is over-expressed in one-third of human HCC and confers resistance to hepatoma cells toward a variety of apoptotic insults generated by serum starvation and *p53* activation [13]. Patients with Bcl-xL-overexpressing HCC were shown to have significantly shorter disease-free survival after surgery [14]. Recently, it was proposed that autophagy defect is another mechanism of the malignant phenotype of Bcl-xL-overexpressing HCC through interaction between Bcl-xL and Beclin1 [15]. The underlying mechanisms of Bcl-xL over-expression in HCC are not clearly understood. Several reports show that transcription factors such as NF-κB [16] and STAT3 [17] could upregulate Bcl-xL expression in hepatoma cells. In addition, hepatitis C virus-related proteins, such as core [18] and NS5A [19], could upregulate Bcl-xL at a transcriptional level. However, we noticed that Bcl-xL-overexpressing hepatocarcinoma tissues do not always display upregulation of *bcl-xl* mRNA [13]. This observation led us to examine the possibility that post-transcriptional regulation by miRNAs may be involved in Bcl-xL expression in human HCC.

In the present study, we demonstrate that *let-7* family miRNAs, a prototype of human miRNAs [20], negatively regulate Bcl-xL expression in human HCC. *let-7* miRNAs are downregulated in human hepatoma cells and tissues in association with enhanced expression of Bcl-xL. Over-expression of *let-7* miRNAs in hepatoma cells downregulates Bcl-xL in a *bcl-xl* 3′UTR sequence-specific manner and enhances apoptosis induced by sorafenib, a recently approved anti-cancer drug for HCC [21]. The present study demonstrates for the first time that *let-7* miRNAs directly target Bcl-xL and induce apoptosis in cooperation with an anti-cancer drug targeting Mcl-1 in HCC.

Materials and methods

miRNA target predictions

The algorithms miRanda (http://www.microrna.org/), Pictar (http://pictar.mdc-berlin.de/), and TargetScan (http://www.targetscan.org/) were used to predict miRNAs that could potentially bind to *bcl-xl* mRNA.

Cell lines and tissues

Primary human hepatocytes were obtained from ScienCell Research Laboratories (Carlsbad, CA) and cultured with the provided medium. Human hepatoma cell lines, Huh7 and HepG2, were cultured with Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated foetal bovine serum (Sigma, St. Louis, MO). HCCs and adjacent non-tumour counterparts were obtained at the time of surgical resection. Written informed consent was obtained from each patient. All tissues were stored at −80 °C until the time of use.

RNA extractions

Total RNA including the miRNA fraction was isolated from cell lines and tissue samples using the miRNeasy Mini Kit (QIAGEN, Valencia, CA). After extraction, the quality of each RNA sample was checked using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA).

miRNA microarray analysis

RNA labelling and hybridisation were performed using a human miRNA microarray kit and a miRNA complete labelling and hybridisation kit (Agilent Technologies). After washing with Gene Expression Wash Buffer, the slides were scanned with an Agilent Microarray Scanner and analysed by GeneSpring GX software.

Western blot

Cells or tissues were lysed and Western blotted as previously described [22]. For immunodetection, the following antibodies were used: anti-Bcl-xL polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Mcl-1 polyclonal antibody (Santa Cruz Biotechnology), anti-Bak polyclonal antibody (Millipore, Billerica, MA), anti-Bax polyclonal antibody (Cell Signaling Technology, Danvers, MA). Optical densities of bands in each blot were analysed using ImageJ 1.40 g (NIH, Bethesda, MD).

Real time reverse transcription (RT)-PCR assays for mature miRNAs

To quantify the expression of mature miRNA, we synthesised cDNA from 10 ng of RNA sample using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Quantitative PCR was performed with TaqMan MicroRNA Assays (Applied Biosystems) specific for *let-7c* (P/N 4373167) and *let-7g* (P/N 4395393). To normalise the expression levels of miRNAs, we used TaqMan MicroRNA Assays specific for RNU6B (P/N 4373381) as the endogenous control.

Real time RT-PCR assays for bcl-xl mRNA

We reverse-transcribed RNA with High Capacity RNA-to-cDNA Master Mix (Applied Biosystems), and *bcl-xl* mRNA expression was measured using TaqMan Gene Expression Assays (Applied Biosystems, Assay ID: Hs99999146_m1). We also quantified *β-actin* mRNA as an endogenous control (Assay ID: Hs99999903_m1).

