Gene expression profiling of liver metastases from colorectal cancer as potential basis for treatment choice

MA Pantaleo¹, A Astolfi², M Nannini¹, P Paterini³, G Piazzì³, G Ercolani⁴, G Brandi¹, G Martinelli¹, A Pession², AD Pinna³ and G Biasco*,¹

¹Institute of Hematology and Medical Oncology ‘L. A. Seragnoli’, S. Olsa-Malpighi Hospital, University of Bologna, Bologna, Italy; ²Pediatric Oncology and Hematology, University of Bologna, Bologna, Italy; ³Centre of Applied Biomedical Research (CRBA), S.Orlsola-Malpighi Hospital, University of Bologna, Bologna, Italy; ⁴Liver and Multi-organ Transplantation Unit, University of Bologna, Bologna, Italy

At present no reports on gene expression profiling of liver metastases from colorectal cancer are available. We identified two different signatures using Affymetrix platform: epidermal growth factor receptor pathway was upregulated in metachronous lesions, whereas the pathway mainly related to angiogenesis was in synchronous lesions. Synchronous or metachronous liver metastases could be treated differently on the basis of different molecular pathways.

British Journal of Cancer (2008) 99, 1729 – 1734. doi:10.1038/sj.bjc.6604681 www.bjcancer.com

Published online 30 September 2008

© 2008 Cancer Research UK

Keywords: gene expression profiling; microarrays; colorectal cancer; liver metastases; epidermal growth factor receptor; cyclooxygenase-2.

The liver is the most common site of metastases from colorectal cancer. About 25% of patients presented liver metastases at diagnosis and about 70% of patients develop a liver recurrence after radical surgery of colorectal tumours (50% of patients with stage III and 20% with stage II cancer) (Penna and Nordlinger, 2002). The treatment of metastases from colorectal cancer is complicated and still controversial (Biasco et al, 2006; Ercolani et al, 2006). For unresectable lesions, the medical systemic treatment is considered the standard option. During the last few years, novel biological agents such as monoclonal antibodies inhibiting growth factor receptors or angiogenesis have been combined with chemotherapy to improve the outcome of patients affected by colorectal cancer (Cunningham et al, 2004; Hurwitz et al, 2004; Saltz et al, 2004, 2007; Giontonio et al, 2007; Tabernero et al, 2007; Van Cutsem et al, 2007). In clinical practice the choice of treatments are based only on published clinical data with the respect to first-, second- and third-line therapy. However, the natural history, the clinical scenario and the prognosis of liver metastases may be different. From a general clinical point of view, liver metastases are classified as ‘synchronous’ lesions if they are present at diagnosis of disease or if they occur less than 6 months after surgery of primary tumour and ‘metachronous’ lesions if they occur after more than 6 months. At present, the systemic therapy is not differentiated for these two clinical settings. The aim of this study is to study the gene expression profiling of synchronous and metachronous liver metastases using Affymetrix platform to identify molecular patterns as a possible basis for the choice of systemic therapies and for response prediction.

MATERIALS AND METHODS

Patients and tissues

This study was approved by the local Ethical Committee (approval number: 6/2005/U/Tess). Fresh tissue specimens from liver metastases of 18 patients who had undergone liver surgery were collected after written consent. The specimens were obtained from a single lesion for each patient to avoid the inter-lesion biological variability and immediately frozen in the operating room in liquid nitrogen. The lesions were classified as 10 synchronous and 8 metachronous lesions. The patient’s characteristics are described in Table 1.

