# Expression profiling and long lasting responses to chemotherapy in metastatic gastric cancer

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**Abstract.** Current palliative chemotherapy (CT) regimens achieve clinical benefits in less than 50% of patients treated for metastatic gastric cancers, and long-term survivals are anecdotical. Genetic polymorphisms and differences at the level of transcription in genes involved in biological processes of drug metabolism, DNA repair and drug resistance can explain the observed individual differences in response to drugs, in survival and in different susceptibility to the toxic effects of CT. The possibility to classify patients on the basis of genetic signatures could help in choosing the CT regimen. We present herein an analysis of genetic and expression profiling of three patients affected by metastatic gastric cancer, treated with CT and alive, disease-free, at 66-82 months. Four patients with typical clinical outcome represented the control group. Expression profiling from paraffin-embedded tumor tissues was performed on an *ad hoc* set of genes involved in drug metabolism and resistance, DNA repair, cell cycle regulation and growth factors signalling. Genetic polymorphism analysis on DNA extracted from peripheral blood was done by pyrosequencing of genetic markers predictive of drug response. Expression analysis in long-term survivors revealed a significant upregulation of PTEN, TP63, GADD45a and MAPK1 genes. We found also an up-regulation of CYP1A1, CYP3A4 and ERBB4 genes. EGF was found to be down-regulated in long-term survivors. ERCC1 C8092A polymorphism seems to be associated with survival in our set of patients. The present study shed light on a set of genes, which could have a predictive role in survival of patients with metastatic gastric tumors.

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*Key words:* metastatic gastric cancer, chemotherapy, expression profiling, single nucleotide polymorphisms, survival

## Introduction

Gastric cancer is among the most frequent malignancies, it accounts for roughly 800,000 new cases every year worldwide and represents the second cause of cancer death (1). It is a heterogeneous disease both histologically and genetically, and patient outcome is difficult to predict using classical histological and molecular classification.

In spite of the great therapeutic enhancements, many patients treated for gastric cancer do not reach recovery. Disease is often diagnosed in advanced stages and is therefore associated with poor prognosis. Current palliative chemotherapy (CT) regimens achieve clinical benefits in less than 50% of patients treated for metastatic gastric cancers, and long-term survivals are rare (2).

The significant survival benefit of 5-fluorouracil (5-FU)-based CT for metastatic gastric cancer compared with best supportive care has been reported (3). In Western countries, 5-FU combined with cisplatin, plus/minus epirubicin, have been referred to as the standard chemotherapy for metastatic gastric cancer, with the median survival time ranging from 7.3 to 10.5 months (3). Combination CT of those drugs has increased response rates to over 50%, but has not increased the median survival time to over 11 months, and the therapies are often related to a higher frequency of severe adverse events than older regimens (3). Thus, there is a need for diagnostic methods that allow the prediction of clinical outcome and enable the pre-therapeutic discrimination of treatment effect.

Currently, there are no efficient clinical, pathological or molecular markers to distinguish between responders and non-responders, and between long and normal survivors, in relation to chemotherapy regimens used in gastric cancer (4).

A growing body of evidence suggests that the intratumor protein expression, single nucleotide polymorphisms and gene expression of drug-metabolizing enzymes, DNA repair enzymes, or angiogenic enzymes may have important implications for anticancer drug efficacy. Few studies have investigated the predictive or prognostic value of these genetic markers in patients with advanced gastric cancer (4).

Table I. Clinical characteristics and survival of the patients.[a]

| No. | Date of diagnosis | Tumor type | Site of metastasis | Treatment | Start therapy | End therapy | Last follow-up | Status | PFS (months) | Long/ normal survivor | Median survival (months) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 02/2004 | Gastric | Liver | ECF x 8 | 03/04 | 08/2004 | 01/2010 | NED | 66 | Long | |
| 2 | 08/2004 | Gastric | Liver | ECF x 6 | 09/03 | 12/2003 | 11/2009 | NED | 75 | Long | 75 |
| 3 | 05/2003 | Cardias | Non-regional nodes | TCF x 4 followed by surgery due to the observed CR | 06/03 | 09/2003 | 03/2010 | NED | 82 | Long | |
| 4 | 10/2000 | Gastric | Liver | ECF x 6 | 09/05 | 01/2006 | 11/2006 | Exitus | 14 | Normal | |
| 5 | 03/2001 | Gastric | Liver | ECF x 6 | 04/01 | 08/2001 | 05/2004 | Exitus | 33 | Normal | |
| 6 | 08/2001 | Gastric | Liver and lymph nodes | ECF x 6 | 08/01 | 01/2002 | 05/2002 | Exitus | 9 | Normal | 13 |
| 7 | 02/2002 | Gastric | Liver and cns | ECF x 6 | 02/02 | 06/2002 | 02/2003 | Exitus | 12 | Normal | |

[a]ECF, epirubicin, cisplatin and 5-FU; TCF, taxanes, cisplatin and 5-FU; cns, central nervous system; NED, no evidence of disease; PFS, progression-free survival; CR, complete response.

