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Molecular determinants of outcome in sorafenib-treated patients with hepatocellular carcinoma

Nicola Personeni · Lorenza Rimassa · Tiziana Pressiani · Annarita Destro ·
Claudia Ligorio · Maria Chiara Tronconi · Silvia Bozzarelli · Carlo Carnaghi ·
Luca Di Tommaso · Laura Giordano · Massimo Roncalli · Armando Santoro

Abstract

Purpose Preclinical studies show that sorafenib, a multitarget kinase inhibitor, displays anti-proliferative, anti-angiogenic, and pro-apoptotic properties in hepatocellular carcinoma (HCC). However, the determinants of sorafenib sensitivity in vivo remain largely unknown.

Methods We assessed the expression of Mcl-1, activated/phosphorylated extracellular signal-regulated kinase (pERK) 1/2, and activated/phosphorylated AKT (pAKT) in pretreatment tumor specimens from 44 patients with advanced HCC who received sorafenib. Furthermore, we assessed *MYC* and *MET* gene copy numbers (GCN) by fluorescence in situ hybridization.

Results Poorer overall survival (OS) times were correlated with pERK expression [hazard ratio (HR) 1.013; 95 % CI 1.003–1.035] and Mcl-1 expression (HR 1.016; 95 % CI 1.002–1.030) in pretreatment tumor samples. Expression levels of pERK and Mcl-1, however, were not correlated with time to tumor progression (TTP). Increased pERK expression was positively associated with higher Cancer of Liver Italian Program scores ($P = 0.012$) and was prognostic in patients with scores 2–6 but not in those with scores 0–1. pERK expression was significantly less frequent in specimens sourced from previous surgical procedures compared to biopsy samples (9.6 vs. 92.3 %, respectively; $P < 0.0001$). Analysis of pAKT expression, *MET* and *MYC* GCN, did not indicate any prognostic nor predictive values for these biomarkers in terms of survival.

Conclusions Expression levels of Mcl-1 and pERK are associated with reduced OS in HCC patients treated with sorafenib and might be useful markers for risk stratification. However, in contrast to previous findings, pERK expression levels, as well as other biomarkers tested, did not affect TTP.

Keywords Sorafenib · Hepatocellular carcinoma · Fluorescence in situ hybridization · pERK · Prognosis · Mcl-1

N. Personeni (✉) · L. Rimassa · T. Pressiani · C. Ligorio ·
M. C. Tronconi · S. Bozzarelli · C. Carnaghi · A. Santoro
Department of Oncology-Hematology, Humanitas Clinical and
Research Center, Via Manzoni 56, 20089 Rozzano (Mi), Italy
e-mail: nic_personeni@hotmail.com

A. Destro · L. Di Tommaso · M. Roncalli
Department of Pathology, Humanitas Clinical and Research
Center, Via Manzoni 56, 20089 Rozzano (Mi), Italy

L. Giordano
Humanitas Clinical and Research Center, Via Manzoni 56,
20089 Rozzano (Mi), Italy

Introduction

For patients with advanced hepatocellular carcinoma (HCC) not responsive to curative or locoregional therapies, few therapeutic options exist, as common cytotoxic chemotherapeutic regimes are ineffective. Two Phase III clinical trials found that sorafenib, an inhibitor of tyrosine kinases involved in tumor proliferation and angiogenesis, conferred significant survival advantages over placebo (Llovet et al. 2008; Cheng et al. 2009). In HCC cell lines, sorafenib inhibits the RAF/mitogen-activated protein kinase (MAPK) kinase (MEK)/extracellular signal-regulated kinase 1/2 (ERK) signaling pathway, and it displays pro-apoptotic effects by downregulating the anti-apoptotic protein Mcl-1 (Liu et al. 2006). Due to the inhibition of such diverse pathways, critical questions have been raised as to which of the mechanisms of action are responsible for sorafenib-mediated clinical benefits. In a Phase II study, higher levels of ERK phosphorylation (pERK) in tumor samples were

more likely to predict a longer time to tumor progression (TTP) (Abou-Alfa et al. 2006), thereby suggesting that pERK levels might predict sorafenib efficacy. These findings were further paralleled by preclinical evidences suggesting a correlation between pERK levels and sensitivity to sorafenib in different HCC cell lines (Zhang et al. 2009).