Microarray analysis

Total RNA was extracted from frozen tumour specimens using TRIzol Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), labelled and hybridised to HG-U133Plus 2.0 Affymetrix arrays following the manufacturer’s instructions. Data shown in this publication have been deposited in the NCBI Gene Expression Omnibus database. Raw data were background-subtracted, normalised and summarised with the robust multi-array average (RMA) algorithm implemented in the affy package of Bioconductor (http://www.bioconductor.org). Routine quality controls available in the affy and affyPLM packages of Bioconductor were performed to check for the presence of artifacts and for the consistency of normalisation across arrays. Probes poorly
expressed in more than 8 samples out of 18 or not changing among the samples, based on interquartile range (IQR) calculation, were excluded from further analysis. Genes differentially expressed between synchronous and metachronous lesions were selected by the permutation-based t-statistics implemented in the SAM algorithm (Tusher et al, 2001). SAM computes the false discovery rate (FDR, the proportion of false positives in output list of differential genes) by permutations of the sample labels. We set the FDR threshold for significance at 5%. All the analyses were performed with R 2.6.0 and Bioconductor packages. Heatmap representation of differentially expressed genes was performed with MeV software (http://www.tm4.org/mev.html), and pathway analysis with EASE tool (http://david.abcc.ncifcrf.gov), calculating the significance of enrichment of a pathway by the EASE score.

Following the suggestions from the microarrays analysis and in order to confirm the data, quantitative determinations were performed of most clinical relevant proteins such as cyclooxygenase-2 (COX-2) and epidermal growth factor receptor (EGFr).

**Real-time PCR quantification of COX-2 and EGFr**

Total RNA was reverse transcribed using Superscript II (Invitrogen Life Technologies) with oligo-dT primers, according to the manufacturer’s guidelines. Gene-specific primers and TaqMan probes were designed with the Beacon Designer 2.0 Software (Premier Biosoft International, Palo Alto, CA, USA) and real-time PCR was performed using an iCycler apparatus (Bio-Rad Laboratories, Hercules, CA, USA). The cycle numbers were recorded when the accumulated PCR products crossed an arbitrary threshold (CT or threshold cycle) and CT values were used to calculate the expression levels of COX-2 and EGFr relative to the average of two housekeeping genes (β-actin and 18S rRNA).

**Protein extraction and COX-2 western blot analysis**

Frozen tissues were homogenised using lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 2 mM MgCl2, 1% Triton X-100, 10% glycerol, 2 mM EGTA, 1 mM DTT) containing protease inhibitors (10 mgml⁻¹ aprotinin and leupeptin, 5 mgml⁻¹ pepstatin, 1 mM PMSF) and phosphatase inhibitors (50 mM NaF, 10 mM Na₂P₂O₇, 1 mM Na₂VO₃, 3 mM H₂O₂). Samples were processed according to the standard procedures: anti COX-2 antibody (BD Transduction Laboratories, Lexington, KY, USA), incubations conditions: 1 : 500 in TBS-TB buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Tween 20 and 3% bovine serum albumin) at 4°C o.n.

**ELISA quantification of EGFr**

The concentration of total EGFr was assessed using the ELISA kits purchased from Biosource International Inc. (Camarillo, CA, USA). Protein lysates from A431 and SW620 cell lines were used respectively as positive and negative controls, to verify the specificity of the EGFr ELISA assays. Protein quantification was expressed using box plots. Significance was analysed by non-parametric log-rank test (Mann-Whitney test). A P-value less than 0.05 was considered significant. The statistical calculations were performed using StatView 5.0 statistical software (SAS Institute Inc., Cary NC, USA). (Details on Materials and Methods are available in Supplementary Information).

**RESULTS**

The gene expression analysis identified 49 genes upregulated in metachronous and 55 genes upregulated in synchronous metastases with a FDR <5% (Figure 1). Among these, functional analysis of differential genes showed two main deregulated pathways of clinical interest in medical oncology: EGFr signalling pathway (P = 0.065, modified Fisher’s exact test) and eicosanoid metabolism (P = 0.012). Key genes belonging to these pathways are EGFr, PIK3R1, the regulatory subunit 1 (p85–) of the phosphoinositide-3-kinase, COX-2, COX1 and ALOX5AP, the activating protein of the arachidonate 5-lipoxygenase. In particular, EGFr was overexpressed in metachronous lesions (P = 0.046, Mann-Whitney test) and COX-2 gene was overexpressed in synchronous lesions (P = 0.012) (Figure 2A). To confirm the differential expression of these two genes, a quantitative analysis of EGFr and COX-2 mRNA with real-time PCR, and of protein levels by western blot (COX-2) and ELISA test (EGFr) were performed. Both analyses showed that COX-2 was overexpressed in synchronous lesions (P = 0.033 and P = 0.034) and EGFr was overexpressed in metachronous lesions (P = 0.013 and P = 0.043), respectively, at the mRNA and protein levels (Figure 2B and C).

