Application of array technology for identification of colon cancer specific biomarkers

PhD thesis

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INTRODUCTION

Incidence of colorectal cancer

Nowadays, diagnostics and therapy of colorectal cancer (CRC) are under huge development – mainly due to the help of high-throughput molecular techniques such as microarray. High incidence of the disease emphasizes the necessity of further experiments to more extensively discover its pathogenetic, molecular background and origin. The prevalence is significantly higher in modern western societies, the colorectal cancer the second most common cancer type in both genders. According to the estimation of World Health Organization, 1.361.000 new CRC cases are diagnosed and 694.000 people die in every year (Ferlay et al. 2012). Colorectal cancer is the second common malignant disease and leading cause of cancer-related mortality in Europe, as well. In 2012, 447.000 new cases were diagnosed and 215.000 people died in CRC (Ferlay et al. 2013). In Hungary, CRC is also among the leading cause of mortality and morbidity in both genders. In 2012, 4800 and 3700 new cases were identified and 2600 and 2100 people died, in case of males and females, respectively. The CRC prevalence is one of the highest in Europe, since the occurrence is 87 and 45 per hundred thousand citizens, in case of males and females, respectively. The above-mentioned data show the importance of the early detection, the discrimination of genetically different tumors and of improvement of the therapeutic efficacy. The five-year survival data supremely emphasize the relevance of early diagnosis: the five-year survival rate is 80-90% in early stage CRC, 60% in case of lymph node metastases, while under 10% in advanced CRC with distant organ metastasis (O'Connell et al. 2004).

Simultaneous expression analysis of large number of genes can be performed on high-throughput platforms suitable for measurement of systematic mRNA expression changes such as spotted cDNA, high-density oligonucleotide microarrays. The mRNA expression microarrays give an opportunity for comparison the expression of huge number of genes in different cells and tissues.

With the measurement of whole genomic expression changes during the colorectal adenoma-dysplasia-carcinoma transition, more comprehensive data can be obtained about the molecular background of malignant transformation which can contribute to the early recognition of CRC.

In my PhD thesis I have identified a transcript set which could be suitable for characterization of colorectal dysplasia-carcinoma transition. During my PhD work, the gene expression analyses by whole genomic microarray and RT-PCR were performed using complex bioinformatic analysis, the results were also confirmed at protein level.

AIMS

The key questions of my PhD work were:

- Can we determine transcript set which show characteristic expression changes according to the Vogelstein model of CRC development and progression?
- From this set are there transcripts whose expression changes can help to differentiate healthy, adenoma and CRC samples sensitively and specifically? Can we determine their epithelial and/or stromal origin?
- Can we differentiate the clinically important high-grade dysplastic adenoma and early colon cancer samples?
- Can we validate the classification efficacy of the identified transcript set on independent samples from Gene Expression Omnibus (GEO) database?
- Can we confirm the expression changes of this transcript set between the different diagnostic groups on independent samples using conventional RT-PCR?
- Further purpose was to confirm the mRNA expression results on tissue samples at protein level using immunohistochemical method.
- I also aimed to determinate the effectiveness of transcript set in blood samples.

MATERIALS AND METHODS

Microarray analysis

Using gene expression microarrays, it became possible to determinate adenoma-dysplasia-carcinoma transition pathways. High-throughput microarray approach is suitable for characterization of gene expression pattern in the actual tumor milieu. Altogether 147 biopsy samples were involved. In case of "training" microarray set, 53 samples (11 healthy, 9 low-grade dysplastic adenoma, 11 high-grade dysplastic adenoma, 10 early CRC, 12 late CRC) were analyzed. Gene expression data are available at Gene Expression Omnibus database (GSE reference: GSE4183). Subsequently other microarray sample set was involved into our experiment as "test" group. In total 94 (38 healthy, 16 low-grade dysplastic adenoma, 13 high-grade dysplastic adenoma, 14 early CRC, 13 late CRC) samples were hybridized. Data are available at Gene Expression Omnibus database (GSE reference: GSE37364). Altogether 47 blood samples (16 healthy, 12 adenoma and 19 CRC –7 early, 12 late CRC) were examined using microarrays. Regional and Institutional Committee of Science and Research and Ethics (TUKEB reference: 69/2008) approved our experiments.

Independent data from Gene Expression Omnibus database

Independent microarray data were downloaded from Gene Expression Omnibus (GEO) database. The same microarray platform (HGU133 Plus2.0) was applied for comparison. Both research teams published their original data according to the MIAME criteria (identifiers: GSE8671 (Sabates-Bellver et al. 2007), GSE18105 (Matsuyama et al. 2010). In case of GSE8671, 64 samples (32 healthy and 32 adenoma) and in case of GSE18105, 111 samples (17 healthy and 94 CRC) were involved.

