eds.), 2003, p. 54, Antoniou et al., 2001). Tumours with either BRCA1 or BRCA2 mutation differ from each other in some clinical and histopathological features (Lakhani et al., 1998, Loman et al., 1998), but these features are not sufficient for classification of individual patients. The understanding of pathogenesis in hereditary breast cancer has been improved by Hedenfalk et al. (2001), who compared expression profiles in breast cancer specimens from BRCA1 mutation carriers, BRCA2 mutation carriers, and sporadic tumours applying cDNA microarrays, and identified a subset of differently expressed genes between these forms of breast cancer. Recently, molecular signatures have been published also for the prediction of hormone treatment failure (Vendrell et al., 2008) and the identification of different histological types of breast cancer (Bertucci et al., 2008).

Among the first to apply cDNA microarrays in the evaluation of breast cancer survival, Bertucci et al. (2000) revealed with the help of gene expression profiling, a histologically and clinically undistinguishable subgroup of patients with a poor outcome. Soon after this, prognostic subgroups of breast cancer patients were determined by a gene expression signature in two different studies (Sørlie T et al., 2001, Bertucci et al., 2002). In a paper by Bertucci et al. (2002), they could, with the help of gene expression profiling, further refine tumour classification by identifying three additional prognostic subgroups among a homogeneous group of breast cancer patients who, according to the established prognosticators, all had a similar poor prognosis of the disease.

2.3.4. Examples of cDNA microarrays in colorectal carcinoma research

In the beginning of this millennium, Kitahara et al. (2001), Takemasa et al. (2001) and Williams et al. (2003) made attempts to survey deregulated gene expression in colorectal cancer in order to identify significant genes influencing the pathogenesis, and constituting potential new targets for diagnosis and therapy of the disease. However, comparison and evaluation of the lists of up- and downregulated genes in these studies is hindered by the many differing attributes of methodology and study design, such as genes included in the microarrays, tissue material collected and prepared, and methods of data normalization. Also, the sample size in these studies was rather limited with a maximum of twenty patients. A little later, Bertucci et al. (2004) profiled fifty tissue samples and published gene expression signatures distinguishing various clinically relevant subgroups, and also a signature associated with patient outcome. Also Eschrich et al. (2005) studied the gene expression of colorectal cancer in a somewhat larger patient material, where the

expression profiles could be correlated with patient outcome. Recently, Yamasaki et al. (2007) studied over one hundred colorectal samples to identify sets of genes differently expressed during different stages of oncogenic development. Applying independent smaller patient material, they were able to validate gene expression signatures classifying tumours originally diagnosed as local ones into localized and metastasized classes with different outcome.

2.3.5. Examples of TMAs in breast carcinoma research

Quite soon after the concept of TMA was initially introduced, the validity of the technique was tested in the evaluation of estrogen receptors, progesterone receptors and Her2/neu-oncogene expression – the established clinical markers of breast cancer. In 2000, Gillett et al. found a strong association between both separate TMA cores and TMA and traditional whole sections. Furthermore, they observed that the inclusion of more than one core improved the correlations only marginally. (Gillett et al., 2000) To the contrary, the study carried out by Camp et al. (2000), suggested the use of two cores, one from the centre and the other from the periphery of the tumour, for accurate tissue representation. Later on, Torhorst et al. (2001) showed that the number of cores needed for an accurate representation of the biomarker profile is dependent upon the marker in question. Since the first validation studies, many other reports have been published on breast cancer, confirming the good concordance of established markers between TMA and whole sections (Sapino et al., 2006, Kyndi et al., 2008, Zhang et al., 2003). In addition, many large-scale evaluations have proven the feasibility of TMA to produce clinically relevant correlations between the established biomarkers of breast cancer and patient outcome (Kyndi et al., 2008, Wärnberg et al., 2008, Gillett et al., 2000, Torhorst et al., 2001). In spite of the promises in research settings, TMAs have not been recommended for routine use in patient care, since perfect agreement between TMA and whole sections has not been proven (Gillett et al., 2000, Selvarajan et al., 2006).

2.3.6. Examples of TMAs in colorectal carcinoma research

In colorectal cancer, the TMA technique has been validated with relatively small-scale materials comparing the expression of range of biomarkers. Generally, a good correlation has been reached between TMA and whole sections, as well as between central and peripheral cores. These studies have suggested the use of at least three, or preferably four cores per case. (Fernebro et al., 2002, Jourdan et al., 2003) In the evaluation of clinical outcome, TMA has proven useful in some works (Prall et al., 2004), but not in all (Hoos et al., 2002).

2.4. REVIEW OF SECURIN, CARBONIC ANHYDRASE IX AND P120 CATENIN

2.4.1. Review of securin

2.4.1.1. Introduction

In 1997, securin (pituitary tumor-transforming, Pttg) was cloned from a rat pituitary tumour cDNA library employing the differential display PCR technique (Pei and Melmed, 1997). The next year, Dominguez et al., cloned a human counterpart from a Jurkat cell cDNA library (Domínguez et al., 1998), and the gene was localized to chromosome 5 (Kakar, 1998.). Almost at the same time, PTTG cDNA was independently cloned by Kakar and Jennes from human testis (1999). Soon after, the product of the PTTG gene was identified as a human securin protein capable of binding separase, and securin destruction was found to be essential for normal sister-chromatid separation (Zou et al., 1999). Previously, the interplay of the yeast counterparts of securin and separase had been studied along with sister-chromatid separation (Cohen-Fix et al., 1996, Funabiki et al., 1996, Ciosk et al., 1998). In 2000, the previously cloned human PTTG was reported as one of the three members of the PTTG-family, and renamed PTTG1 (Chen et al., 2000).

Originally, the human PTTG1 gene was shown to contain five exons in the coding region (Zhang et al., 1999b). Later, Clem et al. (2003) reported the existence of a sixth exon upstream of the translation start site, containing transcription factor binding sites. Sequence analysis has revealed an open reading frame for a 22 kDa protein (Domínguez et al., 1998). However, in sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) analysis, securin has been shown to migrate as approximately 29 kDa protein (Domínguez et al., 1998), suggesting some post-translational modifications occur (Vlotides et al., 2007). Particularly during mitosis, securin seems to have a potential to migrate as doublet, possibly due to cell-cycle dependent phosphorylation (Ramos-Morales et al., 2000). PTTG1 mRNA is ubiquitously expressed in a normal adult testis and thymus (Zhang et al., 1999b). Weak expression has been found in the colon, small intestine, placenta, brain, pancreas and lung (Zhang et al., 1999b, Domínguez et al., 1998). Furthermore, fetal liver has been shown to express PTTG1 mRNA (Zhang et al., 1999b).

Securin protein has been shown to localize both in the nucleus and cytoplasm (Domínguez et al., 1998, Yu et al., 2000b), and also minor membranous localization of expression has been reported (Yu et al., 2000b). Sub-cellular distribution of expression has been suggested to be cell type- (Mu et al., 2003), tumour type- (Sáez et al., 1999), and cell cycle-dependent (Yu et al., 2000b). In the studies concerning pituitary and breast cancer,

nuclear localization in particular has been suggested to associate with tumorous phenotype or aggressive disease (Wierinckx et al., 2007, Ogbagabriel et al., 2005). In colorectal cancer cells, chemically induced DNA damage has been shown to decrease, especially nuclear securin expression, and thus shift the expression ratio from the nucleus towards the cytoplasm (Kim et al., 2007). Phosphorylation, along with the PTTG-binding factor, may facilitate the transport of securin into the nucleus (Pei, 2000, Chien and Pei, 2000). The ratio of the expression in different locations remains unclear, as is the case also with the role of cytoplasmic securin. However, nuclear expression has been suggested to be consistent with the biological activity of securin as a transcriptional activator and also with the activity of securin protein. (Panguluri et al., 2008, Vlotides et al., 2007, Salehi et al., 2008)

2.4.1.2. Cellular functions

At present, securin is known to have several important roles in the cell. Among the plethora of functions, securin has the most prominent role during the cell cycle, where it starts to accumulate at the onset of the S phase and peaks at the G2 – M phases. Then the level of securin drops quickly upon ubiquitinylation and degradation by the anaphase promoting complex. Thereby, sister-chromatid separation is enabled and the following anaphase triggered. (Zou et al., 1999) At the molecular level, two sister-chromatids are held together by a cohesion complex, consisting of multiple subunits (Michaelis et al., 1997), one of them dissociating from the chromosome after cleavage by separase and, consequently, releasing the chromatids. The function of securin is to prevent the premature activation of separase. (Ciosk et al., 1998, Uhlmann et al., 1999) Recently, securin has been related to mediation of G1- to S-phase transition (Tong et al., 2007). In the control of sister-choromatid separation, securin has been proposed with a dual function on separase. On one hand, it helps to transport separase to the nucleus and, on the other hand, it inhibits the catalytic activity of separase until the onset of anaphase by preventing the access of substrates to separase and possibly preventing separase to activate itself. (Waizenegger et al., 2002, Hornig et al., 2002) Securin has also been suggested with roles in activation of transcription (Domínguez et al., 1998), DNA repair (Romero et al., 2001, Kim et al., 2007), metabolism (Wang et al., 2003), and cell migration (Ishikawa et al., 2001).

The published evidence is ambiguous about the role of securin overexpression in cell proliferation and apoptosis. While some researchers support the interpretation that upregulation of securin causes increased proliferation (Kakar and Jennes, 1999, Hamid et al., 2005), others report on the inhibition of proliferation (Pei and Melmed, 1997, Mu et al., 2003). Yu et al. (2003) have suggested that PTTG1 overexpression *in vitro* is associated with prolonged prophase and metaphase, indicating securin with a role in blocking mitosis. Securin overexpression has also been suggested to cause apoptosis

(Yu et al., 2000a, Yu et al., 2000b), although reduction of apoptosis due to an increase of securin has been shown to occur as well (Bernal et al., 2002). According to Vlotides et al. (2007), the effect of securin overexpression on cell fate may be phosphorylation- or dose-dependent.

In vivo, PTTG1 overexpression has been suggested to be associated with tumour-inducing capability (Zhang et al., 1999b, Kakar and Jennes, 1999), and angiogenic effects (Ishikawa et al., 2001). Mice lacking the Pttg1 gene have been shown to be viable and fertile, but with some tissue-specific defects, such as spleen, testis and pancreatic beta-cell hypoplasia, and thymic hyperplasia (Wang Z. et al., 2001, Wang et al., 2003). Human cells, devoid of securin, are also viable (Jallepalli et al., 2001). It has been suggested that PTTG1-negative non-lethal phenotype proves the existence of a compensatory mechanism for sister-chromatid separation independent of securin (Wang Z. et al., 2001).

2.4.1.3. Examples of expression in carcinoma

Already upon cloning, PTTG1 was shown to be overexpressed in samples from patients with different hematopoietic malignancies, such as leukemia and lymphoma (Domínguez et al., 1998). At the same time, Kakar and Jennes reported that various human tumours, including pituitary, adrenal, kidney, liver and ovarian tumours have a high expression of PTTG1 (Kakar and Jennes, 1999). Currently, PTTG1 is suggested to be the best available marker for pituitary adenomas (Tfelt-Hansen et al., 2006). PTTG1 mRNA, or securin protein overexpression, has also been related to at least breast (Solbach et al., 2004), colorectal (Heaney et al., 2000), thyroid (Sáez et al., 2006), esophageal (Shibata et al., 2002), testicular (Puri et al., 2001), prostate (Zhu et al., 2006) and lung (Rehfeld et al., 2006) cancers, as well as multiple myeloma (Chiriva-Internati et al., 2008) and glioma (Genkai et al., 2006). In relation to disease phenotype and patient outcome, Zhang et al. suggested already in 1999(a) that PTTG1 mRNA overexpression is associated with the aggressiveness of the pituitary tumours. Parallel results have been obtained also, for example, for hepatocellular (Fujii et al., 2006) and esophageal cancer (Shibata et al., 2002), as well as squamous cell carcinoma of the head and neck (Solbach et al., 2006).

In colorectal tumours, PTTG1 has been shown to be upregulated at the mRNA and protein level (Heaney et al., 2000, Hlubek et al., 2006). mRNA has been suggested to be even more highly expressed in those tumours with a more aggressive disease (Heaney et al., 2000, Kim et al., 2007). According to their studies of the underlying mechanism behind colorectal tumorigenesis, Hlubek et al. (2006) have proposed securin to be a target of the beta-catenin transcriptional activator. This is a downstream signalling molecule of a well-known adenomatous polyposis coli tumour suppressor gene, frequently mutated in colorectal cancer. In addition, it has been reported that PTTG1 upregulation is not due to

gene amplification, and no mutations in the coding region of the gene have been found (Heaney et al., 2000).

In breast cancer, PTTG1 mRNA overexpression has been related to invasive phenotype, with more advanced disease and higher risk of tumour recurrence (Solbach et al., 2004, Ogbagabriel et al., 2005). The PTTG1 mRNA expression level has been shown to correlate with the amount of securin protein (Ghayad et al., 2009). Association between upregulation of securin protein and tumour aggressiveness is apparent also, as assessed by IHC in a relatively small series of breast cancer cases (Ogbagabriel et al., 2005). Interestingly, PTTG1 has been reported as one of four marker mRNAs capable of detecting circulating cancer cells in the blood of breast cancer patients (Chen et al., 2006).

It has been suggested that securin could represent a therapeutic target for colorectal and breast cancer, and that measurement of PTTG1 upregulation could be used as a tool in oncological decision-making (Tfelt-Hansen et al., 2006, Ogbagabriel et al., 2005).

2.4.2. Review of carbonic anhydrase IX

2.4.2.1. Introduction

Carbonic anhydrase 9 (CA9) was originally cloned from a HeLa cell library in 1994, using a specific monoclonal antibody against MN protein. Analysis of the deduced amino acid sequence revealed a partial similarity to carbonic anhydrases. Furthermore, *in vitro* studies suggested a role in the control of cell growth. (Pastorek et al., 1994) In previous studies, expression of MN had been associated with tumorigenicity and it had been found to appear as oligomers and glycosylated forms (Pastoreková et al., 1992, Závada et al., 1993, Liao et al., 1994). In 1996, Hewett-Emmet and Tashian published a revised version of MN cDNA, concluding that MN belonged to an alpha-carbonic anhydrase family, and proposed naming it carbonic anhydrase IX (CAIX). The same year, another revised cDNA sequence of CA9, along with a genomic organization was published, uncovering a coding region with 11 exons encoding a multidomain protein (Opavský et al., 1996). Two years later, the gene for CAIX was localized in chromosome 9 (Nakagawa et al., 1998). In 2000, cDNA encoding for a previously identified tumour-related G250 antigen was isolated, showing perfect identity to MN/CA9 (Grabmaier et al., 2000). Already in 1986, tumour-specific immunopositivity in renal-cell carcinomas had been found by staining with the monoclonal antibody G250. This has resulted in testing G250 antigen as a potential therapeutic target in metastatic renal cell carcinoma. (Oosterwijk et al., 1986, Divgi et al., 1998) At present, it is known that carbonic anhydrases form a large protein family (Hewett-Emmett and Tashian, 1996), and among these proteins, CAIX is the one that shows exceptionally

high catalytic activity (Hilvo et al., 2008). Historically, CAIX has been appointed as the first tumour-associated carbonic anhydrase isoenzyme (Pastoreková et al., 1997). Later, at least CAXII has been shown to be tumour-related, even if it has not been associated with such aggressive tumour types as CAIX (Ivanov et al., 2001, Li et al., 2009b). Mutations in the coding region seem to not be the cause for differential expression between neoplastic and non-neoplastic tissues (Pastoreková et al., 1997). Instead, elevated expression of CAIX has been shown to occur in cancer cells exposed to hypoxic conditions (Ivanov et al., 2001). According to the literature, many factors, such as hypoxia inducible factor 1 alpha (Potter and Harris, 2004), influence CAIX expression (Grabmaier et al., 2004, Oosterwijk, 2008, Kaluz et al., 2009).

Originally, CA9 was shown to be transcribed into a single 1.5 kb mRNA (Pastorek et al., 1994). Later, a hypoxia-independent constitutively expressed transcription variant was found, encoding a truncated protein lacking the C-terminal part of the catalytic domain, and thus showing diminished enzymatic activity (Barathova et al., 2008). The physiological role of the truncated isoform remains to be established (Kaluz et al., 2009). The full length CAIX appears as doublet bands of 58/54 kDa in western analysis (Pastorek et al., 1994). According to the literature, CAIX is a transmembrane protein, having two extracellular domains in addition to a cytoplasmic part – one having carbonic anhydrase activity and the other one involved in cell-cell adhesion (Hilvo et al., 2008, Závada et al., 2000). However, already upon the first identification as MN, nuclear expression was recognized in addition to membranous localization (Pastoreková et al., 1992, Závada et al., 1993). Also cytoplasmic and stromal staining has been reported in cancer (Liao et al., 1997, Ivanov et al., 2001, Bartosová et al., 2002).

During fetal development, CAIX is expressed in many organs and there are also high expression levels in the placenta. Instead, after birth the expression is often silenced. Especially from one year of age and throughout adult life, CAIX expression is limited to only certain non-neoplastic tissues (Liao et al., 2009), especially the stomach and gallbladder and, to some extent, also the heart, liver, colon, small intestine and bile and pancreatic ducts (Pastoreková et al., 1997, Ivanov et al., 2001). Instead, an abundant CA9/CAIX expression has been observed in several tumour types (Liao et al., 1994, Ivanov et al., 2001) and tumour cell lines (Závada et al., 1993, Ivanov et al., 2001), apart from gastric carcinoma cell lines and tumours which are reported with a diminished expression (Pastoreková et al., 1997, Oosterwijk et al., 1986). For many tumours showing abundant CA9/CAIX expression, no expression was detected in corresponding normal tissue, suggesting a tumour-specific activation. In tumours, CAIX expression has been reported to be very heterogeneous in distribution and intensity in association with hypoxia. (Ivanov et al., 2001)

2.4.2.2. Cellular functions

Carbonic anhydrases catalyze a reversible hydration of carbon dioxide. This reaction plays a part in acid-base balance, ion exchange and carbon dioxide transfer, and thus is implicated in many vital physiological processes, for example, biosynthetic reactions, respiration, bone resorption and the production of saliva and gastric acid. (Pastoreková et al., 1997, Supuran, 2008) *In vitro* studies have suggested that upregulation of CA9 expression correlates with increased extracellular acidification (Ivanov et al., 2001). Also in cancer tissue, CAIX has been suggested to take part in acidifying extracellular milieu surrounding the cancer cells and thereby promoting tumour growth and spread (Ivanov et al., 1998, Ivanov et al., 2001, Swietach et al., 2007). Reduction of pH has been suggested to cause enhanced proteolytic activity, which in turn is associated with the increased breakdown of the extracellular matrix, ultimately promoting tumour cell motility (Webb et al., 1999, Martínez-Zaguilán et al., 1996, Rozhin et al., 1994). In colorectal cancer, the expression of CAIX has been suggested to correlate with cell proliferation, but concerning other tumours, contradictory results have been reported (Saarnio et al., 1998a, Wykoff et al., 2001, Proescholdt et al., 2005).

2.4.2.3. Examples of expression in carcinoma

Already before its designation as CAIX, an elevated expression of this enzyme was associated with the aggressiveness of cervical carcinoma (Liao et al., 1994). Later, expression changes were reported in association with poor patient outcome in gastric carcinomas (Leppilampi et al., 2003, Chen et al., 2005). At present, high CAIX expression has been linked to disease aggressiveness or poor prognosis in many tumour types, such as brain, breast and lung cancers (Proescholdt et al., 2005, Chia et al., 2001, Giatromanolaki et al., 2001). On the other hand, the majority of evidence suggests that low CAIX expression is an independent predictor of poor outcome in renal cell carcinoma (Bui et al., 2003, Bui et al., 2004, Sandlund et al., 2007). Also, low expression of CA9 has been related to a worse outcome in acute myeloid leukaemia (Greiner et al., 2006).

In colorectal cancer, CAIX has been reported to be variably expressed (Kivela et al., 2001, Kivela et al., 2005), while non-neoplastic colon and rectum are negative or show only low expression levels (Saarnio et al., 1998b). Hypoxic tumour cells respond poorly to radiotherapy, an important treatment modality of rectal cancer and of many other cancers too. Consequently, CAIX as an indicator of hypoxia may have a role in predicting the response to radiotherapy (Rockwell et al., 2009). Breast tumours have been shown to frequently express the CAIX protein (Chia et al., 2001, Bartosová et al., 2002). Specifically, abundant expression has been related to aggressive carcinoma with necrosis (Ivanov et al., 2001, Wykoff et al., 2001, Chia et al., 2001). The association of CAIX expression has been shown with breast cancer survival (Brennan et al., 2006). Besides the suggested utilization in disease prognosis and treatment prediction, it was early

proposed that CAIX could be considered as a target for therapeutic applications (Ivanov et al., 1998). In addition, preliminary studies have raised hopes for the development of a vaccination-induced cytotoxic T-cell response against CAIX positive cells as a cancer treatment strategy (Uemura et al., 2006). Furthermore, selective blockage of CAIX enzymatic activity or a construction of fusion proteins, including effector molecules with a CAIX antibody for targeting, may play a role in the future interests of cancer treatment and *in vivo* imaging (Oosterwijk, 2008, Supuran, 2008, Swietach et al., 2007, Pastorekova et al., 2008).

2.4.3. Review of p120 catenin

2.4.3.1. Introduction

cDNA for murine catenin delta-1 (Ctnnd1, p120 catenin) was cloned in 1992 (Reynolds et al., 1992). A gene for the human counterpart was localized in chromosome 11 four years later (Reynolds et al., 1996b), and the corresponding full length cDNA cloned from a fetal kidney in 1998 by Keirsebilck et al. Human CTNND1 was determined to contain 21 exons, potentially encoding up to 32 distinct protein isoforms, by the usage of four different initiation codons and the alternative splicing of three exons. The isoforms that arose from the alternative use of initiation codons, were named 1 to 4, whereas the additional isoforms due to alternative usage of exons 18, 20 and 11, were designated as A, B and C, respectively. Thus, the name of the longest isoform became 1ABC. (Keirsebilck et al., 1998) A year before that, cDNA corresponding to the later designated isoform 1AC, had been cloned from the brain and identified as KIAA0384 (Nagase et al., 1997, Keirsebilck et al., 1998). It has been suggested that additional alternation is produced by the alternative splicing of exon 4, designated as exon D, and residing between the putative initiation codons 2 and 3 (Aho et al., 1999, van Hengel et al., 2007). p120 catenin (p120(ctn)) is a member of a protein family containing the so-called Armadillo-repeat domain. This domain enables p120(ctn) interaction with the cytoplasmic juxtamembrane domain in cadherins and the transcription factor Kaiso, as well as binding to microtubules. (Keirsebilck et al., 1998, Yap et al., 1998, Daniel and Reynolds, 1999, Franz and Ridley, 2004)

Generally, CTNND1 mRNA seems to be ubiquitously expressed in human tissues. Abundant expression has been demonstrated in the placenta, lung, liver, and kidney, and somewhat weaker expression in the pancreas, prostate, testis, ovary, and small intestine. (Nagase et al., 1997) In addition, tissue- and cell type specific expression of various isoforms has been reported (Keirsebilck et al., 1998, Reynolds et al., 1994, Mo and Reynolds, 1996). However, the expression pattern has been suggested to differ significantly between neoplastic and non-neoplastic tissues (Aho et al., 1999). Epithelial cells have been regularly associated with the expression of the type 3 shorter variant

of p120(ctn) (Mo and Reynolds, 1996). Type 1 long isoforms have been variably demonstrated in epithelial cells, whereas types 2 and 4 have been suggested as common but not abundant (Keirsebilck et al., 1998). *In vitro* studies have associated long isoforms particularly with invasive phenotype (Yanagisawa et al., 2008, Silva Neto et al., 2008). p120(ctn) has been found to localize in cell-cell junctions at the plasma membrane, at least in E-, N- and P-cadherin-positive cells. However, in cadherin-deficient cell lines, and in tumours, p120(ctn) has been found in cytosol. (Thoreson et al., 2000, Sarrió et al., 2004, Shibata et al., 2004) Occasional nuclear localization has also been demonstrated (Mayerle et al., 2003, Shibata et al., 2004, Silva Neto et al., 2008). However, the exact signal or signals triggering p120(ctn) nuclear translocation or export, remains to be elucidated (Daniel, 2007).