Here, we report on a long lasting response to epirubicin/cisplatin/5-FU combination CT in 3 metastatic gastric cancer patients. We further provide information on gene expression profiling and genetic analysis demonstrating its potential as a predictor for survival in metastatic gastric cancer.

## Patients and methods

*Patients*. In the clinical practice of the Medical Oncology Department of S. Croce General Hospital, 3 long-term survival cases with metastatic gastric cancer after chemotherapy, who were treated with a standard ECF regimen (epirubicin, cisplatin, 5-FU) and have shown prolonged disease-free and overall survival, have been observed. The 3 patients had histologically confirmed metastatic disease. We refer to them as long survivors. All the patients referring to our Department are asked to give a written informed consent for conservation of biological material that could be use for research propose.

In the study reported herein, we selected 4 patients with metastatic gastric cancer, treated with the same therapeutic regimen, who have not reached clinical remission or have presented early disease relapse. We refer to them as normal survivors. Clinical characteristics of patients are reported in Table I. Long survivors present a median disease-free and overall survival of 75 months (66-82 months), while normal responders die within one year (median 13 months; 9-33 months).

*Expression profiling*

*Custom Taqman Low Density Array construction*. Bioinformatic procedure for the design of the database of genes to be evaluated was based on medical literature reporting studies already performed in gastric cancer evaluating polymorphisms and expression profiles of a selection of genes related to the response to CT and survival. This was done through GeneCards database (www.genecards.org), integrated with a literature mining approach both software assisted (MedMiner) (5) and manually edited. Ninety-three genes were selected and used to create the Custom Taqman Low Density array (TLDA, Applied Biosystem) (Table II and Fig. 1).

*RNA extraction*. RNA was extracted from paraffin-embedded slices, using Recover All Total Nucleic Acid Isolation Kit (Ambion), following manufacturer's instructions. For each patient, three different samples of the biopsy were identified by the pathologist as proper cancer tissues and RNA was extracted independently for each sample, in order to be more representative of the tumoral tissue.

*Quantitative real-time RT-PCR on TLDA*. Custom TLDA containing a set of 93 cancer-related genes (and 3 endogenous control genes: ß$_2$M-Hs99999907_m1, GAPDH-Hs99999905_m1, GUSB-Hs99999908_m1) were used for expression studies. cDNAs for real-time RT-PCR were produced using the High Capacity cDNA Archive kit (Applied Biosystem) following the manufacturer's protocol. Inventoried assays were chosen up to 85 bp, of _m type, being assay designed at the exon-exon boundaries of the genes. Amplifications on the TLDA were done on a 7900HT instrument (Applied Biosystem). Forty-five cycles of PCR were performed. Each cDNA was tested in three independent PCR reactions.

*Gene expression data analysis*. Quantitative real-time RT-PCR data were extracted using SDS 2.2.2 software (Applied Biosystem). Microsoft Excel file containing SDS results was acquired and analyzed in R v2.10.1 software environment.

Table II. List of 93 cancer-related genes investigated on Taqman Low Density Array.

