USAGE OF LOW-DENSITY OLIGONUCLEOTIDE MICROARRAYS FOR PROGNOSIS PREDICTION OF COLORECTAL CANCER PATIENTS

[1]Slabý O., [2]Garajová I., [2]Svoboda M., [1]Fabian P., [1]Svoboda M., [1]Šmerdová T., [2]Vyzula R.

[1] Department of Clinical and Experimental Pathology, Masaryk Memorial Cancer Institute, Brno, Czech Republic

[2] Department of Comprehensive Cancer Care, Masaryk Memorial Cancer Institute, Brno, Czech Republic

INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignancies. Unfortunately a significant proportion of surgically cured patiens in the early stage of the disease develop progression and die from the disease. DNA microarrays technology was used in more than sixty studies [1] focused on colorectal cancer during last five years. High-density DNA microarrays showed good analytical ability also in colorectal cancer prognosis [2,3]. However, comparability and reproducibility of studies based on high-density DNA microarrays are notably affected by their technological diversity, and recent findings are not conclusive [4]. This study aimed to find individual up/down-regulated genes associated with progression and metastatic potential of colorectal cancers using low-density oligonucleotide microarrays spotted with genes known to be involved in process of metastasis development. We suppose that focusing on a particular biological pathway may be more useful than genome-wide screening for our purposes. Molecular characterization of patients at high risk of cancer progression using this more economical and productive expression profiling method may improve our knowledge about cancer progression and dissemination and also assist to oncologists in treatment decision by selecting those patients who will need adjuvant chemotherapy.

PATIENTS AND METHODS

PATIENTS

Twelve patients aged 52-76 years, who had histologically confirmed sporadic colon adenocarcinoma with a volume fraction showing at least 70% of malignant tumor cells were included. Only stage II-III patients (pT3-pT4, pN0 or pT2-pT4, pN+) according to IUCC with no prior chemotherapy or radiotherapy were eligible for this study (summary in Table 1). Six patients were poor prognosis cases width disease free survival (DFS) lower then 36 months (median 13) and six were good prognosis cases with DFS>36 (median 43) months. Imunohistochemistry analysis of mismatch repair proteins MSH2, MLH1 and PMS2 were done and colorectal cancers with probable microsatellite instability were excluded [5].

RNA ISOLATION AND QUALITY CONTROL

Samples of primary colon cancers from surgical excisions were immediately stored in liquid nitrogen until RNA extraction. Samples were homogenized (Retch MM301) in sterile conditions before total RNA isolation. RNA was isolated by means of TriReagent under manufacturer's recommendation (MRC Inc., Cincinnati, USA). RNA concentration and purity was controlled by UV spectrophotometry (A260:A280>2.0; A260:A230>1.8). RNA integrity was checked using Agilent 2100 Bioanalyzer and only non-degraded RNA characterized by RIN (RNA Integrity Number) higher than 7 with no DNA contamination signs was processed.

OLIGO GEARRAYS PROTOCOL

For gene expression profiling were used low-density oligonucleotide microarrays Tumor Metastasis Oligo GEArray (OHS-028) obtained from Superarray Bioscience Corp. (Bethesda, MD) and designed for gene-expression relative quantification of 128 genes potentially involved in the metastatic process. Total RNA was converted to amplified- and biotin-labeled cRNA target using TrueLabeling-AMP 2.0 (SuperArray Bioscience Corp.) and purified by ArrayGrade™ cRNA Cleanup Kit (SuperArray Bioscience Corp.) according to manufacturer's instructions. cRNA probes were subsequently denaturated, and hybridization was carried out in GEAHyb solution to nylon membranes spotted with gene-specific 60-mer oligonucleotides. Arrays were then washed and chemiluminescence detection obtained by alkaline phosphatase-conjugated streptavidin and CDP-Star chemiluminescent substrate (Tropix, Inc., Bedford, MA) was captured by 12-bit cooled CCD camera.

IMAGE ANALYSIS AND DATA PROCESSING

Web-based software GEArray Expression Analysis Suite (SuperArray Bioscience Corp.) was used for the raw image analysis, background substraction (spot with minimum value) and data normalization. Data were normalized by housekeeping genes expression levels (GAPDH, ACTB, HSPCB and B2M) and spots with lower intensity than 10% of median value were excluded. Analyses and visualization of normalized data were carried out using TIGR MultiExperiment Viewer version 3.1 (The Institute for Genomic Research, 2003) and genes with statistically significant changes in expression were obtained by SAM (Significance Analysis of Microarrays) and t-test (α =0,01) methods (with condition of at least 2-fold difference in expression).