Transfections with miRNAs

Huh7 and HepG2 cells were transfected with 50 nM Pre-miR miRNA precursor molecules (Ambion, Austin, TX) of either *let-7c* or *let-7g* using RNAiMAX (Invitrogen, Carlsbad, CA) in six-well plates according to the manufacturer's instructions. Pre-miR negative control (Ambion) was also used as a control.

Luciferase assay

To generate the pMIR-Bcl-xL-3′UTR construct that contains the putative binding site of *bcl-xl* 3′UTR downstream of the firefly luciferase gene, we synthesised oligonucleotides to mimic the target sequence and inserted them into the SpeI–HindIII site of pMIR-REPORT Luciferase vector (Ambion). We also generated a pMIR-Bcl-xL-3′UTR mutant that has a point mutation in the putative binding site, using the QuickChange Multi Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA).

Each of these constructs was transfected into Huh7 cells together with 50 nM Pre-miR miRNA precursor molecules and pMIR-REPORT β-Gal vector (Ambion), which contains the β-galactosidase gene for normalisation of transfection efficiency. Transfection was performed using Lipofectamine 2000 (Invitrogen). We measured firefly luciferase activity 24 h after transfection using the Luciferase Assay System (Promega, Madison, WI) and normalised it to the β-galactosidase expression level.

In vitro staurosporine or sorafenib treatment

Huh7 cells were transfected with 50 nM Pre-miR miRNA precursor molecules as described above, and 48 h after transfection, the medium was changed to DMEM containing staurosporine (Calbiochem, Gibbstown, NJ) or sorafenib. Sorafenib was kindly provided by Bayer HealthCare Pharmaceuticals Inc. (Wayne, NJ). Cells were additionally cultured and assayed for apoptosis by monitoring the activity of caspase-3/7 using a luminescent substrate assay for caspase-3 and caspase-7 (Caspase-Glo assay, Promega, Madison, WI), or by flow cytometry using the Annexin V-PE Apoptosis Detection Kit I (BD Biosciences, San Jose, CA). We defined apoptotic cells as Annexin V-PE-positive and 7-amino-actinomycin D (7-AAD)-negative cells. Cell viability was determined by the WST assay using cell count reagent SF (Nacalai Tesque, Kyoto, Japan).

Statistical analysis

Data are presented as mean ± SD. Comparisons between two groups were performed by the unpaired *t*-test. Multiple comparisons were performed by ANOVA with the Scheffe post hoc test. $p < 0.05$ was considered statistically significant.

Results

let-7 miRNAs were downregulated in hepatoma cells with upregulated expression of Bcl-xL

As observed in human HCC tissues, Bcl-xL was over-expressed, according to Western blot analysis, in Huh7 and HepG2 human hepatoma cell lines compared to normal hepatocytes (Fig. 1A). Previous research established that 30 and 32 kDa species are original and post-translationally modified Bcl-xL, respectively [23]. Mcl-1 was also over-expressed in human hepatoma cells, but the levels of expression of Bak and Bax did not differ between hepatoma cells and normal hepatocytes. We reasoned that miRNA regulating Bcl-xL expression would be downregulated in those hepatoma cell lines. To search for the candidate miRNA, microarray analysis was performed. More specifically, miRNA expression in Huh7 cells and normal hepatocytes was compared. When levels of expression less than 50% were considered significant, 26 miRNAs were identified as being downregulated in Huh7 cells: *let-7b*, *let-7g*, *let-7i*, *miR-127-3p*, *miR-214*, *miR-376a*, *miR-381*, *miR-409-3p*, *miR-376c*, *miR-493**, *miR-432*, *miR-487b*, *let-7d*, *let-7a*, *let-7f*, *let-7c*, *miR-200a*, *let-7e*, *miR-134*, *miR-503*, *miR-34a*, *miR-638*, *miR-150**, *miR-1225-5p*, *miR-21**, and *miR-223*. Among them, *in silico* analysis revealed that only the *let-7* family is capable of potentially targeting the 3′UTR of the *bcl-xl* mRNA. To confirm the results of the microarray analysis, quantitative real time RT-PCR was performed to evaluate the expression of *let-7c* and *let-7g* (Fig. 1B). After normalisation to endogenous RNU6B expression levels, the expression levels of both miRNAs were substantially lower in Huh7 cells than in normal hepatocytes. These results were consistent with the results of microarray analysis. Furthermore, the expression levels of both miRNAs were found to be downregulated in another human hepatoma cell line, HepG2, compared to normal hepatocytes.