| Gene symbol | Gene description | Assay no. (AB) |
|---|---|---|
| ABCB1 | ATP-binding cassette, sub-family B (MDR/TAP), member 1 | Hs00184500_m1 |
| ABCC1 | ATP-binding cassette, sub-family C (CFTR/MRP), member 1 | Hs00219905_m1 |
| ABCC2 | ATP-binding cassette, sub-family C (CFTR/MRP), member 2 | Hs00166123_m1 |
| ABCC3 | ATP-binding cassette, sub-family C (CFTR/MRP), member 3 | Hs00358656_m1 |
| ABCC5 | ATP-binding cassette, sub-family C (CFTR/MRP), member 5 | Hs00194701_m1 |
| ABCG2 | ATP-binding cassette, sub-family G (WHITE), member 2 | Hs00184979_m1 |
| AKT1 | v-akt murine thymoma viral oncogene homolog 1 | Hs00178289_m1 |
| APEX1 | APEX nuclease (multifunctional DNA repair enzyme) 1 | Hs00172396_m1 |
| ATM | ataxia telangiectasia mutated | Hs00175892_m1 |
| BAX | BCL2-associated X protein | Hs00180269_m1 |
| BCL2 | B-cell CLL/lymphoma 2 | Hs00608023_m1 |
| BCL2L1 | BCL2-like 1 | Hs00169141_m1 |
| BIRC4 | baculoviral IAP repeat-containing 4 | Hs00236913_m1 |
| CALD1 | caldesmon 1 | Hs00189021_m1 |
| CASP3 | caspase 3, apoptosis-related cysteine peptidase | Hs00234385_m1 |
| CASP9 | caspase 9, apoptosis-related cysteine peptidase | Hs00154261_m1 |
| CCNB1 | cyclin B1 | Hs00259126_m1 |
| CCND1 | cyclin D1 | Hs00277039_m1 |
| CDH1 | cadherin 1, type 1, E-cadherin (epithelial) | Hs00170423_m1 |
| CDKN1A | cyclin-dependent kinase inhibitor 1A (p21, Cip1) | Hs00355782_m1 |
| CDKN1B | cyclin-dependent kinase inhibitor 1B (p27, Kip1) | Hs00153277_m1 |
| CHFR | checkpoint with forkhead and ring finger domains | Hs00217191_m1 |
| CYP1A1 | cytochrome P450, family 1, subfamily A, polypeptide 1 | Hs00153120_m1 |
| CYP2C8 | cytochrome P450, family 2, subfamily C, polypeptide 8 | Hs00946140_g1 |
| CYP3A4 | cytochrome P450, family 3, subfamily A, polypeptide 4 | Hs00430021_m1 |
| DDIT3 | DNA-damage-inducible transcript 3 | Hs00358796_g1 |
| DHFR | dihydrofolate reductase | Hs00758822_s1 |
| DPYD | dihydropyrimidine dehydrogenase | Hs00559279_m1 |
| E2F1 | E2F transcription factor 1 | Hs00153451_m1 |
| ECGF1 | endothelial cell growth factor 1 (platelet-derived) | Hs00157317_m1 |
| EGF | epidermal growth factor (beta-urogastrone) | Hs00153181_m1 |
| EGFR | epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian) | Hs00193306_m1 |
| ERBB2 | v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian) | Hs00170433_m1 |
| ERBB3 | v-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian) | Hs00176538_m1 |
| ERBB4 | v-erb-a erythroblastic leukemia viral oncogene homolog 4 (avian) | Hs00171783_m1 |
| ERCC1 | excision repair cross-complementing rodent repair deficiency, complementation group 1 | Hs00157415_m1 |
| ERCC2 | excision repair cross-complementing rodent repair deficiency, complementation group 2 (xeroderma pigmentosum D) | Hs00361161_m1 |
| FGFR4 | fibroblast growth factor receptor 4 | Hs00242558_m1 |
| GADD45A | growth arrest and DNA-damage-inducible, alpha | Hs00169255_m1 |
| GSTP1 | glutathione S-transferase pi | Hs00168310_m1 |
| GSTT1 | glutathione S-transferase theta 1 | Hs00184475_m1 |
| HGF | hepatocyte growth factor (hepapoietin A; scatter factor) | Hs00300159_m1 |
| HIF1A | hypoxia-inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor) | Hs00153153_m1 |

Table II. Continued.

| Gene symbol | Gene description | Assay no. (AB) |
|---|---|---|
| HPRT1 | hypoxanthine phosphoribosyltransferase 1 (Lesch-Nyhan syndrome) | Hs99999909_m1 |
| HRAS | v-Ha-ras Harvey rat sarcoma viral oncogene homolog | Hs00610483_m1 |
| JUN | jun oncogene | Hs99999141_s1 |
| KDR | kinase insert domain receptor (a type III receptor tyrosine kinase) | Hs00176676_m1 |
| KIT | v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog | Hs00174029_m1 |
| KRAS | v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog | Hs00364282_m1 |
| MAPK1 | mitogen-activated protein kinase 1 | Hs00177066_m1 |
| MAPK3 | mitogen-activated protein kinase 3 | Hs00385075_m1 |
| MAPK8 | mitogen-activated protein kinase 8 | Hs00177083_m1 |
| MDK | midkine (neurite growth-promoting factor 2) | Hs00171064_m1 |
| MET | met proto-oncogene (hepatocyte growth factor receptor) | Hs00179845_m1 |
| MGMT | O-6-methylguanine-DNA methyltransferase | Hs00172470_m1 |
| MMP1 | matrix metallopeptidase 1 (interstitial collagenase) | Hs00899658_m1 |
| MMP3 | matrix metallopeptidase 3 (stromelysin 1, progelatinase) | Hs00968308_m1 |
| MRE11A | MRE11 meiotic recombination 11 homolog A (*S. cerevisiae*) | Hs00271551_m1 |
| MTHFR | 5,10-methylenetetrahydrofolate reductase (NADPH) | Hs00195560_m1 |
| MUC1 | mucin 1, cell surface associated | Hs00159357_m1 |
| MVP | major vault protein | Hs00245438_m1 |
| OGG1 | 8-oxoguanine DNA glycosylase | Hs00213454_m1 |
| PDGFRA | platelet-derived growth factor receptor, alpha polypeptide | Hs00998026_m1 |
| PIK3CA | phosphoinositide-3-kinase, catalytic, alpha polypeptide | Hs00180679_m1 |
| PLK1 | polo-like kinase 1 (*Drosophila*) | Hs00153444_m1 |
| POR | P450 (cytochrome) oxidoreductase | Hs00287016_m1 |
| PRNP | prion protein (p27-30) (Creutzfeldt-Jakob disease, Gerstmann-Strausler-Scheinker syndrome, fatal familial insomnia) | Hs00175591_m1 |
| PTEN | phosphatase and tensin homolog (mutated in multiple advanced cancers 1) | Hs01920652_s1 |
| PTGS2 | prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase) | Hs00153133_m1 |
| RAD50 | RAD50 homolog (*S. cerevisiae*) | Hs00194871_m1 |
| REG4 | regenerating islet-derived family, member 4 | Hs00230746_m1 |
| RRM1 | ribonucleotide reductase M1 polypeptide | Hs00168784_m1 |
| SMAD4 | SMAD family member 4 | Hs00232068_m1 |
| SOD2 | superoxide dismutase 2, mitochondrial | Hs00167309_m1 |
| SRC | v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian) | Hs00178494_m1 |
| TGFA | transforming growth factor, alpha | Hs00608187_m1 |
| TGFB1 | transforming growth factor, beta 1 | Hs00171257_m1 |
| TNF | tumor necrosis factor (TNF superfamily, member 2) | Hs00174128_m1 |
| TOP1 | topoisomerase (DNA) I | Hs00243257_m1 |
| TOP2A | topoisomerase (DNA) II alpha 170 kDa | Hs00172214_m1 |
| TOP2B | topoisomerase (DNA) II beta 180 kDa | Hs00172259_m1 |
| TP53 | tumor protein p53 (Li-Fraumeni syndrome) | Hs00153349_m1 |
| TP63 | tumor protein p63 | Hs00186613_m1 |
| TP73 | tumor protein p73 | Hs00232088_m1 |
| TSG101 | tumor susceptibility gene 101 | Hs00173072_m1 |
| TUBB | tubulin, beta | Hs00742828_s1 |
| TYMS | thymidylate synthetase | Hs00426591_m1 |
| UGT1A1 | UDP glucuronosyltransferase 1 family, polypeptide A1 | Hs00153559_m1 |
| UGT1A10 | UDP glucuronosyltransferase 1 family, polypeptide A10 | |