2.4.3.2. Cellular functions

Interaction between p120(ctn) and cadherins was demonstrated already in the 1990's. It was shown that p120(ctn) is a component of the multiprotein cell adhesion complex, containing also E-cadherin, alpha-catenin, beta-catenin and gamma-catenin. (Reynolds et al., 1994, Shibamoto et al., 1995) The role of the other catenins, except p120(ctn), is to connect the cell adhesion molecule cadherin to the actin cytoskeleton. p120(ctn), however, has been reported to regulate cell-cell adhesion via controlling cadherin stability and turnover at the cell surface. (Ireton et al., 2002, Davis et al., 2003, Reynolds and Roczniak-Ferguson, 2004) It has been suggested that p120(ctn) promotes tumour-suppression in the presence of E-cadherin, and cell growth in the absence of it (Soto et al., 2008). A model where p120(ctn) has a dual role in cancer has been presented in literature. According to the hypothesis, E-cadherin levels at the cell surface drop if p120(ctn) is lost first, and thus, p120(ctn) ultimately acts on tumour suppression. However, if E-cadherin is lost first, p120(ctn) may accumulate in the cytoplasm, enter the nucleus and actively promote metastasis. (Sarrió et al., 2004, Thoreson and Reynolds, 2002) Between p120(ctn) isoforms no difference has been detected in E-cadherin binding (Reynolds et al., 1996a). p120(ctn) has been reported to interact also with the transcription factor Kaiso (Daniel and Reynolds, 1999). Recent study reports the existence of the cytoplasmic complex of Kaiso and p120(ctn) in cancer cells and tissues (Dai et al., 2010), and nuclear interaction has been suggested too, but the modulation of Kaiso activity by p120 is not fully understood (van Roy and McCrea, 2005).

In vitro overexpression of p120(ctn) has been reported to be capable of inducing the loss of normal cell-cell contact (Aho et al., 2002). Studies have also associated p120(ctn) with morphological changes related to invasive capacity (Shibata et al., 2004, Silva Neto et al., 2008), and with aberrant mitosis and polyploidy (Chartier et al., 2007). The effects on invasion and proliferation have recently been reported to be p120(ctn) isoform-specific, where type 1 isoform was particularly associated with the invasive capacity and

type 3 with cell cycle and proliferation (Liu et al., 2009). However, the mechanisms of the action concerning these functions still remain unclear (Anastasiadis, 2007).

2.4.3.3. Examples of expression in carcinoma

Lost, decreased, abnormally localized and/or heterogeneous p120(ctn) expression has been demonstrated in various tumours (Dabbs et al., 2007a, Bremnes et al., 2002, Kallakury et al., 2001), and the correlation between abnormal expression and unfavourable outcome has been suggested for some of them (Silva Neto et al., 2008, Wijnhoven et al., 2005, Stefansson et al., 2004). In breast cancer, the expression of p120(ctn) has been described as heterogeneous, cytoplasmic, membranous or reduced membranous, using antibodies recognizing all isoforms (Nakopoulou et al., 2002, Sarrió et al., 2004, Dabbs et al., 2007a, Dabbs et al., 2007b, Paredes et al., 2008). Also, minor nuclear expression has been reported (Sarrió et al., 2004). Some researchers have also reported complete loss of p120(ctn) protein in up to 10% of the studied cases of invasive ductal breast cancer (Dillon et al., 1998, Nakopoulou et al., 2002, Sarrió et al., 2004). Dillon et al. (1998) have also tested antibodies specific for long isoform, and reported a similar pattern of lost expression as obtained with those recognizing all isoforms. A more distinct shift of expression from membrane to cytoplasm has been observed in specific histological types of breast cancer (Sarrió et al., 2004, Paredes et al., 2008, Dabbs et al., 2007a, Dabbs et al., 2007b). Cytoplasmic accumulation of p120(ctn) has been associated with poor survival in the subgroup of E- and P-cadherin positive breast cancers (Paredes et al., 2008). In colorectal cancer, a heterogeneous and frequently altered p120(ctn) expression has been reported (Skoudy et al., 1996, Karayiannakis et al., 1999, Gold et al., 1998). Cytoplasmic localization of p120(ctn) seems to play a role in the outcome of colorectal cancer (Bellovin et al., 2005). According to Gold et al. (1998), loss of p120(ctn) is involved with poor outcome of colorectal cancer.

It has been speculated that the aberrant expression of p120(ctn) isoforms may lead to cancer progression through dysregulation of cadherin-mediated adhesion, or promoting cell motility and invasion (Anastasiadis and Reynolds, 2000). Differential expression of the various isoforms of p120(ctn) has generally been correlated to disease aggressiveness and outcome (Yanagisawa et al., 2008, Liu et al., 2007, Miao et al., 2009), but also contradictory implications exist (Ishizaki et al., 2004).

3. AIMS OF THE STUDY

This thesis concentrates on the use of two selected methods of molecular pathology, cDNA and tissue microarrays. Studies I, II and V apply cDNA microarrays, studies I, IV and V apply tissue microarrays, and studies I and V apply them both. Study III confirms the selected results of cDNA microarray analysis. In all, experimental studies I – V identifies potential tumour markers and test their applicability to predict the outcome of two common human malignancies, breast and colorectal carcinomas. The aims of this summary are, furthermore, to enlighten the strengths and shortcomings of cDNA and tissue microarray methods in cancer research.

1. To summarize the methodological features of cDNA and tissue microarrays, influencing their applicability in clinical pathology using breast and colorectal cancer as examples

2. To screen, with cDNA microarrays, aberrant gene expression (I, II, V)

3. To explore the value of selected gene expression changes at the protein level to patient outcome (I –V)

4. To analyse the correlations of the selected expression changes to patient outcome on tissue microarrays (I, IV, V), or on whole sections (II, III).

4. MATERIALS AND METHODS

4.1. PATIENT AND TISSUE MATERIAL

The cDNA microarray experiments are comprised of tissue specimens representing a total of ten patients with breast carcinoma (II, V), and six patients with colon carcinoma (I). All tissue material for the cDNA microarray experiments was obtained fresh from the operating theatre at the Department of Surgery, and prepared at the Departments of Pathology and Surgery, in Turku University Hospital, Turku, Finland. Immunohistochemical analysis involved the histological samples of a total of 413 patients with breast carcinoma (II, IV, V), 116 patients with colon carcinoma (I), and 166 patients with rectal carcinoma (III). In addition, western blotting was performed in four colon carcinoma patients out of the cDNA microarray patient material, followed by immunohistochemistry. Breast cancer tissue material for immunohistochemical analyses was obtained from the pathology archives of the Departments of Pathology, at Turku University Hospital, Turku, Finland (for whole sections), and at Jyväskylä Central Hospital, Jyväskylä, Finland (for TMAs). All tissue material for the analysis of colorectal carcinomas originated from the Department of Pathology, at Turku University Hospital. For cDNA microarray experiments of breast carcinoma, normal tissue controls were obtained from five cases of histologically verified non-cancerous breast tissue specimens. In the case of colon carcinomas, normal tissue controls were collected from normal mucosa outside the tumour. For immunohistochemistry of breast and colorectal carcinomas, normal tissue areas outside the tumours were applied. Prognostic analyses of both breast and colorectal carcinomas were based on complete (clinical) patient data from the time of diagnosis, and a maximum of follow-up information of 19.9, years and 9.5 years for breast and colorectal carcinoma patients, respectively. Tables I and II summarize the description of the patients and tissue materials applied in publications I – V.

Table I. The clinicopathological characteristics of the patients in publications I – V.

	Breast cancer				Colon cancer		Rectal cancer
	cDNA microarray	IHC			cDNA microarray	IHC	IHC
	II, V	II	IV	V	I	I	III
Gender (%)							
male / female	0 / 100	0 / 100	0 / 100	0 / 100	50 / 50	40 / 60	59 / 41
Age (years)							
mean	69	63	58	58	67	70	68
Tumour grade (%)							
I	20	18	25	26	17	23	15
II	30	41	44	43	66	60	64
III	50	41	26	25	17	18	17
unknown	0	0	5	6	0	0	3
Nodal status (%)							
negative	30	54	46	46	67	35	56
positive	70	46	45	44	17	28	42
unknown	0	0	9	10	17	37	2
Tumour diameter (cm)							
mean (s.d.)	3.2 (1.45)	2.6 (1.68)	2.3 (1.4)	2.3 (1.5)			
Histological type (%)							
ductal	100	100	81	80			
lobular	0	0	11	10			
other	0	0	7	7			
unknown	0	0	1	2			

s.d. standard deviation

Table II. Summary of patients, histological material and methods of analysis in publications I – V.

Publication	Cancer type	Analysis method	Histological material	n	Follow-up (mean)	Cancer specific death (%)
I	colon	cDNA microarray		6		
	colon	IHC	TMA	114	4 y 5 m	45
II	breast	cDNA microarray		10		
	breast	IHC	whole sections	44	4 y 1 m	14
III	colon	western analysis		4		
	rectal	IHC	whole sections	166	3 y 4 m	17
IV	breast	IHC	TMA	310	10 y 6 m	18
V	breast	cDNA microarray		10		
	breast	IHC	TMA	341	10 y 7 m	17

The present study has the approval of the Ethical Committee of Turku University Hospital, or Jyväskylä Central Hospital, and the National Authority for Medicolegal Affairs. Each investigated specimen was included in the cDNA microarray study only after the written consent of the patient. The research was carried out in accordance with the Declaration of Helsinki.

4.2. THE cDNA MICROARRAY METHOD

In cDNA microarray analysis, special emphasis was placed on the high quality of tissue material. Fresh samples of breast tissue were dissected by a pathologist and carefully prepared macroscopically from fat and connective tissue. A consecutive histological slide was prepared from all the cases to verify the diagnosis. In addition, normal tissue reference was obtained from benign breast tissue outside the tumours of five patients. In the case of colon cancer, neoplastic and non-neoplastic mucosa from each patient was obtained. Both types of cancer samples contained in addition to carcinoma cells, also non-neoplastic epithelium and non-epithelial cells. All samples were fresh frozen in liquid nitrogen within thirty minutes after their surgical removal, and stored at -70 °C until used for RNA isolation. The maximum time of storage was 5 months and 5 days, for breast and colon tissue samples, respectively.

Total RNA was isolated using the Chomczynski & Sacchi (1987) method. Briefly, frozen tissues were homogenized using ultra-turrax T25 (Janke & Kunkel, IKA-Labortechnik) in a solution, including 4 M guanidinium thiocyanate, 0.5% (w/v) sarcosyl, 25 mM sodium citrate, and 0.7% (v/v) 2-mercaptoethanol. Tissue homogenates were kept on an ice bath during RNA isolation. The homogenizer was taken into pieces and washed after each tissue sample. 0.1 volume of 2 M sodium acetate (pH 4.1), an equal volume of saturated phenol (pH 4.3), and 0.2 volume of chloroform:isoamyl alcohol (24:1), were sequentially added to the homogenate and mixed. The mixture was cooled on an ice bath for five minutes and centrifuged 10 000 g for twenty minutes at 4 °C. The organic phase, containing DNA and protein, was stored at -20 °C for possible later use. The aqueous phase was transferred to a fresh tube, mixed with an isopropanol volume corresponding to the volume of the original homogenate, and placed at -20 °C for at least two hours. The total RNA was sedimented by centrifugation at 14 500 g for twenty minutes at 4 °C. The pellet was washed with ice-cold ethanol (70%). After a brief dry, it was dissolved in RNase-free water. The total RNA was further purified using a RNeasy kit (Qiagen) according to the manufacturer's instructions and, finally, eluted in RNase-free water. The concentration and purity of the RNA was determined spectrophotometrically (Ultrospec 1100pro, Amersham Pharmacia Biotech and/or NanoDrop ND-1000, NanoDrop Technologies). In the evaluation of purity, the ratios of 260 nm:280 nm and 260 nm:230 nm were used. To be considered pure, and to be included in the study, the measured ratio 260 nm:280 nm had to be from 1.9 to 2.1, and ratio 260 nm:230 nm had to exceed 2.0. In addition, the integrity of the RNA was evaluated after standard denaturing AGE and ethidium bromide staining. The intensity ratio of the ribosomal bands 28S:18S had to be at least nearly 2:1 in visual inspection. The RNA samples were stored at -70 °C until used for cDNA microarray labelling.

For cDNA microarray analysis, tumour and reference RNAs were fluorescently labelled with Cy5 and Cy3, respectively (CyDye, Amershambiosciences). For colon cancer, a

paired tissue sample from the corresponding normal mucosa of each patient was used as reference material. In the breast cancer study, an equal quantity of the purified RNA obtained from benign breast tissue outside the tumour of five patients was used to prepare a reference pool. Cy-labelled cDNA targets were generated as described by Sillanpää et al. (2004), with minor modifications. Briefly, 25 and 21.5 µg of denatured RNA were reverse transcribed using 2 µg oligo$(dT)_{12\text{-}18}$ primer in colon and breast cancer studies, respectively. The reaction mixture included also 0.5 mM dATP, dCTP and dGTP each, 0.2 mM dTTP, 0.08 mM Cy-labelled dUTP, 30 U RNase inhibitor, and 400 U Superscript II reverse transcriptase (Gibco BRL Life Technologies) in the 1st strand buffer (75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 50 mM tris(hydroxymethyl)aminomethane, pH 8.3). The reaction was allowed to proceed for two hours at 42 °C, and then stopped by adding ethylenediaminetetraacetic acid. RNA was hydrolyzed from the prepared cDNA mixture by a sodium hydroxide treatment at 65 °C for 15 minutes. After neutralization, combined labelled cDNA targets were purified and concentrated with Microcon-30 columns (Millipore). The sufficient efficiency of labelling, purity of labelled cDNA, and the amount of cDNA produced were checked using spectrophotometer (NanoDrop ND-1000). The labelled cDNA targets were hybridized to the cDNA microarrays without delay.

A custom cDNA microarray was produced at Turku Centre for Biotechnology (BTC) in 2002 (University of Turku and Åbo Akademi University). The microarray included a collection of proposed cancer-related genes. They were chosen according to the knowledge of the beginning of the 2000's, concerning the pathogenesis, progression and prognosis of cancer. The microarray contained 4 080 spots, the majority of which included probes for cancer-related genes. Among those were also empty control spots, non-human control probes, and 35 spots for human housekeeping genes. Multiple clones were included for some genes. All the spots were arrayed in triplicate. The microarrays were poly-l-lysine coated. Prior to target hybridization, the microarrays were crosslinked with ultraviolet radiation (90 mj/cm^2). Furthermore, the presence of spots was checked by scanning at 633 nm (Scanarray Express 5000, PerkinElmer), and the microarrays were prehybridized in preheated 5xSSC (1xSSC: 150 mM sodium chloride, 15 mM tri-sodium citrate, pH 7), 0.1% SDS, 1% bovine serum albumin (Fraction V) at 50 °C for 30 minutes. After prehybridization, the microarrays were sequentially washed with 2xSSC and 0.2xSSC at room temperature, and finally spin-dried. The preheated hybridization solution consisted of labelled targets with 1.3xDenhardt's solution, 3xSSC, 0.3% SDS, 125 µg/ml human COT-1 DNA (Invitrogen), 250 µg/ml polyadenylic acid (Sigma), 52.5 µg/ml yeast tRNA (Invitrogen), and 0.5xDIG-blocking buffer (Roche). Hybridization was performed at 65 °C overnight in the volume of 80 µl in a humified chamber under LifterSlips (24 x 60 mm, Erie Scientific Company). A maximum of three microarrays were hybridized at a time. Each sample was hybridized once. The next day, the

microarrays were sequentially washed. The most stringent washing solution contained 0.06xSSC. Finally, the microarrays were spin-dried. They were stored in a dark and dust-free enclosure between washing and signal detection with a maximum delay of 5.5 hours before scanning.

The fluorescent signals from hybridized targets were determined by scanning with a confocal laser scanner (Scanarray Express 5000) according to the procedure recommended by the BTC in 2003. For initial scanning, 10 μm resolution, 150 μm spot size, and automatic sensitivity calibration using default values and fixed laser power, were chosen as scanning parameters. In repeated scannings, different values for the photomultiplier tube were tested to bring Cy-labels in interrelated balance and to avoid saturated spots. A maximum of five repeated scans were performed in order to avoid substantial photobleaching of the Cy-dyes. The images were checked for a uniform signal across the slide. Spot registration, quantitation and normalization were performed using QuantArray software (Packard Bioscience) according to the procedure of the BTC in 2003. Briefly, the spot diameter was set as 150 μm, and the histogram method with the default foreground and background values as proposed by the software, was applied for spot segmentation. The foreground mean values subtracted with background median values were used to calculate spot intensities. For each sample, the raw data consisted of ~4080 transcripts, where the expression ratio between the study case and the reference case was determined for each transcript. The expression ratios were $\log_2$-transformed and intensity-normalized with a locally weighted scatterplot smoothing (LOWESS, Yang et al., 2002) to remove systematic variation from the cDNA microarray data. The quality of each spot was determined first automatically by the footprint option (max 100 μm), and then confirmed visually spot by spot. Only spots with acceptable quality were taken into account. Additionally, background subtracted MA-plots [$\log_2$Cy5 – $\log_2$Cy3 vs ½ × ($\log_2$Cy5 + $\log_2$Cy3), Dudoit et al., 2002] were visually checked for sufficient data quality and success of normalization.

There were three technical replicate spots for each transcript on the array. In the data analysis, the median of them was used to calculate the expression ratio of the transcript in question, when there were three proper values. In the case of two proper values, the mean of them was used, and in the case of only one proper value, it was used as such. If no acceptable value was found, the transcript was marked as a missing value for the sample under study. Only transcripts with three or more non-missing signal values among the studied samples were subjected to statistical analyses. For each transcript, the preprocessed log-ratios were compared to zero with the one-sample Student's *t*-test. A transcript was considered upregulated if its mean expression ratio was larger than 0.5, and the *p*-value of the *t*-test was less than 0.05. Similarly, a transcript was considered downregulated if its mean value was less than -0.5, and the *p*-value was less than 0.05. If a transcript did not fulfil the criteria for up- or downregulation, it was marked as

non-deregulated. The GoMiner program package was used to explore the functions of the genes identified (Zeeberg et al., 2003). Mitotic cell cycle and proliferation related genes are known to be crucial for the progression of breast cancer (Tavassoli and Devilee (Eds.), 2003, p. 57). Furthermore, there are implications for the significance of cell-cell adhesion proteins (Wheelock et al., 2001, Gould Rothberg and Bracken, 2006, Brennan et al., 2010). Therefore, validation of gene expression changes and further study of breast cancer in relation to patient outcome was concentrated to these functions. The same strategy was adapted for colon cancer, since at that time, such strong associations between particular functions and disease progression were not as evident for colorectal as it was for breast cancer. In addition, a single significantly upregulated gene in colon cancer was chosen for further study. The authenticity of the selected up- and downregulated genes was verified by DNA sequencing (service by BTC), applying cycle sequencing with fluorescence dye terminator chemistry. A capillary electrophoretic separation of the labelled products was performed with an Applied Biosystems 3130xl Genetic Analyzer. When the obtained sequences were of sufficient quality, they were compared to human RefSeq RNA, or non-RefSeq RNA, databases at the National Center for Biotechnology Information (NCBI), using a Basic Local Alignment Search Tool (BLAST) with megablast or blastn options and default parameters (Altschul et al., 1990, Zhang et al., 2000).

4.3. IMMUNOHISTOCHEMISTRY

The number of breast and colorectal cancer cases involved in immunohistochemical analyses are reported in publications I – V. Fourty-four cases of breast carcinomas, and 166 cases of rectal carcinomas, were analysed with whole sections. In the analyses of TMAs, the corresponding numbers were originally 369 and 116 for breast and colon carcinomas, respectively. Table II summarizes the cases available for correlations with outcome, along with information on the type of histological material and follow-up data. In addition, whole tissue sections from four colon cancer patients included in the cDNA microarray analysis (I) were immunohistochemically stained, using CAIX antibody (III), in order to verify overexpression of CA9 at the protein level. Standard hospital procedures were followed in the fixation, embedding and storage of paraffin blocks.

Tissue microarrays were prepared as described by Kononen et al. (1998), with minor modifications. Briefly, the histological material of each cancer patient was evaluated to identify a representative cancer area from archival formalin fixed and paraffin embedded blocks of breast and colon cancers. Medically qualified professional personnel performed or supervised the construction of the TMAs. In the case of breast cancer (IV, V), two core biopsies (diameter 0.6 mm, min height 5 mm), typically one from the centre and the other from the edge of the cancer area of each paraffin block, were punched and

precisely arranged into three TMA blocks with 238 – 258 cores in each, using a Beecher Instruments MTA-1 Manual Tissue Arrayer. Two cores in each tumour were prepared in order to improve the representativity of the TMA. For practical reasons, such as small tumour size, in the minority of cases the cores couldn't be obtained from different parts of the tumour. In these cases, both of the two cores were punched, either from the edge (85 cases) or the centre (7 cases) of the tumour. Each TMA had an empty line running horizontally and vertically through the array, dividing it into four separate, asymmetric sections. In addition, each TMA had an asymmetric sample position to mark the first sample of the array. In the case of the colon tissue material (I), five blocks including 13 – 55 cores in each, were constructed using a manual tissue arrayer (Beecher Instruments). The TMAs included a tissue core of both tumour and benign mucosa of each colon cancer patient. The cores were 3 mm in diameter, and minimum 5 mm in height. The cancerous tissue core was punched from the centre of the tumour. In addition, each TMA had an empty position to create asymmetry.

FFPE tissue material was sectioned with microtome at 3 – 5 μm, and placed on adhesive treated microscope slides (Dako S2024 in study I or Menzel J1800AMNZ SuperFrost® Plus in studies II – V). Sections were allowed to adhere to the slides at 65 °C for at least an hour. The sections were usually stained within days of cutting. In that case, they were stored at room temperature. If immunohistochemical staining was performed later (maximally within a few weeks), the sections were stored at 4 °C. Immunohistochemical stainings for securin, p120(ctn), and CAIX, were performed using standard procedures. The optimization of protocols was performed using a set of whole tissue sections. The details of the stainings are summarized in Table III. Heat induced epitope retrieval (HIER) in 10 mM sodium citrate (pH 6) was used in all stainings. Different compositions of epitope retrieval solution were tested for securin. For CAIX, citrate was recommended by the manufacturer. In p120(ctn) stainings, HIER in citrate gave satisfactory results and, therefore, was used without further optimization according to the practice of the Department of Pathology, of the University of Turku, at that time. In addition, heating time (ten minutes) was optimized in the breast cancer study, applying securin antibody on TMAs (IV). In that study, epitope retrieval was performed in a scientific microwave oven (Micromed T/T Mega, Milestone), with a steady temperature of 99 °C. A fourteen minute heating (two seven minute cycles) in a regular household microwave oven (650 W), in a standardized volume of buffer and with an equal number of slides, was used in the other studies (I, II, III and V). Care was taken to keep the temperature of the solution just below boiling. Endogenous peroxidase activity was blocked with hydrogen peroxide. The manufacturers' instructions for positive tissue controls were followed in securin, p120C, and CAIX stainings. For p120N IHC, the staining of benign and neoplastic breast epithelium was used instead of the mouse embryonic fibroblast cell line suggested. Information provided by the manufacturers was adapted for specificity of

monoclonal antibodies. The specificity of the polyclonal CAIX antibody was confirmed by western blotting. Separate stainings, without primary antibody, and using whole tissue sections of breast and colorectal cancer, were used as negative controls. Furthermore, normal colorectal epithelium, devoid of immunopositivity, was used as an internal negative control in CAIX IHC. The methods of immunohistochemical stainings of Ki-67 (I, II, IV and V), estrogen and progesterone receptors (V), E-cadherin (V), smad2 (V), and CDC25B (I), are presented in orginal publications.