Genome-wide mapping of gene copy numbers (GCNs) alterations in HCC has identified recurrently altered chromosomal regions in HCC. Interestingly, large-size regional changes leading to copy number gains have been detected on 7 and 8q (Kim et al. 2008; Schlaeger et al. 2008), which contain deregulated oncogenes including *MET* and *MYC*, respectively. Increased *MET* GCN, detected by fluorescence in situ hybridization (FISH) and protein overexpression, are predictors of poor prognosis in nonsmall cell lung cancer (Cappuzzo et al. 2009) and in HCC (Ueki et al. 1997; Santoro et al. 2013; Ke et al. 2009), respectively. Furthermore, a prognostic model established according to *MET*-dependent gene expression signatures previously identified a subset of HCC characterized by an aggressive phenotype and poor prognosis (Kaposi-Novak et al. 2006).

Gains of *MYC* GCN, as assessed by FISH, have been detected in approximately 30 % of HCCs (Chan et al. 2004) and frequently associate with HBV, HCV infection, and alcohol abuse (Schlaeger et al. 2008). A *MYC* activation signature has been identified as a prominent feature of a molecular subclass of HCC displaying elevated of phosphorylated AKT (pAKT) (Hoshida et al. 2009). Interestingly, in MYC-dependent tumors, the anti-apoptotic and the mitochondrial functions of Mcl-1 and Bcl-xL, which belong to the Bcl-2 family proteins, may play relevant roles in sustaining tumor growth fueled by concomitant oncogenic stresses (Perciavalle and Opferman 2013). Meanwhile, Mcl-1 also counteracts Bak and/or Bax, which are pro-apoptotic effectors induced in response to cellular stress and death signals (Cheng et al. 2001). Mounting evidence indicates that human HCC and HCC cell lines often overexpress Mcl-1 (Sieghart et al. 2006; Fleischer et al. 2006), which is considered a predictor of resistance to chemotherapy-induced apoptosis. Given these data, to further clarify the predictive potential of selected biomarkers, we examined the expression of pERK, pAKT, and Mcl-1 in pretreatment samples from a cohort of patients with advanced HCC treated with sorafenib. Additionally, *MYC* and *MET* GCN were evaluated by FISH analysis for their contribution.

Methods

Patients and treatment

We retrospectively evaluated the clinical records of patients with advanced HCC treated with sorafenib in one of three clinical trials conducted at Humanitas Cancer Center (Llovet et al. 2008; Abou-Alfa et al. 2006; Pressiani et al. 2013). Patients included in these trials had metastatic or locally advanced, pathologically confirmed HCC not amenable to surgery or locoregional therapies, including transcatheter arterial (chemo)embolization, and local ablation. The three trials were approved by the Institutional review board of Humanitas Cancer Center. Written informed consent for analysis of clinical records had been given by the patients at the time of enrollment onto these trials. For each patient, we obtained pretreatment data regarding age, gender, cause of disease, Child-Pugh class, Eastern Cooperative Oncology Group performance status, Cancer of Liver Italian Program (CLIP) stage, and presence of extrahepatic disease. Tumor response to sorafenib was evaluated every 8 weeks by computed tomography (CT) according to RECIST (Therasse et al. 2000). Formalin-fixed paraffin-embedded specimens were sourced from previous surgical procedures or obtained from diagnostic biopsies prior to enrollment. Patients were required to have measurable progressive disease and were rated as either Child-Pugh A or B. Sorafenib treatment was continued at 400 mg bid until progression of disease or toxicity occurred.

Immunohistochemistry

Standard 2-µm-thick sections were submitted to antigen retrieval and incubated with mouse monoclonal Mcl-1 Ab-1 antibody (1:25 dilution; Thermo Scientific, Waltham, MA), polyclonal Phospho-ERK1/2 [phospho p44/42 Map kinase (Erk1/2) threonine 202; tyrosine 204, 1:100 dilution; Cell Signaling Technology, Danvers, MA, USA], and Phospho-Akt [Phospho-Akt (1/2/3) ser 473, 1:25 dilution; Cell Signaling Technology, Danvers, MA]. Slides were then incubated with the secondary antibody using the DAKO EnVision Universal kit (DAKO Corporation, Carpinteria, CA, USA). Staining was performed with 3,3′-diaminobenzidine (DAB) as a chromogen, and sections were then counterstained with hematoxylin. Negative control slides were incubated with nonimmune solution instead of the primary antibody. The constant positive staining of smooth muscle cells of tumor vessels served as the positive internal control.

In the absence of validated scoring systems to interpret staining of Mcl-1, pERK, and pAKT in HCC, the percentage of immunoreactive nuclei and intensity of immunoreactivity for each marker, as scored in ten high-power fields, were considered. In agreement with the previous biomarker investigations into HCC (Schmitz et al. 2008; Xiong et al. 2010), tumors exhibiting pERK, Mcl-1, and pAKT staining in less than 1 % cells were considered negative. With respect to pERK expression, a previously

published semiquantitative scoring system was also considered (Abou-Alfa et al. 2006). Mcl-1, pERK, and pAKT immunostaining was assessed by two pathologists (MR and LDT) in a blind-trial fashion without knowledge of the clinical outcome.