**DISCUSSION**

Over the few last years, the gene expression profiling analysis with microarray technology has shown a great potential for clinical application in medical oncology (Bittner et al, 2000; Perou et al, 2000; Dhanasekaran et al, 2001; Garber et al, 2001; Takahashi et al, 2001; van ’t Veer et al, 2002; Van de Vijver et al, 2002; Ayers et al, 2004; Jones et al, 2005; Dressman et al, 2006; Bonnefoi et al, 2007; Bueno-de-Mesquita et al, 2007; Kim et al, 2007). Several data have already been published on the powerful prognostic role of the gene signature in many tumours (Bittner et al, 2000; Dhanasekaran et al, 2001; Garber et al, 2001; Takahashi et al, 2001; van ’t Veer et al, 2002; Van de Vijver et al, 2002; Jones et al, 2005; Bueno-de-Mesquita et al, 2007) and also on the predictor role of complete response to neoadjuvant chemotherapy especially in breast cancer (Ayers et al, 2004; Dressman et al, 2006; Bonnefoi et al, 2007; Kim et al, 2007). Concerning colorectal cancer, several reports are available but they are mostly aimed at improving the diagnosis on a molecular basis differentiating between cancer, adenoma and...
**Figure 1** Heatmap representation of genes differentially expressed between metachronous (M) and synchronous (S) liver metastases: in blue (underexpressed genes, log2 ratio = −3) and in red (overexpressed genes, log2 ratio = 3) representing the two extremities of gene expression. Log ratios are referred to average expression level in all samples for each gene.
normal mucosa, and also to evaluating the potential for metastases developing (Alon et al., 1999; Notterman et al., 2001; Yanagawa et al., 2001; Bertucci et al., 2004; Koehler et al., 2004; Li et al., 2004; Wang et al., 2004; Eschrich et al., 2005). Moreover, the studies have been mainly conducted on primary tumours and only very few data are available on metastases (Yanagawa et al., 2001; Koehler et al., 2004).

This study first reports the gene expression profiling of liver metastases from colorectal cancer. Our results showed that the molecular background of liver metastases may be different and that EGFR and COX-2 are overexpressed in metachronous and synchronous metastases, respectively. These findings improve the current knowledge on biological background of colorectal liver metastases. Furthermore, they may also have some clinical implications, because they suggest that medical treatments of patients with liver metastases may be differentiated in these two clinical setting according to their different biological background. Therapies based on EGFr pathway inhibition may be considered for metachronous metastases (such as monoclonal antibodies cetuximab or panitumumab-based treatments except for K-ras mutations (De Roock et al., 2008; Lièvre et al., 2008). In fact, it is already well known that morphological expressions of the receptor studied with immunohistochemistry is inadequate and other evaluations did not reach any conclusive data (Dei Tos and Ellis, 2005; Moroni et al., 2005; Vallböhmer et al., 2005; Lenz et al., 2006). In our study, the expression of EGFr has been evaluated using three different molecular techniques that provide quantitative information that, in our opinion, may be a more accurate and reliable study of EGFr status in colorectal cancer as a predictor of response to EGFr inhibitors.

The COX-2 upregulation in synchronous metastases supports its association with tumour invasiveness and metastatic process because COX-2 affects cell proliferation, tumour growth, angiogenesis, apoptosis resistance and immune response (Sheng et al., 1997; Chen et al., 2001). COX-2 overexpression may suggest a more aggressive phenotype of this kind of metastases that require a treatment preferentially directed against tumour angiogenesis, such as bevacizumab-based combinations or a treatment creating an unfavourable environment for tumour growth as recently published with COX-2 inhibitors (de Heer et al., 2008).