Table III. Details of the immunohistochemical methods in publications I – V.

Publication	**I**	**II**	**III**	**IV**	**V**	**V**
Antibody						
Name	securin	securin	CAIX	securin	p120(ctn), P120N	p120(ctn), P120C
Type	monoclonal	monoclonal	polyclonal	monoclonal	monoclonal	monoclonal
Clone	DCS-280	DCS-280		DCS-280	6H11	15D2
Source	Abcam ab3305	Abcam ab3305	Abcam ab15086	Abcam ab3305	Santa Cruz Biotechnology sc-23873	Santa Cruz Biotechnology sc-23872
Dilution	1:20	1:20	1:8000	1:50	1:500	1:50
Incubation	overnight 4 °C	overnight 4 °C	1h RT•	1h RT•	overnight 4 °C	overnight 4 °C
Detection	manual *	manual *	automated #	automated #	manual *	manual *

* Vectastain ABC reagent
\# LabVision Autostainer, PowerVision+ Poly-HRP IHC kit
• Room temperature

The evaluation of IHC was performed under a light microscope. In the case of breast carcinomas, the evaluation of securin (II, IV), and p120N (V), immunoreactivity was registered by the fraction of positively stained nuclei, and p120C (V) by cytoplasmic and membrane-bound positivity (low/high). The evaluations were performed in the whole tissue area of TMA cores, and at the area of the most pronounced staining in whole tumour sections. In the prognostic analyses of the classified values, the observed immunopositivities were stratified according to the observed proliferative activity and patient survival. In colon carcinomas, the evaluation of securin (I) was registered by the fraction of positively stained nuclei. Three high power fields of each TMA core were included in the evaluation. In the prognostic analyses, each cancer specimen was compared to its paired control from a normal mucosa. The tumours were classified as positive if at least 12% of the cancer cell nuclei were stained as positive, and the number of positive cells was at least double, compared to its paired control. CAIX (III) was evaluated by estimating the predominant membrane-bound staining intensity in whole tissue sections. The slides were assessed as negative, if the proportion of positive carcinoma cells in the sections was less than 10%. For positive cases, the slides were evaluated for staining intensity (low/moderate/high). For prognostic analyses, negative cases were combined with low intensity cases and moderate with strong intensity ones. An analysis of the immunohistochemical stainings of Ki-67 (I, II, IV and V), estrogen

and progesterone receptors (V), E-cadherin (V), smad2 (V), and CDC25B (I), and the evaluation of Mitotic Activity Index (II), are presented in origninal publications.

In the statistical analyses, prognostic associations between securin, CAIX, p120N or p120C, and disease-specific survival, were analysed by Kaplan-Meier method using log-rank statistics and Cox's regression analysis. Survival time was defined from the day of surgical diagnosis to death, or to the end of the follow-up. The differences were quantified by hazard ratios, with 95% confidence intervals. *P*-values less than 0.05 were considered statistically significant. To adjust the results for the selected clinicopathological variables, Cox's regression analysis with multiple explanatory variables were applied. More detailed descriptions of the statistical analyses has been represented in original publications I – V.

Concerning breast cancer TMA, immunopositivity of the central and peripheral tumour areas were compared using descriptive figures, intraclass correlation coefficients (ICC), kappa (κ) coefficient, and McNemar's test of marginal homogeneity. Furthermore, if the variation between the different tissue cores appeared high, the mean difference in tissue cores was also tested using linear mixed models. If these tests showed concordance between the central and peripheral cores, prognostic analyses were performed, using either the mean of the results of the two tissue cores, or a single observation in the case of only one assessable core (securin, p120C). If disconcordance between the central and peripheral cores was observed, immunopositivities were also treated as separate values in the prognostic analyses (p120N). In the case of a discrepant classification of p120C between each pair of tissue cores, the lower expression value was applied in concordance with the literature, showing a regional loss of p120(ctn) expression, to predict disease outcome (Gold et al., 1998).

In order to assess the quality of the interpretations of IHC, evaluations of two independent observers, and/or repeated evaluations by a single observer, were applied without knowledge of the patients' clinical data for each antibody used. Reproducibilities between observers and repeated immunoevaluations were expressed for continuous variables as ICC, and for categorized variables as Cohen's κ- or weighted kappa (κ_w) coefficients. In the present study, the following limits were applied: for ICC, value <0.4 was considered poor, 0.40 – 0.75 fair to good and >0.75 excellent. Correspondingly, for κ or κ_w, value <0.2 was considered poor, 0.20 – 0.40 fair, >0.40 but ≤0.60 moderate, >0.60 but ≤0.80 good and >0.80 very good. (Marx et al., 1998, Brennan and Silman, 1992) Statistical computations were performed using SAS System for Windows (release 8.2.2001, versions 9.1.3 and 9.2), SAS Enterprise Guide 4.1 (SAS Institute Inc), SPSS for Windows (version 16.0.2, SPSS Inc), and STATA/SE (version 10.1, Stata Corp.) software packages.

4.4. WESTERN ANALYSIS

Four out of six colon cancer patients from the cDNA microarray experiment were included in the western analysis. DNA and protein fractions were sequentially precipitated from the remaining phenol phases after the isolation of total RNA. Protocols recommended for commercial TRIzol LS and TRI reagents (http://tools.invitrogen.com/content/sfs/manuals/10296010.pdf, http://www.mrcgene.com/tri.htm 3.8.2010) were followed with minor modifications. Briefly, DNA was precipitated by adding 0.4 volumes of ethanol to an organic alcohol-phenol phase and recovered by centrifugation. Protein was then isolated from the supernatant by the addition of three volumes of acetone. After pelleting, washing and drying, proteins were dissolved in 1% SDS in 1 M tris(hydroxymethyl)aminomethane, pH 8.5. The total protein was quantified for each sample in duplicate using Bio-Rad Protein Assay Dye (Bio-Rad Laboratories), and the quality was verified by SDS-PAGE, followed by Coomassie blue staining. For the western blotting, equal amounts of denaturated protein samples were size fractioned using 10% SDS-PAGE, and electroblotted onto a nitrocellulose membrane (Whatman Protran, Perkin Elmer). Uniform loading and blotting was checked with Ponceau S staining. The CAIX primary antibody (ab15086, Abcam) was diluted 1:1000 for western detection. Horseradish peroxidase conjugated anti-rabbit immunoglobulins (Dako) and Pierce ECL Western Blotting Substrate (Thermo Scientific) was used according to the manufacturers' instructions for the visualization of the signal.

5. RESULTS

5.1. cDNA MICROARRAY

The results of cDNA microarrays as up- and downregulated differentially expressed genes are visualized in Figure 1, and summarized in Tables IV – VI, for breast and colon carcinomas. The genes have been allocated into groups according to the functions of the mitotic cell cycle and cell-cell adhesion. The obtained lists of deregulated genes were associated with biological functions at different times and, therefore, different versions of gene ontology databases were used. As a consequence, CTNND1 has been related to the mitotic cell cycle in colon cancer, but not in breast cancer. The tables also include the results of the DNA sequencing in order to verify the authenticity of the corresponding genes. At the time of analysis, there were no established biomarkers for the survival of colorectal carcinoma and, therefore, CA9, as the strongest among 66 upregulated genes in colon cancer was selected for further analysis. DNA sequencing confirmed the authenticity of the CA9 gene.

Concerning breast carcinoma, Figure 1A presents genes related to the mitotic cell cycle and 1B genes related to cell-cell adhesion. Figure 1C presents genes related to the mitotic cell cycle in colon carcinoma. Patient cases are represented in x-axis and deregulated genes in y-axis. Colour scale bar indicates differential regulation between cancer and reference tissues. Upregulation is depicted in beige and downregulation in blue.

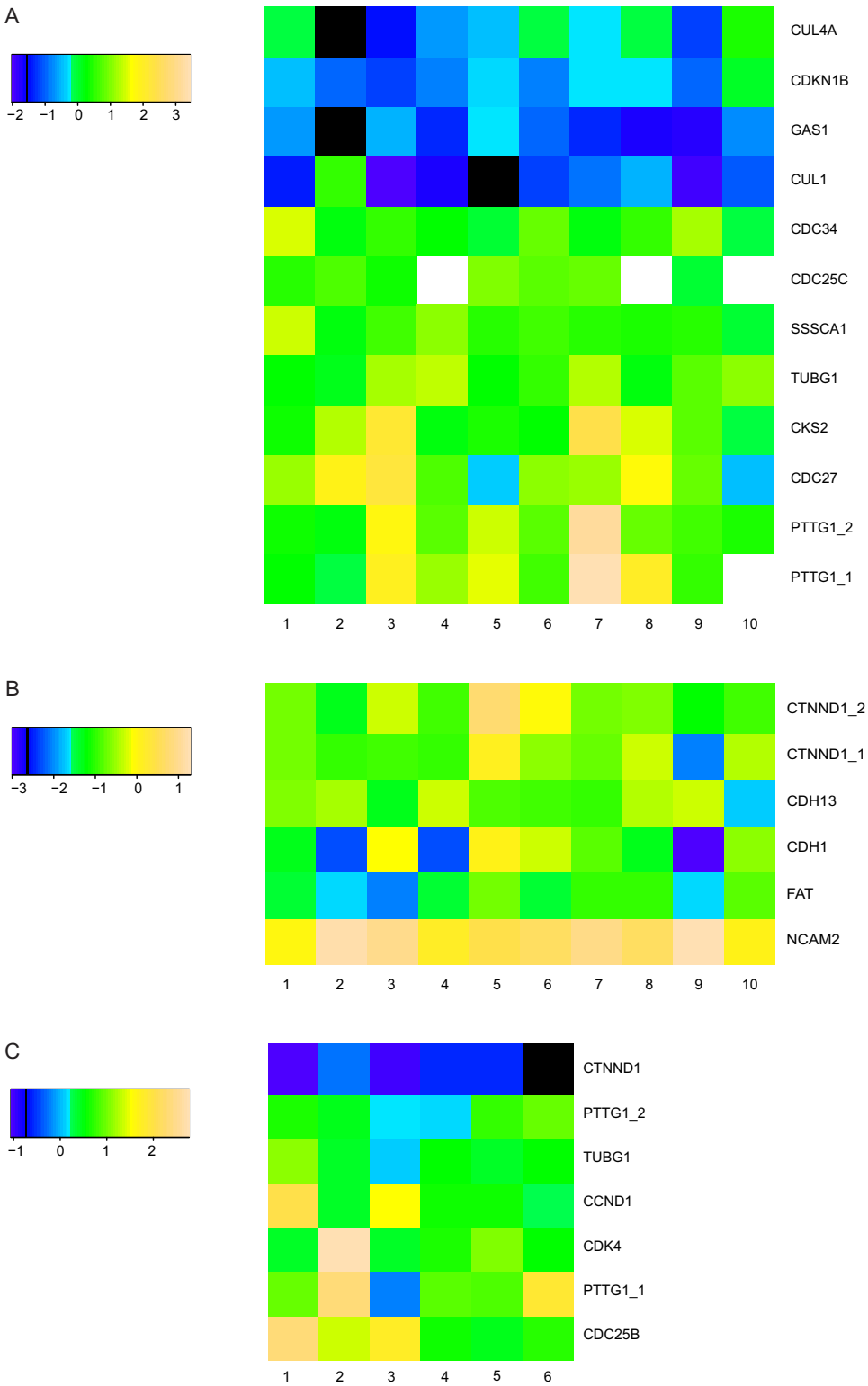

Figure 1. Heat maps of differentially expressed genes in breast and colon carcinomas.

Table IV. Example of breast carcinoma. List of up- and downregulated genes in cDNA microarray analysis (II) associated with mitotic cell cycle. The genes have been arranged in order of decreasing relative expression change.

Gene description	Gene symbol	Deregulation	P-value	Fold change	Authenticity verified
pituitary tumor-transforming 1 (clone 1)	PTTG1	up	0.0070	2.5	yes
pituitary tumor-transforming 1 (clone 2)	PTTG1	up	0.0060	2.1	yes*
cell division cycle 27	CDC27	up	0.0060	2.0	yes
CDC28 protein kinase 2	CKS2	up	0.0080	1.9	yes
tubulin, gamma 1	TUBG1	up	0.0010	1.6	yes
Sjogren's syndrome/scleroderma autoantigen 1	SSSCA1	up	0.0010	1.5	yes
cell division cycle 25C	CDC25C	up	0.0050	1.5	yes
cell division cycle 34	CDC34	up	0.0100	1.4	yes
cullin 1	CUL1	down	0.0010	2.2	no
growth arrest-specific 1	GAS1	down	0.0001	2.1	yes
cyclin-dependent kinase inhibitor 1B (p27, Kip1)	CDKN1B	down	0.0010	1.5	no
cullin 4A	CUL4A	down	0.0320	1.4	yes

*PTTG1 is the most prominent species, but includes also other minor species

Table V. Example of breast carcinoma. List of up- and downregulated genes in cDNA microarray analysis (II, V) associated with cell-cell adhesion. The genes have been arranged in order of decreasing relative expression change.

Gene description	Gene symbol	Deregulation	P-value	Fold change	Authenticity verified
neural cell adhesion molecule 2	NCAM2	up	0.0020	1.5	yes
FAT tumor suppressor (Drosophila) homolog	FAT	down	5.4E-06	2.5	no*
cadherin 1, type 1, E-cadherin (epithelial)	CDH1	down	0.0060	2.3	yes
cadherin 13, H-cadherin (heart)	CDH13	down	0.0010	1.7	yes
catenin (cadherin-associated protein), delta 1 (clone 1)	CTNND1	down	0.0030	1.7	yes
catenin (cadherin-associated protein), delta 1 (clone 2)	CTNND1	down	0.0130	1.5	yes

*SPARCL1 is the most prominent species, but includes also other minor species

Table VI. Example of colon carcinoma. List of up- and downregulated genes in cDNA microarray analysis (I) associated with mitotic cell cycle. The genes have been arranged in order of decreasing relative expression change.

Gene description	Gene symbol	Deregulation	P-value	Fold change	Authenticity verified
cell division cycle 25B	CDC25B	up	0.0111	2.3	no
pituitary tumor-transforming 1 (clone 1)	PTTG1	up	0.0263	2.2	yes
cyclin-dependent kinase 4	CDK4	up	0.0485	2.0	yes
cyclin D1 (PRAD1: parathyroid adenomatosis 1)	CCND1	up	0.0254	1.9	yes
tubulin, gamma 1	TUBG1	up	0.0133	1.4	yes
pituitary tumor-transforming 1 (clone 2)	PTTG1	up	0.0111	1.4	yes*
catenin (cadherin-associated protein), delta 1 (clone 1)	CTNND1	down	0.0020	1.6	yes

*PTTG1 is the most prominent species, but includes also other minor species

Among those up- and downregulated genes, which are related to the mitotic cell cycle and cell-cell adhesion, the number of measured signal values exceeded the original requirement set for a transcript to be regarded as deregulated. Among upregulated genes, the signal could be measured from at least seven out of ten breast cancer patients. The concordance of the direction of expression change was at least 80%. Respectively, the signal was measured from all ten breast cancer samples among downregulated genes. These genes showed at least 90% concordance. In colon cancer, the signal was measurable from all six patient samples among deregulated genes related to the mitotic cell cycle. There was at least 83% concordance in the direction of expression change in genes considered as upregulated. In downregulated ones the same figure was 100%.

As a quality assessment of the cDNA microarray method, the expression of housekeeping genes was evaluated separately. In breast cancer, expression change was detected in seven housekeeping genes among 343 deregulated genes in total. However, in sixteen housekeeping genes no change in expression was detected. In the case of colon cancer, five housekeeping genes out of 150 deregulated genes showed change in expression, while 17 housekeeping genes were specified as non-deregulated.

5.2. IMMUNOHISTOCHEMISTRY

The results of immunohistochemical stainings of securin, CAIX, p120N and p120C are presented in Table VII for breast and colorectal carcinomas. The observed immunohistochemical expression is depicted in either whole tissue sections, or in TMA tissue cores punched from different locations on the tumour. In statistical analyses, securin and p120C immunopositivities showed concordance between the two tissue cores punched from the edge and the centre of the tumour. Concerning p120N, however, statistically significant difference between the central and peripheral cores was found. The results of immunohistochemical stainings of Ki-67 (I, II and IV), smad2 (V), and CDC25B (I), and the evaluation of the Mitotic Activity Index (II), are presented in original publications.

Table VII. Evaluation of securin, CAIX, p120N and p120C immunopositivity in prognostic analyses of breast and colorectal carcinomas.

	Breast carcinoma				Colon carcinoma	Rectal carcinoma
	Whole section	TMA			TMA	Whole section
	Hotspot	Edge	Centre	Prognostic results	Centre	
Securin (%) (II, IV, I)						
Fraction (mean)	10.92	6.11	5.86	6.13	6.14	
Classified (cases <1,5% / ≥1,5%)		35.9 / 64.1	38.1 / 61.9	30.6 / 69.4		
CAIX (%) (III)						
Intensity (cases low / high)						71.0 / 29.0
p120N (%) (V)						
Fraction (mean)		10.02*	7.15*	10.02		
Classified (cases <5% / ≥5%)		56.8 / 43.2*	64.4 / 35.6*	56.8 / 43.2		
p120C (%) (V)						
Intensity (cases low / high)		80.0 / 20.0	83.7 / 16.3	84.0 / 16.0		

*statistically significant difference between the central and peripheral cores

In order to evaluate the value of securin, p120(ctn) and CAIX, for the survival of breast and colorectal carcinoma, the results of IHC were correlated with follow-up information from the patient material (Table VIII). The most significant prognostic value of p120N was associated with the immunopositivity at the edge of the tumour.

Table VIII. Summary of correlations with outcome of securin, CAIX and p120(ctn) immunohistochemistry to predict disease-specific survival among breast and colorectal carcinoma patients.

	Breast cancer		Colon cancer		Rectal cancer	
	Correlation with outcome in		Correlation with outcome in		Correlation with outcome in	
	univariate analysis	multivariate analysis	univariate analysis	multivariate analysis	univariate analysis	multivariate analysis
Securin - whole section (II)	yes	ND*				
Securin - TMA (IV, I)	yes	yes	no	no		
CAIX - whole section (III)					yes	yes
p120N - TMA (V)	yes	yes				
p120C - TMA (V)	yes	yes				

* not determined

Fractions of evaluable tissue core biopsies in immunohistochemical stainings of TMAs for breast and colon cancer are summarized in Table IX. In both cancers, the loss of cores was equally evident in the central and the peripheral locations of the TMAs.

Table IX. Fraction (%) of successful and unsuccessful evaluations of TMA core biopsies in immunohistochemical analyses of securin, p120N and p120C in breast and colon carcinomas.

	Breast carcinoma			Colon carcinoma		
	Evaluable result	Unrepresentative core	Lost core	Evaluable result	Unrepresentative core	Lost core
Securin (IV, I)	77	23	0	96	0	4
p120N (V)	82	16	2			
p120C (V)	84	16	0			

As a quality measure for the interpretation of immunopositivity, repeated evaluations by one observer and two independent observes were applied. Statistical analyses showed good to excellent intraobserver reproducibility in securin IHC, using whole breast cancer sections. Interobserver reproducibility, however, was somewhat lower and considered as fair to good. Very good or excellent reproducibility between observers was achieved for CAIX expression in rectal carcinoma using whole tissue sections, and for p120(ctn) expression in breast carcinoma applying TMAs.

5.3. WESTERN ANALYSIS

Western analysis of samples from colon cancer patients revealed a single faint band with the CAIX antibody in all four studied samples of normal colonic mucosa. While a similar single band was found on two of the corresponding tumour specimens of the same patients, two others showed an additional larger band. In three cases, the visual intensities of the detected bands from tumour samples were stronger than those found in the corresponding normal mucosa, which was interpreted as an upregulation of CAIX protein expression in colon cancer. In one case, the intensity was approximately similar in both normal and cancer samples. The upregulation of CAIX according to the western analysis of each patient paralleled the results obtained from the cDNA microarray analysis and immunohistochemistry of the same patients.

5.4. CORRELATION WITH PATIENT OUTCOME

In the following sections, some of the most significant prognostic associations found in studies I – V for securin, p120(ctn), and CAIX are summarized.

In the present material (II, IV), securin immunohistochemistry identified patients with favourable and unfavourable outcome. In the Kaplan-Meier analysis, the classified fraction of securin positivity was correlated with breast cancer survival (IV) among all patients ($p = 0.004$), among patients with a specific histological type of breast carcinoma ($p = 0.010$), and among small and large tumour size ($p = 0.033$ and 0.034 for tumour diameter $\leq$3cm and >3cm, respectively). Also, in the univariate Cox's regression analysis, securin predicted survival with a statistical significance ($p = 0.006$ and 0.014 for all cases and cases representing a specific histological type, respectively). Among all breast cancer cases, securin immunopositivity was associated with a 2.9- or 2.3-fold risk of breast cancer death in univariate or multivariate analysis, respectively. In the multivariate Cox's regression model, the established prognosticators of breast cancer were included. In the multivariate analyses, the highest prognostic value for securin immunopositivity was observed as a 13.1-fold risk of breast cancer death among a specific histological

type of breast carcinoma, showing low proliferative activity. In colon cancer, similar association of securin immunopositivity with patient outcome could not been found (I).

p120(ctn) predicted up to a 3.7-fold risk for breast cancer death when analysed as p120C immunoreactivity (V). p120C predicted breast cancer death, both in the univariate (p = 0.023) and in the multivariate analyses, when adjusted for the established prognosticators of breast carcinoma (p = 0.039). The statistically significant prognostic correlations applied for the univariate analysis of all patients and a subgroup of specific breast cancer type. In addition, p120N predicted survival at a statistically significant level among subgroups of specific histological and hormonal breast cancer types, with up to a 3.6-fold risk of breast cancer death in the univariate analyses (V). In the multivariate analyses involving the established prognosticators of breast carcinoma, p120N did not predict breast cancer survival.

In rectal cancer, the staining intensity of CAIX correlated with patient outcome (III). In the univariate analysis, patients with negative or weak staining intensity had a significantly longer disease-specific survival (p = 0.009) than patients with moderate or strong staining intensity. In Cox's multivariate analysis, involving the established prognosticators of rectal carcinoma, CAIX intensity was the strongest statistically significant predictor of disease-specific death (hazard ratio 9.23) of rectal carcinoma.

6. DISCUSSION

The current study presents a survey on the applicability of fairly modern high throughput methods – cDNA and tissue microarrays – to identify novel biomarkers. Breast and colorectal cancers were chosen as examples in the experimental works. In the cDNA microarray analyses presented here, several deregulated genes were identified and linked to their biological functions known at the time of analysis. Among these functions, the mitotic cell cycle and cell-cell adhesion, the phenomena which are known to be crucial for breast cancer progression (Tavassoli and Devilee (Eds.), 2003, p 57, Wheelock et al., 2001, Gould Rothberg and Bracken, 2006, Brennan et al., 2010), were considered as potential for the identification of meaningful novel biomarkers. Recently published evidence has further fortified the significance of adhesion, especially in the early stages of breast tumorigenesis (Emery et al., 2009). Among these genes, securin (I, II, IV) and p120(ctn) (V) were selected for further immunohistochemical studies. In addition, carbonic anhydrase IX was chosen for futher analysis in colorectal cancer as the most upregulated gene in the colon cancer material used (I, III). At the time when this study was initiated, the outcome of colorectal carcinoma had not been significantly associated with any particular cellular functions.