Fluorescence in situ hybridization (FISH) analysis

Unstained 4-μm sections, obtained from tumor biopsies or resections, were submitted to a dual-color FISH assay. *MET* and *MYC* GCN were investigated using the *MET*-specific (Jiang et al. 2008) SpectrumOrange LSI D7S522/SpectrumGreen CEP7 probe set (Vysis/Abbott Molecular, Des Plaines, IL, USA) and a mixture probe composed of SpectrumOrange LSI c-myc (Vysis/Abbott Molecular, Des Plaines, IL, USA) and SpectrumGreen CEP8 alpha (Vysis/Abbott Molecular, Des Plaines, IL, USA). The presence of invasive carcinoma was confirmed by a pathologist from a slide sequentially stained with hematoxylin and eosin (H&E). Following deparaffinization and dehydration, pretreatment and enzyme digestion were performed using $2\times$ SSC at 75 °C and Proteinase-K for 12–15 min at 45 °C. Captures were acquired using an epifluorescence microscope with single (orange, green, DAPI)-, dual (orange/green)-, and triple-band (orange/green/DAPI) filters and equipped with a charge-coupled device (CCD) camera and dedicated software (CytoVision, Santa Clara, CA, USA).

FISH interpretation

Probe signals were counted in individual nuclei if they were bright, distinct, and easily assessable against a dark background relatively free of fluorescent particles and haziness. GCN and chromosome centromere numbers per nucleus were determined in at least 60 nuclei distributed within two to three contiguous microscope fields inside tumor areas selected in the proximity of relevant histological features identifiable on the H&E slide. Chromosome polysomy was defined as >2 centromere signals with a mean gene/chromosome centromere ratio <2. A mean gene/chromosome centromere ratio ≥2 was considered as gene amplification.

Statistical analysis

To verify whether the mean GCN per cell and the percentage of staining cells were sufficiently informative to predict survival outcomes, an univariate Cox proportional hazards model was constructed with OS as the primary endpoint, in order to calculate hazards ratios (HR), 95 % confidence intervals (95 % CIs), and *P* values. The prognostic significance of such parameters in terms of diagnostic performance was depicted by the area under the curve (AUC), with values ranging between 0.5 (fortuitous prediction) and 1 (perfect discrimination). If the model was deemed to be informative, then cutoff points would be determined by means of the receiver operating characteristics (ROC) curve analysis. The value being related to the greatest sensitivity and specificity would be used as the cutoff point. Chi-square test (continuity adjusted) or Fisher's exact test, when appropriate, was used to calculate the *P* values for the associations between marker expression level and clinical and demographic characteristics and disease control rate following sorafenib treatment. Pearson's correlation coefficient was used to measure the strength of the association between the markers' expression. Univariate survival analyses were depicted using the Kaplan–Meier method, while a multivariate model was built to assess the effect of confounding factors. The level of statistical significance was set at $P = 0.05$.

TTP was defined as the time from the first day of sorafenib treatment to the day of tumor progression. Data from patients who died without tumor progression were censored at the time of their last radiological assessment. Overall survival was measured from the first day of sorafenib treatment until the date of death from any cause, or the date of the last follow-up at which the patients were censored.

Results

Patient characteristics and clinical outcomes

Review of clinical records of HCC patients treated at Humanitas Cancer Center in trials of sorafenib permitted the identification of 44 patients with available samples. Thirteen specimens were sourced from previous surgical procedures, while 31 originated from diagnostic biopsies. After median treatment duration with sorafenib of 3.4 months, median OS was 10.2 months (1.5–46.9) and median TTP was 6.0 months (1.4–46.9). Survival rate at 1 year was 50.8 %. Overall, 41 patients were evaluable for radiological outcome. Among them, one patient (9 %) had a partial response (PR), and 28 (68 %) had stable disease. The radiological outcome of three patients could not be evaluated due to global deterioration of health status requiring early discontinuation of treatment. The association between the most relevant clinical characteristics and OS in univariate analysis is shown in Table 1. Only the dichotomized CLIP score and alpha-fetoprotein (AFP) logarithmic values analyzed as a continuous trait were significantly associated with survival.