Table II. Continued.

| Gene symbol | Gene description | Assay no. (AB) |
|---|---|---|
| UGT1A3 | UDP glucuronosyltransferase 1 family, polypeptide A3 | |
| UGT1A4 | UDP glucuronosyltransferase 1 family, polypeptide A4 | |
| UGT1A5 | UDP glucuronosyltransferase 1 family, polypeptide A5 | |
| UGT1A6 | UDP glucuronosyltransferase 1 family, polypeptide A6 | |
| UGT1A7 | UDP glucuronosyltransferase 1 family, polypeptide A7 | |
| UGT1A8 | UDP glucuronosyltransferase 1 family, polypeptide A8 | |
| UGT1A9 | UDP glucuronosyltransferase 1 family, polypeptide A9 | |
| UMPS | uridine monophosphate synthetase (orotate phosphoribosyl transferase and orotidine-5'-decarboxylase) | Hs00923517_m1 |
| VEGFA | vascular endothelial growth factor A | Hs00900054_m1 |
| XPC | xeroderma pigmentosum, complementation group C | Hs00190295_m1 |
| XRCC1 | X-ray repair complementing defective repair in Chinese hamster cells 1 | Hs00959834_m1 |
| XRCC3 | X-ray repair complementing defective repair in Chinese hamster cells 3 | Hs00193725_m1 |

In order to investigate the expression level of the 93 cancer-related genes, an analysis of Ct determined (CDA) and an analysis of Ct undetermined (CUA) were performed. In CDA the data transformations were applied in the following order: i) pre-processed for quality assessment. To perform this, genes with <3 out of ten replicates and Ct undetermined results were removed. For each cDNA, the values of the endogenous control $ß_2$M more than 70th percentile were not considered in the subsequent analysis. For each patient, the Ct values were obtained as mean of the replicates of each gene; genes with >30% missing values were filtered out. ii) Data were normalized using the delta method (Applied Biosystem) towards the endogenous control gene $ß_2$M. iii) Differential gene expression analysis was carried out in the two groups of cases and controls using the t-test.

The genes, which were removed from the CDA after being pre-processed for quality assessment, were considered in the CUA. In particular, the frequency of Ct undetermined was tested between cases and controls throughout the Fisher's exact test. As to the genes resulting statistically significant, the Ct undetermined was set to 45 (as the number of PCR cycles performed) and the differential gene expression analysis using the t-test was performed again.

In all analyses, the tests were adjusted for multiple comparisons using the false discovery rate (FDR) method (6) and genes with an estimated FDR <0.05 were selected as significant. Similarities among groups of patients and among groups of genes were studied using hierarchical clustering analysis (Ward method, Euclidean distance) on significant genes in each study comparison.

*Genotyping of SNPs*

*DNA extraction*. DNA was extracted from leukocytes of peripheral blood using EZ1 DNA blood kit (Qiagen) and from paraffin-embedded slices using standard xylene-phenol protocol. In order to empower statistical analysis, DNA was extracted from peripheral blood of additional control groups: i) 10 normal survivors affected by gastric cancer; ii) 115 patients affected by various cancers; iii) 232 patients affected by breast and head-neck cancer.

*Pyrosequencing*. Single nucleotide polymorphism (SNP) analysis was done through pyrosequencing using the chemotherapy response kits (Diatech). The following SNPs were characterized: MTHFR-C677T, MTHFR-A1298C, DPYD IVS14+1G>A, the 5' UTR region of TYMS (TSER), the 6-bp deletion at nucleotide 1494 within the 3'UTR of TYMS (+6 bp/-6 bp 3'UTR), Ile105Val in GSTP1, C8092A (rs3212986) and T19007C (rs11615) in ERCC1, G28152A in XRCC1 (Arg399Gln), C1236T and C3435T in ABCB1, CYP3A4*1B -392A>G and CYP3A5*3 22893G>A, ABCC2 -24C>T.