6.1. ABERRANT EXPRESSION OF THE SELECTED GENES AND PROTEINS

According to the presented analyses, securin was found upregulated at the mRNA and protein level in both breast and colon cancers. Prior to this study, overexpression of mRNA and/or protein had been demonstrated in a few reports (Ogbagabriel et al., 2005, Solbach et al., 2004, Kakar et al., 2001, Puri et al., 2001, Heaney et al., 2000). Furthermore, aberrant expression of securin protein was associated with disease outcome in two different breast cancer materials, using both whole tissue and TMA sections. However, in colon cancer material using TMAs, the same association could not be demonstrated. In previous literature concerning breast or colon cancer, no association between securin and disease outcome had been reported.

In the current study, carbonic anhydrase 9 mRNA was shown as significantly upregulated in colon cancer, and the same was demonstrated also at the protein level in colon and rectal cancer. This was already known from previous literature (Ivanov et al., 2001, Kivela et al., 2001, Kivela et al., 2005). According to the results presented here, expression of CAIX could also be associated with rectal cancer outcome, a finding, which to my knowledge, has not been reported in the literature.

The adhesion-related molecule, p120(ctn), was identified as underexpressed at the mRNA level in breast and colon cancer. The lists of deregulated genes obtained from cDNA microarray analyses were arranged according to the internet database of biological functions at different times for breast and colon cancers. According to cumulating research, the database is updated from time to time, and therefore, p120(ctn) was also associated with the mitotic cell cycle in colon, but not in breast cancer. Immunohistochemical stainings of breast cancer specimens showed the same trend of diminished expression also at the protein level, and the altered expression was associated with disease outcome in the present study. Loss of p120(ctn) expression in some colorectal tumours had been demonstrated previously using IHC (Bellovin et al., 2005, Karayiannakis et al., 1999, Skoudy et al., 1996, Gold et al., 1998). In addition, at least partial loss of p120 catenin expression had also been shown by earlier breast cancer studies (Dillon et al., 1998, Nakopoulou et al., 2002, Sarrió et al., 2004). However, no clear association of p120(ctn) protein with breast cancer outcome had been reported prior to this study, although there was a published report available, suggesting on the basis of univariate analysis, that altered localization of p120(ctn) predicts unfavourable outcome in the subgroup of P- and E-cadherin positive breast cancers (Paredes et al., 2008). The current study was also the first one reporting the use of the isoform specific antibody (p120N) in breast cancer IHC, and describing the nuclear distribution of p120N-immunopositivity in this disease. In previous breast cancer literature, this antibody had only been used in western analyses (Sarrió et al., 2004), or for the staining of a few patient cases (Dillon et al., 1998). Nuclear p120 catenin expression had been shown in the minority of a specific histological type of breast cancer cases, but using the isoform unspecific antibody (Sarrió et al., 2004). Therefore, no direct comparisons on nuclear p120 catenin expression can be made to the present results applying different antibodies and histological types of breast cancer.

6.2. EXPERIENCES ON cDNA MICROARRAY

Many things have changed since the present research was started. The number of probes that can easily be deposited on cDNA microarray have increased and the methodology has advanced tremendously. Today, even the whole genome can be included on a single DNA microarray (Barry et al., 2010, Ki et al., 2007, Ocak et al., 2009). At the same time, prices have dropped. The prices were still rather high when the first plans to use DNA microarrays had arisen in the present research group. This was especially true for oligonucleotide-based commercial chips, which were the only alternative for the non-commercial cDNA microarrays of BTC in those days in Turku. For the present study, the application of cDNA microarrays was the only realistic choice. In small research units like ours, the material resources are limited. That is why we could use cDNA microarrays only for screening purposes in small patient materials. The previous use of

cDNA microarray screening for new biomarkers supported our choice (Zembutsu et al., 2002).

The microarray used in the analyses presented here included a selection of genes, which were at the time considered significant for cancer development and progression, according to the expertise of Turku Centre for Biotechnology. At the beginning of this study, there had not been even attempts to standardize this line of research. It required step-by-step efforts to develop accurate and workable procedures, for example in tissue sampling, RNA labelling, bioinformatics, and data mining. For example, biological functions for the identified deregulated genes had to be manually searched from databases after working out different alternatives for the naming of these genes. This kind of information is nowadays more trivial and often included in the annotation files of the microarrays (http://www.switchtoi.com/annotationfiles.ilmn, http://www.phalanxbiotech.com/tech_support/faqs.html 5.8.2010). Carrying out this study required close co-operation with experts in different research areas, which led to some difficulties in fully comprehending each other. However, the development of procedures through the study can be seen as an advantage, since the researcher can then have better insight into the technicalities of the methodology, obtained data, and ultimately to the results.

The applicability of the microarrays used in the current study can be supported by the observed comparable immunohistochemical expression and verified prognostic value in cancer. When evaluating the quality of the gene expression analyses in breast and colon cancer, most of the housekeeping genes were listed as non-deregulated, although some also showed expression changes. This is in line with the present knowledge of housekeeping genes, which are traditionally regarded as stably expressed (Khimani et al., 2005, Chambers, 2002). This study also confirmed what is known about the fidelity of the clones used in the construction of cDNA microarrays (Halgren et al., 2001, Taylor et al., 2001). In the current study, the gene identity declared in the annotation of the microarray was verified by DNA sequencing for most of the probes. However, there were also a few mixed clones containing more than one probe, and some probes whose gene identity changed in the sequencing.

At the moment, a variety of commercial industrial alternatives for DNA microarray methods and software and service packages are available at a reasonable price. The present-day simplicity and fairly low costs makes possible the use of modern microarrays and the surveying of enormous patient materials for the whole research community. Today, the oligonucleotide-based chips are often the method of choice, but next generation sequencing methods are most probably coming into general use. (http://www.biocompare.com/Documents/surveys_files/ExecSumm/DNAMicro_2006_execsumm.pdf, http://www.biocompare.com/Articles/FeaturedArticle/1111/Microarray-Instrumentation-And-Software.html 5.8.2010) It has been suggested that NGS may

supersede hybridization-based techniques within a few years (Marguerat and Bähler, 2010). It is also possible that microarrays will remain in combination with modern sequencing techniques used, for example, to enrich protein-coding regions with microarray prior to sequence analysis (Hurd and Nelson, 2009). Nevertheless, it is inevitable that novel diagnostic, prognostic, predictive and pharmacogenomic markers will be discovered with increasing rate in the next few years by applying NGS. Also the price to study human cancer will still lower through the application of this method. (Aparicio and Huntsman, 2010, Marguerat and Bähler, 2010) Especially the identification of relevant mutations and biological pathways in early-stage cancer, and progress in screening for mutations in general, have been anticipated. As well, advances in the understanding of intra- and intertumoural heterogeneity have been envisioned. (Aparicio and Huntsman, 2010) In breast cancer, NGS has been used, for example, to identify unique serum biomarkers associated with the disease. These markers have been suggested to provide a basis for the development of a minimally invasive routine laboratory test for breast cancer screening and monitoring (Beck et al., 2010). However, there are some obstacles that have to be overcome in order to translate novel discoveries into better diagnostics, treatment, and prevention of cancer (Mardis and Wilson, 2009). For example, better bioinformatics tools have to be developed to manage, store, and analyse huge amounts of data produced by NGS. Also, more technical and clinical validation studies in large patient materials have been urged as well as advances in quality control and ethics. (Hurd and Nelson, 2009, Aparicio and Huntsman, 2010) In conclusion, eventually nucleotide sequence-based technologies are expected to become part of routine histopathological practice, but this probably will still take many years (Aparicio and Huntsman, 2010).

Currently, there are no population-based, accurate, and cost-efficient screening methods for cancer. The available methods are expensive, labour-intensive, and their sensitivity and specificity may in some cases be compromised. It has been speculated that new approaches in cancer screening may involve microarray or other multiplex technologies in the identification of specific biomarkers for diagnosis, prognosis, and therapeutic targets leading to the personalization of medicine. However, different studies report on highly variable gene signatures. In colorectal carcinoma, expression profiles have been linked to some specific features of the course and prognosis of the disease (Hao et al., 2010). Concerning breast carcinoma, some researchers suggest that in the future, routine patient care may be guided by the molecular signatures of individual patients (Slodkowska and Ross, 2009).

6.3. IMMUNOHISTOCHEMISTRY AND WESTERN ANALYSIS

Immunohistochemical staining is an established method, and may even be considered as a ”gold standard” in the validation of biomarkers for clinical use (Hewitt 2006, Takikita

et al., 2007). It can be considered a routine method in laboratories of clinical pathology – being nowadays reliable, fairly uncomplicated, as well as automated and cheap. IHC has been used to validate results obtained from cDNA microarrays, and it has been shown to be a method of choice when screening and developing possible future biomarkers for cancer patients (Hao et al., 2004, Prall et al., 2004). In spite of the controversialities associated with cDNA microarray technology at the start of the present research, all selected gene expression changes could be verified by IHC, improving the value of this study. Additionally, many previously unpublished associations with patient outcome could be demonstrated, using independent series of patients. Especially in breast cancer reports IV and V, the special advantages also include the large patient material originating from the era of mammographic screening and a long follow-up (mean app. 10.5 years). In the present research, IHC was applied both in whole sections and in different types of tissue microarrays, thus giving some experience from both.

Among the antibodies used in this study, securin, p120C, and p120N, were monoclonal, and CAIX was polyclonal. Published literature and information provided by the manufacturers supported the specificity of the monoclonal antibodies. The specificity of the polyclonal antibody (ab15086, Abcam) was checked by western blotting, since at the time of the immunohistochemical analyses, the manufacturer provided no information on it. Monoclonal antibody M75 has been used in most of the published reports on CAIX, and it has been considered a gold standard for CAIX expression (Pastorek et al., 1994, Li et al., 2009a), but it was not commercially available for this study. The results of the western analysis using ab15086, showed double bands of approximately the same size as observed previously with the M75 antibody in the corresponding analyses (Pastorek et al., 1994, Liao et al., 1997, Proescholdt et al., 2005, Brennan et al., 2006). Similarly, the results from IHC were compared to the previous literature (Ivanov et al., 2001). Presently, there are published results comparing M75 to another commercially available antibody, NB100-417 (Novus Biologicals). In the immunohistochemical analyses, M75 and NB100 have shown comparable results (Al-Ahmadie et al., 2008), but other analyses have suggested a cross-reactivity with cytoplasmic beta-tubulin (Li et al., 2009a). M75 and NB100 are raised against different epitopes, but the epitopes of NB100 and ab15806 are at least partially overlapping (http://www.abcam.com/Carbonic-Anhydrase-IX-antibody-ab15086.html; Li et al., 2009a). Therefore, it can't be ruled out that also ab15086, used in the present study, could detect beta-tubulin. In prognostic analyses, however, only membrane-bound staining was taken into account, although cytoplasmic staining was also sometimes seen. So, the potential cross-reactivity to cytoplasmic protein should not diminish the value of the results presented in the current study.

In the literature, the non-automated evaluation of IHC has been accused of a potential bias, due to subjective interpretation (Camp et al., 2002, Giltnane and Rimm 2004). In the present study, a good or close to excellent reproducibility between observers was

reached in the majority of the stainings, using both TMAs and whole tissue sections. The concordance between the interpretations of different observers was lower in the evaluation of securin immunopositivity, using whole breast carcinoma sections. However, intraobserver reproducibility was good in the same staining. In these analyses, the most intensive areas of staining, the so-called hot spots, were selected. The subjective choice of the hot spot may have had an influence on the lower concordance.

6.4. EXPERIENCES ON TMAs

In the present research, different types of TMAs were prepared and applied. In the case of breast cancer, two tissue cores at 0.6 mm in diameter were punched from the centre and periphery of the tumour area in order to acknowledge the known heterogeneity of breast cancer tissue (Rosen (Ed.), 2001, p. 326). TMAs of colon cancer included one larger core from the centre of the tumours, in order to minimize the possibility of cores devoid of cancer cells. These TMAs included also a paired normal control tissue as separate cores, mimicking the situation in the cDNA microarray analysis, where a paired control of each patient was also included. Missing invasive cancer tissue in the core was the major reason for non-evaluable results in breast cancer TMAs. In colon cancer, however, none of the cores had to be dismissed due to unrepresentativeness. Respectively, detaching of the tissue core from the slide was the only reason for loosing evaluable results in colon cancer TMAs. In breast cancer, detaching was never seen in securin and p120C stainings, and it was the reason for loosing evaluable results in only 9% of the lost cases in p120N stainings. In the studies applying TMAs (I, IV, V), different types of slides and microwave ovens were used, possibly affecting the higher number of detached cores in colon cancer compared to breast cancer.

Prior to the current study, TMAs had been used in the analysis of securin, p120(ctn), and CAIX expression, using a variety of malignant tumours (Winnepenninckx et al., 2006, Sarrió et al., 2004, Crabb et al., 2008). In the present study, however, whole tissue sections were chosen for the immunostaining of CAIX. This was because, firstly, previous literature has pointed out that hypoxia-related markers may show heterogeneous and clinically relevant focal perinecrotic staining, which could be missed in small TMA cores (van Diest et al., 2005, Leibovich et al., 2007). Secondly, in light of previous literature (Iakovlev et al., 2007, Goethals et al., 2006), at least four cores from different tumour regions are required for the adequate representation of CAIX. Thirdly, the experiences of previous and current studies on the heterogeneous expression of proliferation markers favoured the use of whole sections in place of TMAs (Beliën et al., 1999, Salminen et al., 2005). Concerning the present study, no statistically significant association with patient outcome could be found applying TMAs with only one core punched from the centre of colon tumours (I). Concerning breast cancer, staining of the whole sections

showed heterogeneous staining of securin and, therefore, the areas of most intensive staining at the infiltrating border of tumours were used for evaluations (II). In study IV of securin expression in breast cancer, these previous experiences were acknowledged, and TMAs with two tissue cores, one punched from the edge and the other from the centre of tumour, were constructed. However, no significant difference between the two cores could be found in statistical analyses concerning the securin expression.

In the literature, also p120(ctn) has been reported with heterogeneous expression (Nakopoulou et al., 2002, Bremnes et al., 2002). In the current study, the heterogeneous pattern of p120(ctn) immunoexpression was evaluated in TMAs consisting of two tissue punches from different locations of the tumour. Statistically significant difference between the different tumour locations was not found, using the p120C antibody raised against all p120(ctn) isoforms. There was, however, a statistically significant difference in the nuclear expression of p120(ctn) long isoform (p120N) at the edge and the centre of the tumour. More significant association with patient outcome was related to the expression of p120N at the tumour edge compared to the centre. The association between breast cancer outcome and nuclear p120(ctn) staining at the edge of the tumour are parallel with previous literature on beta-catenin, which also has been demonstrated both in adherens junctions and in the nucleus (Brabletz et al., 2005). In colorectal cancer, especially the nuclear beta-catenin expression at the invasive front has been associated with the metastasizing capacity of cancer (Suzuki et al., 2008). In addition to adhesion related proteins, the significance of the immunostaining at the invasive margin concerns also other tumour markers. Indeed, a recent study has shown that the immunostaining of hypoxia markers in colorectal cancer TMA could be especially promising, if the used TMA reflects the expression status at the invasive margin (Rajaganeshan et al., 2009).

In general, the prognostic correlations obtained from the TMA methodology of the current study appeared to show higher significance in breast than in colon cancer. This may reflect the differences between the TMAs used – that is, the number of cores included and the location where they were punched. It can also be linked with the method used in the evaluation of immunohistochemical reactions, but the sole reason can also be in the different cell biological properties of these cancers concerning the significance of the studied biomarkers. Based on the experiences from the present research, several aspects should be taken into account in the application of TMA-methodology. Careful consideration should be put on the selection of the antibodies used on TMA, with a special emphasis when antibodies show an uneven expression pattern. This concerns, for example, proliferation markers, which according to the literature have their highest expression at the edge of the tumour (Beliën et al., 1999, Jenner et al., 1996). It would be advisable that these types of markers are studied on specially planned and constructed TMAs, with cores taken also from the invasive front of the tumour. In an optimal setting, a pilot study using a small material of the whole sections should be performed for

unestablished stainings in order to achieve information of the expression pattern of the particular antibody.

The combination of TMA and IHC has been established in the validation of novel biomarkers (Hao et al., 2004). However, in the discovery of novel disease markers mass spectrometry (MS) has been referred to as one of the most important high throughput proteomic tools (Sikaroodi et al., 2010). Furthermore, it has been suggested that this method may bypass the bottleneck created by the need for sensitive and reliable antibodies required in biomarker validation using IHC (Simpson et al., 2009). MS has produced, for example, candidate markers and signatures for breast cancer diagnosis, as well as correlations to clinicopathological factors and patient outcome. However, large-scale clinical validation of these markers is still required. (Gast et al., 2009, Nakagawa et al., 2006, Goncalves et al., 2006) Either body fluids, such as serum, or tissue samples can be used to identify and quantitate both proteins and their post-translational modifications by MS (Sikaroodi et al., 2010, Simpson et al., 2009, Fang et al., 2009). Complications caused by the heterogeneity of tumour tissue have been reduced by the advances in sample preparation techniques, such as tumour microdissection. Thus far, tissue handling methods applied in routine clinical use still hamper the application of MS for the archival patient materials, but techniques are evolving also in this field. (Fang et al., 2009, Gast et al., 2009)

7. CONCLUSIONS

The focus of the present study is to test the applicability of cDNA and tissue microarray methodologies for the screening of potential novel tumour markers. Common human malignancies, breast and colorectal carcinomas, were selected to be used as examples. On the basis of the present study, the following conclusions can be made:

1. In order to identify potential biomarkers in cancer, cDNA microarray methodology can be applied in the screening for aberrant gene expression, and tissue microarrays for validating the obtained results.

2. For reliable and relevant results, the principles of the methods should be thoroughly understood, their technical details carefully planned, and their performance tested in sufficient pilot experiments prior to application in full scale.

3. On the basis of experience from the present study, in applying cDNA microarrays, special attention should be placed on the representativeness of the tissue samples, the homogeneity of tissue material, quality issues of specimen processing, and the criteria of aberrant gene expression. Therefore, validation of the results obtained from cDNA microarrays is essential.

4. Applicability of TMA is primarily dependent on the studied expression pattern in the lesion and structure of the TMA. The number and size of the tissue cores influence the results, as a balance between the number of cases obtained and the fraction of material lost during tissue processing.

5. The application of cDNA microarrays and TMAs may increase the understanding of the prognostic value of novel biomarkers in cancer. The present study, applying cDNA microarrays and immunohistochemistry on TMAs or on whole tissue sections, revealed that securin and p120(ctn) predict breast cancer outcome, and CAIX the outcome in rectal carcinoma.

8. ACKNOWLEDGEMENTS

The studies for this thesis were carried out during the years 2002-2009 at the Department of Pathology, in the University of Turku. I am grateful to Professors Olli Carpén, Timo Nevalainen and Yrjö Collan for the opportunity to work at the department and for creating an inspirational atmosphere towards scientific work during the course of the studies. Professor Timo Nevalainen is additionally acknowledged for being a initiator of the application of microarrays at the Department of Pathology. I wish to express my warmest thanks to all those who have assisted and supported me during these years.

I owe my deepest gratitude to my supervisors Docent Pauliina Kronqvist, and M.D., Ph.D. Jari Sundström, for introducing me to the world of cancer research. I wish to also thank them for their endless support and great sense of humour, which became invaluable during the ups and downs of this study.

I am grateful to the reviewers, Professor Veli-Matti Kosma, from University of Eastern Finland, and Ph.D. Annika Brandt, from Turku University of Applied Sciences, for the critical comments and valuable corrections they have made on the manuscript of this thesis.

I wish to express my gratitude to my co-authors from the Departments of Pathology, Docent Pirkko Hirsimäki and M.D. Henna Karra, at the University of Turku, and Docent Teijo Kuopio, M.D., Jorma Anttinen, M.Sc., Marjukka Friman (maiden name Nykänen), and M.Sc. Anssi Nieminen, in Jyväskylä Central Hospital. I also thank Professors Seppo Pyrhönen, Heikki Minn, and Kari Syrjänen, Docent Panu Jaakkola, and M.D. Eija Korkeila, from the Department of Oncology and Radiotherapy, at the University of Turku, for their collaboration with studies concerning CAIX. My sincere thanks belong to Professor Olli Nevalainen, Docent Tero Aittokallio, and M.Sc. Johannes Tuikkala, from the Department of Information Technology, at the University of Turku; M.Sc. Saija Hurme and M.Sc. Heikki Hiekkanen, from the Department of Biostatistics, at the University of Turku, as well as, Docents Juha O. Grönroos and Arto Rantala, and M.D., Ph.D. Heikki Huhtinen, from the Department of Surgery, at the University of Turku. Henno Parks is thanked for editing the language of this thesis and Daniel Laajala for creating heat maps.

My warmest thanks belong to the personnel and my colleagues who have worked at the Department of Pathology during the last thirteen years. Especially, Docent Heikki Peuravuori, is thanked for his guidance in the academic world, and for keeping my computer running; Jaakko Liippo, for his professional skills in photography; Hannele Nysten, Maija Ahlholm and Sirpa Fjäder, for helping with all the paper work; and Erja Ettanen and Anja Viitanen, for keeping the lab equipment and working environment

clean and tidy; Ph.L. Zhou Qi and the researches of ezrin/palladin-group (Professor Olli Carpén's group) are thanked for their scientific discussions. My sincere thanks belong to Sinikka Kollanus, for all the hints she gave me in immunohistochemistry, but also for the daily coffee breaks. They have been absolutely necessary. Last, but definitely not least, Pekka Ojala, is greatly acknowledged for the laboratory tutoring he gave me at the beginning of my career. He was of help when I needed it most.

I wish to express my gratitude to the Turku Center for Biotechnology and the Finnish Microarray and Sequencing Centre. This thesis would not have been completed without the expertise of their personnel and their willingness to help. I want to especially thank Eveliina Virtanen, Päivi Junni, Marjo Linja, Arja Reinikainen, and Katja Kimppa, for their valuable help.

My parents, Marja and Ilpo, have supported me in all my endeavours - not only in my education and work, but also in life in general. Thank you for always being there. I also want to thank Esa for the joy of having a big brother, and Esa's family, Salme, Tuulikki and Arto, for all the nice and relaxing summers in Askainen.

Finally, I want to thank the person who has supported me probably the most, my husband, Tomi. You must have had an enormous amount of patience every day. And my beloved son, Visa, my true sunshine. Thank you for your constant observations on our life – so sharp and realistic. Despite every step forward in my scientific career, it is you two who make my life worth living.

This work was supported by Turku University Central Hospital (EVO grant), Turku University Foundation, the Cancer Society of South-Western Finland, the Finnish Society of Histotechnology, the Lilly Foundation and the Finnish Oncology Society.

Turku, September 2010

Kati Talvinen

9. REFERENCES

Abdullah-Sayani A, Bueno-de-Mesquita JM, van de Vijver MJ. Technology Insight: tuning into the genetic orchestra using microarrays--limitations of DNA microarrays in clinical practice. Nat Clin Pract Oncol 2006;3:501-516.

Aguilar-Mahecha A, Hassan S, Ferrario C, Basik M. Microarrays as validation strategies in clinical samples: tissue and protein microarrays. OMICS 2006;10:311-326.

Aho S, Levänsuo L, Montonen O, Kari C, Rodeck U, Uitto J. Specific sequences in p120ctn determine subcellular distribution of its multiple isoforms involved in cellular adhesion of normal and malignant epithelial cells. J Cell Sci 2002;115:1391-1402.

Aho S, Rothenberger K, Uitto J. Human p120ctn catenin: tissue-specific expression of isoforms and molecular interactions with BP180/type XVII collagen. J Cell Biochem 1999;73:390-399.

Al-Ahmadie HA, Alden D, Qin LX, Olgac S, Fine SW, Gopalan A, Russo P, Motzer RJ, Reuter VE, Tickoo SK. Carbonic anhydrase IX expression in clear cell renal cell carcinoma: an immunohistochemical study comparing 2 antibodies. Am J Surg Pathol 2008;32:377-382.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol 1990;215:403-410.

Anastasiadis PZ. p120-ctn: A nexus for contextual signaling via Rho GTPases. Biochim Biophys Acta 2007;1773:34-46.