Real-time RT-PCR examinations

Commercially available RT-PCR assays were applied for confirmation of mRNA expression of 11 transcripts. 68 independent samples (20 healthy, 13 low-grade dysplastic adenoma, 11 high-grade dysplastic adenoma, 10 early CRC, 10 late CRC and 4 unknown stage CRC) were included in the RT-PCR validation. After reverse transcription of 2.5 µg total RNA of each samples using Transcriptor First Strand cDNA Synthesis Kit (Roche), real-time RT-PCR was performed on 384-well plates. Gene specific forward and reverse primers and fluorescently labeled hydrolysis probes (Universal ProbeLibrary, F. Hoffmann-La Roche Ltd, Switzerland,

Basel) were lyophilized on PCR plates. 7 housekeeping genes (GAPDH, B2M, ACTB, HPRT1, RPL13A, 18S and YWHAZ) were tested in order to select the most appropriate reference gene.

Protein level analysis

mRNA microarray and RT-PCR techniques provide information about gene function and activity, however they do not serve data about protein expression and the phenotype. Protein level confirmation is essential to validate microarray results. Selected transcripts were validated on tissue microarrays (TMA) at protein level. For protein level examination of the seven selected markers, healthy (n=10-12), adenoma (tubular and tubulovillosus; n=37-64) and CRC (n=13-30) formalin fixed paraffin embedded tissue samples were applied. For evaluation of slides, digital microscopy (Pannoramic Viewer v:1.15.2) was used with the following semi-quantitative score system:

- no sign of immunoreaction in cell cytoplasm (in case of epithelium or stroma) (-2)
- weak staining in cell cytoplasm (in case of epithelium or stroma) (0)
- medium staining in stroma (+1)
- strong immunoreactions in cell cytoplasm (in case of epithelium or stroma) (+2).

In further immunohistochemical examination, 35 healthy, 75 adenoma (37 low-grade dysplastic and 38 high-grade dysplastic adenoma) and 58 CRC (29 Dukes' A-B and 29 Dukes' C-D CRC) samples were tested on tissue microarray (Sipos et al. 2014).

Statistical evaluation

Determination of differently expressed genes was done by Significance Analysis of Microarray (SAM) in case of microarrays. Beside this method „nearest shrunken centroid" approach (Prediction Analysis for miroarrays - PAM) was also applied for gene expression classification. Using this method, we search for subset/transcript groups which can characterize the different diagnostic groups (Tibshirani et al. 2002). Pre-processing, data mining and statistical analysis were performed by R 2.15.0 (R Development Core Team, 2011) with Bioconductor libraries. Hierarchical cluster analysis, discriminant analysis, PCA and logistic regression techniques were applied for the determination of most effective separative transcript set and for testing its classificatory power. During the evaluation of RT-PCR results, relative quantification of gene expression was made. For fold change evaluation $\Delta\Delta$CT method was applied. 18S ribosomal RNA was applied for normalization as inner

control (ΔCT). For the determination of diagnostic variables, logistic regression was applied (with binary system: 0-control, 1-unhealthy stage) both in case of microarray and RT-PCR analysis. For proportion of diagnosis (P) the following equation used:

$$X = \text{logit}(P) = \ln (P/(1-P) = b_0 + b_1\Delta Ct_1 + b_2\Delta Ct_2 + ...+ b_n\Delta Ct_n$$

During „maximum-likelihood" fit method (empirical) coefficients {bi} were used to specify relationship between X and the experimental measurements {ΔCti}. For „Receiving operating characteristic" (ROC) evaluation Medcalc 12.1 software was used in order to determinate differentiation capability of transcript set (sensitivity and specificity). For discriminant analysis SPSS 20.0 used. Differentiation of different stages was examined. For accuracy and power determination leave-one-out classification was used. For visualization, classification tables were created showing the numbers and percentages of properly and improperly ranked samples.

Results

11 markers were identified as the best differentiating transcript group from 58.000 transcripts. These markers (IL8, MMP3, IL1B, CHI3L1, GREM1, IL1RN, CXCL1, CXCL2, CA7 and SLC7A5) – with exception of (COL12A1) – are proved to be involved in development and progression of colorectal carcinoma. CHI3L1 and CXCL1 are associated with carcinogenesis, IL8, CXCL1, SLC7A5 and MMP3 with tumor growth and development, IL8, CHI3L1 and SLC7A5 with angiogenesis and metastatic invasion. During microarray experiments, diagnostic groups could be efficiently discriminated which was also confirmed on independent samples using RT-PCR.