Direct sequencing and restriction enzyme analysis was used for the G/C SNP at the 12th nucleotide in the second repeat of the 3R allele (3RG>3RC). A custom assay for pyrosequencing was designed for the genotyping of p53 codon 72 polymorphism (forward and sequencing primer: 5'-caacgttctggtaaggacaagg-3'; reverse primer: 5'-ccggtgtaggag ctgctgg-3').

*SNPs statistical analysis*. In SNP analysis long survivors were compared with the 3 different sets of controls above reported. When the frequency distribution of a polymorphism was known both in cases and controls, the association between the long survival and the polymorphic variants of selected genes was tested throughout the Fisher's exact test. A binomial exact test was performed to compare the genomic profile of long survivors with data from literature.

## Results

*Expression profiles*. The expression study was performed in the three long survivors and the four normal survivors on the TLDA platform of 93 cancer-related genes (Table II and Fig. 1). The inclusion criteria of pre-processed for quality

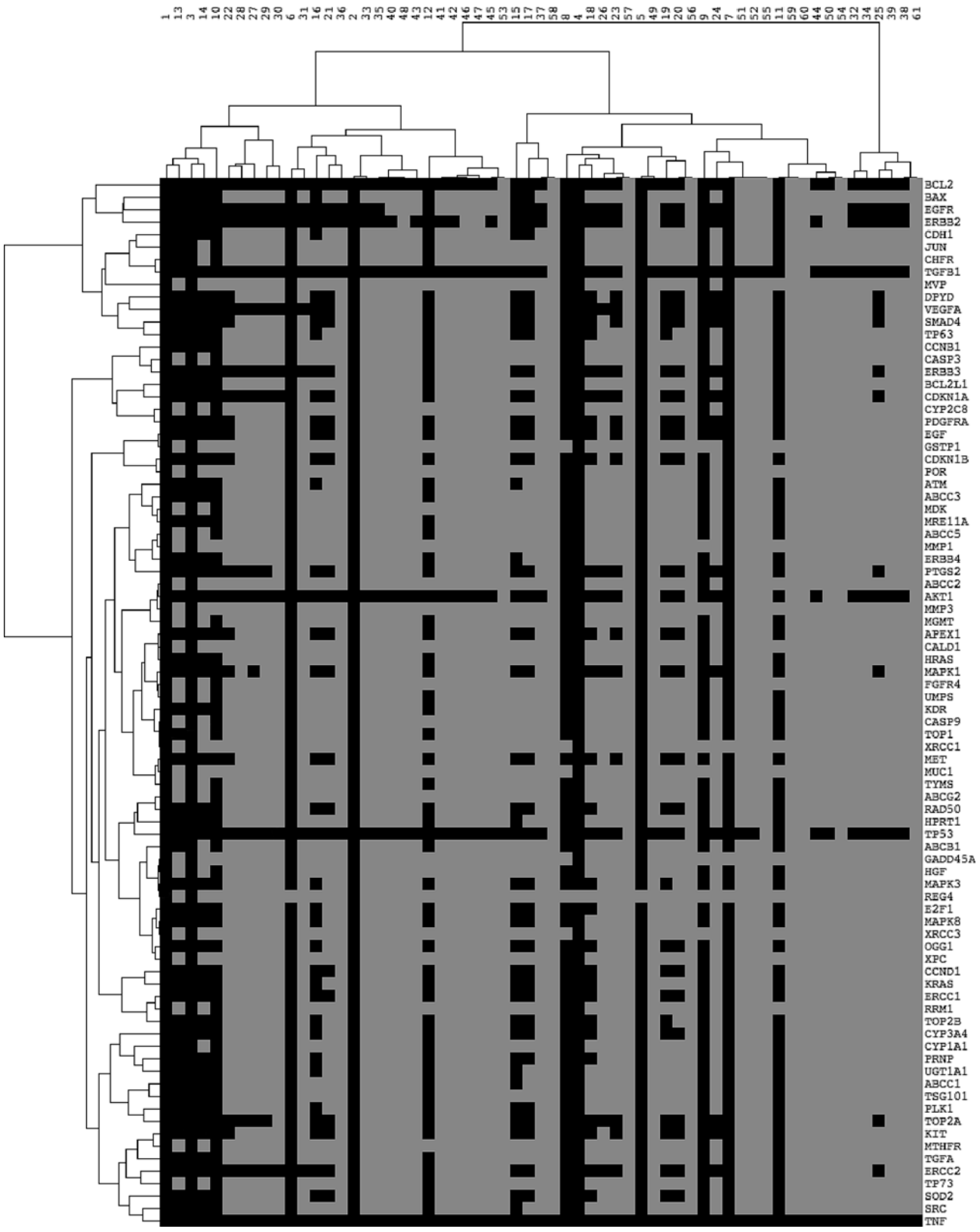


Figure 1. Clustering of selected genes according to Gene Ontology (GO) classification (http://www.geneontology.org/). The genes are classified according to GO categories (biological process). Hierarchical clustering was then performed owing to the hierachical structure GO entries for grouping together genes involved in similar biological pathways.

assessment (see *Gene expression data analysis* paragraph) were not met by five genes (i.e., CYP1A1, CYP3A4, ERBB4, EGF and TP63). Those genes were removed from the CDA and were considered in the CUA.