Anastasiadis PZ, Reynolds AB. The p120 catenin family: complex roles in adhesion, signaling and cancer. J Cell Sci 2000;113:1319-1334.

Andersen CL, Monni O, Wagner U, Kononen J, Bärlund M, Bucher C, Haas P, Nocito A, Bissig H, Sauter G, Kallioniemi A. High-throughput copy number analysis of 17q23 in 3520 tissue specimens by fluorescence in situ hybridization to tissue microarrays. Am J Pathol 2002;161:73-79.

Andras SC, Power JB, Cocking EC, Davey MR. Strategies for signal amplification in nucleic acid detection. Mol Biotechnol 2001;19:29-44.

Antoniou AC, Pharoah PD, McMullan G, Day NE, Ponder BA, Easton D. Evidence for further breast cancer susceptibility genes in addition to BRCA1 and BRCA2 in a population-based study. Genet Epidemiol 2001;21:1-18.

Aparicio SA, Huntsman DG. Does massively parallel DNA resequencing signify the end of histopathology as we know it? J Pathol 2010;220:307-315.

Bammler T, Beyer RP, Bhattacharya S, Boorman GA, Boyles A, Bradford BU, Bumgarner RE, Bushel PR, Chaturvedi K, Choi D, Cunningham ML, Deng S, Dressman HK, Fannin RD, Farin FM, Freedman JH, Fry RC, Harper A, Humble MC, Hurban P, Kavanagh TJ, Kaufmann WK, Kerr KF, Jing L, Lapidus JA, Lasarev MR, Li J, Li YJ, Lobenhofer EK, Lu X, Malek RL, Milton S, Nagalla SR, O'malley JP, Palmer VS, Pattee P, Paules RS, Perou CM, Phillips K, Qin LX, Qiu Y, Quigley SD, Rodland M, Rusyn I, Samson LD, Schwartz DA, Shi Y, Shin JL, Sieber SO, Slifer S, Speer MC, Spencer PS, Sproles DI, Swenberg JA, Suk WA, Sullivan RC, Tian R, Tennant RW, Todd SA, Tucker CJ, Van Houten B, Weis BK, Xuan S, Zarbl H; Members of the Toxicogenomics Research Consortium. Standardizing global gene expression analysis between laboratories and across platforms. Nat Methods 2005;2:351-356.

Barathova M, Takacova M, Holotnakova T, Gibadulinova A, Ohradanova A, Zatovicova M, Hulikova A, Kopacek J, Parkkila S, Supuran CT, Pastorekova S, Pastorek J. Alternative splicing variant of the hypoxia marker carbonic anhydrase IX expressed independently of hypoxia and tumour phenotype. Br J Cancer 2008;98:129-136.

Barrett JC, Kawasaki ES. Microarrays: the use of oligonucleotides and cDNA for the analysis of gene expression. Drug Discov Today 2003;8:134-141.

Barry WT, Kernagis DN, Dressman HK, Griffis RJ, Hunter JD, Olson JA, Marks JR, Ginsburg GS, Marcom PK, Nevins JR, Geradts J, Datto MB. Intratumor heterogeneity and precision of microarray-based predictors of breast cancer biology and clinical outcome. J Clin Oncol 2010;28:2198-2206.

Bartosová M, Parkkila S, Pohlodek K, Karttunen TJ, Galbavý S, Mucha V, Harris AL, Pastorek J, Pastoreková S. Expression of carbonic anhydrase IX in breast is associated with malignant tissues and is related to overexpression of c-erbB2. J Pathol 2002;197:314-321.

Battifora H, Mehta P. The checkerboard tissue block. An improved multitissue control block. Lab Invest 1990;63:722-724.

Battifora H. The multitumor (sausage) tissue block: novel method for immunohistochemical antibody testing. Lab Invest 1986;55:244-248.

Beck J, Urnovitz HB, Mitchell WM, Schütz E. Next generation sequencing of serum circulating nucleic acids from patients with invasive ductal breast cancer reveals differences to healthy and nonmalignant controls. Mol Cancer Res 2010;8:335-342.

Beliën JA, van Diest PJ, Baak JP. Relationships between vascularization and proliferation in invasive breast cancer. J Pathol 1999;189:309-318.

Bellovin DI, Bates RC, Muzikansky A, Rimm DL, Mercurio AM. Altered localization of p120 catenin during epithelial to mesenchymal transition of colon carcinoma is prognostic for aggressive disease. Cancer Res 2005;65:10938-10945.

Bernal JA, Luna R, Espina A, Lázaro I, Ramos-Morales F, Romero F, Arias C, Silva A, Tortolero M, Pintor-Toro JA. Human securin interacts with p53 and modulates p53-mediated transcriptional activity and apoptosis. Nat Genet 2002;32:306-311.

Bertucci F, Bernard K, Loriod B, Chang YC, Granjeaud S, Birnbaum D, Nguyen C, Peck K, Jordan BR. Sensitivity issues in DNA array-based expression measurements and performance of nylon microarrays for small samples. Hum Mol Genet 1999;8:1715-1722.

Bertucci F, Houlgatte R, Benziane A, Granjeaud S, Adélaïde J, Tagett R, Loriod B, Jacquemier J, Viens P, Jordan B, Birnbaum D, Nguyen C. Gene expression profiling of primary breast carcinomas using arrays of candidate genes. Hum Mol Genet 2000;9:2981-2991.

Bertucci F, Nasser V, Granjeaud S, Eisinger F, Adelaïde J, Tagett R, Loriod B, Giaconia A, Benziane A, Devilard E, Jacquemier J, Viens P, Nguyen C, Birnbaum D, Houlgatte R. Gene expression profiles of poor-prognosis primary breast cancer correlate with survival. Hum Mol Genet 2002;11:863-872.

Bertucci F, Orsetti B, Nègre V, Finetti P, Rougé C, Ahomadegbe JC, Bibeau F, Mathieu MC, Treilleux I, Jacquemier J, Ursule L, Martinec A, Wang Q, Bénard J, Puisieux A, Birnbaum D, Theillet C. Lobular and ductal carcinomas of the breast have distinct genomic and expression profiles. Oncogene 2008;27:5359-5372.

Bertucci F, Salas S, Eysteries S, Nasser V, Finetti P, Ginestier C, Charafe-Jauffret E, Loriod B, Bachelart L, Montfort J, Victorero G, Viret F, Ollendorff V, Fert V, Giovaninni M, Delpero JR, Nguyen C, Viens P, Monges G, Birnbaum D, Houlgatte R. Gene expression profiling of colon cancer by DNA microarrays and correlation with histoclinical parameters. Oncogene 2004;23:1377-1391.

Bier FF, von Nickisch-Rosenegk M, Ehrentreich-Förster E, Reiss E, Henkel J, Strehlow R, Andresen D. DNA microarrays. Adv Biochem Eng Biotechnol 2008;109:433-453.

Boone J, van Hillegersberg R, van Diest PJ, Offerhaus GJ, Rinkes IH, Kate FJ. Validation of tissue microarray technology in squamous cell carcinoma of the esophagus. Virchows Arch 2008;452:507-514.

Bowtell D, Sambrook J (Eds). DNA microarrays: a molecular cloning manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 2003.

Brabletz T, Hlubek F, Spaderna S, Schmalhofer O, Hiendlmeyer E, Jung A, Kirchner T. Invasion and metastasis in colorectal cancer: epithelial-mesenchymal transition, mesenchymal-epithelial transition, stem cells and beta-catenin. Cells Tissues Organs 2005;179:56-65.

Brazma A, Hingamp P, Quackenbush J, Sherlock G, Spellman P, Stoeckert C, Aach J, Ansorge W, Ball CA, Causton HC, Gaasterland T, Glenisson P, Holstege FC, Kim IF, Markowitz V, Matese JC, Parkinson H, Robinson A, Sarkans U, Schulze-Kremer S, Stewart J, Taylor R, Vilo J, Vingron M. Minimum information about a microarray experiment (MIAME)-toward standards for microarray data. Nat Genet 2001;29:365-371.

Brazma A, Robinson A, Cameron G, Ashburner M. One-stop shop for microarray data. Nature 2000;403:699-700.

Bremnes RM, Veve R, Gabrielson E, Hirsch FR, Baron A, Bemis L, Gemmill RM, Drabkin HA, Franklin WA. High-throughput tissue microarray analysis used to evaluate biology and prognostic significance of the E-cadherin pathway in non-small-cell lung cancer. J Clin Oncol 2002;20:2417-2428.

Brennan DJ, Jirstrom K, Kronblad A, Millikan RC, Landberg G, Duffy MJ, Rydén L, Gallagher WM, O'Brien SL. CA IX is an independent prognostic marker in premenopausal breast cancer patients with one to three positive lymph nodes and a putative marker of radiation resistance. Clin Cancer Res 2006;12:6421-6431.

Brennan K, Offiah G, McSherry EA, Hopkins AM. Tight junctions: a barrier to the initiation and

progression of breast cancer? J Biomed Biotechnol 2010;2010:460607.

Brennan P, Silman A. Statistical methods for assessing observer variability in clinical measures. BMJ 1992;304:1491-1494.

Brooks SA, Leathem AJ. Expression of the CD15 antigen (Lewis x) in breast cancer. Histochem J 1995;27:689-693.

Bubendorf L, Kolmer M, Kononen J, Koivisto P, Mousses S, Chen Y, Mahlamäki E, Schraml P, Moch H, Willi N, Elkahloun AG, Pretlow TG, Gasser TC, Mihatsch MJ, Sauter G, Kallioniemi OP. Hormone therapy failure in human prostate cancer: analysis by complementary DNA and tissue microarrays. J Natl Cancer Inst 1999;91:1758-1764.

Bucca G, Carruba G, Saetta A, Muti P, Castagnetta L, Smith CP. Gene expression profiling of human cancers. Ann N Y Acad Sci 2004;1028:28-37.

Bui MH, Seligson D, Han KR, Pantuck AJ, Dorey FJ, Huang Y, Horvath S, Leibovich BC, Chopra S, Liao SY, Stanbridge E, Lerman MI, Palotie A, Figlin RA, Belldegrun AS. Carbonic anhydrase IX is an independent predictor of survival in advanced renal clear cell carcinoma: implications for prognosis and therapy. Clin Cancer Res 2003;9:802-811.

Bui MH, Visapaa H, Seligson D, Kim H, Han KR, Huang Y, Horvath S, Stanbridge EJ, Palotie A, Figlin RA, Belldegrun AS. Prognostic value of carbonic anhydrase IX and KI67 as predictors of survival for renal clear cell carcinoma. J Urol 2004;171:2461-2466.

Callagy G, Cattaneo E, Daigo Y, Happerfield L, Bobrow LG, Pharoah PD, Caldas C. Molecular classification of breast carcinomas using tissue microarrays. Diagn Mol Pathol 2003;12:27-34.

Camp RL, Charette LA, Rimm DL. Validation of tissue microarray technology in breast carcinoma. Lab Invest 2000;80:1943-1949.

Camp RL, Chung GG, Rimm DL. Automated subcellular localization and quantification of protein expression in tissue microarrays. Nat Med 2002;8:1323-1327.

Camp RL, Neumeister V, Rimm DL. A decade of tissue microarrays: progress in the discovery and validation of cancer biomarkers. J Clin Oncol 2008;26:5630-5637.

Cardillo MR, Yap E, Castagna G. Molecular genetic analysis of TGF beta1 in breast cancer. J Exp Clin Cancer Res 1997;16:57-63.

Chambers RC. Gene expression profiling: good housekeeping and a clean message. Thorax 2002;57:754-756.

Chartier NT, Oddou CI, Lainé MG, Ducarouge B, Marie CA, Block MR, Jacquier-Sarlin MR. Cyclin-dependent kinase 2/cyclin E complex is involved in p120 catenin (p120ctn)-dependent cell growth control: a new role for p120ctn in cancer. Cancer Res 2007;67:9781-9790.

Cheang MC, Treaba DO, Speers CH, Olivotto IA, Bajdik CD, Chia SK, Goldstein LC, Gelmon KA, Huntsman D, Gilks CB, Nielsen TO, Gown AM. Immunohistochemical detection using the new rabbit monoclonal antibody SP1 of estrogen receptor in breast cancer is superior to mouse monoclonal antibody 1D5 in predicting survival. J Clin Oncol 2006;24:5637-5644.

Chen CC, Chang TW, Chen FM, Hou MF, Hung SY, Chong IW, Lee SC, Zhou TH, Lin SR. Combination of multiple mRNA markers (PTTG1, Survivin, UbcH10 and TK1) in the diagnosis of Taiwanese patients with breast cancer by membrane array. Oncology 2006;70:438-446.

Chen JJ, Wu R, Yang PC, Huang JY, Sher YP, Han MH, Kao WC, Lee PJ, Chiu TF, Chang F, Chu YW, Wu CW, Peck K. Profiling expression patterns and isolating differentially expressed genes by cDNA microarray system with colorimetry detection. Genomics 1998;51:313-324.

Chen J, Röcken C, Hoffmann J, Krüger S, Lendeckel U, Rocco A, Pastorekova S, Malfertheiner P, Ebert MP. Expression of carbonic anhydrase 9 at the invasion front of gastric cancers. Gut 2005;54:920-927.

Chen L, Puri R, Lefkowitz EJ, Kakar SS. Identification of the human pituitary tumor transforming gene (hPTTG) family: molecular structure, expression, and chromosomal localization. Gene 2000;248:41-50.

Chia SK, Wykoff CC, Watson PH, Han C, Leek RD, Pastorek J, Gatter KC, Ratcliffe P, Harris AL. Prognostic significance of a novel hypoxia-regulated marker, carbonic anhydrase IX, in invasive breast carcinoma. J Clin Oncol 2001;19:3660-3668.

Chien W, Pei L. A novel binding factor facilitates nuclear translocation and transcriptional activation function of the pituitary tumor-transforming gene product. J Biol Chem 2000;275:19422-19427.

Chiriboga L, Osman I, Mikhail M, Lau C. Tissue microarrays, tread carefully. Lab Invest 2004;84:1677.

Chiriva-Internati M, Ferrari R, Prabhakar M, Yu Y, Baggoni L, Moreno J, Gagliano N, Portinaro N, Jenkins MR, Frezza EE, Hardwicke F, D'Cunha N, Kast W, Cobos E. The pituitary tumor transforming gene 1 (PTTG-1): an immunological target for multiple myeloma. J Transl Med 2008;6:15.

Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987;162:156-159.

Chowdary D, Lathrop J, Skelton J, Curtin K, Briggs T, Zhang Y, Yu J, Wang Y, Mazumder A. Prognostic gene expression signatures can be measured in tissues collected in RNAlater preservative. J Mol Diagn 2006;8:31-39.

Chuaqui RF, Bonner RF, Best CJ, Gillespie JW, Flaig MJ, Hewitt SM, Phillips JL, Krizman DB, Tangrea MA, Ahram M, Linehan WM, Knezevic V, Emmert-Buck MR. Post-analysis follow-up and validation of microarray experiments. Nat Genet 2002;32 Suppl:509-514.

Chung JY, Braunschweig T, Hewitt SM. Optimization of recovery of RNA from formalin-fixed, paraffin-embedded tissue. Diagn Mol Pathol 2006;15:229-236.

Churchill GA. Fundamentals of experimental design for cDNA microarrays. Nat Genet 2002;32 Suppl:490-495.

Ciosk R, Zachariae W, Michaelis C, Shevchenko A, Mann M, Nasmyth K. An ESP1/PDS1 complex regulates loss of sister chromatid cohesion at the metaphase to anaphase transition in yeast. Cell 1998;93:1067-1076.

Clem AL, Hamid T, Kakar SS. Characterization of the role of Sp1 and NF-Y in differential regulation of PTTG/securin expression in tumor cells. Gene 2003;322:113-121.

Cohen-Fix O, Peters JM, Kirschner MW, Koshland D. Anaphase initiation in Saccharomyces cerevisiae is controlled by the APC-dependent degradation of the anaphase inhibitor Pds1p. Genes Dev 1996;10:3081-3093.

Couvelard A, Deschamps L, Ravaud P, Baron G, Sauvanet A, Hentic O, Colnot N, Paradis V, Belghiti J, Bedossa P, Ruszniewski P. Heterogeneity of tumor prognostic markers: a reproducibility study applied to liver metastases of pancreatic endocrine tumors. Mod Pathol 2009;22:273-281.

Crabb SJ, Bajdik CD, Leung S, Speers CH, Kennecke H, Huntsman DG, Gelmon KA. Can clinically relevant prognostic subsets of breast cancer patients with four or more involved axillary lymph nodes be identified through immunohistochemical biomarkers? A tissue microarray feasibility study. Breast Cancer Res 2008;10:R6.

Cronin M, Pho M, Dutta D, Stephans JC, Shak S, Kiefer MC, Esteban JM, Baker JB. Measurement of gene expression in archival paraffin-embedded tissues: development and performance of a 92-gene reverse transcriptase-polymerase chain reaction assay. Am J Pathol 2004;164:35-42.

Dabbs DJ, Bhargava R, Chivukula M. Lobular versus ductal breast neoplasms: the diagnostic utility of p120 catenin. Am J Surg Pathol 2007a;31:427-437.

Dabbs DJ, Kaplai M, Chivukula M, Kanbour A, Kanbour-Shakir A, Carter GJ. The spectrum of morphomolecular abnormalities of the E-cadherin/catenin complex in pleomorphic lobular carcinoma of the breast. Appl Immunohistochem Mol Morphol 2007b;15:260-266.

Dai SD, Wang Y, Jiang GY, Zhang PX, Dong XJ, Wei Q, Xu HT, Li QC, Zhao C, Wang EH. Kaiso is expressed in lung cancer: its expression and localization is affected by p120ctn. Lung Cancer 2010;67:205-215.

Daniel JM. Dancing in and out of the nucleus: p120(ctn) and the transcription factor Kaiso. Biochim Biophys Acta 2007;1773:59-68.

Daniel JM, Reynolds AB. The catenin p120(ctn) interacts with Kaiso, a novel BTB/POZ domain zinc finger transcription factor. Mol Cell Biol 1999;19:3614-3623.

Datta MW, Kahler A, Macias V, Brodzeller T, Kajdacsy-Balla A. A simple inexpensive method for the production of tissue microarrays from needle biopsy specimens: examples with prostate cancer. Appl Immunohistochem Mol Morphol 2005;13:96-103.

Davis MA, Ireton RC, Reynolds AB. A core function for p120-catenin in cadherin turnover. J Cell Biol 2003;163:525-534.

Dennis J, Westra J, Bell A, Montgomery K, Oien K. The type and quality of paraffin wax is important when constructing tissue microarrays. Mol Pathol 2003;56:306.

DeRisi J, Penland L, Brown PO, Bittner ML, Meltzer PS, Ray M, Chen Y, Su YA, Trent JM. Use of a cDNA microarray to analyse gene expression patterns in human cancer. Nat Genet 1996;14:457-460.

Desai KV, Kavanaugh CJ, Calvo A, Green JE. Chipping away at breast cancer: insights from microarray studies of human and mouse mammary cancer. Endocr Relat Cancer 2002;9:207-220.

Deyholos MK, Galbraith DW. High-density microarrays for gene expression analysis. Cytometry 2001;43:229-238.

van Diest PJ, Vleugel MM, van der Wall E. Expression of HIF-1alpha in human tumours. J Clin Pathol 2005;58:335-336.

Dietel M, Sers C. Personalized medicine and development of targeted therapies: The upcoming challenge for diagnostic molecular pathology. A review. Virchows Arch 2006;448:744-755.

Dillon DA, D'Aquila T, Reynolds AB, Fearon ER, Rimm DL. The expression of p120ctn protein in breast cancer is independent of alpha- and beta-catenin and E-cadherin. Am J Pathol 1998;152:75-82.

Divgi CR, Bander NH, Scott AM, O'Donoghue JA, Sgouros G, Welt S, Finn RD, Morrissey F, Capitelli P, Williams JM, Deland D, Nakhre A, Oosterwijk E, Gulec S, Graham MC, Larson SM, Old LJ. Phase I/II radioimmunotherapy trial with iodine-131-labeled monoclonal antibody G250 in metastatic renal cell carcinoma. Clin Cancer Res 1998;4:2729-2739.

DiVito KA, Charette LA, Rimm DL, Camp RL. Long-term preservation of antigenicity on tissue microarrays. Lab Invest 2004;84:1071-1078.

Domínguez A, Ramos-Morales F, Romero F, Rios RM, Dreyfus F, Tortolero M, Pintor-Toro JA. hpttg, a human homologue of rat pttg, is overexpressed in hematopoietic neoplasms. Evidence for a transcriptional activation function of hPTTG. Oncogene 1998;17:2187-2193.

Dudoit S, Yang YH, Speed TP, Callow MJ. Statistical methods for identifying differentially expressed genes in replicated cDNA microarray experiments. Stat Sin 2002;12:111-140.

Eguíluz C, Viguera E, Millán L, Pérez J. Multitissue array review: a chronological description of tissue array techniques, applications and procedures. Pathol Res Pract 2006;202:561-568.

Emerson JW, Dolled-Filhart M, Harris L, Rimm DL, Tuck DP. Quantitative assessment of tissue biomarkers and construction of a model to predict outcome in breast cancer using multiple imputation. Cancer Inform 2009;7:29-40.

Emery LA, Tripathi A, King C, Kavanah M, Mendez J, Stone MD, de las Morenas A, Sebastiani P, Rosenberg CL. Early dysregulation of cell adhesion and extracellular matrix pathways in breast cancer progression. Am J Pathol 2009;175:1292-1302.

Emmert-Buck MR, Strausberg RL, Krizman DB, Bonaldo MF, Bonner RF, Bostwick DG, Brown MR, Buetow KH, Chuaqui RF, Cole KA, Duray PH, Englert CR, Gillespie JW, Greenhut S, Grouse L, Hillier LW, Katz KS, Klausner RD, Kuznetzov V, Lash AE, Lennon G, Linehan WM, Liotta LA, Marra MA, Munson PJ, Ornstein DK, Prabhu VV, Prange C, Schuler GD, Soares MB, Tolstoshev CM, Vocke CD, Waterston RH. Molecular profiling of clinical tissue specimens: feasibility and applications. Am J Pathol 2000;156:1109-1115.

Eschrich S, Yang I, Bloom G, Kwong KY, Boulware D, Cantor A, Coppola D, Kruhøffer M, Aaltonen L, Orntoft TF, Quackenbush J, Yeatman TJ. Molecular staging for survival prediction of colorectal cancer patients. J Clin Oncol 2005;23:3526-3535.

Fang X, Balgley BM, Lee CS. Recent advances in capillary electrophoresis-based proteomic techniques for biomarker discovery. Electrophoresis 2009;30:3998-4007.

Fergenbaum JH, Garcia-Closas M, Hewitt SM, Lissowska J, Sakoda LC, Sherman ME. Loss of antigenicity in stored sections of breast cancer tissue microarrays. Cancer Epidemiol Biomarkers Prev 2004;13:667-672.

Fernebro E, Dictor M, Bendahl PO, Fernö M, Nilbert M. Evaluation of the tissue microarray technique for immunohistochemical analysis in rectal cancer. Arch Pathol Lab Med 2002;126:702-705.

Fons G, Hasibuan SM, van der Velden J, ten Kate FJ. Validation of tissue microarray technology in endometrioid cancer of the endometrium. J Clin Pathol 2007;60:500-503.

Franz CM, Ridley AJ. p120 catenin associates with microtubules: inverse relationship between microtubule binding and Rho GTPase regulation. J Biol Chem 2004;279:6588-6594.

Frederiksen CM, Aaboe M, Dyrskjøt L, Laurberg S, Wolf H, Ørntoft TF, Kruhøffer M. Technical evaluation of cDNA microarrays. APMIS Suppl 2003;109:96-101.

Fujii T, Nomoto S, Koshikawa K, Yatabe Y, Teshigawara O, Mori T, Inoue S, Takeda S, Nakao A. Overexpression of pituitary tumor transforming gene 1 in HCC is associated with angiogenesis and poor prognosis. Hepatology 2006;43:1267-1275.