Table 1 Univariate Cox proportional hazard ratios, 95 % confidence intervals (95 % CI), and *P* values for the contribution of clinical factors to overall survival patients with advanced hepatocellular carcinoma

	Hazard ratio	95 % CI	*P*
Gender, male versus female	0.91	0.28–2.99	0.881
Age	0.99	0.95–1.03	0.616
Bilirubin, abnormal versus normal	1.77	0.86–3.63	0.121
Child-Pugh status, B versus A	1.96	0.88–4.31	0.096
Cause of disease, hepatitis C versus other causes	1.51	0.79–2.89	0.210
ECOG performance status, 2 versus 0–1	1.24	0.68–2.25	0.490
Baseline alpha-fetoprotein levels (logarithmic values)	1.29	1.02–1.63	0.036
Sampling, biopsy versus surgery	1.41	0.73–2.72	0.313
CLIP[a] staging, score 2–6 versus 0–1	2.50	1.24–5.07	0.011
Extrahepatic disease, yes versus no	0.83	0.43–1.57	0.560

[a] Cancer of the liver Italian program

Immunohistochemistry analyses and association with clinico-pathological features

Thirty of 44 samples (68 %) displayed positive pERK staining to some extent. According to scoring criteria previously published by Abou-Alfa et al. (2006), pERK staining was deemed as moderate to intense in five of 44 cases (11.3 %). Furthermore, of 42 samples available for analysis of Mcl-1 expression, 24 (57 %) displayed a positive staining (Fig. 1), while 24/44 samples (62 %) displayed positive pAKT expression patterns. Consistent with a previous report (Domina et al. 2004), expressions of Mcl-1 and pERK were found to be directly correlated ($P < 0.001$). The relationship of pERK and Mcl-1 expression with most relevant clinico-pathological parameters is summarized in Table 2. Interestingly, 12 of 13 (92.3 %) surgical specimens did not exhibit pERK immunoreactivity in neoplastic tissue, compared with three of 31 (9.7 %) biopsy samples ($P < 0.001$) (Fig. 2). Additionally, increased pERK expression levels, when analyzed as a
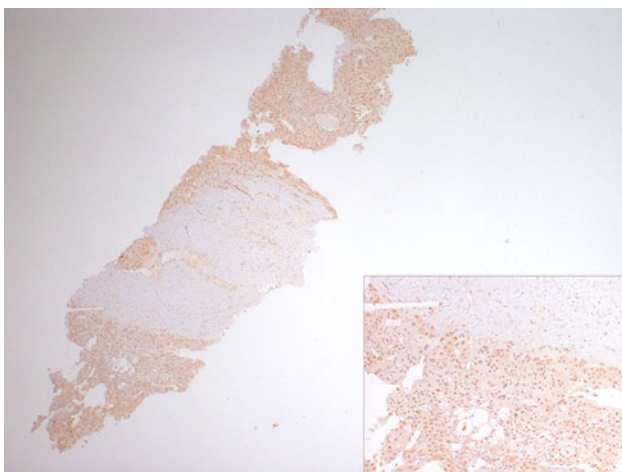
continuous trait, were positively associated with higher CLIP scores (odds ratio 1.46; 95 % CI 1.09–1.95, $P = 0.012$). This association prompted us to further analyze the expression of pERK and Mcl-1 in noncancerous liver tissue retrieved from surgical samples. Although this analysis was restricted to only eight samples with surrounding available liver tissue, in keeping with previous investigations (Krajewski et al. 1995; Schmitz et al. 2008), we observed a weak homogeneous Mcl-1 staining throughout normal hepatocellular tissue in all samples, whereas no pERK staining was detected.

Correlation of pERK, pAKT, and Mcl-1 immunostaining with overall survival and time to progression

When assessed as a continuous trait, higher pERK expression levels were associated with a slight increase in the risk of death (HR 1.013; 95 % CI 1.003–1.035; $P = 0.025$). After correction for the logarithmic basal values of alpha-fetoprotein (AFP), in patients with CLIP scores between 2 and 6, higher pERK levels conferred a significant increase in the risk of death (HR 1.02; 95 % CI 1.00–1.03; $P = 0.040$), but not in patients with CLIP scores 0 or 1 (HR 0.94; 95 % CI 0.72–1.22; $P = 0.620$). Using previously published scoring criteria (Abou-Alfa et al. 2006), moderate to intense pERK staining was associated with a markedly greater risk of death (HR 4.5; 95 % CI 1.7–12.2; $P = 0.003$). However, pERK expression, regardless of the scoring method, was not associated with TTP. No association between pAKT expression and OS or TTP was detected (data not shown). In contrast, Mcl-1 expression, when analyzed as a continuous trait, conferred a slightly increased risk of death (HR 1.016; 95 % CI 1.002–1.030; $P = 0.021$), but was not associated with TTP ($P = 0.818$). We tried a time-dependent ROC curve analysis in the attempt to generate a cutoff point for Mcl-1 expression that predicted survival. The AUC for Mcl-1 expression was 0.72. Thus, with Mcl-1 expression dichotomized as positive or negative, after 12-month follow-up,