let-7c and let-7g downregulate Bcl-xL expression by directly targeting the 3′UTR of bcl-xl mRNA

To examine whether *let-7* miRNAs are capable of suppressing translation of Bcl-xL, hepatoma cell lines were transfected with *let-7c* or *let-7g* or the negative control. Three days after transfection, Huh7 cells showed a decrease in Bcl-xL protein levels in both the *let-7c*-transfected group and the *let-7g*-transfected group in comparison with the negative control group (Fig. 2A). The transfection of *let-7c* and *let-7g* showed suppression of Bcl-xL protein levels in HepG2 cells as well (Fig. 2B). It did not affect expression of Bak and Bax, but increased Mcl-1 expression, which may be a secondary phenomenon of suppression of Bcl-xL. Normal hepatocytes were also transfected with *let-7c* or *let-7g* (Suppl. Fig. 1). The transfection led to a decrease in Bcl-xL expression in normal hepatocytes, but the decline was lesser than that observed in hepatoma cells. This finding may be explained by the observation that endogenous expression of *let-7c* and *let-7g* was extremely high in normal hepatocytes.

To examine whether the downregulation of Bcl-xL by *let-7c* or *let-7g* is caused by direct binding to a putative targeting site in the *bcl-xl* mRNA, we constructed the luciferase reporter plasmid pMIR-Bcl-xL-3′UTR containing the putative *let-7* binding site of *bcl-xl* 3′UTR downstream of the luciferase open reading frame (Fig 3A). The pMIR-Bcl-xL-3′UTR construct was cotransfected with the control pMIR-REPORT β-gal vector into Huh7 cells together with *let-7c* or *let-7g* or the negative control. When *let-7c* or *let-7g* Pre-miR was cotransfected with pMIR-Bcl-xL-3′UTR, the expression of firefly luciferase was significantly reduced compared to the negative control cotransfected group. There was no difference in firefly luciferase expression levels when pMIR-REPORT, which does not contain the putative *let-7* binding site, was cotransfected with *let-7c*, *let-7g* or the negative control (Fig. 3B). We also generated a pMIR-Bcl-xL-3′UTR mutant with a single base mutation in the seed region of the putative binding sequence to investigate whether the downregulation of firefly luciferase can be attributed to the insert (Fig. 3A). A single base mutation prevented the downregulation of firefly luciferase

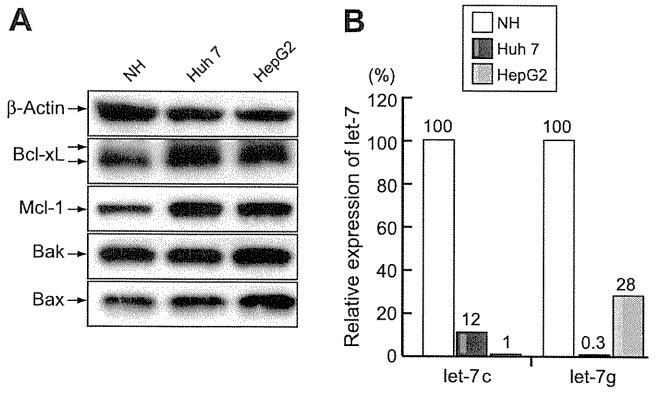

Fig. 1. Expression of Bcl-xL and *let-7* miRNAs in cultured human hepatocytes and hepatoma cells. Human hepatoma cell lines, Huh7 and HepG2, and normal hepatocytes (NH) were cultured and then lysed. (A) Western blot analysis for Bcl-2 family proteins. Bcl-xL migrates as a doublet band (see text). (B) Real time RT-PCR analysis for *let-7c* and *let-7g* expression. After normalisation to endogenous RNU6B expression, the expression of each miRNA in hepatoma cells was expressed in comparison to the levels observed in normal hepatocytes.