Funabiki H, Kumada K, Yanagida M. Fission yeast Cut1 and Cut2 are essential for sister chromatid separation, concentrate along the metaphase spindle and form large complexes. EMBO J 1996;15:6617-6628.

García JF, Camacho FI, Morente M, Fraga M, Montalbán C, Alvaro T, Bellas C, Castaño A, Díez A, Flores T, Martin C, Martinez MA, Mazorra F,

Menárguez J, Mestre MJ, Mollejo M, Sáez AI, Sánchez L, Piris MA; Spanish Hodgkin Lymphoma Study Group. Hodgkin and Reed-Sternberg cells harbor alterations in the major tumor suppressor pathways and cell-cycle checkpoints: analyses using tissue microarrays. Blood 2003;101:681-689.

Gast MC, Schellens JH, Beijnen JH. Clinical proteomics in breast cancer: a review. Breast Cancer Res Treat 2009;116:17-29.

Genkai N, Homma J, Sano M, Tanaka R, Yamanaka R. Increased expression of pituitary tumor-transforming gene (PTTG)-1 is correlated with poor prognosis in glioma patients. Oncol Rep 2006;15:1569-1574.

Geyer FC, Marchio C, Reis-Filho JS. The role of molecular analysis in breast cancer. Pathology 2009;41:77-88.

Ghayad SE, Vendrell JA, Bieche I, Spyratos F, Dumontet C, Treilleux I, Lidereau R, Cohen PA. Identification of TACC1, NOV, and PTTG1 as new candidate genes associated with endocrine therapy resistance in breast cancer. J Mol Endocrinol 2009;42:87-103.

Giatromanolaki A, Koukourakis MI, Sivridis E, Pastorek J, Wykoff CC, Gatter KC, Harris AL. Expression of hypoxia-inducible carbonic anhydrase-9 relates to angiogenic pathways and independently to poor outcome in non-small cell lung cancer. Cancer Res 2001;61:7992-7998.

Gillett CE, Springall RJ, Barnes DM, Hanby AM. Multiple tissue core arrays in histopathology research: a validation study. J Pathol 2000;192:549-553.

Giltnane JM, Rimm DL. Technology insight: Identification of biomarkers with tissue microarray technology. Nat Clin Pract Oncol 2004;1:104-111.

Goethals L, Perneel C, Debucquoy A, De Schutter H, Borghys D, Ectors N, Geboes K, McBride WH, Haustermans KM. A new approach to the validation of tissue microarrays. J Pathol 2006;208:607-614.

Gold JS, Reynolds AB, Rimm DL. Loss of p120ctn in human colorectal cancer predicts metastasis and poor survival. Cancer Lett 1998;132:193-201.

Gonçalves A, Esterni B, Bertucci F, Sauvan R, Chabannon C, Cubizolles M, Bardou VJ, Houvenaegel G, Jacquemier J, Granjeaud S, Meng XY, Fung ET, Birnbaum D, Maraninchi D, Viens P, Borg JP. Postoperative serum proteomic profiles may predict metastatic relapse in high-risk primary breast cancer patients receiving adjuvant chemotherapy. Oncogene 2006;25:981-989.

Gould Rothberg BE, Bracken MB. E-cadherin immunohistochemical expression as a prognostic factor in infiltrating ductal carcinoma of the breast: a systematic review and meta-analysis. Breast Cancer Res Treat 2006;100:139-148.

Grabmaier K, A de Weijert MC, Verhaegh GW, Schalken JA, Oosterwijk E. Strict regulation of CAIX(G250/MN) by HIF-1alpha in clear cell renal cell carcinoma. Oncogene 2004;23:5624-5631.

Grabmaier K, Vissers JL, De Weijert MC, Oosterwijk-Wakka JC, Van Bokhoven A, Brakenhoff RH, Noessner E, Mulders PA, Merkx G, Figdor CG, Adema GJ, Oosterwijk E. Molecular cloning and immunogenicity of renal cell carcinoma-associated antigen G250. Int J Cancer 2000;85:865-870.

Greiner J, Schmitt M, Li L, Giannopoulos K, Bosch K, Schmitt A, Dohner K, Schlenk RF, Pollack JR, Dohner H, Bullinger L. Expression of tumor-associated antigens in acute myeloid leukemia: Implications for specific immunotherapeutic approaches. Blood 2006;108:4109-4117.

Grotzer MA, Patti R, Geoerger B, Eggert A, Chou TT, Phillips PC. Biological stability of RNA isolated from RNAlater-treated brain tumor and neuroblastoma xenografts. Med Pediatr Oncol 2000;34:438-442.

Gulmann C, Sheehan KM, Kay EW, Liotta LA, Petricoin EF 3rd. Array-based proteomics: mapping of protein circuitries for diagnostics, prognostics, and therapy guidance in cancer. J Pathol 2006;208:595-606.

Halgren RG, Fielden MR, Fong CJ, Zacharewski TR. Assessment of clone identity and sequence fidelity for 1189 IMAGE cDNA clones. Nucleic Acids Res 2001;29:582-588.

Hamid T, Malik MT, Kakar SS. Ectopic expression of PTTG1/securin promotes tumorigenesis in human embryonic kidney cells. Mol Cancer 2005;4:3.

Hao JM, Chen JZ, Sui HM, Si-Ma XQ, Li GQ, Liu C, Li JL, Ding YQ, Li JM. A five-gene signature as a potential predictor of metastasis and survival in colorectal cancer. J Pathol 2010;220:475-489.

Hao X, Sun B, Hu L, Lähdesmäki H, Dunmire V, Feng Y, Zhang SW, Wang H, Wu C, Wang H, Fuller GN, Symmans WF, Shmulevich I, Zhang W. Differential gene and protein expression in primary breast malignancies and their lymph node metastases as revealed by combined cDNA microarray and tissue microarray analysis. Cancer 2004;100:1110-1122.

Heaney AP, Singson R, McCabe CJ, Nelson V, Nakashima M, Melmed S. Expression of pituitary-

tumour transforming gene in colorectal tumours. Lancet 2000;355:716-719.

Hecht JL, Kotsopoulos J, Gates MA, Hankinson SE, Tworoger SS. Validation of tissue microarray technology in ovarian cancer: results from the Nurses' Health Study. Cancer Epidemiol Biomarkers Prev 2008;17:3043-3050.

Hedenfalk I, Duggan D, Chen Y, Radmacher M, Bittner M, Simon R, Meltzer P, Gusterson B, Esteller M, Kallioniemi OP, Wilfond B, Borg A, Trent J, Raffeld M, Yakhini Z, Ben-Dor A, Dougherty E, Kononen J, Bubendorf L, Fehrle W, Pittaluga S, Gruvberger S, Loman N, Johannsson O, Olsson H, Sauter G. Gene-expression profiles in hereditary breast cancer. N Engl J Med 2001;344:539-548.

van Hengel J, van Roy F. Diverse functions of p120ctn in tumors. Biochim Biophys Acta 2007;1773:78-88.

Henshall S. Tissue microarrays. J Mammary Gland Biol Neoplasia 2003;8:347-358.

Hewett-Emmett D, Tashian RE. Functional diversity, conservation, and convergence in the evolution of the alpha-, beta-, and gamma-carbonic anhydrase gene families. Mol Phylogenet Evol 1996;5:50-77.

Hewitt SM. Design, construction, and use of tissue microarrays. Methods Mol Biol 2004;264:61-72.

Hewitt SM. The application of tissue microarrays in the validation of microarray results. Methods Enzymol 2006;410:400-415.

Hilvo M, Baranauskiene L, Salzano AM, Scaloni A, Matulis D, Innocenti A, Scozzafava A, Monti SM, Di Fiore A, De Simone G, Lindfors M, Jänis J, Valjakka J, Pastoreková S, Pastorek J, Kulomaa MS, Nordlund HR, Supuran CT, Parkkila S. Biochemical characterization of CA IX, one of the most active carbonic anhydrase isozymes. J Biol Chem 2008;283:27799-27809.

Hlubek F, Pfeiffer S, Budczies J, Spaderna S, Jung A, Kirchner T, Brabletz T. Securin (hPTTG1) expression is regulated by beta-catenin/TCF in human colorectal carcinoma. Br J Cancer 2006;94:1672-1677.

Hoos A, Cordon-Cardo C. Tissue microarray profiling of cancer specimens and cell lines: opportunities and limitations. Lab Invest 2001;81:1331-1338.

Hoos A, Nissan A, Stojadinovic A, Shia J, Hedvat CV, Leung DH, Paty PB, Klimstra D, Cordon-Cardo C, Wong WD. Tissue microarray molecular profiling of early, node-negative adenocarcinoma of the rectum: a comprehensive analysis. Clin Cancer Res 2002;8:3841-3849.

Hoos A, Urist MJ, Stojadinovic A, Mastorides S, Dudas ME, Leung DH, Kuo D, Brennan MF, Lewis JJ, Cordon-Cardo C. Validation of tissue microarrays for immunohistochemical profiling of cancer specimens using the example of human fibroblastic tumors. Am J Pathol 2001;158:1245-1251.

Hornig NC, Knowles PP, McDonald NQ, Uhlmann F. The dual mechanism of separase regulation by securin. Curr Biol 2002;12:973-982.

Huang J, Qi R, Quackenbush J, Dauway E, Lazaridis E, Yeatman T. Effects of ischemia on gene expression. J Surg Res 2001;99:222-227.

Huddleston HG, Wong KK, Welch WR, Berkowitz RS, Mok SC. Clinical applications of microarray technology: creatine kinase B is an up-regulated gene in epithelial ovarian cancer and shows promise as a serum marker. Gynecol Oncol 2005;96:77-83.

Hudelist G, Pacher-Zavisin M, Singer CF, Holper T, Kubista E, Schreiber M, Manavi M, Bilban M, Czerwenka K. Use of high-throughput protein array for profiling of differentially expressed proteins in normal and malignant breast tissue. Breast Cancer Res Treat 2004;86:281-291.

Hurd PJ, Nelson CJ. Advantages of next-generation sequencing versus the microarray in epigenetic research. Brief Funct Genomic Proteomic 2009;8:174-183.

Iakovlev VV, Pintilie M, Morrison A, Fyles AW, Hill RP, Hedley DW. Effect of distributional heterogeneity on the analysis of tumor hypoxia based on carbonic anhydrase IX. Lab Invest 2007;87:1206-1217.

Ireton RC, Davis MA, van Hengel J, Mariner DJ, Barnes K, Thoreson MA, Anastasiadis PZ, Matrisian L, Bundy LM, Sealy L, Gilbert B, van Roy F, Reynolds AB. A novel role for p120 catenin in E-cadherin function. J Cell Biol 2002;159:465-476.

Irizarry RA, Warren D, Spencer F, Kim IF, Biswal S, Frank BC, Gabrielson E, Garcia JG, Geoghegan J, Germino G, Griffin C, Hilmer SC, Hoffman E, Jedlicka AE, Kawasaki E, Martínez-Murillo F, Morsberger L, Lee H, Petersen D, Quackenbush J, Scott A, Wilson M, Yang Y, Ye SQ, Yu W. Multiple-laboratory comparison of microarray platforms. Nat Methods 2005;2:345-350.

Ishibashi T, Takagi Y, Mori K, Naruse S, Nishino H, Yue BY, Kinoshita S. cDNA microarray analysis of gene expression changes induced by

dexamethasone in cultured human trabecular meshwork cells. Invest Ophthalmol Vis Sci 2002;43:3691-3697.

Ishikawa H, Heaney AP, Yu R, Horwitz GA, Melmed S. Human pituitary tumor-transforming gene induces angiogenesis. J Clin Endocrinol Metab 2001;86:867-874.

Ishizaki Y, Omori Y, Momiyama M, Nishikawa Y, Tokairin T, Manabe M, Enomoto K. Reduced expression and aberrant localization of p120catenin in human squamous cell carcinoma of the skin. J Dermatol Sci 2004;34:99-108.

Ivanov S, Liao SY, Ivanova A, Danilkovitch-Miagkova A, Tarasova N, Weirich G, Merrill MJ, Proescholdt MA, Oldfield EH, Lee J, Zavada J, Waheed A, Sly W, Lerman MI, Stanbridge EJ. Expression of hypoxia-inducible cell-surface transmembrane carbonic anhydrases in human cancer. Am J Pathol 2001;158:905-919.

Ivanov SV, Kuzmin I, Wei MH, Pack S, Geil L, Johnson BE, Stanbridge EJ, Lerman MI. Down-regulation of transmembrane carbonic anhydrases in renal cell carcinoma cell lines by wild-type von Hippel-Lindau transgenes. Proc Natl Acad Sci U S A 1998;95:12596-12601.

Jallepalli PV, Waizenegger IC, Bunz F, Langer S, Speicher MR, Peters JM, Kinzler KW, Vogelstein B, Lengauer C. Securin is required for chromosomal stability in human cells. Cell 2001;105:445-457.

Jenner K, Darnton SJ, Billingham L, Warfield AT, Matthews HR. Tumour heterogeneity: a problem in biopsy assessment of the proliferation index of oesophageal adenocarcinomas. Clin Mol Pathol 1996;49:M61-63.

de Jong D, Xie W, Rosenwald A, Chhanabhai M, Gaulard P, Klapper W, Lee A, Sander B, Thorns C, Campo E, Molina T, Hagenbeek A, Horning S, Lister A, Raemaekers J, Salles G, Gascoyne RD, Weller E. Immunohistochemical prognostic markers in diffuse large B-cell lymphoma: validation of tissue microarray as a prerequisite for broad clinical applications (a study from the Lunenburg Lymphoma Biomarker Consortium). J Clin Pathol 2009;62:128-138.

Jordan B. Historical background and anticipated developments. Ann N Y Acad Sci 2002;975:24-32.

Jourdan F, Sebbagh N, Comperat E, Mourra N, Flahault A, Olschwang S, Duval A, Hamelin R, Flejou JF. Tissue microarray technology: validation in colorectal carcinoma and analysis of p53, hMLH1, and hMSH2 immunohistochemical expression. Virchows Arch 2003;443:115-121.

Jubb AM, Landon TH, Burwick J, Pham TQ, Frantz GD, Cairns B, Quirke P, Peale FV, Hillan KJ. Quantitative analysis of colorectal tissue microarrays by immunofluorescence and in situ hybridization. J Pathol 2003;200:577-588.

Kafatos FC, Jones CW, Efstratiadis A. Determination of nucleic acid sequence homologies and relative concentrations by a dot hybridization procedure. Nucleic Acids Res 1979;7:1541-1552.

Kakar SS. Assignment of the human tumor transforming gene TUTR1 to chromosome band 5q35.1 by fluorescence in situ hybridization. Cytogenet Cell Genet 1998;83:93-95.

Kakar SS, Chen L, Puri R, Flynn SE, Jennes L. Characterization of a polyclonal antibody to human pituitary tumor transforming gene 1 (PTTG1) protein. J Histochem Cytochem 2001;49:1537-1546.

Kakar SS, Jennes L. Molecular cloning and characterization of the tumor transforming gene (TUTR1): a novel gene in human tumorigenesis. Cytogenet Cell Genet 1999;84:211-216.

Kallakury BV, Sheehan CE, Winn-Deen E, Oliver J, Fisher HA, Kaufman RP Jr, Ross JS. Decreased expression of catenins (alpha and beta), p120 CTN, and E-cadherin cell adhesion proteins and E-cadherin gene promoter methylation in prostatic adenocarcinomas. Cancer 2001;92:2786-2795.

Kallioniemi OP, Wagner U, Kononen J, Sauter G. Tissue microarray technology for high-throughput molecular profiling of cancer. Hum Mol Genet 2001;10:657-662.

Kaluz S, Kaluzová M, Liao SY, Lerman M, Stanbridge EJ. Transcriptional control of the tumor- and hypoxia-marker carbonic anhydrase 9: A one transcription factor (HIF-1) show? Biochim Biophys Acta 2009;1795:162-172.

Karayiannakis AJ, Syrigos KN, Alexiou D, Kalahanis N, Rosenberg T, Bastounis E, Pignatelli M. Expression patterns of the novel catenin p120cas in gastrointestinal cancers. Anticancer Res 1999;19:4401-4405.

Keirsebilck A, Bonné S, Staes K, van Hengel J, Nollet F, Reynolds A, van Roy F. Molecular cloning of the human p120ctn catenin gene (CTNND1): expression of multiple alternatively spliced isoforms. Genomics 1998;50:129-146.

Kent AJ, Woolf D, McCue J, Greenfield SM. The use of symptoms to predict colorectal site. Can we

reduce the pressure on our endoscopy services? Colorectal Dis 2010;12:114-118.

Khan J, Wei JS, Ringnér M, Saal LH, Ladanyi M, Westermann F, Berthold F, Schwab M, Antonescu CR, Peterson C, Meltzer PS. Classification and diagnostic prediction of cancers using gene expression profiling and artificial neural networks. Nat Med 2001;7:673-679.

Khimani AH, Mhashilkar AM, Mikulskis A, O'Malley M, Liao J, Golenko EE, Mayer P, Chada S, Killian JB, Lott ST. Housekeeping genes in cancer: normalization of array data. Biotechniques 2005;38:739-745.

Ki DH, Jeung HC, Park CH, Kang SH, Lee GY, Lee WS, Kim NK, Chung HC, Rha SY. Whole genome analysis for liver metastasis gene signatures in colorectal cancer. Int J Cancer 2007;121:2005-2012.

Kim DS, Franklyn JA, Smith VE, Stratford AL, Pemberton HN, Warfield A, Watkinson JC, Ishmail T, Wakelam MJ, McCabe CJ. Securin induces genetic instability in colorectal cancer by inhibiting double-stranded DNA repair activity. Carcinogenesis 2007;28:749-759.

Kitahara O, Furukawa Y, Tanaka T, Kihara C, Ono K, Yanagawa R, Nita ME, Takagi T, Nakamura Y, Tsunoda T. Alterations of gene expression during colorectal carcinogenesis revealed by cDNA microarrays after laser-capture microdissection of tumor tissues and normal epithelia. Cancer Res 2001;61:3544-3549.

Kivela AJ, Parkkila S, Saarnio J, Karttunen TJ, Kivela J, Parkkila AK, Bartosova M, Mucha V, Novak M, Waheed A, Sly WS, Rajaniemi H, Pastorekova S, Pastorek J. Expression of von Hippel-Lindau tumor suppressor and tumor-associated carbonic anhydrases IX and XII in normal and neoplastic colorectal mucosa. World J Gastroenterol 2005;11:2616-2625.

Kivela AJ, Saarnio J, Karttunen TJ, Kivelä J, Parkkila AK, Pastorekova S, Pastorek J, Waheed A, Sly WS, Parkkila TS, Rajaniemi H. Differential expression of cytoplasmic carbonic anhydrases, CA I and II, and membrane-associated isozymes, CA IX and XII, in normal mucosa of large intestine and in colorectal tumors. Dig Dis Sci 2001;46:2179-2186.

Kononen J, Bubendorf L, Kallioniemi A, Bärlund M, Schraml P, Leighton S, Torhorst J, Mihatsch MJ, Sauter G, Kallioniemi OP. Tissue microarrays for high-throughput molecular profiling of tumor specimens. Nat Med 1998;4:844-847.

Kraaz W, Risberg B, Hussein A. Multiblock: an aid in diagnostic immunohistochemistry. J Clin Pathol 1988;41:1337.

Kramer MW, Merseburger AS, Hennenlotter J, Kuczyk M. Tissue microarrays in clinical urology--technical considerations. Scand J Urol Nephrol 2007;41:478-484.

Kuo WP, Jenssen TK, Butte AJ, Ohno-Machado L, Kohane IS. Analysis of matched mRNA measurements from two different microarray technologies. Bioinformatics 2002;18:405-412.

Kyndi M, Sørensen FB, Knudsen H, Overgaard M, Nielsen HM, Andersen J, Overgaard J. Tissue microarrays compared with whole sections and biochemical analyses. A subgroup analysis of DBCG 82 b&c. Acta Oncol 2008;47:591-599.

Lakhani SR, Jacquemier J, Sloane JP, Gusterson BA, Anderson TJ, van de Vijver MJ, Farid LM, Venter D, Antoniou A, Storfer-Isser A, Smyth E, Steel CM, Haites N, Scott RJ, Goldgar D, Neuhausen S, Daly PA, Ormiston W, McManus R, Scherneck S, Ponder BA, Ford D, Peto J, Stoppa-Lyonnet D, Bignon YJ, Struewing JP, Spurr NK, Bishop DT, Klijn JG, Devilee P, Cornelisse CJ, Lasset C, Lenoir G, Barkardottir RB, Egilsson V, Hamann U, Chang-Claude J, Sobol H, Weber B, Stratton MR, Easton DF. Multifactorial analysis of differences between sporadic breast cancers and cancers involving BRCA1 and BRCA2 mutations. J Natl Cancer Inst 1998;90:1138-1145.

Larkin JE, Frank BC, Gavras H, Sultana R, Quackenbush J. Independence and reproducibility across microarray platforms. Nat Methods 2005;2:337-344.

Leibovich BC, Sheinin Y, Lohse CM, Thompson RH, Cheville JC, Zavada J, Kwon ED. Carbonic anhydrase IX is not an independent predictor of outcome for patients with clear cell renal cell carcinoma. J Clin Oncol 2007;25:4757-4764.

Lennon GG, Lehrach H. Hybridization analyses of arrayed cDNA libraries. Trends Genet 1991;7:314-317.

Leppilampi M, Saarnio J, Karttunen TJ, Kivelä J, Pastoreková S, Pastorek J, Waheed A, Sly WS, Parkkila S. Carbonic anhydrase isozymes IX and XII in gastric tumors. World J Gastroenterol 2003;9:1398-1403.

Li R, Ni J, Bourne PA, Yeh S, Yao J, di Sant'Agnese PA, Huang J. Cell culture block array for immunocytochemical study of protein expression in cultured cells. Appl Immunohistochem Mol Morphol 2005;13:85-90.

Li Y, Wang H, Oosterwijk E, Selman Y, Mira JC, Medrano T, Shiverick KT, Frost SC. Antibody-specific detection of CAIX in breast and prostate cancers. Biochem Biophys Res Commun 2009a;386:488-492.

Li Y, Wang H, Oosterwijk E, Tu C, Shiverick KT, Silverman DN, Frost SC. Expression and activity of carbonic anhydrase IX is associated with metabolic dysfunction in MDA-MB-231 breast cancer cells. Cancer Invest 2009b;27:613-623.

Liao SY, Aurelio ON, Jan K, Zavada J, Stanbridge EJ. Identification of the MN/CA9 protein as a reliable diagnostic biomarker of clear cell carcinoma of the kidney. Cancer Res 1997;57:2827-2831.

Liao SY, Brewer C, Závada J, Pastorek J, Pastorekova S, Manetta A, Berman ML, DiSaia PJ, Stanbridge EJ. Identification of the MN antigen as a diagnostic biomarker of cervical intraepithelial squamous and glandular neoplasia and cervical carcinomas. Am J Pathol 1994;145:598-609.

Liao SY, Lerman MI, Stanbridge E. Expression of transmembrane carbonic anhydrases, CAIX and CAXII, in human development. BMC Developmental Biology 2009;9:22.

Linderoth J, Ehinger M, Akerman M, Cavallin-Ståhl E, Enblad G, Erlanson M, Jerkeman M. Tissue microarray is inappropriate for analysis of BCL6 expression in diffuse large B-cell lymphoma. Eur J Haematol 2007;79:146-149.

Liu Y, Dong QZ, Zhao Y, Dong XJ, Miao Y, Dai SD, Yang ZQ, Zhang D, Wang Y, Li QC, Zhao C, Wang EH. P120-catenin isoforms 1A and 3A differently affect invasion and proliferation of lung cancer cells. Exp Cell Res 2009;315:890-898.

Liu Y, Xu HT, Dai SD, Wei Q, Yuan XM, Wang EH. Reduction of p120(ctn) isoforms 1 and 3 is significantly associated with metastatic progression of human lung cancer. APMIS 2007;115:848-856.

