Correlation Between mRNA Detected by Microarrays and qRT-PCR and Protein Detected by Immunohistochemistry of Cyclins in Tumour Tissue from Colonic Adenocarcinomas

K. Jonsdottir[*,1], R. Størkson[1,2], A. Krog[1] and I. R. K. Bukholm[3,4]

[1]*Institute of Epidemiology and Molecular Biology (EpiGen), Faculty Division Akershus University Hospital, University of Oslo, Oslo, Norway*

[2]*Department of Surgery, Hospital of Østfold, Fredrikstad, Norway*

[3]*Department of Surgery, Akershus University Hospital, Lørenskog, Norway*

[4]*Faculty Division Akershus University Hospital, University of Oslo, Oslo, Norway*

Abstract: There are several studies evaluating the prognostic value of overexpression of cyclin proteins, detected by immunohistochemistry. Overexpression detected by microarray and real-time PCR techniques is now applied in analyzing the prognostic value of different genes on patient outcome. In this study we wanted to evaluate the correlation between these three methods for detection of cyclins (A2, B1, D1, D3, E and H) overexpression, as well as correlation between overexpression and gene amplification of the cyclin genes. We used both normal mucosa and tumour tissue obtained from 22 patients at the time of surgery. None of the cyclins were defined to be amplified at the gene level. qRT-PCR and cDNA microarray results are comparable, but no correlation between gene expression and protein expression, measured by immunohistochemistry was observed. This may indicate that immunohistochemistry detects some other parameters than gene expression analysis measured by cDNA microarray and qRT-PCR. It is of interest to evaluate which of the three methods may give the most important information about patient prognosis.

Keywords: Colonic adenocarcinomas, real-time PCR, microarray, immunohistochemistry.

INTRODUCTION

Colorectal cancer, as with all other solid cancers, is a heterogeneous disease, both in terms of its molecular properties as well as in its diverse clinical behavior. Currently, histological staging is the only satisfactory classification method for predicting treatment outcome following surgical resection [1]. However, there is considerable prognostic heterogeneity within each solid tumor stage, as tumor with similar histopathological characteristics can have distinct clinical outcome [2]. Thus, clinical and histopathological staging system often fails to discriminate the biological behavior of different tumors, resulting in an inadequate treatment. Consequently, the urgent need for new prognostic strategies has engendered the search for alternative techniques that allow rapid, accurate and personalized detection of cancer markers for prognosis.

Several sensitive, specific and potentially high-throughput techniques for cancer screening have been developed. Micro array technology is one of them and is widely used to detect gene expression levels in biology and in the clinical setting [3, 4]. The benefit obtained with the method is the large amount of information that can be retrieved at the same time, but there are some doubts if this method is reliable [5]. Micro array results are influenced by array production, RNA extraction, probe labeling, hybridization conditions and image analysis [5-7]. Because of the inherent limitations in reliability, the genes are identified as differentially expressed, especially those of interest must be validated with other methods.

Real-time polymerase chain reaction (real-time PCR) is an *in vitro* method for enzymatically amplifying defined sequences of DNA or RNA [8, 9]. The method is sensitive and very flexible quantitative and makes it possible to determine gene duplications or deletions, and also expression level of target genes.

Immunohistochemistry is a widely used method to study protein expression of specific antigens. The method permits use of archive material, such as paraffin-embedded formalin fixed tissue. Although the method is sensitive, immunohistochemistry results are not quantitative and there are no standardized scoring systems and no uniformly accepted threshold for positivity.

Since immunohistochemistry is a commonly used method to determine cancer patient's prognosis, it is important to validate the method with other molecular methods. Real-time PCR and microarray are more rapid and accurate methods for analysing many genes at the same time. However, limited information about correlation between immunohistochemistry, microarray and real-time PCR methods exists [10, 11]. If these methods are comparable, it will be possible to evaluate results regarding the impact of a potential cancer marker at DNA, RNA and protein level at the same time. In the present study we have analyzed expression

*Address correspondence to this author at the Institute of Epidemiology and Molecular Biology (EpiGen), Faculty Division Akershus University Hospital, University of Oslo, Oslo, Norway; Tel: +47 67984421;
Fax: 47 67927803; E-mail: kristin.jonsdottir@medisin.uio.no

1874-3757/08

of six different cyclins using three different methods of detection and correlate the results between these. Expression of cyclins was chosen for this study because cyclins are fundamental in cell cycle regulation and deregulation of cyclins may be apparent in all stages of the colorectal cancer.

