Biomarkers of Apoptosis and Necrosis in Patients with Hepatocellular Carcinoma Treated with Sorafenib

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Abstract. Background/Aim: Sorafenib is the medical reference for treatment of hepatocellular carcinoma (HCC). Multiple forms of cytotoxicity are induced by sorafenib in HCC cells in vitro but it is unclear what extent of apoptosis and necrosis is induced in HCC patients receiving sorafenib. Patients and Methods: The M30 and M65 biomarkers, which reflect the release of cytokeratin-18 and its apoptotic cleavage fragments, were measured in patients with HCC (n=36) and matched patients with cirrhosis (n=47). A serum sample was collected from 20 patients with HCC four weeks after the onset of treatment with sorafenib. Results: Basal serum levels of M30 and M65 were increased in patients with HCC compared to those with uncomplicated cirrhosis. No statistically significant increase in the level of M30 or M65 was found in the sera of patients with HCC after sorafenib. Conclusion: The findings indicate that sorafenib is not a potent inducer of HCC cell death in most patients.

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It is unclear to which extent the two forms of cell death, apoptosis and necrosis, take place in patients with HCC treated with sorafenib.

In the present study, our aim was to measure the induction of apoptosis and necrosis in patients with HCC receiving sorafenib. We analysed two biomarkers reflecting the induction of programmed cell death in epithelial cells, namely the M30 and M65 antigens. Both antigens are carried by cytokeratin-18 (CK18) and its cleavage products, and they have been extensively used to measure the level of cell death in various pathological states of the liver (12). Here, we validated the use of M30 and M65 antigens in the culture medium of HCC cells exposed to sorafenib and investigated their levels in serum samples obtained from patients with HCC and matched controls presenting with cirrhosis.

Patients and Methods

Cell culture and reagents. Huh7 cells were a kind gift from Dr. Wychowski (Institut de Biologie de Lille, France) and were authenticated using profiling of short tandem repeats at 16 loci (LGC Standards, Strasbourg, France). Cells were cultured in Dulbecco’s Modified Eagle’s Medium (Sigma, Saint Quentin Fallavier, France) supplemented with 10% fetal calf serum (Jacques Boy, Reims, France), 2 mM glutamine, and penicillin/Streptomycin. Sorafenib and ABT-737 were purchased from Selleck chemicals (Houston, TX, USA). Erastin, doxorubicin and all other reagents were purchased from Sigma and used as previously reported (7).

Patients. Two groups of patients were studied: The first group comprised of 36 consecutive patients with advanced HCC treated with sorafenib (800 mg/day) on behalf of the weekly multidisciplinary meeting “Primary liver tumours” from the Paris-Seine-Saint-Denis University Hospitals between January 2012 and October 2014. Basal serum samples were collected from these patients within three months before the onset of treatment. A second serum sample was collected from 20 of these patients four to 24 weeks after the onset of treatment with sorafenib. The results of the periodical evaluation performed at 4, 12 or 24 weeks after the beginning of treatment (abdominal tomodensitometry or magnetic resonance imaging, as well as biological tests including measurement of serum alpha feto-protein) showed that none of these 20 patients responded to sorafenib according to the Modified Response Evaluation Criteria In Solid Tumours (mRECIST) (13). Disease was stable in seven patients and progressive in 13 patients.

The second group comprised 47 patients with cirrhosis but without detectable HCC, matched for age, sex, cirrhosis severity (Child-Pugh score) and the main aetiology of cirrhosis, who were prospectively followed up by the Liver Unit of Paris-Seine-Saint-Denis University Hospitals (Bondy, France) between October 2010 and May 2013. Clinical and biological characteristics of the patients are summarized in Table I. Frozen serum samples (stored at −80°C) were provided by the Paris-Seine-Saint-Denis University Hospitals liver Biobank.

Biomarkers of programmed cell death. M30 Apoptosense and M65 kits were purchased from Enzo Life Sciences (Villeurbanne, France) and used according to the manufacturer’s recommendations. The M30- and M65-based sandwich ELISAs determine the circulating levels of the different forms of CK18; the M30 antibody recognizes a neo-epitope mapped to positions 387 to 396 of CK18 that appears only after caspase cleavage of CK18 and, therefore, reflects the induction of apoptosis (12, 14). The M65 epitope is carried by full-length CK18 and its cleaved fragments. The cellular release of an immunoreactive product recognized by M65 but not M30, i.e. of uncleaved CK18, reflects the occurrence of necrosis (12, 14, 15).