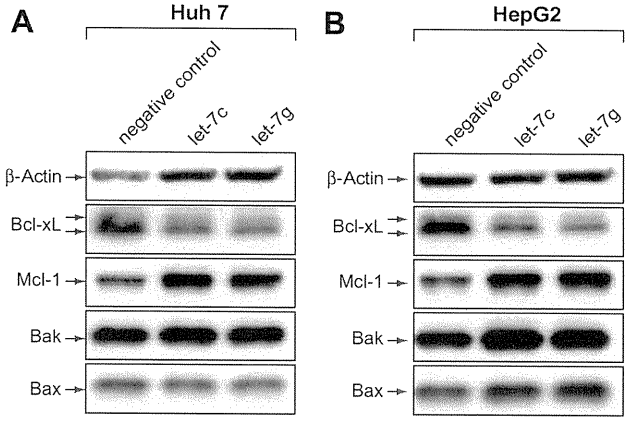

Fig. 2. Over-expression of *let-7* miRNAs downregulates Bcl-xL expression in hepatoma cells. Hepatoma cell lines Huh7 (A) and HepG2 (B) were transfected with *let-7c*, *let-7g*, or negative control miRNA at 50 nM and cultured for 3 days. Expression levels of Bcl-2 family proteins were determined by Western blot analysis.

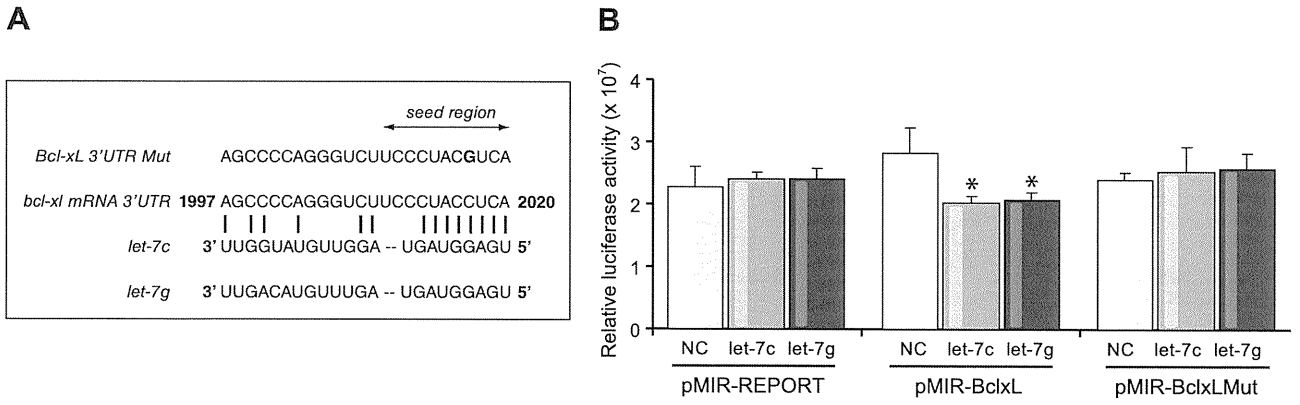

Fig. 3. Sequence-specific suppression of *bcl-xl* gene expression by *let-7c* or *let-7g* miRNAs. (A) The putative target site of *bcl-xl* mRNA 3′UTR determined by computational predictions. The target sequence was cloned into pMIR-REPORT vector (pMIR-Bcl-xL-3′UTR). pMIR-Bcl-xL-3′UTR mutant was also generated with a single mutation (indicated by a bold character) in the target site. (B) Each of these constructs was transfected into Huh7 cells together with *let-7c*, *let-7g* or negative control miRNA (NC). At 24 h after transfection, the activity of firefly luciferase was measured and normalised to β-galactosidase expression levels ($n = 3$). $^*p < 0.05$.

induced by *let-7c* or *let-7g*, which strongly suggests a direct inhibitory effect of *let-7* on Bcl-xL expression (Fig. 3B).

Downregulation of let-7c miRNA in human HCC tissues overexpressing Bcl-xL but not bcl-xl mRNA