MATERIALS AND METHODS

Tissue Samples

In this study we used colorectal tissue as representative for solid tumors. Twenty-two patients who were operated for colorectal cancer were included in the study, 11 women and 11 men. Median age at time of surgery was 74.5 years (Range 35-86 years). Representative samples of tumor tissue and normal mucosa were taken from the resected colon/rectum and the samples were immediately stabilized and stored in a tube containing RNAlaterTM (Ambion Inc., Austin, Texas) and kept at 4°C for up to 2 weeks, before the RNAlater was removed from the tissue and tumor samples stored at -80°C.

DNA/RNA Isolation

Colorectal tissue was homogenized in 1 ml of TRIzol Reagent (Life Technologies, Inc., Gaithersburg, MD) with a dispergierstation T8.10 (IKA labortechnik Inc., Staufen, Germany). Total RNA was extracted according to a hybrid protocol of Trizol Reagent and RNeasy Mini Kit (Qiagen, Valencia, CA), described by Wei and Khan [12]. RNA was dissolved in diethylpyrocarbonate treated water (Milli-Q Synthesis A10, Millipore, USA). The quality and quantity of total RNA isolated was determined using Agilent's 2100 Bioanalyser with RNA Nano LabChip (Agilent Technologies, Palo Alta, CA, USA). Samples were stored at -80°C. The DNA was extracted using the Qiagen's DNeasy Tissue Kit (Qiagen, Hilden, Germany), dissolved in diethylpyrocarbonate treated water (Millipore) and the concentration was measured by NanoDrop ND-1000 Spectrophotometer (Nano Drop Technologies Wilmington, Delaware, USA).

Real-Time PCR

Real-time PCR was performed on DNA and complementary DNA (cDNA) for the genes ccn A2, ccn B1, ccn D1, ccn D3, ccn E1 and ccn H. Primers and probes was design as described by Bondi *et al*. [13]. Sequences for DNA primers and probes are shown in the same article [13] and sequences for cDNA primers and probes are shown in Table **1**.

Real-time PCR was performed using the ABI Prism 7900HT Sequences detection system (Applied Biosystems, Foster City, CA, USA) with the software program SDS2.1. The PCR mix consisted of 1 µl DNA or 1 µl total RNA (10-50ng/µl), 2x TaqMan Universal PCR Master Mix or Taqman One-Step RT-PCR Master Mix Reagents Kit (both from Applied Biosystems), 600 nM of forward and reverse and 200 nM probes (except for HSA forward and reverse primers that were used at 700nM). We added water to the total reaction volume of 25µl. In every experiment all samples were run as triplicates. The calibrator sample was analyzed on every assay together with a patient sample (tumor and normal) and a negative control (water). Default thermal cycling conditions were used in the PCR (Applied Biosystems). Threshold cycle number, Ct, for the real time quantification was defined to be in the exponential phase of the PCR amplification. The calibrator used in the real-time PCR experiments was the patient normal mucosa tissue. To determinate the relative gene copy number of DNA, we used the $2^{-\Delta\Delta Ct}$ – method [14]. To determinate the relative expression of cDNA we used relative standard curve (Applied Biosystems User Bulletin No. 2 (P/N 4303859)). Since we did not find GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as a suitable reference gene, we normalized all copy numbers to total RNA concentration, which we measured accurately using NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies Wilmington, Delaware, USA). All concentrations were measured three times and the mean was calculated. Copy number of target gene was then recorded as copy number per µg total RNA. To generate the relative expression levels we divided the normalized target values by the calibrator normalized target value [15].