Table 1: Clinical features of patients in the study

Sample	Age	Gender	Clinical Stage (IUCC)	pTNM stage	Grade	Anatomical Localization	CEA [ug/l]	DFS [month]	Disease status
dp1	73	female	III.B	pT3N1M0	G2	C184 (P)	3,4	58	remission
dp2	73	male	II.A	pT3N0M0	G2	C185 (D)	2,1	54	remission
dp3	61	female	II.A	pT3N0M0	G1	C182 (P)	1,9	41	remission
dp4	56	female	II.A	pT3N0M0	G2	C187 (D)	0,5	44	remission
dp5	62	female	III.A	pT2N1M0	G2	C190 (D)	0,7	36	remission
dp6	61	male	III.B	pT3N1M0	G2	C186 (D)	1,9	36	remission
sp1	52	male	II.A	pT3N0M0	G2	C190 (D)	9,4	20	mesenterium metastasis
sp2	72	male	II.A	pT3N0M0	G2	C183 (P)	4,8	6	liver metastasis
sp3	60	female	III.C	pT4N2M0	G3	C180 (P)	1,4	13	liver metastasis
sp4	73	male	II.A	pT3N0M0	G1	C182 (P)	1,7	7	skeletal metastasis
sp5	52	male	II.A	pT3N0M0	G1	C190 (D)	41	18	local progression
sp6	76	male	III.B	pT3N1M0	G1	C190 (D)	22,6	12	local progression

Abbreviations: dp – good prognosis, sp – bad prognosis, P – tumors proximal to splenic flexure, D – tumors distal to splenic flexure, CEA – carcinoembryogenic antigen (cut-off value fixed at 4,6 μg/l), colorectal cancer dissemination and prognosis marker

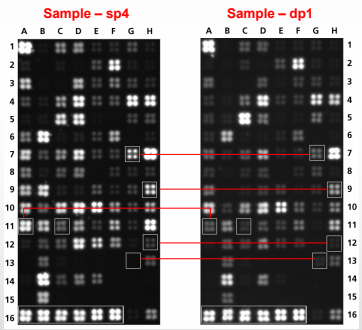

Figure 1: Examples of oligonucleotide arrays of tumors with different prognosis. *(positioning in Table 2)*

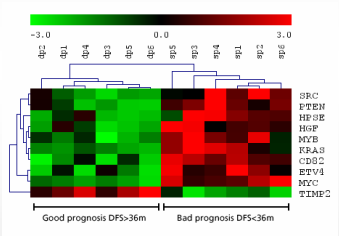

Figure 2: Cluster analysis of 10 selected genes (red color represents overexpressed genes relative to green, underexpressed genes). The data suggest that genes can discriminate good from poor prognosis.

RESULTS AND DISCUSSION

Relative gene expression levels of 128 genes potentially involved in cancer progression and dissemination were obtained by low-density oligonucleotide microarrays from 12 primary colon cancer samples of patients clinically characterized in Table 1. Positive signal intensities (higher than 10% of median value) were detected in more than 40% of spots on the array (range 41-75%) for all twelve tumor samples. Gene expression data analysis based on the SAM and t-test (α = 0,01) methods identified 10 genes (9 up-regulated, 1 down-regulated) with significantly different expression in primary tumors of patients with poor prognosis (Figure 2 and Table 2). In subsequent cluster analysis this group of genes was able to discriminate good from poor prognosis. The functional categories of up-regulated genes belong to oncogenes (MYC, MYB, KRAS, SRC), cell cycle regulation (PTEN), transcriptional regulation (ETV4), cell adhesion and extracellular matrix molecules (CD82, HPSE) and growth factors (HGF). The only down-regulated gene in the group of patients with poor prognosis was antiangiogenic metalloproteinase inhibitor 2 (TIMP2). Some of these genes (MYC, KRAS, SRC, TIMP2) were frequently studied in connection with colorectal cancer carcinogenesis and their altered expression was associated with poor prognosis and adverse clinical outcome [6, 7]. Co-expression of MYB and MYC oncogenes was inversely correlated with apoptotic rate in tumor tissue [8]. Moreover, MYB was associated with expression of antiapoptotic gene Bcl-X and poor prognosis of colorectal cancer patients [9]. Extracellular matrix enzyme heparanase (HPSE) disturbs proteoglycans protection (particularly heparane sulfate) of vascular surfaces and directly participates in hematogenic metastatic process. Up-regulation of HPSE gene was correlated with significantly lower overall survival of colorectal cancer patients [10]. Altered expressions of hepatocyte growth factor (HGF) and transcription factor ETV4 (E1AF) genes were associated with poor prognosis of colorectal cancer patients for the first time. ETV4 gene encodes Ets-related transcription factor stimulating transcription of several matrix metalloproteinases, mainly matrilysin MMP7 known to be involved in colorectal cancer dissemination [11]. Hepatocyte growth factor (HGF) is potent pro-angiogenic factor. HGF mediates angiogenesis through positive regulation of VEGF and urokinase plasminogen activator receptor (uPAR) [12]. All of these expression differences are consistent with previous reports and molecular and cellular aspects of cancer progression and metastasis.