Fig. 1 Mcl-1 staining in hepatocellular carcinoma. The immunoreactivity is limited to the malignant cells avoiding the fibrous septa within the lesion (low magnification). As highlighted in the *inset*, Mcl-1 exhibits nuclear reactivity

Table 2 pERK1/2 and Mcl-1 expression and baseline clinicopathological characteristics of study patients ($N = 44$) with advanced hepatocellular carcinoma treated with sorafenib

Characteristics	pERK expression		P	Mcl-1 expression		P
	Positive	Negative		Positive	Negative	
Age, years (range)	68 (48–83)	68 (57–77)	1.000	68.5 (45–83)	67.5 (57–77)	0.587
Cause of disease, N (%)			0.828			1.00
Hepatitis C only	16 (55.2)	7 (46.7)		13 (54.2)	9 (50.0)	
Other	13 (44.8)	8 (53.3)		11 (45.8)	9 (50.0)	
Sex, N (%)			0.264			1.00
Male	28 (96.6)	13 (86.7)		22 (91.7)	17 (94.4)	
Female	1 (3.4)	2 (13.3)		2 (8.3)	1 (5.6)	
Extrahepatic spread, N (%)			0.176			0.680
Present	8 (27.6)	8 (53.3)		8 (33.3)	8 (44.4)	
Absent	21 (72.4)	7 (46.7)		16 (66.7)	10 (55.6)	
Child-Pugh status, N (%)			0.037			0.109
A	21 (72.4)	15 (100)		17 (70.8)	17 (94.4)	
B	8 (27.6)	0		7 (29.2)	1 (5.6)	
Baseline total bilirubin levels, N (%)			0.065			0.262
Normal	18 (64.3)	14 (93.3)		17 (70.8)	15 (88.2)	
Abnormal	10 (35.7)	1 (6.7)		7 (29.2)	2 (11.8)	
Performance status, N (%)			0.285			0.154
0	10 (34.5)	9 (60.0)		8 (33.3)	11 (61.1)	
1	17 (58.6)	6 (40.0)		14 (53.3)	7 (38.9)	
2	2 (6.9)	0		2 (8.3)	0	
CLIP[a] stage, N (%)			<0.001			0.078
0–1	6 (21.4)	11 (78.6)		7 (29.2)	10 (62.5)	
2–6	22 (78.6)	3 (21.4)		17 (70.8)	6 (37.5)	
Sampling, N (%)			<0.001			<0.001
Surgery	1 (3.4)	12 (80.0)		1 (4.2)	12 (66.7)	
Biopsy	28 (96.6)	3 (20.0)		23 (95.8)	6 (33.3)	
Baseline alpha-fetoprotein levels[b], N (%)			0.235			1.00
Normal	4 (13.8)	5 (33.3)		5 (20.8)	4 (22.2)	
Abnormal	25 (86.2)	10 (66.7)		19 (79.2)	14 (77.8)	

Values are median (range) unless otherwise indicated

[a] Cancer of the liver Italian program

[b] Alpha-fetoprotein levels >8 ng/ml are considered abnormal

26.2 % of patients with Mcl-1-positive tumors were alive compared with 76.1 % of patients with Mcl-1-negative tumors. The Kaplan–Meier survival curves for OS according to Mcl-1 expression are shown in Fig. 3; positive Mcl-1 expression conferred a markedly increased risk of death (HR 4.6; 95 % CI 1.5–13.8, $P = 0.007$). In the multivariate model, only Mcl-1 expression and CLIP dichotomized score (0–1 vs. 2–6) were found independent prognostic factors for overall survival (data not shown). No differences in terms of partial response or stable disease were observed with respect to Mcl-1 or pERK expression. In order to assess the combined effect of Mcl-1 and pERK expression on survival, a composite Mcl-1/pERK score to define three groups of patients was generated: One group

had tumors showing negative Mcl-1 and negative pERK expression (14 patients), the second group had tumors with either negative Mcl-1 and positive pERK expression or positive Mcl-1 and negative pERK expression (five patients), and the third group (23 patients) had tumors expressing both Mcl-1 and pERK. Patients in the latter group experienced a shorter OS ($P = 0.014$; Fig. 4), but not TTP ($P = 0.836$).