Sorafenib measurement assay. Sorafenib trough concentrations were assayed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Chromatographic separation was performed with a kinetex C18 column (100x4.6 mm; 2.6 μm particle size; Prominence; Shimadzu, Marne La Vallée, France). Detection was performed with a triple quadrupole mass spectrometer (3200 QTRAP; ABsciex, Les Ulis, France).

Statistical analyses. Student’s t-test, non-parametric ANOVA, and Spearman correlation coefficient were used as appropriate and a value of p<0.05 was considered as threshold for significance. Statistical analyses were performed with R3.02 (http://www.r-project.org/).

Results

In vitro validation of the biomarkers of cell death in HCC cells exposed to sorafenib. In order to validate the use of the CK18-based biomarkers for programmed cell death in HCC, cultures of the human HCC reference cell line Huh7 were exposed to sorafenib in vitro under apoptotic and necrotic conditions. The effects induced by sorafenib alone (10 μM for 18h) were compared to a strong pro-apoptotic regimen, consisting of doxorubicin and the compound ABT-737 (9). The chemical compound erastin was also applied to Huh7 cells (5 μM for 18h) in order to promote ferroptosis (10). The levels of the M30 and M65 antigens were measured in the culture medium of Huh7 cells (Figure 1). The exposure of HCC cells to doxorubicin and ABT-737 resulted in a massive release of the caspase-cleaved form of CK18, detected as strong...
immunoreactivity for M30. Conversely, erastin promoted a massive release of the uncleaved form of CK18, detected as immunoreactivity in the M65 assay (Figure 1). Sorafenib applied under the same conditions promoted a release of uncleaved CK18, although the extent of this release was modest in comparison to the effect produced by erastin. Based on these experiments, we concluded that the measurement of M30 and M65 reflects the extent of apoptotic and necrotic cell death in HCC cells exposed to sorafenib, respectively.

**Figure 1.** In vitro release of M30 and M65 in the culture medium of Huh7 cells exposed to apoptotic and ferroptotic (necrotic) conditions. Huh7 cells in culture were exposed to erastin (5 μM), doxorubicin (2 μM) plus ABT-737 (5 μM) or sorafenib (10 μM). Cell culture supernatants were collected after 18h and M30 and M65 were measured as indicated in the Materials and Methods section. The ratio M30/M65 was calculated from the corresponding values. *p<0.05 compared to control using the Student’s t-test.

**Figure 2.** Serum levels of M30 and M65 in patients with hepatocellular carcinoma (HCC) compared to patients with cirrhosis. M30 and M65 levels were determined as indicated in the Materials and Methods section and the ratio M30/M65 was calculated from these values. The graph represents the distribution between the 5th and 95th percentiles. *p<0.05 compared to the cirrhosis group using non-parametric ANOVA. ns, Lack of statistical significance compared to the cirrhosis group.

In order to explore the basal levels of M30 and M65 in patients with HCC, we compared the sera of patients with advanced HCC and those of patients with uncomplicated cirrhosis. An analysis performed with non-parametric ANOVA revealed that the plasma levels of the antigens M30 and M65 were both significantly increased in HCC compared to cirrhosis (Figure 2). The levels of both markers were higher in patients with HCC compared to those with cirrhosis (M30:

**Biomarkers of cell death in HCC compared to cirrhosis.** In order to explore the basal levels of M30 and M65 in patients with HCC, we compared the sera of patients with advanced HCC and those of patients with uncomplicated cirrhosis. An analysis performed with non-parametric ANOVA revealed that the plasma levels of the antigens M30 and M65 were both significantly increased in HCC compared to cirrhosis (Figure 2). The levels of both markers were higher in patients with HCC compared to those with cirrhosis (M30:
median=263.6 U/l in the HCC group vs. 179.9 U/l in cirrhosis, p<0.05; M65: median=1,470.3 U/l in HCC vs. 873.9 U/l, p<0.005). However, there was no significant difference between the two groups in terms of the M30:M65 ratio (median=0.191 in the HCC group vs. 0.191 in cirrhosis, p=0.44). We attempted to correlate the severity of cirrhosis with the levels of the two markers, although most patients in this study presented with well-compensated cirrhosis (Child A). The levels of M30 and M65 were not significantly different between patients with Child A and those with Child B cirrhosis. No correlation was apparent between the levels of the two markers and the aetiology of liver disease (alcohol, non-alcoholic steatohepatitis, viral hepatitis).