Table 2: Summary of genes differentially expressed in tumors with progressive phenotype

Accession Number	Symbol	Gene name	Position	p-value
Genes up-regulated in primary tumors with poor prognosis				
NM_005417	*SRC*	V-src sarcoma viral oncogene homolog	H12	**0,004**
NM_000314	*PTEN*	Phosphatase and tensin homolog	C11	**0,001**
NM_006665	*HPSE*	Heparanase	D6	**0,004**
NM_000601	*HGF*	Hepatocyte growth factor (hepapoietin A; scatter factor)	E6	**0,003**
NM_005375	*MYB*	V-myb myeloblastosis viral oncogene homolog	H9	**0,001**
NM_004985	*KRAS*	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	G7	**0,002**
NM_002231	*CD82*	CD82 antigen (KAI1)	E7	**<0,001**
NM_001986	*ETV4*	Ets variant gene 4 (E1A enhancer binding protein, E1AF)	E4	**0,002**
NM_002467	*MYC*	V-myc myelocytomatosis viral oncogene homolog	A11	**<0,001**
Gene down-regulated in primary tumors with poor prognosis				
NM_003255	*TIMP2*	TIMP metalloproteinase inhibitor 2	G13	**<0,001**

CONCLUSIONS

Our preliminary data suggest that low-density oligonucleotide microarray technology should contribute to a better understanding of the progression of colorectal cancers and facilitate prediction of their metastatic potential. Expression profiling outperformed previously reported genetic markers of prognosis such as MYC, KRAS or TIMP2. Up-regulation of ETV4 and HGF genes in colorectal cancer patients with poor prognosis, identified for the first time, is consistent with molecular and cellular aspects of cancer progression and metastasis. Analysis of gene expression data from larger group of colorectal cancer patients and validation of the results by other more accurate technology (RT-qPCR, imunohistochemistry) will enable us to identify distinct prognostic subsets of patients based on molecular characteristics in the near future.

REFERENCES

[1] Shih W, Chetty R, Tsao MS: Expression profiling by microarrays in colorectal cancer (Review). Oncol Rep 13:517-24, 2005

[2] Wang Y, Jatkoe T, Zhang Y, et al: Gene expression profiles and molecular markers to predict recurrence of Dukes' B colon cancer. J Clin Oncol 22:1564-71, 2004

[3] Eschrich S, Yang I, Bloom G, et al: Molecular staging for survival prediction of colorectal cancer patients. J Clin Oncol 23:3526-35, 2005

[4] Draghici S, Khatri P, Eklund AC, et al: Reliability and reproducibility issues in DNA microarray measurements. Trends Genet 22:101-9, 2006

[5] Plevova P, Krepelova A, Papezova M, et al: Immunohistochemical detection of the hMLH1 and hMSH2 proteins in hereditary non-polyposis colon cancer and sporadic colon cancer. Neoplasma 51:275-84, 2004

[6] Anwar S, Frayling IM, Scott NA, et al: Systematic review of genetic influences on the prognosis of colorectal cancer. Br J Surg 91:1275-91, 2004

[7] Summy JM, Gallick GE: Src family kinases in tumor progression and metastasis. Cancer Metastasis Rev 22:337-58, 2003

[8] Greco C, Alvino S, Buglioni S, et al: Activation of c-MYC and c-MYB proto-oncogenes is associated with decreased apoptosis in tumor colon progression. Anticancer Res 21:3185-92, 2001

[9] Biroccio A, Benassi B, D'Agnano I, et al: c-Myb and Bcl-x overexpression predicts poor prognosis in colorectal cancer: clinical and experimental findings. Am J Pathol 158:1289-99, 2001

[10] Sato T, Yamaguchi A, Goi T, et al: Heparanase expression in human colorectal cancer and its relationship to tumor angiogenesis, hematogenous metastasis, and prognosis. J Surg Oncol 87:174-81, 2004

[11] Horiuchi S, Yamamoto H, Min Y, et al: Association of ets-related transcriptional factor E1AF expression with tumour progression and overexpression of MMP-1 and matrilysin in human colorectal cancer. J Pathol 200:568-76, 2003

[12] Rudmik LR, Magliocco AM: Molecular mechanisms of hepatic metastasis in colorectal cancer. J Surg Oncol 92:347-59, 2005

This work was supported by IGA MZ ČR NR/9076 - 4.