Marker set examination on „training" microarray samples

In case of „test" sample set (53 microarrays: 11 healthy, 20 adenoma and 22 CRC samples) the marker set containing 11 markers could be successfully applied for discrimination of different diagnostic groups. According to the result of the discriminant analysis, 96.2% of the samples could be correctly classified, while only 2 CRC samples (4,5-4,5% of the whole samples set) classified inappropriately into the healthy and the adenoma groups.

Multiple logistic regression was used for determination of differentiation capability of the identified transcript set. ROC analysis resulted in 100% sensitivity and specificity in healthy vs. adenoma samples. In comparison of healthy and CRC samples these values were 95.5%

and 100%, respectively. In case of adenoma vs. CRC samples the same specificity and sensitivity were observed. Younden index was applied for power determination of ROC, these values were between 0.95 and 1.

Marker set examination on „test" microarray samples

In case of „test" sample set (94 microarrays: 38 healthy, 29 adenoma and 27 CRC) discriminant analysis resulted in 93.6% appropriate group classification. Altogether 4 adenoma and 2 CRC samples classified incorrectly which means that 86.2% and 92.6% of the samples could be correctly classified. Multiple logistic regression was used for determination of differentiation power. ROC analysis showed 100% sensitivity and specificity in case healthy vs. CRC samples. In comparison of healthy and adenoma samples these values were 96.6% and 100%, respectively. When the benign adenoma and malignant CRC samples were compared, the specificity decreased (89.7%), while the sensitivity remained 100%. Younden index was applied for power determination of ROC, these values were between 0.89 and 1.

Application of GEO database samples as independent samples set

32 healthy and 32 adenoma samples were involved in experiment "GSE8671". ROC analysis resulted in 100% sensitivity and specificity in healthy vs. adenoma samples. Further *in silico* examination was performed on experiment "GSE18105" containing 17 healthy and 94 CRC samples. ROC analyses showed 100% sensitivity and specificity in healthy vs. CRC samples. For further *in silico* analysis – GSE39582 – was carried out in order to determinate CRC specificity. Beside 19 healthy mucosa, 24 CIMP-, 13 CIMP+,14 MSI and 22 MSS CRC samples were involved.

Expression analysis of the identified markers on independent samples using RT-PCR

The classificatory power of set of 11 transcripts was validated on independent biopsy samples (68 samples: 20 healthy, 24 adenoma and 24 CRC). 18S ribosomal RNA was chosen for internal control from 7 potential housekeeping genes due to its smallest standard deviation. According to the results of the discriminant analysis, 95.6% of the samples could be properly classified. ROC analysis resulted in 100% sensitivity and specificity in healthy vs. CRC samples. In comparison of healthy and adenoma samples these values were 95.8% and 95%, respectively. When the benign adenoma and malignant carcinoma samples were compared, similarly high sensitivity and specificity were detected (95.8% and 100%, respectively).

Younden index was applied for power determination of ROC, these values were between 0.958 and 1.

Comparison of high-grade dysplastic adenoma and early CRC samples

In case of "training" sample set, the high-grade dysplastic adenoma (n=11) and early CRC (n=10) biopsy samples could be separated well (sensitivity: 100% and specificity: 90.9%) according to the expression changes of set of 11 transcripts The analysis of the independent "test" sample set confirmed these results, since high-grade dysplastic adenoma (n=13) and early CRC (n=14) biopsy samples could be classified with 100% sensitivity and 92.3% specificity. With the simultaneous analysis of two above mentioned sample sets – 24 high-grade dysplastic adenoma and 24 early CRC –, sensitivity was 100%, beside 83.3% of specificity. The RT-PCR validation results (11 high-grade dysplastic adenoma vs. 10 early CRC) showed quite similar differentiation.

According to the results of hierarchical cluster analysis, 10 early CRC samples classified correctly, however 3 adenoma samples (reference number: 6, 10 and 11) misclassified into the CRC sample group. After patient follow-up, samples with reference number 6 and 11 diagnosed as *in situ* carcinoma. During independent RT-PCR validation, sensitivity was 100% beside 90.9% of specificity. Patient follow-up slightly changed the sample groups (9 high-grade dysplastic adenoma vs. 12 early CRC), hence ROC and logistic regression equation recalculation were essential. According to the new grouping, both sensitivity and specificity reached 100%. The power of the transcript set showed quite strong differentiation among sample groups considering the diagnostic changes found during patient follow-up.