Expression analysis in long survivors revealed a significant upregulation of PTEN. Moreover, a consistent upregulation of GADD45a, MAPK1, TP63 genes and a consistent downregulation of the EGF gene, which presented

| Genes | ΔCt cases | ΔCt controls | -ΔΔCt | P.value | P.value.adjust | |
|---|---|---|---|---|---|---|
| PTEN-Hs01920652_s1 | 8.0705 | 13.1724 | 5.1019 | 0.0005 | 0.0430 | ** |
| GADD45A-Hs00169255_m1 | 10.0543 | 14.1033 | 4.0491 | 0.0053 | 0.2182 | ** |
| MAPK1-Hs00177066_m1 | 7.5945 | 10.2277 | 2.6332 | 0.0073 | 0.2182 | ** |
| EGF-Hs00153181_m1 | 21.1744 | 14.2098 | -6.9647 | 0.0120 | 0.0600 | * |
| TP63-Hs00186613_m1 | -6.6869 | 18.2299 | 24.9168 | 0.0350 | 0.0875 | * |
| CYP3A4-Hs00430021_m1 | 16.9003 | 21.1641 | 4.2637 | 0.1191 | 0.1824 | * |
| CYP1A1-Hs00153120_m1 | 16.8978 | 21.1641 | 4.2663 | 0.1459 | 0.1824 | * |
| ERBB4-Hs00171783_m1 | 16.8254 | 20.7188 | 3.8934 | 0.1998 | 0.1998 | * |

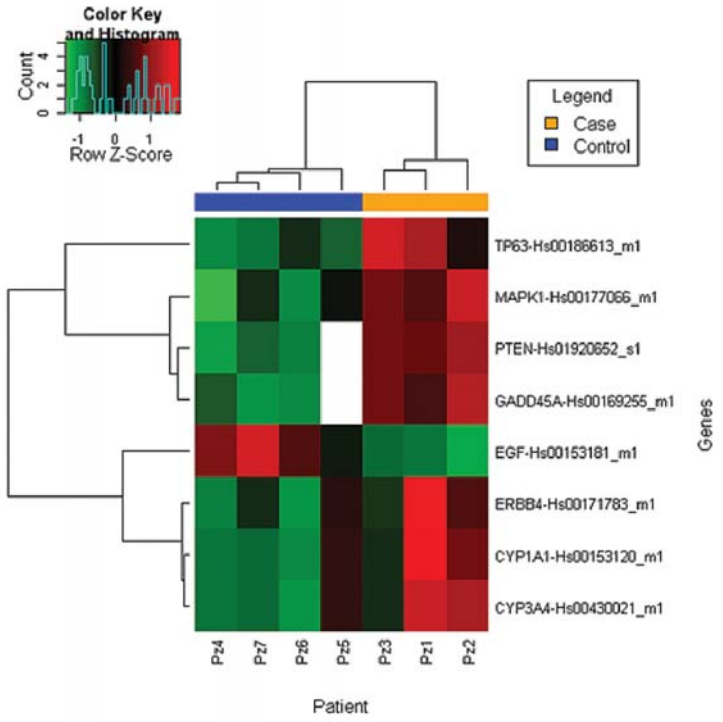


Figure 2. Expression analysis in long survivors (cases) versus normal survivors (controls). ΔCt cases, mean Delta Ct cases; ΔCt controls, mean Delta Ct controls; -ΔΔCt, Delta Delta Ct, P-value, p-value of the t-test; P-value.adjust, p-value adjusted; **results from CDA (Ct determined) analysis; *results from CUA (Ct undetermined) analysis.

a $p<0.05$, were found (Fig. 2). As regards CYP1A1, CYP3A4 and ERBB4 genes, the Fisher's exact test showed a higher frequency of Ct undetermined in controls, revealing that those genes were upregulated in long survivors. Having re-done the t-test analysis by replacing the Ct undetermined data with Ct = 45, differential expression was seen for CYP1A1, CYP3A4 and ERBB4 genes (Fig. 2), although not reaching statistical significance.

*Genetic screening*. In addition to genes involved in 5-FU (TYMS, DYPD, MTHFR) and cisplatin (GSTP, ERCC1, XRCC1) metabolism, we studied selected polymorphisms in CYP3A4, CYP3A5, ABCC2, ABCB1 genes, in order to explain, at least partially, the expression data we observed in the tissues analysed. Moreover, we analysed p53 codon 72 SNP in our cohort of patients and in additional 232 blood samples from cancer patients.