Table 1. List of cDNA Primer and Probe Sequences Used in the qRT-PCR of the Indicated Genes

Gene	Primer and Probe Sequence
Cyclin A2-sense	CATTGGTCCCTCTTGATTATCCA
-antisense	GGCTTTTCATCTTCTAATACAATTGACA
-probe	TGGATGGTAGTTTTGAGTCACCACATACTATGGAC
Cyclin B1-sense	CATGGCGCTCCGAGTCA
-antisense	GCGCCTGCCATGTTGATC
-probe	CGCCTTATTTTCAGCATTAATTTTCGAGTTCCTG
Cyclin D1-sense	CCGTCCATGCGGAAGATC
-antisense	GAAGACCTCCTCCTCGCACTT
-probe	CTCGCAGACCTCCAGCATCCAGGT
Cyclin D3-sense	CAGGCCTTGGTCAAAAAGCA
-antisense	GCGGGTACATGGCAAAGGTA
-probe	AGACCTTTTTGGCCCTCTGTGCTACAGATT
Cyclin E1-sense	TCCAGGAAGAGGAAGGCAAAC
-antisense	CCTGTCGATTTTGGCCATTT
-probe	CATCTGGATCCTGCAAAAAAACGGTCA
Cyclin H-sense	CAACCGCAAATTCAGATGCA
-antisense	GAAAGACTGGATCATTCGGAAGA
-probe	CCTTCCCGTTGGCCACGGCT
GADPH -sense	CCACATCGCTCAGACACCAT
-antisense	CCAGGCGCCCAATACG
-probe	CAAATCCGTTGACTCCGACCTTCACCT

Microarray

From isolated total RNA samples we amplified total RNA by using MessageAmpTM aRNA Kit (Ambion, Austin, Texas, USA). To determine the quality and amount of ampli-

Table 2. Antibodies and Protocol Used in Immunohistochemistry

Antibody	Sources	Dilution	Pre-Treatment (Microwave Oven, 850W)
Cyclin A2	Novocastra Laboratories (Newcastle-upon-Tyne, UK)	1:75	2 x 5 min, 1mM EDTA (pH 8)
Cyclin B1	BioSource International (Canarillo, CA)	1:200	5 min + 15 min 350W, 10mM Citrate buffer (pH 6)
Cyclin D1	Oncogene Reasearch (San Diego, CA)	1:100	2 x 10 min 350W, 1mM Citrate buffer (pH 6)
Cyclin D3	Dako (Carpinteria, CA)	1:50	2 x 5 min, 1mM EDTA (pH 8)
Cyclin E	Novocastra Laboratories (Newcastle-upon-Tyne, UK)	1:75	2 x 5 min, 1mM EDTA (pH 8)
Cyclin H	Santa Cruz Biotechnology(Santa Cruz, CA)	1:300	5 min + 15 min 350W, Tris-EDTA (pH 9)

fied RNA (aRNA) synthesised, we used the 2100 Bioanalyser (Agilent Technologies, Palo Alto, CA). The aRNA samples (tumor and normal) were labeled with Cyanine3-dCTP or Cyanine5-dCTP (Perking-Elmer Life Sciences, Inc., Boston, MA, USA) using the Flourescent Direct Label Kit (Agilent). Instead of using 200 ng of aRNA, we used 600 ng. The labelled cDNA samples were hybridised to Agilents Human 1 cDNA microarrays (Agilent) with 12 814 unique clones. The tumor cDNA was hybridized with the normal cDNA from the same patient. For every experiment there was preformed a dye swap. The slides were scanned on an Agilent Technologies G2565BA Micro array Scanner (Agilent).

Statistical Analysis

Feature Extraction and data normalization were conducted with Agilent Feature Extraction software version 7.5. Spots that did not pass quality control procedures were flagged by the software and removed from the analysis. The normalized data was transferred to Rosetta Luminator software (Rosetta Biosoftware, Seattle, WA, USA). The relative expression level was measured by normalized ratio of signal intensities for Cy5/Cy3 fluorescence. The cut off value for gene over expression, as well as for gene amplification was set at level $l > 2$. The statistical software program SPSS, was used for the correlation analysis. Pearson correlation coefficient was calculated for logarithmically transformed data obtained with qRT-PCR and microarray. For correlations with immunohistochemistry Kendall's tau was used. The predefined significance level was set to $p < 0.05$.