Protein level validation on tissue microarrays (TMAs) using immunohistochemistry

During protein level experiments, immunhistochemical method was applied to determinate protein expression profiles on tissue microarray slides. Healthy, adenoma and CRC samples were involved in this analysis. Single antibodies were chosen for each potential protein marker (CA7, COL12A1, IL8, MMP3, SLC7A5, CXCL1-2-3 and IL1RN). Evaluation of immunhistochemical results were performed using an empirical score system.

MMP3 and CXCL1 protein expression in adenoma and CRC samples

Altogether 35 healthy, 75 adenoma (37 low-grade and 38 high-grade dysplastic adenoma) and 58 CRC (29 Dukes' A-B and 29 Dukes' C-D) samples were examined on tissue microarray (Sipos et al. 2014). Differentiation between benign and malignant tumors is clinically

relevant. Expression level of MMP3 in lamina propria showed significant differences ($p<0.001$) during dysplasia-carcinoma transition. Application of MMP3 alone is enough to differentiate benign and malignant samples with 98.2% accuracy. CXCL1 was found to be similarly effective marker in both lamina propria and epithelium. Combination of the two markers provided outstanding differentiation. MMP3 protein expression increased during the colorectal adenoma-dysplasia-carcinoma transition. Expression of CXCL1 was higher in CRC than in adenoma in both epithelium and lamina propria.

According to the results of Fisher's egzact test the semi quantitative CXCL1 and MMP3 protein expression showed the highest positive correlation with the transition in lamina propria. From 139 benign and malignant tumor samples, 113 showed valuable expression results. According to the results of the discriminant analysis, 98.6% of adenoma (n=69) and 97.7% of CRC (n=44) samples classified correctly which means that only 1-1 samples classified incorrectly.

CXCL1 and MMP3 protein expression in lamina propria provided clear separation of high-grade dysplastic adenoma and early CRC samples. In case of CXCL1 11.43% of high-grade dysplastic adenoma and 11.11% of early-CRC samples misclassified. In case of MMP3 marker there was no misclassification.

Differentiation capability of transcript set on microarray blood samples

After confirmation that the transcript set is suitable for differentiating biopsy samples, discriminant analysis was applied on blood samples. Application of the set of 11 markers resulted in 58.3-73.7% of differentiation among the diagnostic groups. Adenoma samples had the largest misclassification rate, as approximately 40% of adenoma samples misclassified into healthy or CRC groups. The majority of previously publish studies focused on healthy and CRC comparison only, hence in the next step only these two groups were compared, where 93.8% of healthy and 84.2% of CRC samples classified correctly. When benign adenoma and malignant CRC samples were handled together as "diseased" group and were compared with the healthy group, results showed above 80% differentiation.

ROC analysis resulted in 84.2% sensitivity and 100% specificity in healthy vs. CRC samples. In comparison of healthy and adenoma samples these values were 78.9% and 100%, respectively. When the benign adenoma and malignant carcinoma were compared, the sensitivity and specificity were moderate, 91.7% and 75%, respectively.

CONCLUSIONS

According to our results the following conclusions can be made:

- Significant gene expression changes were determined using whole genome microarray analysis in healthy, benign adenoma and malignant CRC biopsy samples.
- Different microarray experiments ("training" and "test" sets) were suitable for determination of 11 transcripts which show mRNA expression differences between healthy samples and disease stages. These markers showed higher gene expression – only exception is CA7 where decreased expression level was observed – in benign and malignant stages.
- The above mentioned transcript set was also suitable for differentiation between high-grade dysplastic adenoma and early CRC samples.
- Results were confirmed with involvement of *in silico* data, however comparable results were available only in healthy vs. benign adenoma and healthy vs. malignant CRC comparisons. Appropriate separating capabilities of the 11 marker was confirmed by *in silico* approaches.
- The application the set of markers was proved to differentiate diagnostic groups with high accuracy in both microarray and independent RT-PCR experiments.
- Similar protein expression patterns were observed during immunohistochemical validation than were found in microarray experiments.
- Using the set of tissue markers, significant distinction could be obtained between healthy and diseased blood samples. However, when adenoma and CRC samples were handled separately, large proportion of benign adenoma samples tend to be misclassified into the other two diagnostic groups. Therefore, the marker set could be applied for differentiation of diagnostic blood sample groups only with restrictions.

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