### Biomarkers of cell death in HCC treated with sorafenib

We analyzed the levels of the M30 and M65 antigens in the sera of 20 patients from whom blood samples were obtained before the start of treatment and at four weeks after the beginning of treatment with sorafenib (Figure 3A). We found no significant difference between the levels of M30 or M65 in samples obtained before and after sorafenib treatment: the median M30 level decreased from 468.9 U/l before sorafenib to 368 U/l after four weeks of sorafenib (p=0.62). M65 level was relatively stable before and after sorafenib, with respective median values of 1,429.6 and 1,229.3 U/l (p=0.81).

In an attempt to examine possible correlation between the levels of M30 and M65 antigens and the clinical response to sorafenib, we performed a separate analysis in the groups of patients with stable (n=7) and those with progressive (n=13) disease receiving sorafenib (Figure 3B). In patients with progressive disease, a direct comparison found no statistically significant differences in M30 and M65 antigen levels either before or after treatment with sorafenib (median M30 level before...
sorafenib = 571.8 U/l vs. 630.3 U/l after sorafenib, \( p = 0.54 \); M65 level before sorafenib = 1423.4 U/l vs. 1769.6 U/l after sorafenib, \( p = 0.45 \) (Figure 3B). In patients with stable disease under sorafenib, M30 or M65 levels were either stable or decreased during the course of treatment (median M30 = 262.7 U/l before vs. 269.5 U/l after sorafenib treatment, \( p = 0.21 \); M65 = 1,435.7 U/l before vs. 877.5 U/l after sorafenib treatment, \( p = 0.57 \)).

Serum concentrations of sorafenib were highly heterogeneous among patients, with a more than 80-fold difference between the minimum and the maximum concentrations (min. concentration: 0.5 \( \mu \)M; max.: 41.3 \( \mu \)M; median: 7.1 \( \mu \)M). We examined the possibility that there might be a correlation between the concentration of sorafenib and the level of cell death after four weeks of treatment with sorafenib. However, neither the plasma levels of M30 nor those of M65 correlated with the concentration of sorafenib (M30: \( r = -0.313 \), \( p = 0.18 \); M65: \( r = -0.346 \), \( p = 0.14 \) using Spearman correlation coefficient).

**Discussion**

In the present study, we analyzed the serum levels of CK18 (M65) and its apoptotic cleavage product (M30) with the aim of exploring the regulation of cell death in patients with HCC. The two CK18-based biomarkers, M30 and M65, have been extensively used to analyze the cytotoxic response of liver cells to various insults and pathological conditions (12). Recently, the M30 and M65 markers were also reported to be of potential clinical value for the biological diagnosis of HCC (15). Our findings constitute, to our knowledge, the first report using these biomarkers to investigate cell death in HCC treated by sorafenib. We validated the use of M30 and M65 for the quantification of the extent of cell death in HCC and subsequently measured their levels in the sera of a small series of patients with HCC. We found that patients with advanced HCC present higher basal levels of M30 and M65 than matched patients with cirrhosis. We found no significant increase in M30 and M65 in the sera of patients with HCC treated with sorafenib. In addition, our attempt to relate the clinical efficacy of sorafenib to the induction of programmed cell death was also clearly negative.

Although the relatively small number of patients enrolled in the study constitutes a limit that needs to be mentioned at this stage, our findings already indicate that sorafenib is not a potent inducer of HCC cell death in most patients. These findings are in agreement with the fact that sorafenib essentially stabilizes the growth of HCC, rather than promoting tumor regression (2, 5). Tumor regression, thought to occur through massive death of HCC cells, occurs in fewer than 3% of the total population of those with HCC receiving sorafenib (2, 5). Although sorafenib is probably not a potent inducer of cancer cell death in most patients with HCC, it remains to be seen whether biomarkers of cell death could help identify the small sub-population of patients that are responders to sorafenib.

**Conflicts of Interest**

Jean-Claude Barbare, Jean-Claude Trinchet and Nathalie Ganne received honoraria as clinical consultants from Bayer. The other Authors have not declared any conflict of interest.

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**References**


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