Immunohistochemistry

The immunohistochemistry methods have previously been described [13]. Tumour tissues were preserved in RNAlater, then formalin fixed and paraffin wax embedded. Sections (4-6 μm thick) were applied to coated slides. After antigen retrieval by microwaving, immunostaining was performed in an Optimax plus automated cell stained (model 1.5; BioGenex, San Ramon, California, USA), using the biotin-streptavin-peroxidase method (Supersensitive Immunodetection system, LP-UL; Biogenex). The slides were incubated at room temperature for 30 minutes and counterstained with Mayer`s haematoxylin for visualization of tissue structures. Immunostaining protocol and antibodies used, see Table **2**. All series included positive and negative controls. A semi quantitative score for immunoreactivity was used as follows; grade 0 immunoreactivity as detected in the normal mucosa; + up to 30% positive cells, ++ 31-70% positive cells and +++ more than 70% positive cells. Only cells with convincing nuclear staining were scored as positive. However, if present, also cytoplasmic staining was observed and noted. For all samples at least 100 cells (if available more than 1000) were analyzed in higher power microscopic fields from all parts of the tumor tissue in each slide. We also had excess to formalin fixed embedded tumor tissue which not had been in contact with RNAlater. This material was used for control of a possible effect of RNAlater.

RESULTS

The samples were analyzed at the DNA, RNA and at the protein level. The results for all the samples are shown in Table **3**. None of the tumor samples had any amplification of cyclins at the DNA level. As can be seen from the Table **3** the numbers of samples with gene expression recorded by qRT-RCR was as follows: cyclin A2 (11 of 22 cases), B1 (14 of 22 cases), D1 (17 of 22 cases), D3 (2 of 22 cases), E (13 of 22 cases), H (1 of 22 cases) and GAPDH (10 of 22 cases). Upregulation of GAPDH was observed in tumor tissue were also one or more of the cyclins were upregulated, making this gene inappropriate as an endogenous control gene.

The altitude of expression level was lower in cDNA microarray analyses compared to qRT-PCR methods. The gene expression recorded by microarray and by qRT-RCR correlated significantly for cyclins B1, D1, D3 and E, whereas for cyclins A2 and H no significant correlation was detected, Table **4**. Although cyclin H expression level for most of the samples are between 0 - 1.9 for this to methods, they do not have the same or opposite tendency. Values tend to vary randomly for this to variables.

Immunohistochemistry was performed to examine the correlation between RNA expression and the presence of the genetic product in both RNAlater stabilised tumour tissue and paraffin embedded tumour tissue. The immunohistochemistry results correlated strongly between these two methods of preservation. Positive immunoreactivity was observed for only cyclin A2, B1 and H. The correlation between mRNA level and protein expression was significant for only cyclin A2.

DISCUSSION

mRNA was detected using both microarray and qRT-PCR methods. The correlation between the two methods for RNA expression was good for the cyclins that had a significant expression compared to the control. Previously studies have shown a good correlation between these two methods [16-18]. qRT-PCR is generally considered as the "gold-standard" assay for measuring gene expression, while gene

Table 3. Amplification Level, Expression Level and Immunoreactivity of the Cyclins in Colorectal Cancer