FISH analyses

FISH analysis of *MYC* and *MET* GCN was informative in all cases. The range of *MET* and *MYC* signals was 1.27–3.62 (mean 2.01) and 1.37–5.52 (mean 2.73),
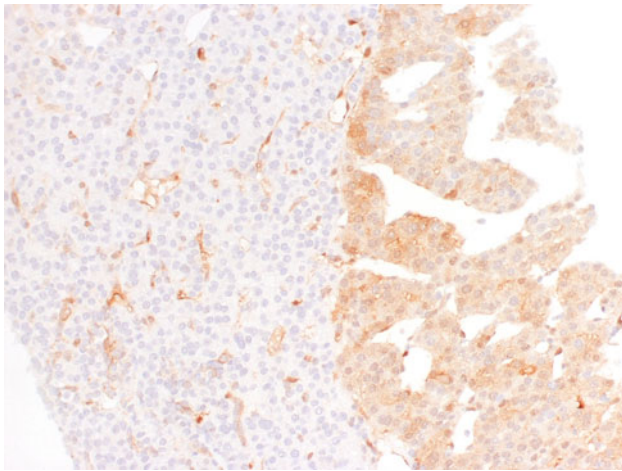
Fig. 2 Heterogeneous pERK expression in HCC: nucleo-cytoplasmic staining in malignant hepatocytes (*right*) as compared to a negative tumoral area with exclusive endothelial staining (*left*)
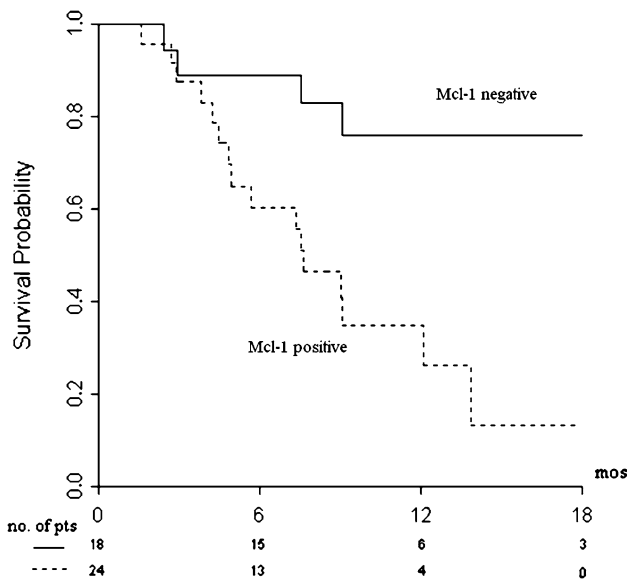

Fig. 3 Survival of patients (*pts*) with advanced hepatocellular carcinoma, according to Mcl-1-positive or Mcl-1-negative tumor expression (threshold identified by the receiver operating characteristic curve analysis). At 12-month (*mo*) follow-up, 26.2 % of patients with Mcl-1-positive tumors were alive compared with 76.1 % of patients with Mcl-1-negative tumors

Fig. 4 Overall survival of patients (*pts*) with advanced hepatocellular carcinoma, according to combined Mcl-1 and pERK tumor expression levels

Fig. 5 Representative results for *MET* GCN analysis by FISH with reference to CEP7 signals. The majority of tumor cells exhibit low-level polysomy (≤4 copies) of CEP7 (*green*) with balanced genomic gains of *MET* (*red*). No tumor cells show *MET* amplification