To investigate the relationship between *let-7* expression levels and Bcl-xL protein levels in human HCC samples, we used 22 pairs of surgically resected human HCC tissue samples and adjacent non-tumour tissue samples with highly preserved RNA. Compared to the non-tumour counterparts, *bcl-xl* mRNA was found to be over-expressed in HCC tissue samples in only two cases; Bcl-xL was also over-expressed at the protein level in these cases. To assess the significance of *let-7* in post-transcriptional regulation of Bcl-xL *in vivo*, we selected 20 pairs of HCC tissue samples that did not over-express *bcl-xl* mRNA. When these samples were divided into two groups according to relative *let-7c* expression levels, the relative expression of Bcl-xL protein was significantly higher in the *let-7c* low expression group than in the *let-7c* high expression group (Fig. 4). By contrast, there was no significant difference in *bcl-xl* mRNA expression between the two groups. We also examined the relationship between relative *let-7g* expression and Bcl-xL expression. The *let-7g* low expression group tended to over-express Bcl-xL protein compared to the *let-7g* high expression group, although the difference did not reach statistical significance (data not shown). These results are consistent with the hypothesis that *let-7* miRNAs negatively regulate Bcl-xL expression independent of transcriptional regulation.

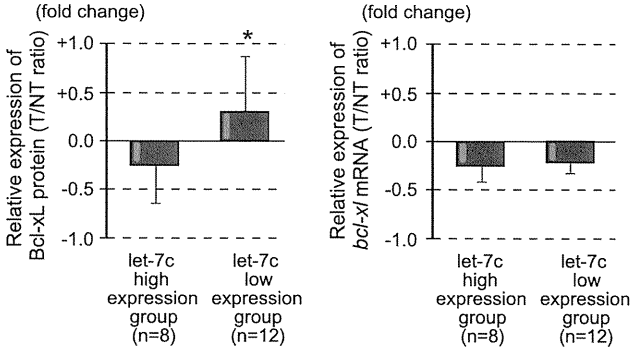

Fig. 4. Expression of Bcl-xL, *bcl-xl* mRNA and *let-7* miRNAs in human HCC tissue. Relationship between *let-7* and Bcl-xL expression in human HCC tissue samples. HCC tissue samples that did not show transcriptional upregulation of *bcl-xl* mRNA were divided into two groups according to relative *let-7c* expression levels with the threshold set at a 0.4-fold change in the tumour to non-tumour (T/NT) ratio. Relative expression of Bcl-xL protein and *bcl-xl* mRNA was calculated as the optical densities of the Bcl-xL blots normalised with the β-actin blots and those of real time RT-PCR assays, respectively, and are shown as the ratio of expression in the tumour to non-tumour expression in $\log_{10}$ scale. $^*p < 0.05$.

let-7c miRNA sensitises human Huh7 cells to sorafenib, which downregulates Mcl-1 expression

To investigate the effect of *let-7* in the resistance of hepatoma cells to apoptosis, we transfected Huh7 hepatoma cells with *let-7c* miRNAs and then subjected them to apoptosis analysis and a cell viability assay. There was no significant difference in caspase-3/7 activation or cell viability between *let-7c* miRNA-transfected Huh7 cells and control miRNA-transfected Huh7 cells (represented by the DMSO-treated group of Fig. 5A and B). These results are in agreement with our previous finding that anti-sense oligonucleotide-mediated knockdown of Bcl-xL sensitised hepatoma cells to apoptotic stimuli, such as serum starvation and *p53* activation, but did not induce apoptosis by itself [13]. Next, we exposed miRNA-transfected Huh7 cells to staurosporine, which is a well-established apoptosis inducer. Staurosporine treatment induced apoptosis, as determined by caspase-3/7 activation and decreased the viability of Huh7 cells by itself, but *let-7c* miRNA-transfected Huh7 cells were more susceptible to staurosporine treatment than control miRNA-transfected cells. *let-7c* miRNA-transfected Huh7 cells showed a significant decrease in cell viability, even upon exposure to low-dose of staurosporine at which control miRNA-transfected Huh7 did not show a significant difference in cell viability (Fig. 5B). In addition, the activation of caspase-3/7 was more intense in *let-7c* miRNA-transfected Huh7 cells than in control miRNA-transfected Huh7 cells (Fig. 5A). Thus, suppression of *let-7* expression leading to over-expression of Bcl-xL, may be a mechanism by which hepatoma cells resist apoptotic stimuli. While normal hepatocytes were more sensitive to staurosporine than hepatoma cells, transfection of *let-7* miRNA did not affect sensitivity to staurosporine