	Amplification Level of Cyclins Measured by qPCR*						RNA Expression Level of Cyclins Measured by qRT-PCR#							RNA Expression Level of Cyclins, Measured by cDNA Microarray¤						Protein Expression Level of Cyclins, Measured by Immunohistochemistry§					
Nr	**A2**	**B1**	**D1**	**D3**	**E1**	**H**	**GAPDH**	**A2**	**B1**	**D1**	**D3**	**E1**	**H**	**A2**	**B1**	**D1**	**D3**	**E1**	**H**	**A2**	**B1**	**D1**	**D3**	**E1**	**H**
1	0.80	1.02	1.18	1.21	1.01	1.01	1.76	1.37	0.95	3.59	1.04	2.86	1.58	0.90	0.69	2.09	0.57	1.23	1.12	1	0	0	0	0	3
2	1.02	0.85	0.87	0.78	0.97	0.99	2.17	1.83	2.90	3.97	0.65	1.24	1.08	1.00	2.40	2.56	0.63	1.22	1.31	1	0	0	0	0	1
3	1.24	1.07	1.46	1.09	0.93	0.96	2.68	15.99	10.74	3.45	2.18	3.00	0.69	1.00	4.27	2.05	1.55	1.88	1.28	1	0	0	0	0	1
4	0.94	1.01	0.85	0.98	1.04	1.02	1.74	1.59	1.56	6.48	1.06	1.08	1.29	1.00	1.04	1.89	0.72	1.03	1.53	0	0	0	0	0	2
5	1.02	0.96	0.79	0.83	1.01	1.01	2.09	1.48	2.49	2.72	0.92	4.80	1.17	1.21	1.10	1.69	0.83	1.61	1.04	2	0	0	0	0	2
6	0.95	1.96	1.48	1.68	1.03	1.02	0.81	0.77	0.91	1.35	1.00	2.01	1.27	0.74	1.10	1.14	0.88	1.01	0.86	1	1	0	0	0	2
7	0.89	0.72	0.50	0.67	1.07	1.04	0.71	0.16	0.28	1.56	0.35	0.25	1.12	0.76	0.97	1.49	0.64	0.88	1.25	-	-	-	-	-	-
8	1.08	0.91	0.87	1.02	1.04	1.02	4.46	16.11	12.10	3.65	1.53	11.47	0.77	1.00	3.57	3.38	1.36	2.48	1.12	0	0	-	0	0	3
9	0.84	0.89	0.73	0.87	1.04	1.02	2.43	1.91	2.62	1.38	0.84	1.02	1.29	1.15	1.21	1.14	0.83	1.00	1.13	1	0	0	0	0	0
10	1.69	1.24	1.12	1.15	1.01	1.01	2.22	2.14	3.91	3.85	1.40	4.85	0.71	1.41	1.50	1.81	1.05	1.37	0.94	1	2	0	0	0	2
11	0.79	0.75	0.57	0.79	1.08	1.04	1.48	2.82	2.22	5.50	0.50	3.72	1.39	1.00	1.69	2.45	0.84	1.50	1.36	2	0	0	0	0	0
12	0.86	0.79	0.80	0.76	0.99	0.99	2.16	2.71	4.03	2.40	1.00	1.80	0.86	1.98	2.68	1.74	0.91	2.29	1.35	2	0	0	0	0	0
13	0.95	0.85	1.29	0.80	0.89	0.94	1.70	3.91	3.62	2.92	0.35	5.72	1.19	2.40	3.25	2.11	0.46	1.41	1.44	2	0	0	0	0	3
14	0.73	0.71	0.56	0.74	0.77	0.94	1.56	0.66	1.60	1.56	0.61	1.01	0.69	1.54	1.58	1.28	0.57	1.18	1.45	3	0	0	0	0	0
15	1.22	0.78	0.91	0.86	1.01	0.27	3.21	49.85	22.05	2.90	1.25	10.05	2.12	1.00	2.46	2.35	1.00	1.37	0.99	1	0	0	0	0	3
16	1.40	0.82	0.90	0.73	0.95	0.97	1.22	6.52	5.72	5.14	1.21	1.05	1.01	1.00	1.42	2.20	1.24	1.30	0.85	1	1	0	0	0	1
17	0.76	1.03	1.10	1.02	0.98	0.99	1.65	2.92	4.73	3.17	1.20	2.41	1.37	2.47	1.95	1.51	0.93	1.88	1.40	2	0	0	0	0	0
18	0.89	0.98	1.75	0.93	0.85	0.92	3.40	10.86	12.26	5.72	1.51	2.77	1.27	3.65	2.38	3.33	1.03	1.33	1.34	2	0	0	0	0	0
19	1.19	1.04	0.98	0.94	0.99	0.99	1.61	2.14	2.38	4.17	0.93	2.24	1.26	0.83	1.64	2.68	0.75	1.15	1.21	1	0	0	0	0	3
20	1.32	0.81	0.84	0.75	0.97	0.99	0.39	0.50	0.59	3.39	0.56	0.23	0.56	0.94	0.85	2.74	0.87	0.79	0.97	1	0	0	0	0	0
21	0.88	1.26	0.95	1.20	1.06	1.03	2.38	1.98	1.01	1.52	0.78	0.73	1.21	0.79	0.84	1.48	0.50	0.84	1.02	0	0	-	0	0	0
22	1.24	0.89	1.09	0.91	0.96	0.98	1.95	1.09	1.44	6.06	2.00	2.23	1.09	0.53	0.69	2.38	1.04	1.38	0.76	0	0	0	0	0	0