Loman N, Johannsson O, Bendahl PO, Borg A, Fernö M, Olsson H. Steroid receptors in hereditary breast carcinomas associated with BRCA1 or BRCA2 mutations or unknown susceptibility genes. Cancer 1998;83:310-319.

Loring JF. Evolution of microarray analysis. Neurobiol Aging 2006;27:1084-1086.

Luo L, Salunga RC, Guo H, Bittner A, Joy KC, Galindo JE, Xiao H, Rogers KE, Wan JS, Jackson MR, Erlander MG. Gene expression profiles of laser-captured adjacent neuronal subtypes. Nat Med 1999;5:117-122.

Makretsov NA, Huntsman DG, Nielsen TO, Yorida E, Peacock M, Cheang MC, Dunn SE, Hayes M, van de Rijn M, Bajdik C, Gilks CB. Hierarchical clustering analysis of tissue microarray immunostaining data identifies prognostically significant groups of breast carcinoma. Clin Cancer Res 2004;10:6143-6151.

Manduchi E, Scearce LM, Brestelli JE, Grant GR, Kaestner KH, Stoeckert CJ Jr. Comparison of different labeling methods for two-channel high-density microarray experiments. Physiol Genomics 2002;10:169-179.

Manley S, Mucci NR, De Marzo AM, Rubin MA. Relational database structure to manage high-density tissue microarray data and images for pathology studies focusing on clinical outcome: the prostate specialized program of research excellence model. Am J Pathol 2001;159:837-843.

Manning AT, Garvin JT, Shahbazi RI, Miller N, McNeill RE, Kerin MJ. Molecular profiling techniques and bioinformatics in cancer research. Eur J Surg Oncol 2007;33:255-265.

MAQC Consortium, Shi L, Reid LH, Jones WD, Shippy R, Warrington JA, Baker SC, Collins PJ, de Longueville F, Kawasaki ES, Lee KY, Luo Y, Sun YA, Willey JC, Setterquist RA, Fischer GM, Tong W, Dragan YP, Dix DJ, Frueh FW, Goodsaid FM, Herman D, Jensen RV, Johnson CD, Lobenhofer EK, Puri RK, Schrf U, Thierry-Mieg J, Wang C, Wilson M, Wolber PK, Zhang L, Amur S, Bao W, Barbacioru CC, Lucas AB, Bertholet V, Boysen C, Bromley B, Brown D, Brunner A, Canales R, Cao XM, Cebula TA, Chen JJ, Cheng J, Chu TM, Chudin E, Corson J, Corton JC, Croner LJ, Davies C, Davison TS, Delenstarr G, Deng X, Dorris D, Eklund AC, Fan XH, Fang H, Fulmer-Smentek S, Fuscoe JC, Gallagher K, Ge W, Guo L, Guo X, Hager J, Haje PK, Han J, Han T, Harbottle HC, Harris SC, Hatchwell E, Hauser CA, Hester S, Hong H, Hurban P, Jackson SA, Ji H, Knight CR, Kuo WP, LeClerc JE, Levy S, Li QZ, Liu C, Liu Y, Lombardi MJ, Ma Y, Magnuson SR, Maqsodi B, McDaniel T, Mei N, Myklebost O, Ning B, Novoradovskaya N, Orr MS, Osborn TW, Papallo A, Patterson TA, Perkins RG, Peters EH, Peterson R, Philips KL, Pine PS, Pusztai L, Qian F, Ren H, Rosen M, Rosenzweig BA, Samaha RR, Schena M, Schroth GP, Shchegrova S, Smith DD, Staedtler F, Su Z, Sun H, Szallasi Z, Tezak Z, Thierry-Mieg D, Thompson KL, Tikhonova I, Turpaz Y, Vallanat B, Van C, Walker SJ, Wang SJ, Wang Y, Wolfinger R, Wong A, Wu J, Xiao C, Xie Q, Xu J, Yang W, Zhang L, Zhong S, Zong Y, Slikker W Jr. The MicroArray Quality Control (MAQC) project shows inter- and intraplatform reproducibility of gene expression measurements. Nat Biotechnol 2006;24:1151-1161.

Mardis ER, Wilson RK. Cancer genome sequencing: a review. Hum Mol Genet 2009;18:R163-168.

Marguerat S, Bähler J. RNA-seq: from technology to biology. Cell Mol Life Sci 2010;67:569-579.

Markowitz SD, Bertagnolli MM. Molecular origins of cancer: Molecular basis of colorectal cancer. N Engl J Med 2009;361:2449-2460.

Martínez-Zaguilán R, Seftor EA, Seftor RE, Chu YW, Gillies RJ, Hendrix MJ. Acidic pH enhances the invasive behavior of human melanoma cells. Clin Exp Metastasis 1996;14:176-186.

Marx RG, Hudak PL, Bombardier C, Graham B, Goldsmith C, Wright JG. The reliability of physical examination for carpal tunnel syndrome. J Hand Surg Br 1998;23:499-502.

Mayerle J, Friess H, Büchler MW, Schnekenburger J, Weiss FU, Zimmer KP, Domschke W, Lerch MM. Up-regulation, nuclear import, and tumor growth stimulation of the adhesion protein p120 in pancreatic cancer. Gastroenterology 2003;124:949-960.

Mecham BH, Klus GT, Strovel J, Augustus M, Byrne D, Bozso P, Wetmore DZ, Mariani TJ, Kohane IS, Szallasi Z. Sequence-matched probes produce increased cross-platform consistency and more reproducible biological results in microarray-based gene expression measurements. Nucleic Acids Res 2004;32:e74.

Medeiros F, Rigl CT, Anderson GG, Becker SH, Halling KC. Tissue handling for genome-wide expression analysis: a review of the issues, evidence, and opportunities. Arch Pathol Lab Med 2007;131:1805-1816.

Mello-Coelho V, Hess KL. A conceptual and practical overview of cDNA microarray technology: implications for basic and clinical sciences. Braz J Med Biol Res 2005;38:1543-1552.

Mengel M, von Wasielewski R, Wiese B, Rüdiger T, Müller-Hermelink HK, Kreipe H. Inter-laboratory and inter-observer reproducibility of immunohistochemical assessment of the Ki-67 labelling index in a large multi-centre trial. J Pathol 2002;198:292-299.

Merseburger AS, Kuczyk MA, Serth J, Bokemeyer C, Young DY, Sun L, Connelly RR, McLeod DG, Mostofi FK, Srivastava SK, Stenzl A, Moul JW, Sesterhenn IA. Limitations of tissue microarrays in the evaluation of focal alterations of bcl-2 and p53 in whole mount derived prostate tissues. Oncol Rep 2003;10:223-228.

Miao Y, Liu N, Zhang Y, Liu Y, Yu JH, Dai SD, Xu HT, Wang EH. p120ctn isoform 1 expression significantly correlates with abnormal expression of E-cadherin and poor survival of lung cancer patients. Med Oncol 2010;27:880-886.

Michaelis C, Ciosk R, Nasmyth K. Cohesins: chromosomal proteins that prevent premature separation of sister chromatids. Cell 1997;91:35-45.

Micke P, Ohshima M, Tahmasebpoor S, Ren ZP, Ostman A, Pontén F, Botling J. Biobanking of fresh frozen tissue: RNA is stable in nonfixed surgical specimens. Lab Invest 2006;86:202-211.

Miller RT, Groothuis CL. Multitumor "sausage" blocks in immunohistochemistry. Simplified method of preparation, practical uses, and roles in quality assurance. Am J Clin Pathol 1991;96:228-232.

Mirlacher M, Kasper M, Storz M, Knecht Y, Dürmüller U, Simon R, Mihatsch MJ, Sauter G. Influence of slide aging on results of translational research studies using immunohistochemistry. Mod Pathol 2004;17:1414-1420.

Mo YY, Reynolds AB. Identification of murine p120 isoforms and heterogeneous expression of p120cas isoforms in human tumor cell lines. Cancer Res 1996;56:2633-2640.

Moch H, Kononen T, Kallioniemi OP, Sauter G. Tissue microarrays: what will they bring to molecular and anatomic pathology? Adv Anat Pathol 2001;8:14-20.

Moch H, Schraml P, Bubendorf L, Mirlacher M, Kononen J, Gasser T, Mihatsch MJ, Kallioniemi OP, Sauter G. High-throughput tissue microarray analysis to evaluate genes uncovered by cDNA microarray screening in renal cell carcinoma. Am J Pathol 1999;154:981-986.

Montgomery K, Zhao S, van de Rijn M, Natkunam Y. A novel method for making "tissue" microarrays from small numbers of suspension cells. Appl Immunohistochem Mol Morphol 2005;13:80-84.

Morozova O, Hirst M, Marra MA. Applications of new sequencing technologies for transcriptome analysis. Annu Rev Genomics Hum Genet 2009;10:135-151.

Mu YM, Oba K, Yanase T, Ito T, Ashida K, Goto K, Morinaga H, Ikuyama S, Takayanagi R, Nawata H. Human pituitary tumor transforming gene (hPTTG) inhibits human lung cancer A549 cell growth through activation of p21(WAF1/CIP1). Endocr J 2003;50:771-781.

Mucci NR, Akdas G, Manely S, Rubin MA. Neuroendocrine expression in metastatic prostate cancer: evaluation of high throughput tissue

microarrays to detect heterogeneous protein expression. Hum Pathol 2000;31:406-414.

Nagase T, Ishikawa K, Nakajima D, Ohira M, Seki N, Miyajima N, Tanaka A, Kotani H, Nomura N, Ohara O. Prediction of the coding sequences of unidentified human genes. VII. The complete sequences of 100 new cDNA clones from brain which can code for large proteins in vitro. DNA Res 1997;4:141-150.

Nakagawa T, Huang SK, Martinez SR, Tran AN, Elashoff D, Ye X, Turner RR, Giuliano AE, Hoon DS. Proteomic profiling of primary breast cancer predicts axillary lymph node metastasis. Cancer Res 2006;66:11825-11830.

Nakagawa Y, Uemura H, Hirao Y, Yoshida K, Saga S, Yoshikawa K. Radiation hybrid mapping of the human MN/CA9 locus to chromosome band 9p12-p13. Genomics 1998;53:118-119.

Nakopoulou L, Gakiopoulou-Givalou H, Karayiannakis AJ, Giannopoulou I, Keramopoulos A, Davaris P, Pignatelli M. Abnormal alpha-catenin expression in invasive breast cancer correlates with poor patient survival. Histopathology 2002;40:536-546.

Nature. Microarray standards at last. Nature 2002;419:323.

Nguyen C, Rocha D, Granjeaud S, Baldit M, Bernard K, Naquet P, Jordan BR. Differential gene expression in the murine thymus assayed by quantitative hybridization of arrayed cDNA clones. Genomics 1995;29:207-216.

Nishizuka S, Chen ST, Gwadry FG, Alexander J, Major SM, Scherf U, Reinhold WC, Waltham M, Charboneau L, Young L, Bussey KJ, Kim S, Lababidi S, Lee JK, Pittaluga S, Scudiero DA, Sausville EA, Munson PJ, Petricoin EF 3rd, Liotta LA, Hewitt SM, Raffeld M, Weinstein JN. Diagnostic markers that distinguish colon and ovarian adenocarcinomas: identification by genomic, proteomic, and tissue array profiling. Cancer Res 2003;63:5243-5250.

Nocito A, Bubendorf L, Tinner EM, Süess K, Wagner U, Forster T, Kononen J, Fijan A, Bruderer J, Schmid U, Ackermann D, Maurer R, Alund G, Knönagel H, Rist M, Anabitarte M, Hering F, Hardmeier T, Schoenenberger AJ, Flury R, Jäger P, Fehr JL, Schraml P, Moch H, Mihatsch MJ, Gasser T, Sauter G. Microarrays of bladder cancer tissue are highly representative of proliferation index and histological grade. J Pathol 2001;194:349-357.

Ocak S, Sos ML, Thomas RK, Massion PP. High-throughput molecular analysis in lung cancer: insights into biology and potential clinical applications. Eur Respir J 2009;34:489-506.

Ogbagabriel S, Fernando M, Waldman FM, Bose S, Heaney AP. Securin is overexpressed in breast cancer. Mod Pathol 2005;18:985-990.

Oosterwijk E. Carbonic anhydrase IX: historical and future perspectives. BJU Int 2008;101 Suppl 4:2-7.

Oosterwijk E, Ruiter DJ, Hoedemaeker PJ, Pauwels EK, Jonas U, Zwartendijk J, Warnaar SO. Monoclonal antibody G 250 recognizes a determinant present in renal-cell carcinoma and absent from normal kidney. Int J Cancer 1986;38:489-494.

Opavský R, Pastoreková S, Zelník V, Gibadulinová A, Stanbridge EJ, Závada J, Kettmann R, Pastorek J. Human MN/CA9 gene, a novel member of the carbonic anhydrase family: structure and exon to protein domain relationships. Genomics 1996;33:480-487.

Packeisen J, Buerger H, Krech R, Boecker W. Tissue microarrays: a new approach for quality control in immunohistochemistry. J Clin Pathol 2002;55:613-615.

Packeisen J, Korsching E, Herbst H, Boecker W, Buerger H. Demystified...tissue microarray technology. Mol Pathol 2003;56:198-204.

Pan CC, Chen PC, Chiang H. An easy method for manual construction of high-density tissue arrays. Appl Immunohistochem Mol Morphol 2004;12:370-372.

Panguluri SK, Yeakel C, Kakar SS. PTTG: an important target gene for ovarian cancer therapy. J Ovarian Res 2008;1:6.

Paredes J, Correia AL, Ribeiro AS, Milanezi F, Cameselle-Teijeiro J, Schmitt FC. Breast carcinomas that co-express E- and P-cadherin are associated with p120-catenin cytoplasmic localisation and poor patient survival. J Clin Pathol 2008;61:856-862.

Pastorek J, Pastoreková S, Callebaut I, Mornon JP, Zelník V, Opavský R, Zat'ovicová M, Liao S, Portetelle D, Stanbridge EJ, et al. Cloning and characterization of MN, a human tumor-associated protein with a domain homologous to carbonic anhydrase and a putative helix-loop-helix DNA binding segment. Oncogene 1994;9:2877-2888.

Pastoreková S, Parkkila S, Parkkila AK, Opavský R, Zelník V, Saarnio J, Pastorek J. Carbonic anhydrase IX, MN/CA IX: analysis of stomach complementary DNA sequence and expression in

human and rat alimentary tracts. Gastroenterology 1997;112:398-408.

Pastorekova S, Ratcliffe PJ, Pastorek J. Molecular mechanisms of carbonic anhydrase IX-mediated pH regulation under hypoxia. BJU Int 2008;101 Suppl 4:8-15.

Pastoreková S, Závadová Z, Kostál M, Babusíková O, Závada J. A novel quasi-viral agent, MaTu, is a two-component system. Virology 1992;187:620-626.

Pei L. Activation of mitogen-activated protein kinase cascade regulates pituitary tumor-transforming gene transactivation function. J Biol Chem 2000;275:31191-31198.

Pei L, Melmed S. Isolation and characterization of a pituitary tumor-transforming gene (PTTG). Mol Endocrinol 1997;11:433-441.

Perou CM, Jeffrey SS, van de Rijn M, Rees CA, Eisen MB, Ross DT, Pergamenschikov A, Williams CF, Zhu SX, Lee JC, Lashkari D, Shalon D, Brown PO, Botstein D. Distinctive gene expression patterns in human mammary epithelial cells and breast cancers. Proc Natl Acad Sci U S A 1999;96:9212-9217.

Perou CM, Sørlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA, Fluge O, Pergamenschikov A, Williams C, Zhu SX, Lønning PE, Børresen-Dale AL, Brown PO, Botstein D. Molecular portraits of human breast tumours. Nature 2000;406:747-752.

Petersen D, Chandramouli GV, Geoghegan J, Hilburn J, Paarlberg J, Kim CH, Munroe D, Gangi L, Han J, Puri R, Staudt L, Weinstein J, Barrett JC, Green J, Kawasaki ES. Three microarray platforms: an analysis of their concordance in profiling gene expression. BMC Genomics 2005;6:63.

Phimister B. Going global. Nat Genet 1999;21:1.

Pires AR, Andreiuolo Fda M, de Souza SR. TMA for all: a new method for the construction of tissue microarrays without recipient paraffin block using custom-built needles. Diagn Pathol 2006;1:14.

Potter C, Harris AL. Hypoxia inducible carbonic anhydrase IX, marker of tumour hypoxia, survival pathway and therapy target. Cell Cycle 2004;3:164-167.

Prall F, Ostwald C, Nizze H, Barten M. Expression profiling of colorectal carcinomas using tissue microarrays: cell cycle regulatory proteins p21, p27, and p53 as immunohistochemical prognostic markers in univariate and multivariate analysis. Appl Immunohistochem Mol Morphol 2004;12:111-121.

Press MF, Hung G, Godolphin W, Slamon DJ. Sensitivity of HER-2/neu antibodies in archival tissue samples: potential source of error in immunohistochemical studies of oncogene expression. Cancer Res 1994;54:2771-2777.

Proescholdt MA, Mayer C, Kubitza M, Schubert T, Liao SY, Stanbridge EJ, Ivanov S, Oldfield EH, Brawanski A, Merrill MJ. Expression of hypoxia-inducible carbonic anhydrases in brain tumors. Neuro Oncol 2005;7:465-475.

Puri R, Tousson A, Chen L, Kakar SS. Molecular cloning of pituitary tumor transforming gene 1 from ovarian tumors and its expression in tumors. Cancer Lett 2001;163:131-139.

Qu Y, Boutjdir M. RNase protection assay for quantifying gene expression levels. Methods Mol Biol 2007;366:145-158.

Quraishi I, Rishi M, Feldman M, Wargovich MJ, Weber B. Clinical validation of breast cancer biomarkers using tissue microarray technology. Appl Immunohistochem Mol Morphol 2007;15:45-49.

Rajaganeshan R, Prasad R, Guillou PJ, Scott N, Poston G, Jayne DG. Expression patterns of hypoxic markers at the invasive margin of colorectal cancers and liver metastases. Eur J Surg Oncol 2009;35:1286-1294.

Ramaswamy S, Golub TR. DNA microarrays in clinical oncology. J Clin Oncol 2002;20:1932-1941.

Ramos-Morales F, Domínguez A, Romero F, Luna R, Multon MC, Pintor-Toro JA, Tortolero M. Cell cycle regulated expression and phosphorylation of hpttg proto-oncogene product. Oncogene 2000;19:403-409.

Rayner TF, Rocca-Serra P, Spellman PT, Causton HC, Farne A, Holloway E, Irizarry RA, Liu J, Maier DS, Miller M, Petersen K, Quackenbush J, Sherlock G, Stoeckert CJ Jr, White J, Whetzel PL, Wymore F, Parkinson H, Sarkans U, Ball CA, Brazma A. A simple spreadsheet-based, MIAME-supportive format for microarray data: MAGE-TAB. BMC Bioinformatics 2006;7:489.

Rehfeld N, Geddert H, Atamna A, Rohrbeck A, Garcia G, Kliszewski S, Neukirchen J, Bruns I, Steidl U, Fenk R, Gabbert HE, Kronenwett R, Haas R, Rohr UP. The influence of the pituitary tumor transforming gene-1 (PTTG-1) on survival of patients with small cell lung cancer and non-small cell lung cancer. J Carcinog 2006;5:4.

Reynolds AB, Daniel J, McCrea PD, Wheelock MJ, Wu J, Zhang Z. Identification of a new catenin: the tyrosine kinase substrate p120cas associates

with E-cadherin complexes. Mol Cell Biol 1994;14:8333-8342.

Reynolds AB, Daniel JM, Mo YY, Wu J, Zhang Z. The novel catenin p120cas binds classical cadherins and induces an unusual morphological phenotype in NIH3T3 fibroblasts. Exp Cell Res 1996a;225:328-337.

Reynolds AB, Herbert L, Cleveland JL, Berg ST, Gaut JR. p120, a novel substrate of protein tyrosine kinase receptors and of p60v-src, is related to cadherin-binding factors beta-catenin, plakoglobin and armadillo. Oncogene 1992;7:2439-2445.

Reynolds AB, Jenkins NA, Gilbert DJ, Copeland NG, Shapiro DN, Wu J, Daniel JM. The gene encoding p120cas, a novel catenin, localizes on human chromosome 11q11 (CTNND) and mouse chromosome 2 (Catns). Genomics 1996b;31:127-129.

Reynolds AB, Roczniak-Ferguson A. Emerging roles for p120-catenin in cell adhesion and cancer. Oncogene 2004;23:7947-7956.

van de Rijn M, Gilks CB. Applications of microarrays to histopathology. Histopathology 2004;44:97-108.

Rocchi P, So A, Kojima S, Signaevsky M, Beraldi E, Fazli L, Hurtado-Coll A,Yamanaka K, Gleave M. Heat shock protein 27 increases after androgen ablation and plays a cytoprotective role in hormone-refractory prostate cancer. Cancer Res 2004;64:6595-6602.

Rockwell S, Dobrucki IT, Kim EY, Marrison ST, Vu VT. Hypoxia and radioation therapy: past history ongoing research, and future promise. Curr Mol Med 2009;9:442-458.

Romero F, Multon MC, Ramos-Morales F, Domínguez A, Bernal JA, Pintor-Toro JA, Tortolero M. Human securin, hPTTG, is associated with Ku heterodimer, the regulatory subunit of the DNA-dependent protein kinase. Nucleic Acids Res 2001;29:1300-1307.

Rosen PP (Ed.). Rosen's Breast Pathology. Lippincott Williams & Wilkins, Philadelphia, 2001.

van Roy FM, McCrea PD. A role for Kaiso-p120ctn complexes in cancer? Nat Rev Cancer 2005;5:956-964.

Rozhin J, Sameni M, Ziegler G, Sloane BF. Pericellular pH affects distribution and secretion of cathepsin B in malignant cells. Cancer Res 1994;54:6517-6525.

Rubin MA, Dunn R, Strawderman M, Pienta KJ. Tissue microarray sampling strategy for prostate cancer biomarker analysis. Am J Surg Pathol 2002;26:312-319.

Russo G, Zegar C, Giordano A. Advantages and limitations of microarray technology in human cancer. Oncogene 2003;22:6497-6507.

Saarnio J, Parkkila S, Parkkila AK, Haukipuro K, Pastoreková S, Pastorek J, Kairaluoma MI, Karttunen TJ. Immunohistochemical study of colorectal tumors for expression of a novel transmembrane carbonic anhydrase, MN/CA IX, with potential value as a marker of cell proliferation. Am J Pathol 1998a;153:279-285.

Saarnio J, Parkkila S, Parkkila AK, Waheed A, Casey MC, Zhou XY, Pastoreková S, Pastorek J, Karttunen T, Haukipuro K, Kairaluoma MI, Sly WS. Immunohistochemistry of carbonic anhydrase isozyme IX (MN/CA IX) in human gut reveals polarized expression in the epithelial cells with the highest proliferative capacity. J Histochem Cytochem 1998b;46:497-504.

Sáez C, Japón MA, Ramos-Morales F, Romero F, Segura DI, Tortolero M, Pintor-Toro JA. hpttg is over-expressed in pituitary adenomas and other primary epithelial neoplasias. Oncogene 1999;18:5473-5476.

Sáez C, Martínez-Brocca MA, Castilla C, Soto A, Navarro E, Tortolero M, Pintor-Toro JA, Japón MA. Prognostic significance of human pituitary tumor-transforming gene immunohistochemical expression in differentiated thyroid cancer. J Clin Endocrinol Metab 2006;91:1404-1409.

Salehi F, Kovacs K, Scheithauer BW, Lloyd RV, Cusimano M. Pituitary tumor-transforming gene in endocrine and other neoplasms: a review and update. Endocr Relat Cancer 2008;15:721-743.

Sallinen SL, Sallinen PK, Haapasalo HK, Helin HJ, Helén PT, Schraml P, Kallioniemi OP, Kononen J. Identification of differentially expressed genes in human gliomas by DNA microarray and tissue chip techniques. Cancer Res 2000;60:6617-6622.