Genetic screening of SNPs did not reveal interesting genotypes correlated with the long survivor phenotype, except ERCC1 C8092A (Table III). ERCC1 C8092A was associated with survival in patients with advanced gastric cancer treated with standard chemotherapy (p-value versus 4 controls = 0.038; versus 10 controls = 0.035; versus 115 controls = 0.027).

All long survivors presented the G/G genotype at codon 72 of the p53 gene, related to a better response rate to CT, in respect to the 4 normal survivors, where the frequency was 40% and to our control population of 232 tumor samples, where frequency was 50% (data not shown). Although the data did not reach statistical significance.

## Discussion

We present a genetic and gene expression analysis of three gastric patients, metastatic at presentation, who showed unexpected long-term survival, still continuing. These cases were compared to similar patients showing a standard behaviour. Our results revealed a common signature among

Table III. Genotyping at selected SNPs of the long survivors.

| No. | p53 Arg72Pro | MTHFR C677T | MTHFR A1298C | DPYD IVS14+1G>A | TYMS TSER | TYMS +6 bp/-6 bp 3'UTR | TYMS 3RG>3RC | GSTP1 A313G |
|---|---|---|---|---|---|---|---|---|
| 1 | G/G | T/T | A/A | G/G | 2R/3R | del/del | 2R/3RC | A/G |
| 2 | G/G | C/C | C/C | G/G | 2R/3R | ins/del | 2R/3RG | A/A |
| 3 | G/G | C/C | C/A | G/G | 2R/3R | ins/del | 2R/3RG | A/G |

| No. | ERCC1 C8092A | ERCC1 T19007C | XRCC1 G28152A | ABCB1 C3435T | ABCB1 C1236T | CYP3A4*1B -392A>G | CYP3A5*3 22893A>G | ABCC2 -24C>T |
|---|---|---|---|---|---|---|---|---|
| 1 | C/A | C/C | A/G | C/C | C/C | A/A | G/G | C/C |
| 2 | A/A | T/C | G/G | C/T | C/T | A/A | G/G | T/T |
| 3 | C/A | T/C | A/G | C/T | C/T | A/A | G/G | C/C |

the long survivors that distinguish them from the control cases. The 3 patients considered are still alive and disease-free at 66-82 months from chemotherapy. Data from the literature suggest that expected 5-year survival at stage IV is of <5% (7). Indeed, the 4 patients used as control group survived 9-33 months. All the 7 patients received epirubicin/cisplatin/5-FU (ECF) regimen and the control group had even a second line CT.

To evaluate the possibility of using molecular profiling as predictor of clinical outcome and survival in metastatic gastric cancer, we used quantitative RT-PCR to measure the expression of 93 genes on a Custom Taqman Low Density Array, designed by literature mining using MedMiner for data retrieval of cancer-treatment-prolonged-survival-related genes. Selected genes are involved in drug metabolism and resistance, DNA repair, cell cycle regulation and growth factors signalling.

Recently, pharmacogenetics and large-scale molecular techniques such as DNA microarrays have contributed to our understanding of the molecular complexity of gastric cancer. Prognostic classification according to gene expression profile has been achieved (4,8) and many candidate genes for the prediction of patient survival have been reported in gastric cancer (9,10). Expression analysis in our long survivors revealed a significant upregulation of PTEN and a consistent upregulation of GADD45a, MAPK1 and TP63 genes.

GADD45a is involved in cell cycle control and stimulates DNA repair. Napieralski *et al* (11) studied pre-therapeutic paraffin-embedded biopsies of 61 advanced gastric cancer patients, who received a 5-FU-cisplatin-based CT. They analysed the 5-FU-related genes TYMS, DPYD and TP and the cisplatin-related genes ERCC1, ERCC4, KU80 and GADD45a by quantitative real-time PCR. GADD45a and TP levels showed weak associations with response, but GADD45a expression correlated with survival.

There is ample evidence that the functions of GADD45 proteins are mediated via interactions with other cellular proteins implicated in cell cycle regulation and the response of cells to extrinsic stress, including p21, Cdc2/cyclin B1 and p38/JNK.

Mitogen-activated protein kinase 1 (MAPK1) also known as p38, ERK or ERK2, is a member of the MAP kinase family, involved in a wide variety of cellular processes such as proliferation, differentiation, transcription regulation and development. Its activity is determined by the upstream Ras/Raf/MEK cascade and the deactivating MAPK phosphatases 1, 2, and 3. Wu *et al* (12) showed that ERK1/2 phosphorylation and c-Jun expression were significantly lowered in gastric cancer compared with the non-cancer adjacent tissues.

The phosphatase and tensin homolog mutated on chromosome ten (PTEN) gene product is a protein tyrosine phosphatase that participates in modulating the phosphoinositide 3-kinase pathway which antagonizes protein tyrosine kinases. Several authors investigated the correlation between clinicopathologic variables, including survival, and the loss of PTEN expression in gastric adenocarcinoma patients. Altered PTEN expression was significantly associated with tumor depth and size, lymphatic invasion, advanced stage, pTNM stage, and patient survival (13-15).