*Amplification levels were determined $2^{-\Delta\Delta Ct}$ – method.

Expression levels were determined by a relative standard curve.

¤ Expression levels are the ratio between intensity of tumor tissue and normal tissue.

§ Immunohistochemistry on formalin-fixed, paraffin-embedded tumor tissue obtained from patients at the time of surgery and was first preserved in RNAlater. 4 categories: Grade 0: < 5 % positive cells; grade +: 5% to 30% positive cells; grade + +: 30% to 60%; grade + + +: > 60% positive cells.

expression measured by microarray has been found to necessitate validation with other methods, like qRT-PCR and protein expression analyses such as immunohistochemistry. In the present study, we performed such a comparison for six cyclin genes. Results show poor correlation between RNA expression and protein expression measured by immunohistochemistry. We only found a correlation between RNA expression using cDNA microarray and proteins detected in the cells by immunohistochemistry for cyclin A2. This may indicate that either the RNA overexpression detected by qRT-PCR and cDNA microarray did not result in a protein because of posttranscriptional regulation or that the protein was not detectable.

Some of the previous studies have indicated that there should be good correlation between qRT-PCR and immuno histochemistry if a gene is strongly upregulated gene [19, 20]. In our study, cyclin D1 was strongly upregulated, at the mRNA level, but no correlation was observed between

Table 4. Pearson Correlation Coefficients (CO) Between qRT-PCR and Microarray

	qRT-PCR *vs* Microarray	
Cyclin	**CO**	**P-Value**
Cyclin A2	0.302	> 0.05
Cyclin B1	0.782	< 0.01
Cyclin D1	0.771	< 0.01
Cyclin D3	0.748	< 0.01
Cyclin E	0.711	< 0.01
Cyclin H	0.037	> 0.05

qRT-PCR and immunohistochemistry for this protein. The reason for lack of correlation between these two methods may be that antibodies against cyclin D1 used for immunohistochemistry are unspecific. However, these antibodies have been used in several previously published studies and are well characterized.

The immunohistochemistry was performed on the same tumor tissue as for mRNA analyses, so the direct comparison between expression detected by mRNA analyses and expression detected by immunohistochemistry could be performed. Otherwise, tumor heterogeneity may be a problem if different part of a tumor has been analysed in different methods. In some studies there has been indicated that RNAlater may alter the tumor cell morphology and immuno histochemstry staining characteristics [21, 22]. However, we were not able to detect changes in tumor cell morphology comparing RNA later conserved tumor tissue and formalin fixed, paraffin embedded tumor tissue, so we do not believe that the lack of correlation is caused by RNAlater preservation of the tumor tissue.

We started our experiments using GAPDH as housekeeping gene because this gene has long been accepted as a control gene for mRNA analyses in colorectal cancer, and several studies have been published using GAPDH as reference gene [23-27]. Expression of housekeeping gene is assumed to remain constant, so normalizing for variation in processing and signal quantitation can be preformed. However, it is well documented that GAPDH expression is upregulated with proliferation, activation and differentiation [28]. In the present study, upregulation of GAPDH was observed making this gene inappropriate as a control gene.