As future perspective, from a biological point of view, it could be interesting to compare gene expression profiling of metastases and primary tumour to better understand the molecular mechanisms involved in the metastatic process and to early
identify main predicting genes of metachronous metastases development.

In conclusion, synchronous and metachronous liver metastases from colorectal cancer have a different gene expression signature and a different expression of EGFR and COX-2 that may be the basis for choosing the medical treatment. These preliminary results need to be confirmed in larger series and, in the future, their role as molecular predictors should be also investigated in clinical trials.

REFERENCES

Alon U, Barkai N, Notterman DA, Gish K, Ybarra S, Mack D, Levine AJ (1999) Broad patterns of gene expression revealed by clustering analysis of tumour and normal colon tissues probed by oligonucleotide arrays. Proc Natl Acad Sci USA 96: 6745–6750

Ayers M, Symmans WF, Stec J, Damokosh AI, Clark E, Hess K, Lecocq M, Metivier J, Booser D, Ibrahim N, Valero V, Royce M, Arun B, Whitman G, Ross J, Sniege N, Hortobagyi GN, Puotzai L (2004) Gene expression profiles predict complete pathologic response to neoadjuvant paclitaxel and fluorouracil, doxorubicin, and cyclophosphamide chemotherapy in breast cancer. J Clin Oncol 22: 2284–2293

Bertucci F, Salas S, Eysterries S, Nasser V, Finetti P, Giniestier C, Charafe-Jauffret E, Lemoi B, Bachelart L, Montfort J, Victorero G, Viret F, Ollendorff M, Bertucci F, Guertens G, de Boeck G, Koudijs MM, Nagelkerke De Roock W, Piessevaux H, De Schutter J, Janssens M, De Hertogh G, Biasco G, Derenzini E, Grazi GL, Ercolani G, Ravaioli M, Pantaleo MA, Bertucci F, Salas S, Eysterries S, Nasser V, Finetti P, Giniestier C, Charafe-Jauffret E, Lemoi B, Bachelart L, Montfort J, Victorero G, Viret F, Ollendorff M, Bertucci F, Guertens G, de Boeck G, Koudijs MM, Nagelkerke De Roock W, Piessevaux H, De Schutter J, Janssens M, De Hertogh G, Biasco G, Derenzini E, Grazi GL, Ercolani G, Ravaioli M, Pantaleo MA, Bonnefoi H, Potti A, Delorenzi M, Mauriac L, Campone M, Tubiana-Hulin REFERENCES

confirmed in larger series and, in the future, their role as molecular
can be also investigated in clinical trials.

Cancer Chemother Pharmacol

Eur J Cancer E2004 Cetuximab monotherapy and cetuximab plus irinotecan in metastatic colorectal carcinoma refractory to irinotecan, Lenz HJ, Van Cutsem E, Khambata-Ford S, Mayer RJ, Gold P, Stella P, Mirtsching B, Cohn AL, Pippas AW, Azainia N, Tsuchihashi Z, Mauro T, Rovinsky KS (2006) Multicenter phase II and translational study of cetuximab in metastatic colorectal carcinoma refractory to irinotecan, oxaliplatin, and fluorouracil, and leucovorin (FOLFOX4) for previously treated metastatic colorectal cancer: results from the Eastern Cooperative Oncology Group Study E3200. J Clin Oncol 23: 1539–1544

Hurwitz H, Fehrenbacher L, Novotny W, Cartwright T, Hainsworth J, Heim W, Berlin J, Baron A, Griffling S, Holmgren E, Ferrara N, Fyfe G, Rogers B, Ross R, Kabbavinar F (2004) Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. N Engl J Med 350: 2335–2342