TP63 gene is a p53 homologue that encodes proteins with transactivation, DNA-binding and tetramerization domains. No clear published data on TP63 expression in gastric cancer exist. A few reports are related to other malignancies. Several authors found that decreased expression of TP63 and decreased p63 immunoreactivity is significantly associated with advanced tumor stage and grade in renal malignancies (16) and in gallbladder cancer (17). Hallack *et al* (18) found that p63 was expressed in more than 50% of malignant cells in diffuse large B-cell lymphomas and p63(+) patients had better disease-free survival than those who were negative.

In addition, in the three long survivors we found also an upregulation of ERBB4, CYP1A1 and CYP3A4 genes. Although these genes are not differentially expressed according to the t-test analysis, results supplied by Fisher's exact test seem sufficient to take them into consideration.

HER1 and HER2 overexpression in gastric cancers are thought to be prognostic factors and targets of novel biological agents. Hayashi and collaborators (19) found that HER3 overexpression was associated with a significantly worse survival and was an independent prognostic factor in gastric cancer, whereas HER1, HER2 or HER4 overexpression did not show any such association. The effect of HER4 expression in gastric cancer has not been fully elucidated. Published data on HER4 in breast cancer reported conflicting results (20,21). However, Sassen *et al* (22) demonstrated that HER4 expression prolongs overall survival in Herceptin-treated breast cancer patients. HER4 expression was not associated with the prognosis of patients with colorectal cancer, although its membranous expression was associated with involved lymph nodes.

Because CYP3As inactivate many anticancer drugs, an overexpression of CYP3As in tumors could result in an increased intratumoral drug inactivation and decreased drug efficacy. Rodríguez-Antona *et al* (23) found that a high tumoral expression of CYP3A4 was significantly associated with a lower complete remission rate in peripheral T-cell lymphomas (PTCL). They concluded that a high CYP3A4 tumoral expression could be useful to predict poor response to the standard PTCL chemotherapy, but it does not affect survival. However, CYP3A4 and CYP3A1 are not the main enzymes involved in the metabolism of the drugs of the ECF regimen used in these patients. Epidermal growth factor (EGF) promotes the growth of cells of both ectodermal and mesodermal origin, and plays an important role in cellular proliferation and differentiation. Studies investigating EGF receptor in gastric cancer patients have shown that an increased level was associated with poor prognosis (24). Gastric cancer patients with EGFR expression and low ligand levels had better outcomes with cetuximab/mFOLFOX6 treatment (25). In accordance with those studies, we found that EGF is down-regulated in our cohort of long survivals.

Since genetic polymorphisms could explain variations in pharmacokinetics, in activity and in expression of the target or of the proteins involved in the mechanism of action of the drug, we analyzed functional genetic variations in metabolizing enzymes of 5-FU (TYMS, DPYD, MTHFR) and of cisplatin (GSTP, ERCC1, XRCC1). We added drug resistance ABCB1 and ABCC2 genes and CYP3A4, that we have found differentially expressed in long survivors versus normal survivors.

Moreover, several studies have already suggested that the p53 codon 72 polymorphism modulates the p53-dependent apoptotic capacity. In particular, the Arg/Arg genotype correlates with better response rate to CT and longer time to progression in patients with advanced gastric cancer (26). Huang and co-authors (27) found same association in gastric cancer patients who benefit from oxaliplatin-based adjuvant CT.

We did not find any statistical difference at this locus between long and normal survivors, although all the long survivors presented the G/G genotype. This lack of significance may be related, of course, to the low number of long survivors.

Genetic screening did not reveal any other interesting genotype correlated with the long survival, a part from SNP C8092A in ERCC1 (excision repair cross complementing 1). Among the members of the nucleotide excision repair system (NER) family, the product of the ERCC1 gene is necessary for the repair of the damaged DNA due by cross-link interchain and intrachain induced by cis-platinum and its derivates. The expression of ERCC1 gene has been correlated with the clinical outcome of non-small cell lung cancer (NSCLC) and colon cancer treated with cisplatinum and oxaliplatinum, in particular increased responsiveness and prolonged survival has been demonstrated in situations of reduced expression of the ERCC1 gene (28).

C8092A (rs3212986) and T19007C (rs11615) are two common polymorphisms of ERCC1, that have been demonstrated to impact clinical outcome of patients receiving platinum-based chemotherapy. In particular, Okuda *et al* (29) studied C8092A polymorphism in advanced NSCLC patients treated with platinum-based chemotherapy and found that C/C genotype at codon 8092 was associated with better prognosis than C/A or A/A and the wild-type C/C of the codon 118 was associated with better prognosis than C/T or T/T types. On the contrary, Kalikaki *et al* (30) found significant association between the ERCC1 C8092A polymorphism and overall survival in advanced NSCLC, suggesting that any copies of the A allele were associated with an improved outcome.

In conclusion, the present study has shed light on explaining long survival in our metastatic gastric cancer patients. We have focused on a set of markers targeting specific pathways or a set of pathways that may be involved in cellular response to cytotoxic agents. However, it is clear that the cancer phenotype is a sum total of genetic and epigenetic alterations and, therefore, the ultimate response to cytotoxic therapy and survival in cancer is also likely to be dictated by these genetic and epigenetic changes involving perhaps several thousands of genes within the cancer genome.

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