None of the six cyclins showed amplification at DNA level. There are only a few studies where gene amplification of cyclins in colorectal cancer has been analyzed [13, 29-31]. Bondi *et al*. have used the same methods as in the present study for detection of gene amplification of the cyclins. They observed gene amplification of several cyclins, while in our study, none of the cyclin exhibited amplification at the DNA level. This difference can be explained by that Bondi *et al*. used one normal colorectal tissue as a reference for all patients, while in our study the normal mucosa from same patient was used as control. It is a possibility of the normal mucosa tissue that may not be normal in patient with CRC, but for this study we thought it was the best choice.

In summary; in this study we have performed correlation analyses between three different methods for detection of expression of six cyclins. We found a statistical significant correlation between qRT-PCR and microarray analyses, but qRT-PCR and microarray poor correlation was observed between RNA overexpression and protein overexpression. Previously, protein expression analyses have mostly been used for identification of prognostic genes. mRNA analyses are now been applied in a more frequent way. Results from the present study indicate that information about the prognostic value of a potential cancer marker, obtained by protein expression analyses, may not necessarily be applicable to mRNA analyses. It is therefore necessary to further investigate if overexpression of mRNA without corresponding protein overexpression is of prognostic value in colorectal cancer patient.

ACKNOWLEDGEMENT

We gratefully acknowledge the excellent technical assistance of Ellen Hellesylt.

REFERENCES

[1] Sobin LH. TNM: Principles, history, and relation to other prognostic factors. Cancer 2001; 91: 1589-92.

[2] Adell G, Boeryd B, Franlund B, *et al.* Occurrence and prognostic importance of micrometastases in regional lymph nodes in Dukes' B colorectal carcinoma: an immunohistochemical study. Eur J Surg 1996; 162: 637-42.

[3] Bertucci F, Salas S, Eysteries S, *et al.* Gene expression profiling of colon cancer by DNA microarrays and correlation with histoclinical parameters. Oncogene 2004; 23: 1377-91.

[4] Howbrook DN, van der Valk AM, O'Shaughnessy MC, *et al.* Developments in microarray technologies. Drug Discov Today 2003; 8: 642-51.

[5] Fathallah-Shaykh HM. Microarrays: applications and pitfalls. Arch Neurol 2005; 62: 1669-72.

[6] Park PJ, Cao YA, Lee SY, *et al.* Current issues for DNA microarrays: platform comparison, double linear amplification, and universal RNA reference. J Biotechnol 2004; 112: 225-45.

[7] Smyth GK, Yang YH, Speed T. Statistical issues in cDNA microarray data analysis. Methods Mol Biol 2003; 224: 111-36.

[8] Bustin SA. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J Mol Endocrinol 2000; 25: 169-93.

[9] Rappolee DA, Mark D, Banda MJ, *et al.* Wound macrophages express TGF-alpha and other growth factors *in vivo*: analysis by mRNA phenotyping. Science 1988; 241: 708-12.

[10] Nishizuka S, Chen ST, Gwadry FG, *et al.* Diagnostic markers that distinguish colon and ovarian adenocarcinomas: identification by genomic, proteomic, and tissue array profiling. Cancer Res 2003; 63: 5243-50.

[11] Van den Eynden GG, Van Laere SJ, Van dA I, *et al.* Overexpression of caveolin-1 and -2 in cell lines and in human samples of inflammatory breast cancer. Breast Cancer Res Treat 2006; 95: 219-28.

[12] Wei JS, Khan J. In: Bowtell D, Sambrook J, Ed. DNA microarrays a molecular cloning manual, New York: Cold Spring Harbor Laboratory Press, 2003: 110-7.

[13] Bondi J, Husdal A, Bukholm G, *et al.* Expression and gene amplification of primary (A, B1, D1, D3, and E) and secondary (C and H) cyclins in colon adenocarcinomas and correlation with patient outcome. J Clin Pathol 2005; 58: 509-14.

[14] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001; 25: 402-8.

[15] Tricarico C, Pinzani P, Bianchi S, *et al.* Quantitative real-time reverse transcription polymerase chain reaction: normalization to rRNA or single housekeeping genes is inappropriate for human tissue biopsies. Anal Biochem 2002; 309: 293-300.