Jones MH, Virtanen C, Honjoh D, Miyoshi T, Satoh Y, Okumura S, Nakagawa K, Nomura H, Ishikawa Y (2005) Two prognostically significant subtypes of high-grade lung neuroendocrine tumours independent of small-cell and large-cell neuroendocrine carcinomas identified by gene expression profiles. Lancet 365: 775–781

Kim JJ, Lim SB, Kang HC, Chang HJ, Ahn SA, Park HW, Jang SG, Park JH, Kim DY, Jung KH, Choi HS, Jeong SY, Sohn DK, Kim DW, Park JG (2007) Microarray gene expression profiling for predicting complete response to neoadjuvant chemoradiotherapy in patients with advanced rectal cancer. Dis Colon Rectum 50: 1342–1353

Koehler A, Bataille F, Schmid C, Ruesmelle F, Waldecker A, Blazsky H, Hartmann A, Hofstaedter F, Dietmaier W (2004) Gene expression profiling of colorectal cancer and metastases divides tumours according to their clinicopathological stage. J Pathol 204: 65–74

Lenz HJ, Van Cutsem E, Khambata-Ford S, Mayer RJ, Gold P, Stella P, Mirtsching B, Cohn AL, Pippas AW, Azainia N, Tsuchihashi Z, Mauro T, Rovinsky KS (2006) Multicenter phase II and translational study of cetuximab in metastatic colorectal carcinoma refractory to irinotecan, oxaliplatin, and fluoropyrimidines. J Clin Oncol 24: 4914–4921

Li M, Lin YM, Hasegawa S, Shimokawa T, Murata K, Kameyama M, Ishikawa O, Katagi T, Tsudana T, Nakamura Y, Furukawa Y (2004) Genes associated with liver metastasis of colon cancer, identified by genome-wide cDNA microarray. Int J Cancer 24: 305–312

Lievre A, Bachelot JR, Boige V, Gaye A, Le Corre D, Bac E, Ychou M, Bouché O, Landi B, Louvet C, André T, Bibeau F, Diebold MD, Rougier P, Durex B, Aléman G, Emile J, Penault-Llorca F, Laurent-Puig P (2008) KRAS mutations as an independent prognostic factor in patients with advanced colorectal cancer treated with cetuximab. J Clin Oncol 26: 374–379

ACKNOWLEDGEMENTS

This study was supported by Italian Minister of University and Scientific Research (PRIN 2005 Project) and by Fondazione Cassa di Risparmio of Bologna (CARISBO).

Supplementary Information accompanies the paper on British Journal of Cancer website (http://www.nature.com/bjc)

© 2008 Cancer Research UK
Moroni M, Veronese S, Benvenuti S, Marrapese G, Sartore-Bianchi A, Di Nicolantonio F, Gambacorta M, Siena S, Bardelli A (2005) Gene copy number for epidermal growth factor receptor (EGFR) and clinical response to anti-EGFR treatment in colorectal cancer: a cohort study. Lancet Oncol 6: 279 – 286

Notterman DA, Alon U, Sierk AJ, Levine AJ (2001) Transcriptional gene expression profiles of colorectal adenoma, adenocarcinoma, and normal tissue examined by oligonucleotide arrays. Cancer Res 61: 3124 – 3130

Penna C, Nordlinger B (2002) Colorectal metastasis (liver and lung). Surg Clin N Am 82: 1075 – 1090

Perou CM, Sørlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, Pollack JR, van de Vijver MJ, Albertson DG,晤 P, Chen Y,引起 T, Wang E, Lee J, RMes B, Ljung BM, Docking Y, Mes C, Stck T, Golub TR, Troyansk VY, Pappo A, Patel AR, Rouss V, Lander ES, Botstein D (2000) Molecular portraits of human breast tumours. Nature 406: 747 – 752