Salminen E, Palmu S, Vahlberg T, Roberts PJ, Söderström KO. Increased proliferation activity measured by immunoreactive Ki67 is associated with survival improvement in rectal/recto sigmoid cancer. World J Gastroenterol 2005;11:3245-3249.

Salvucci O, Bouchard A, Baccarelli A, Deschênes J, Sauter G, Simon R, Bianchi R, Basik M. The role of CXCR4 receptor expression in breast cancer: a large tissue microarray study. Breast Cancer Res Treat 2006;97:275-283.

Sandlund J, Oosterwijk E, Grankvist K, Oosterwijk-Wakka J, Ljungberg B, Rasmuson T. Prognostic impact of carbonic anhydrase IX expression

in human renal cell carcinoma. BJU Int 2007;100:556-560.

Sapino A, Marchiò C, Senetta R, Castellano I, Macrì L, Cassoni P, Ghisolfi G, Cerrato M, D'Ambrosio E, Bussolati G. Routine assessment of prognostic factors in breast cancer using a multicore tissue microarray procedure. Virchows Arch 2006;449:288-296.

Sara H, Kallioniemi O, Nees M. A decade of cancer gene profiling: from molecular portraits to molecular function. Methods Mol Biol 2010;576:61-87.

Sarrió D, Pérez-Mies B, Hardisson D, Moreno-Bueno G, Suárez A, Cano A, Martín-Pérez J, Gamallo C, Palacios J. Cytoplasmic localization of p120ctn and E-cadherin loss characterize lobular breast carcinoma from preinvasive to metastatic lesions. Oncogene 2004;23:3272-3283.

Schena M, Shalon D, Davis RW, Brown PO. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science 1995;270:467-470.

Schena M, Shalon D, Heller R, Chai A, Brown PO, Davis RW. Parallel human genome analysis: microarray-based expression monitoring of 1000 genes. Proc Natl Acad Sci U S A 1996;93:10614-10619.

Selvarajan S, Tan SY, Sii LH, Tan PH. c-erbB-2 (HER-2/neu) immunohistochemistry in invasive breast cancer: is there concordance between standard sections and tissue microarrays? Pathology 2006;38:316-320.

Senft AP, LeVine AM. Basic molecular biology. Paediatr Respir Rev 2005;6:199-208.

Shendure J. The beginning of the end for microarrays? Nat Methods 2008;5:585-587.

Shergill IS, Shergill NK, Arya M, Patel HR. Tissue microarrays: a current medical research tool. Curr Med Res Opin 2004;20:707-712.

Sherlock G. Of fish and chips. Nat Methods 2005;2:329-330.

Shibamoto S, Hayakawa M, Takeuchi K, Hori T, Miyazawa K, Kitamura N, Johnson KR, Wheelock MJ, Matsuyoshi N, Takeichi M, et al. Association of p120, a tyrosine kinase substrate, with E-cadherin/catenin complexes. J Cell Biol 1995;128:949-957.

Shibata T, Kokubu A, Sekine S, Kanai Y, Hirohashi S. Cytoplasmic p120ctn regulates the invasive phenotypes of E-cadherin-deficient breast cancer. Am J Pathol 2004;164:2269-2278.

Shibata Y, Haruki N, Kuwabara Y, Nishiwaki T, Kato J, Shinoda N, Sato A, Kimura M, Koyama H, Toyama T, Ishiguro H, Kudo J, Terashita Y, Konishi S, Fujii Y. Expression of PTTG (pituitary tumor transforming gene) in esophageal cancer. Jpn J Clin Oncol 2002;32:233-237.

Sikaroodi M, Galachiantz Y, Baranova A. Tumor markers: the potential of "omics" approach. Curr Mol Med 2010;10:249-257.

Sillanpää N, Magureanu CG, Murumägi A, Reinikainen A, West A, Manninen A, Lahti M, Ranki A, Saksela K, Krohn K, Lahesmaa R, Peterson P. Autoimmune regulator induced changes in the gene expression profile of human monocyte-dendritic cell-lineage. Mol Immunol 2004;41:1185-1198.

Silva Neto B, Smith GL, Mandeville JA, Vanni AJ, Wotkowicz C, Rieger-Christ KM, Baumgart E, Jacobs MA, Cohen MS, Zeheb R, Loda M, Libertino JA, Summerhayes IC. Prognostic significance of altered p120 ctn expression in bladder cancer. BJU Int 2008;101:746-752.

Simon R, Radmacher MD, Dobbin K. Design of studies using DNA microarrays. Genet Epidemiol 2002;23:21-36.

Simon R, Sauter G. Tissue microarrays for miniaturized high-throughput molecular profiling of tumors. Exp Hematol 2002;30:1365-1372.

Simpson KL, Whetton AD, Dive C. Quantitative mass spectrometry-based techniques for clinical use: biomarker identification and quantification. J Chromatogr B Analyt Technol Biomed Life Sci 2009;877:1240-1249.

Simpson PT, Reis-Filho JS, Gale T, Lakhani SR. Molecular evolution of breast cancer. J Pathol 2005;205:248-254.

Skoudy A, Gomez S, Fabre M, Garcia de Herreros A. p120-catenin expression in human colorectal cancer. Int J Cancer 1996;68:14-20.

Slodkowska EA, Ross JS. MammaPrint 70-gene signature: another milestone in personalized medical care for breast cancer patients. Expert Rev Mol Diagn 2009;9:417-422.

Solbach C, Roller M, Eckerdt F, Peters S, Knecht R. Pituitary tumor-transforming gene expression is a prognostic marker for tumor recurrence in squamous cell carcinoma of the head and neck. BMC Cancer 2006;6:242.

Solbach C, Roller M, Fellbaum C, Nicoletti M, Kaufmann M. PTTG mRNA expression in primary breast cancer: a prognostic marker for

lymph node invasion and tumor recurrence. Breast 2004;13:80-81.

Soto E, Yanagisawa M, Marlow LA, Copland JA, Perez EA, Anastasiadis PZ. p120 catenin induces opposing effects on tumor cell growth depending on E-cadherin expression. J Cell Biol 2008;183:737-749.

Stears RL, Getts RC, Gullans SR. A novel, sensitive detection system for high-density microarrays using dendrimer technology. Physiol Genomics 2000;3:93-99.

Stefansson IM, Salvesen HB, Akslen LA. Prognostic impact of alterations in P-cadherin expression and related cell adhesion markers in endometrial cancer. J Clin Oncol 2004;22:1242-1252.

Stoeckert CJ Jr, Causton HC, Ball CA. Microarray databases: standards and ontologies. Nat Genet 2002;32 Suppl:469-473.

Su Y, Shrubsole MJ, Ness RM, Cai Q, Kataoka N, Washington K, Zheng W. Immunohistochemical expressions of Ki-67, cyclin D1, beta-catenin, cyclooxygenase-2, and epidermal growth factor receptor in human colorectal adenoma: a validation study of tissue microarrays. Cancer Epidemiol Biomarkers Prev 2006;15:1719-1726.

Supuran CT. Carbonic anhydrases: novel therapeutic applications for inhibitors and activators. Nat Rev Drug Discov 2008;7:168-181.

Suzuki H, Masuda N, Shimura T, Araki K, Kobayashi T, Tsutsumi S, Asao T, Kuwano H. Nuclear beta-catenin expression at the invasive front and in the vessels predicts liver metastasis in colorectal carcinoma. Anticancer Res 2008;28:1821-1830

Swietach P, Vaughan-Jones RD, Harris AL. Regulation of tumor pH and the role of carbonic anhydrase 9. Cancer Metastasis Rev 2007;26:299-310.

Sørlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, Hastie T, Eisen MB, van de Rijn M, Jeffrey SS, Thorsen T, Quist H, Matese JC, Brown PO, Botstein D, Eystein Lønning P, Børresen-Dale AL. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A 2001;98:10869-10874.

Takemasa I, Higuchi H, Yamamoto H, Sekimoto M, Tomita N, Nakamori S, Matoba R, Monden M, Matsubara K. Construction of preferential cDNA microarray specialized for human colorectal carcinoma: molecular sketch of colorectal cancer. Biochem Biophys Res Commun 2001;285:1244-1249.

Takikita M, Chung JY, Hewitt SM. Tissue microarrays enabling high-throughput molecular pathology. Curr Opin Biotechnol 2007;18:318-325.

Tan PK, Downey TJ, Spitznagel EL Jr, Xu P, Fu D, Dimitrov DS, Lempicki RA, Raaka BM, Cam MC. Evaluation of gene expression measurements from commercial microarray platforms. Nucleic Acids Res 2003;31:5676-5684.

Tavassoli FA, Devilee P (Eds.). World Health Organization Classification of Tumours. Pathology and Genetics of Tumours of the Breast and Female genital Organs. IARC Press, Lyon, 2003.

Taylor E, Cogdell D, Coombes K, Hu L, Ramdas L, Tabor A, Hamilton S, Zhang W. Sequence verification as quality-control step for production of cDNA microarrays. Biotechniques 2001;31:62-65.

Tfelt-Hansen J, Kanuparthi D, Chattopadhyay N. The emerging role of pituitary tumor transforming gene in tumorigenesis. Clin Med Res 2006;4:130-137.

Thoreson MA, Anastasiadis PZ, Daniel JM, Ireton RC, Wheelock MJ, Johnson KR, Hummingbird DK, Reynolds AB. Selective uncoupling of p120(ctn) from E-cadherin disrupts strong adhesion. J Cell Biol 2000;148:189-202.

Thoreson MA, Reynolds AB. Altered expression of the catenin p120 in human cancer: implications for tumor progression. Differentiation 2002;70:583-589.

Tong Y, Tan Y, Zhou C, Melmed S. Pituitary tumor transforming gene interacts with Sp1 to modulate G1/S cell phase transition. Oncogene 2007;26:5596-5605.

Torhorst J, Bucher C, Kononen J, Haas P, Zuber M, Köchli OR, Mross F, Dieterich H, Moch H, Mihatsch M, Kallioniemi OP, Sauter G Tissue microarrays for rapid linking of molecular changes to clinical endpoints. Am J Pathol 2001;159:2249-2256.

Tothill RW, Kowalczyk A, Rischin D, Bousioutas A, Haviv I, van Laar RK, Waring PM, Zalcberg J, Ward R, Biankin AV, Sutherland RL, Henshall SM, Fong K, Pollack JR, Bowtell DD, Holloway AJ. An expression-based site of origin diagnostic method designed for clinical application to cancer of unknown origin. Cancer Res 2005;65:4031-4340.

Tumor Analysis Best Practices Working Group. Expression profiling--best practices for data generation and interpretation in clinical trials. Nat Rev Genet 2004;5:229-237.

Uemura H, Fujimoto K, Tanaka M, Yoshikawa M, Hirao Y, Uejima S, Yoshikawa K, Itoh K. A phase I trial of vaccination of CA9-derived peptides for HLA-A24-positive patients with cytokine-refractory metastatic renal cell carcinoma. Clin Cancer Res 2006;12:1768-1775.

Uhlmann F, Lottspeich F, Nasmyth K. Sister-chromatid separation at anaphase onset is promoted by cleavage of the cohesin subunit Scc1. Nature 1999;400:37-42.

Umar A, Kang H, Timmermans AM, Look MP, Meijer-van Gelder ME, den Bakker MA, Jaitly N, Martens JW, Luider TM, Foekens JA, Pasa-Tolić L. Identification of a putative protein profile associated with tamoxifen therapy resistance in breast cancer. Mol Cell Proteomics 2009;8:1278-1294.

Van Gelder RN, von Zastrow ME, Yool A, Dement WC, Barchas JD, Eberwine JH. Amplified RNA synthesized from limited quantities of heterogeneous cDNA. Proc Natl Acad Sci U S A 1990;87:1663-1667.

Vani K, Sompuram SR, Fitzgibbons P, Bogen SA. National HER2 proficiency test results using standardized quantitative controls: characterization of laboratory failures. Arch Pathol Lab Med 2008;132:211-216.

Vendrell JA, Robertson KE, Ravel P, Bray SE, Bajard A, Purdie CA, Nguyen C, Hadad SM, Bieche I, Chabaud S, Bachelot T, Thompson AM, Cohen PA. A candidate molecular signature associated with tamoxifen failure in primary breast cancer. Breast Cancer Res 2008;10:R88.

Vlotides G, Eigler T, Melmed S. Pituitary tumor-transforming gene: physiology and implications for tumorigenesis. Endocr Rev 2007;28:165-186.

Voduc D, Kenney C, Nielsen TO. Tissue microarrays in clinical oncology. Semin Radiat Oncol 2008;18:89-97.

Waizenegger I, Giménez-Abián JF, Wernic D, Peters JM. Regulation of human separase by securin binding and autocleavage. Curr Biol 2002;12:1368-1378.

Walker RA. Immunohistochemical markers as predictive tools for breast cancer. J Clin Pathol 2008;61:689-696.

Walther A, Johnstone E, Swanton C, Midgley R, Tomlinson I, Kerr D. Genetic prognostic and predictive markers in colorectal cancer. Nat Rev Cancer 2009;9:489-499.

Wan WH, Fortuna MB, Furmanski P. A rapid and efficient method for testing immunohistochemical reactivity of monoclonal antibodies against multiple tissue samples simultaneously. J Immunol Methods 1987;103:121-129.

Wang S, Saboorian MH, Frenkel EP, Haley BB, Siddiqui MT, Gokaslan S, Wians FH Jr, Hynan L, Ashfaq R. Assessment of HER-2/neu status in breast cancer. Automated Cellular Imaging System (ACIS)-assisted quantitation of immunohistochemical assay achieves high accuracy in comparison with fluorescence in situ hybridization assay as the standard. Am J Clin Pathol 2001;116:495-503.

Wang W, Yang L, Suwa T, Casson PR, Hornsby PJ. Differentially expressed genes in zona reticularis cells of the human adrenal cortex. Mol Cell Endocrinol 2001;173:127-134.

Wang Z, Moro E, Kovacs K, Yu R, Melmed S. Pituitary tumor transforming gene-null male mice exhibit impaired pancreatic beta cell proliferation and diabetes. Proc Natl Acad Sci U S A 2003;100:3428-3432.

Wang Z, Yu R, Melmed S. Mice lacking pituitary tumor transforming gene show testicular and splenic hypoplasia, thymic hyperplasia, thrombocytopenia, aberrant cell cycle progression, and premature centromere division. Mol Endocrinol 2001;15:1870-1879.

Webb SD, Sherratt JA, Fish RG. Alterations in proteolytic activity at low pH and its association with invasion: a theoretical model. Clin Exp Metastasis 1999;17:397-407.

Wei EK, Giovannucci E, Wu K, Rosner B, Fuchs CS, Willett WC, Coldiz GA. Comparison of risk factors for colon and rectal cancer. Int J Cancer 2004;108:433-442.

Wheelock MJ, Soler AP, Knudsen KA. Cadherin junctions in mammary tumors. J Mammary Gland Biol Neoplasia 2001;6:275-285.

Whetzel PL, Parkinson H, Causton HC, Fan L, Fostel J, Fragoso G, Game L, Heiskanen M, Morrison N, Rocca-Serra P, Sansone SA, Taylor C, White J, Stoeckert CJ Jr. The MGED Ontology: a resource for semantics-based description of microarray experiments. Bioinformatics 2006;22:866-773.

Wierinckx A, Auger C, Devauchelle P, Reynaud A, Chevallier P, Jan M, Perrin G, Fèvre-Montange M, Rey C, Figarella-Branger D, Raverot G, Belin MF, Lachuer J, Trouillas J. A diagnostic marker set for invasion, proliferation, and aggressiveness of prolactin pituitary tumors. Endocr Relat Cancer 2007;14:887-900.

Wijnhoven BP, Pignatelli M, Dinjens WN, Tilanus HW. Reduced p120ctn expression correlates with

poor survival in patients with adenocarcinoma of the gastroesophageal junction. J Surg Oncol 2005;92:116-123.

Wilkinson N, Scott-Conner CE. Surgical therapy for colorectal adenocarcinoma. Gastroenterol Clin North Am 2008;37:253-267.

Williams NS, Gaynor RB, Scoggin S, Verma U, Gokaslan T, Simmang C, Fleming J, Tavana D, Frenkel E, Becerra C. Identification and validation of genes involved in the pathogenesis of colorectal cancer using cDNA microarrays and RNA interference. Clin Cancer Res 2003;9:931-946.

Winnepenninckx V, Debiec-Rychter M, Beliën JA, Fiten P, Michiels S, Lazar V, Opdenakker G, Meijer GA, Spatz A, van den Oord JJ. Expression and possible role of hPTTG1/securin in cutaneous malignant melanoma. Mod Pathol 2006;19:1170-1180.

Wolpin BM, Mayer RJ. Systematic treatment of colorectal cancer. Gastroenterology 2008;134:1296-1310.

Wykoff CC, Beasley N, Watson PH, Campo L, Chia SK, English R, Pastorek J, Sly WS, Ratcliffe P, Harris AL. Expression of the hypoxia-inducible and tumor-associated carbonic anhydrases in ductal carcinoma in situ of the breast. Am J Pathol 2001;158:1011-1019.

Wärnberg F, Amini RM, Goldman M, Jirström K. Quality aspects of the tissue microarray technique in a population-based cohort with ductal carcinoma in situ of the breast. Histopathology 2008;53:642-649.

Yamasaki M, Takemasa I, Komori T, Watanabe S, Sekimoto M, Doki Y, Matsubara K, Monden M. The gene expression profile represents the molecular nature of liver metastasis in colorectal cancer. Int J Oncol 2007;30:129-138.

Yanagisawa M, Huveldt D, Kreinest P, Lohse CM, Cheville JC, Parker AS, Copland JA, Anastasiadis PZ. A p120 catenin isoform switch affects Rho activity, induces tumor cell invasion, and predicts metastatic disease. J Biol Chem 2008;283:18344-18354.

Yang SX, Hewitt SM, Steinberg SM, Liewehr DJ, Swain SM. Expression levels of eIF4E, VEGF, and cyclin D1, and correlation of eIF4E with VEGF and cyclin D1 in multi-tumor tissue microarray. Oncol Rep 2007;17:281-287.

Yang XR, Charette LA, Garcia-Closas M, Lissowska J, Paal E, Sidawy M, Hewitt SM, Rimm DL, Sherman ME. Construction and validation of tissue microarrays of ductal carcinoma in situ and terminal duct lobular units associated with invasive breast carcinoma. Diagn Mol Pathol 2006;15:157-161.

Yang YH, Dudoit S, Luu P, Lin DM, Peng V, Ngai J, Speed TP. Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation. Nucleic Acids Res 2002;30:e15.

Yap AS, Niessen CM, Gumbiner BM. The juxtamembrane region of the cadherin cytoplasmic tail supports lateral clustering, adhesive strengthening, and interaction with p120ctn. J Cell Biol 1998;141:779-789.

Yauk CL, Berndt ML, Williams A, Douglas GR. Comprehensive comparison of six microarray technologies. Nucleic Acids Res 2004;32:e124.

Yoshida S, Yashar BM, Hiriyanna S, Swaroop A. Microarray analysis of gene expression in the aging human retina. Invest Ophthalmol Vis Sci 2002;43:2554-2560.

Yu J, Othman MI, Farjo R, Zareparsi S, MacNee SP, Yoshida S, Swaroop A. Evaluation and optimization of procedures for target labeling and hybridization of cDNA microarrays. Mol Vis 2002;8:130-137.

Yu R, Heaney AP, Lu W, Chen J, Melmed S. Pituitary tumor transforming gene causes aneuploidy and p53-dependent and p53-independent apoptosis. J Biol Chem 2000a;275:36502-36505.

Yu R, Lu W, Chen J, McCabe CJ, Melmed S. Overexpressed pituitary tumor-transforming gene causes aneuploidy in live human cells. Endocrinology 2003;144:4991-4998.

Yu R, Ren SG, Horwitz GA, Wang Z, Melmed S. Pituitary tumor transforming gene (PTTG) regulates placental JEG-3 cell division and survival: evidence from live cell imaging. Mol Endocrinol 2000b;14:1137-1146.

Yue H, Eastman PS, Wang BB, Minor J, Doctolero MH, Nuttall RL, Stack R, Becker JW, Montgomery JR, Vainer M, Johnston R. An evaluation of the performance of cDNA microarrays for detecting changes in global mRNA expression. Nucleic Acids Res 2001;29:E41-1.

Závada J, Závadová Z, Pastorek J, Biesová Z, Jezek J, Velek J. Human tumour-associated cell adhesion protein MN/CA IX: identification of M75 epitope and of the region mediating cell adhesion. Br J Cancer 2000;82:1808-1813.

Závada J, Závadová Z, Pastoreková S, Ciampor F, Pastorek J, Zelník V. Expression of MaTu-MN protein in human tumor cultures and in clinical specimens. Int J Cancer 1993;54:268-274.

Zeeberg BR, Feng W, Wang G, Wang MD, Fojo AT, Sunshine M, Narasimhan S, Kane DW, Reinhold WC, Lababidi S, Bussey KJ, Riss J, Barrett JC, Weinstein JN. GoMiner: a resource for biological interpretation of genomic and proteomic data. Genome Biol 2003;4:R28.

Zembutsu H, Ohnishi Y, Tsunoda T, Furukawa Y, Katagiri T, Ueyama Y, Tamaoki N, Nomura T, Kitahara O, Yanagawa R, Hirata K, Nakamura Y. Genome-wide cDNA microarray screening to correlate gene expression profiles with sensitivity of 85 human cancer xenografts to anticancer drugs. Cancer Res 2002;62:518-527.

Zhang D, Salto-Tellez M, Putti TC, Do E, Koay ES. Reliability of tissue microarrays in detecting protein expression and gene amplification in breast cancer. Mod Pathol 2003;16:79-84.

Zhang X, Horwitz GA, Heaney AP, Nakashima M, Prezant TR, Bronstein MD, Melmed S. Pituitary tumor transforming gene (PTTG) expression in pituitary adenomas. J Clin Endocrinol Metab 1999a;84:761-767.

Zhang X, Horwitz GA, Prezant TR, Valentini A, Nakashima M, Bronstein MD, Melmed S. Structure, expression, and function of human pituitary tumor-transforming gene (PTTG). Mol Endocrinol 1999b;13:156-166.

Zhang Z, Schwartz S, Wagner L, Miller W. A greedy algorithm for aligning DNA sequences. J Comput Biol 2000;7:203-214.

Zhu X, Mao Z, Na Y, Guo Y, Wang X, Xin D. Significance of pituitary tumor transforming gene 1 (PTTG1) in prostate cancer. Anticancer Res 2006;26:1253-1259.

Zou H, McGarry TJ, Bernal T, Kirschner MW. Identification of a vertebrate sister-chromatid separation inhibitor involved in transformation and tumorigenesis. Science 1999;285:418-422.

Internet resources

Finnish Cancer Registry: http://www.cancerregistry.fi/

http://las.perkinelmer.com/content/technicalinfo/tch_scanarraymicroarrayspotqc.pdf

http://www.abcam.com/Carbonic-Anhydrase-IX-antibody-ab15086.html

http://www.biocompare.com/Articles/FeaturedArticle/1111/Microarray-Instrumentation-And-Software.html

http://www.biocompare.com/Documents/surveys_files/ExecSumm/DNAMicro_2006_execsumm.pdf

http://www.bioconductor.org/

http://www.fda.gov/ScienceResearch/BioinformaticsTools/MicroarrayQualityControlProject/default.htm

http://www.mged.org/

http://www.mged.org/Workgroups/MIAME/journals.html

http://www.mged.org/Workgroups/MIAME/miame.html

http://www.mged.org/Workgroups/MIAME/miame_2.0.html

http://www.mrcgene.com/tri.htm

http://www.phalanxbiotech.com/tech_support/faqs.html

http://www.switchtoi.com/annotationfiles.ilmn

http://tools.invitrogen.com/content/sfs/manuals/10296010.pdf