[16] Dallas PB, Gottardo NG, Firth MJ, *et al.* Gene expression levels assessed by oligonucleotide microarray analysis and quantitative real-time RT-PCR -- how well do they correlate? BMC Genomics 2005; 6: 59.

[17] Lin YM, Furukawa Y, Tsunoda T, *et al.* Molecular diagnosis of colorectal tumors by expression profiles of 50 genes expressed differentially in adenomas and carcinomas. Oncogene 2002; 21: 4120-8.

[18] Morey JS, Ryan JC, Van Dolah FM. Microarray validation: factors influencing correlation between oligonucleotide microarrays and real-time PCR. Biol Proced Online 2006; 8: 175-93.

[19] Lacueva J, Perez-Ramos M, Soto JL, *et al.* Multidrug resistance-associated protein (MRP1) gene is strongly expressed in gastric carcinomas. Analysis by immunohistochemistry and real-time quantitative RT-PCR. Histopathology 2005; 46: 389-95.

[20] Diegmann J, Junker K, Gerstmayer B, *et al.* Identification of CD70 as a diagnostic biomarker for clear cell renal cell carcinoma by gene expression profiling, real-time RT-PCR and immunohistochemistry. Eur J Cancer 2005; 41: 1794-801.

[21] Su JM, Perlaky L, Li XN, *et al.* Comparison of ethanol versus formalin fixation on preservation of histology and RNA in laser capture microdissected brain tissues. Brain Pathol 2004; 14: 175-82.

[22] Paska C, Bogi K, Szilak L, *et al*. Effect of formalin, acetone, and RNAlater fixatives on tissue preservation and different size amplicons by real-time PCR from paraffin-embedded tissue. Diagn Mol Pathol 2004; 13: 234-40.

[23] Yamada H, Ichikawa W, Uetake H, *et al.* Thymidylate synthase gene expression in primary colorectal cancer and metastatic sites. Clin Colorectal Cancer 2001; 1: 169-73.

[24] Uchida K, Hayashi K, Kuramochi H, *et al.* Changes in intratumoral thymidylate synthase (TS) and dihydropyrimidine dehydrogenase (DPD) mRNA expression in colorectal and gastric cancer during continuous tegafur infusion. Int J Oncol 2001; 19: 341-6.

[25] Pesta M, Holubec L Jr, Topolcan O, *et al.* Quantitative estimation of matrix metalloproteinases 2 and 7 (MMP-2, MMP-7) and tissue inhibitors of matrix metalloproteinases 1 and 2 (TIMP-1, TIMP-2) in colorectal carcinoma tissue samples. Anticancer Res 2005; 25: 3387-91.

[26] Roeb E, Arndt M, Jansen B, *et al.* Simultaneous determination of matrix metalloproteinase (MMP)-7, MMP-1, -3, and -13 gene expression by multiplex PCR in colorectal carcinomas. Int J Colorectal Dis 2004; 19: 518-24.

[27] Kanai Y, Ushijima S, Nakanishi Y, *et al.* Reduced mRNA expression of the DNA demethylase, MBD2, in human colorectal and stomach cancers. Biochem Biophys Res Commun 1999; 264: 962-6.

[28] Sirover MA. New insights into an old protein: the functional diversity of mammalian glyceraldehyde-3-phosphate dehydrogenase. Biochim Biophys Acta 1999; 1432: 159-84.

[29] Kitahara O, Furukawa Y, Tanaka T, *et al.* 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: 3544-9.

[30] Toncheva D, Petrova D, Tzenova V, *et al.* Tissue microarray analysis of cyclin D1 gene amplification and gain in colorectal carcinomas. Tumor Biol 2004; 25: 157-60.

[31] Kitahara K, Yasui W, Kuniyasu H, *et al.* Concurrent amplification of cyclin E and CDK2 genes in colorectal carcinomas. Int J Cancer 1995; 62: 25-8.

Received: May 29, 2008 Revised: July 4, 2008 Accepted: August 15, 2008