Saltz LB, Lenz HJ, Kindler HL, Hochster HS, Wadler S, Hoff PM, Kemeny NE, Hollywood EM, Gonen M, Quinones M, Morse M, Chen HX (2007) Randomized phase II trial of cetuximab, bevacizumab, and irinotecan compared with cetuximab and bevacizumab alone in irinotecan-refractory colorectal cancer: the BOND-2 study. J Clin Oncol 25: 4557 – 4561

Saltz LB, Meropol NJ, Loehrer Sr PJ, Needle MN, Kopit J, Mayer RJ (2004) Phase II trial of cetuximab in patients with refractory colorectal cancer that expresses the epidermal growth factor receptor. J Clin Oncol 22: 1201 – 1208

Sheng H, Shao J, Kirkland SC, Isakson P, Coffey RJ, Morrow J, Beauchamp RD, DuBois RN (1997) Inhibition of human colon cancer cell growth by selective inhibition of cyclooxygenase-2. J Clin Invest 99: 2254 – 2259

Tabernero J, van Cutsem E, Diaz-Rubio E, Cervantes A, Humblet Y, André T, van Laethem JL, Maurel J, Avril P, Wang K, Zhang X, Kingham G, Patel R, Wen Q, de Gramont A (2007) Phase II trial of cetuximab in combination with fluorouracil, leucovorin, and oxaliplatin in the first-line treatment of metastatic colorectal cancer. J Clin Oncol 25: 5225 – 5232

Takahashi M, Rhodes DR, Purge KA, Kanayama H, Kagawa S, Haab BB, Teh BT (2001) Gene expression profiling of clear cell renal cell carcinoma: gene identification and prognostic classification. Proc Natl Acad Sci USA 98: 9754 – 9759

Tusher VG, Tibshirani R, Chu G (2001) Significance analysis of microarrays applied to the ionizing radiation response. Proc Natl Acad Sci USA 98: 10515

Vallböhmer D, Zhang W, Gordon M, Yang DY, Yun J, Press OA, Rhodes KE, Sherrod AE, Isqbal S, Danenberg KD, Grosben S, Lenz HJ (2005) Molecular determinants of Cetuximab efficacy. J Clin Oncol 23: 3536 – 3544

van ‘t Veer LJ, Dai H, van de Vijver MJ, He YD, Hart AA, Mao M, Peterse HL, van der Kooy K, Marton MJ, Witteveen AT, Schreiber GJ, Kerkhoven RM, Roberts C, Linsley PS, Bernards R, Friend SH (2002) Gene expression profiling predicts clinical outcome of breast cancer. Nature 415: 530 – 536

Van Cutsen E, Peeters M, Siena S, Humblet Y, Hendliš A, Neyns B, Canon JL, Van Laethem JL, Maurel J, Richardson G, Wolf M, Amado RG (2007) Open-label phase III trial of panitumumab plus best supportive care compared with best supportive care alone in patients with chemotherapy-refractory metastatic colorectal cancer. J Clin Oncol 25: 1658 – 1664

van de Vijver MJ, He YD, van’t Veer LJ, Dai H, Hart AA, van de Vijver MJ, He YD, Hart AA, van de Vijver MJ, He YD, van de Vijver MJ, He YD, van de Vijver MJ, He YD, van de Vijver MJ, He YD (2002) A gene-expression signature as a predictor of survival in breast cancer. N Engl J Med 347: 1999 – 2009

Wang Y, Jatkoe T, Zhang Y, Mutch MG, Talantov D, Jiang J, McLeod HL, Atkins D (2004) Gene expression profiles and molecular markers to predict recurrence of Dukes’ B colon cancer. J Clin Oncol 22: 1564 – 1571

Yanagawa R, Furukawa Y, Tsunoda T, Kitahara O, Kameyama M, Murata K, Ishikawa O, Nakamura T (2001) Genome-wide screening of genes showing altered expression in liver metastases of human colorectal cancers by cDNA microarray. Neoplasia 3: 395 – 401

British Journal of Cancer (2008) 99(10), 1729 – 1734 © 2008 Cancer Research UK

Genetics and Genomics