respectively. In 17 specimens exhibiting chromosome 7 polysomy (Fig. 5), the mean *MET*/CEP7 ratio was 1.28, indicating that the relative increase in *MET* GCN was due to chromosome 7 polysomy. In contrast, among 32 specimens with extra *MYC* copies, three displayed gene amplification with a mean *MYC*/CEP8 ratio equal to 2.25 (Fig. 6). In the remaining cases, the relative increase in *MYC* GCN was related to chromosome 8 polysomy. For both *MET* and *MYC* GCN, no significant association was
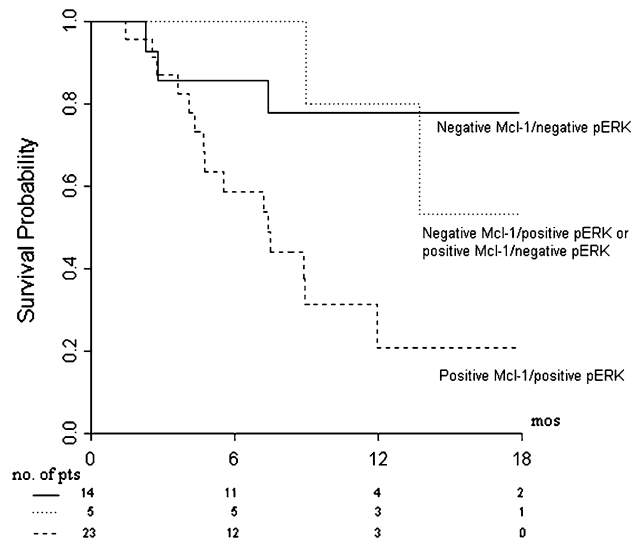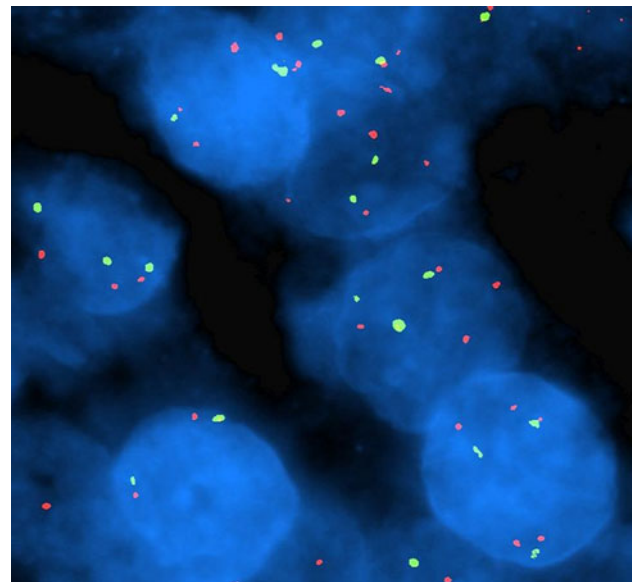
observed with TTP or OS. In particular, OS was not significantly different in three subgroups identified by different tertiles of *MYC* GCN ($P = 0.334$) and *MET* GCN ($P = 0.715$). Similar results were observed using alternative parameters such as the gene/CEP ratio and absolute CEP copy number. With respect to OS and TTP, the outcome of three patients displaying *MYC* amplification in tumor sample did not differ from remaining patients.
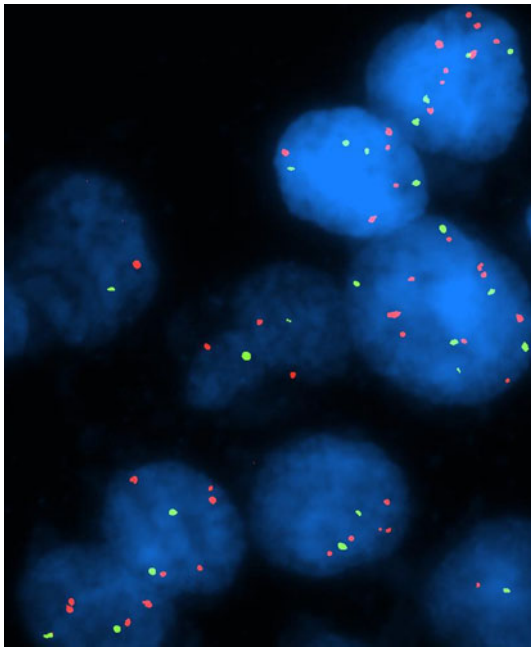
Fig. 6 Representative results for *MYC* GCN analysis by FISH with reference to CEP8 signals. A high level of *MYC* GCN gain (5 or more signals, *red*) relative to the CEP8 signals (*green*) is observed, resulting in a ratio of *MYC* signals to CEP8 ≥2, which is consistent with *MYC* amplification

Discussion

Based on the previous preclinical and clinical investigations, we retrospectively evaluated a panel of relevant prognostic biomarkers to assess their predictive value in a cohort of patients with advanced HCC treated with sorafenib. From a clinical standpoint, we confirm the usefulness of AFP values and CLIP scoring system as valid prognostic tools in HCC patients treated with sorafenib. Our findings are in substantial agreement with previous investigations reporting that increased AFP levels detected at diagnosis correlate with patients' prognosis (Nomura et al. 1989; Stuart et al. 1996; Farinati et al. 2006). Although in the multivariate analysis, among clinical variables we considered, only the CLIP score was significantly associated with survival, the prognostic usefulness of AFP levels is corroborated by the integration of this parameter in most scoring systems used in HCC, including the CLIP score itself. Additionally, we found that expression levels of Mcl-1 and pERK were significantly lower in patients who experienced longer OS following sorafenib treatment. However, with a cutoff similar to that reported by Abou-Alfa et al. (2006), only 11 % of specimens demonstrated high levels of pERK immunoreactivity, compared with 54 % of samples observed in that series. Although the same antibody was used, these discrepancies are likely to reflect the variability in tissue fixation times (Baker et al. 2005), which hampers the reproducibility of

phosphoprotein assessment and, in turn, their systematic use in clinical practice.

Expression of pERK, whichever scoring criterion was considered, was associated with shorter OS, but in contrast to previous investigations (Abou-Alfa et al. 2006), no association was detected with TTP. Thus, the results of the current study do not support the use of pERK expression as a predictive marker of sorafenib efficacy. Likewise, in advanced melanoma, high ERK levels, although they do not necessarily mirror protein activation, indicate shorter OS after treatment with sorafenib and chemotherapy (Jilaveanu et al. 2009). In contrast to previous preclinical findings (Zhang et al. 2009), in this series, the association between high pERK expression and reduced OS times might therefore suggest either resistance to treatment or a biologically more aggressive disease. The latter possibility is supported by previous reports on pERK expression as a marker of poor prognosis in patients with HCC (Schmitz et al. 2008). Indeed, we observed increased pERK levels more frequently in biopsy samples compared to surgical specimens. This observation reflects somehow better prognoses among HCC patients that undergo surgical procedures instead of diagnostic biopsies. Accordingly, we also detected a significant correlation between increased CLIP scores and higher pERK levels, being prognostic in the subset of patients with scores comprised between 2 and 6, but not in patients with scores ranging from 0 to 1.

In agreement with previous figures (Sieghart et al. 2006), the current study revealed that Mcl-1 was expressed in more than half of the tumor samples analyzed. Sorafenib induces Mcl-1 downregulation as a posttranslational event, and this process further mediates cell apoptosis (Yu et al. 2005), whereas the overexpression of Mcl-1 itself might protect from sorafenib-induced apoptosis in tumor cell lines (Yu et al. 2005). Consistently, our findings indicate shorter OS in patients whose tumors express Mcl-1 and might suggest a prognostic effect of this marker, which was previously observed in breast cancer (Ding et al. 2008).

Biomarkers in the current study were analyzed on the basis of radiological endpoints such as TTP. Considering the lack of correlation between radiological response and survival benefits in recent clinical trials of sorafenib (Llovet et al. 2008; Cheng et al. 2009; Personeni et al. 2012), it may be the case that the conventional radiological criteria (Therasse et al. 2000) are not even adequate for depicting potential associations with molecular biomarkers.

A key role of genes such as *MYC* and *MET*, located in recurrently altered chromosomal regions identified by specific cytoband gains, was suggested based on specific clinical features or outcomes (Kim et al. 2008; Schlaeger et al. 2008). In keeping with previous investigations (Chan et al. 2004; Schlaeger et al. 2008; Hoshida et al. 2009), this study confirms frequent increases in *MYC* GCN, which

correspond from a cytogenetic standpoint to low-level chromosome polysomy. On the other hand, *MYC* amplification was observed only in 3 of 44 samples. Analysis of *MYC* GCN was not correlated with survival, even in the small subset of patients displaying *MYC* amplification.

MET is rapidly becoming an attractive target in the clinical arena of advanced HCC (Santoro et al. 2013; Verslype et al. 2012). While in a recent randomized Phase II trial of tivantinib, a MET inhibitor, the increased expression of MET in HCC pretreatment samples was found to confer poor prognosis (Santoro et al. 2013), analysis of *MET* GCN by FISH in the current study did not retain any predictive nor prognostic value. Although extensive genomic surveys of HCC (Kim et al. 2008; Schlaeger et al. 2008; Hoshida et al. 2009) underscore a remarkable prevalence of copy number aberrations concerning chromosomal regions that harbor *MYC* and *MET*, our findings intuitively suggest that MET protein expression, not genomic aberrations, might be relevant in terms of prognosis. Furthermore, the results of the current study are in substantial agreement with a previous report (Kondo et al. 2012) showing a very low rate of *MET* amplification (2 %) in HCC, and no correlation of chromosome 7 polysomy with protein expression.

In conclusion, despite preclinical and clinical investigations (Abou-Alfa et al. 2006; Zhang et al. 2009), our findings do not confirm a role for pERK expression as predictive marker of sorafenib efficacy. Nevertheless, our study suggests a prognostic effect of pERK and Mcl-1 expression levels by immunohistochemistry, which might be useful for risk stratification in future clinical trials. Although we did not test MET expression in this cohort of sorafenib-treated patients due to lack of tissue, analysis of MET protein levels by immunohistochemistry as a predictive and/or prognostic tool is warranted by results of recent trials of MET inhibitors in advanced HCC (Santoro et al. 2013) and gastric cancer (Oliner et al. 2012).

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