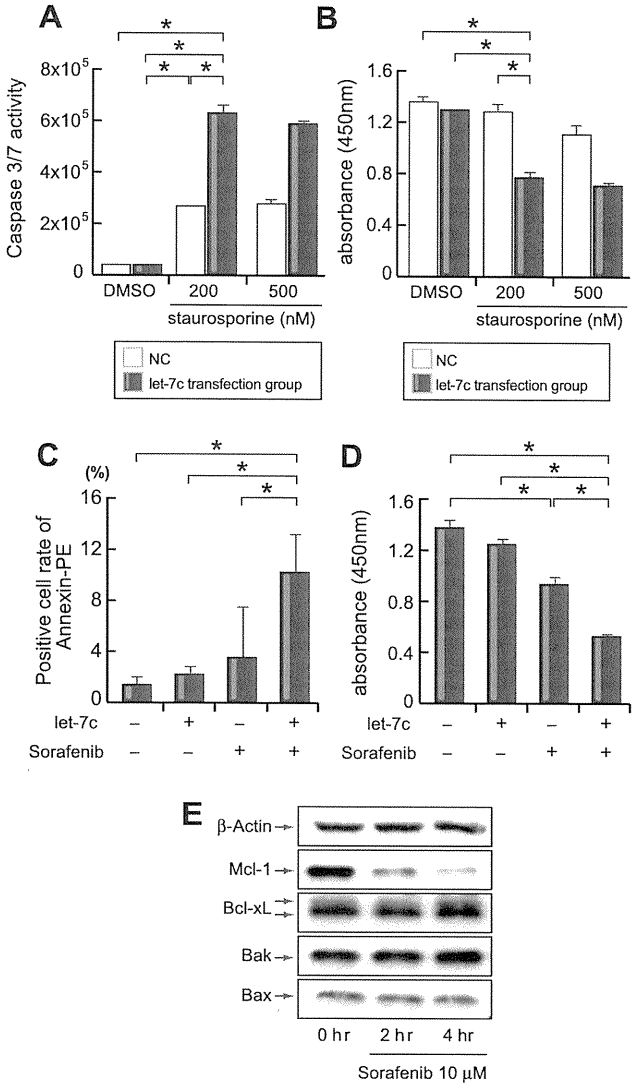

Fig. 5. Introduction of *let-7* miRNAs sensitises hepatoma cells to apoptotic stimuli. (A and B) Response to staurosporine treatment. Huh7 cells were transfected with *let-7c* (grey bars) or control miRNA (white bars) for 48 h and then further treated with staurosporine or DMSO alone for 12 h. The activities of caspase-3 and -7 were determined by luminescent substrate assays for caspase-3 and -7 ($n = 4$) (A). Cell viability was determined by the WST assay ($n = 4$) (B). $^*p < 0.05$. (C and D) Response to sorafenib treatment. Huh7 cells were transfected with *let-7c* or control miRNA for 48 h and then further treated with sorafenib (5 μM) or DMSO alone for 48 h (C) or 72 h (D) 7-AAD negative cells were gated and the positive cell rate for annexin V-PE was determined ($n = 4$) (C). Cell viability was determined by the WST assay ($n = 4$) (D). $^*p < 0.05$. E. Western blot analysis for Bcl-2 family proteins in lysates of Huh7 cells treated with sorafenib.

in normal hepatocytes (Suppl. Fig. 2), which is in agreement with the modest decline of Bcl-xL expression described earlier.

To examine the impact of *let-7* family miRNAs as a therapeutic tool, we investigated the effect of *let-7* miRNAs on apoptosis resistance to sorafenib, a recently approved anti-cancer drug for HCC. It has been reported that sorafenib was capable of downregulating Mcl-1 expression in tumour cells [24], and HCC has been reported to over-express Mcl-1, which is another anti-apoptotic Bcl-2 protein capable of conferring resistance to apoptosis [24–27]. In agreement with these findings, sorafenib treatment clearly downregulated Mcl-1 expression in hepatoma cells, but did not affect Bcl-xL expression (Fig. 5E). In contrast, sorafenib treatment did not affect Mcl-1 expression in normal hepatocytes (Suppl. Fig. 3). We hypothesised that *let-7* miRNA targeting Bcl-xL may induce apoptosis of hepatoma cells in cooperation with sorafenib. Apoptosis determined by Annexin V staining did not increase in *let-7c* miRNA-treated Huh7 cells compared to control miRNA-treated cells (represented by the DMSO-treated group in Fig. 5C). Sorafenib treatment of Huh7 cells led to a slight increase in the annexin V-positive cell rate, although the difference did not reach statistical significance levels under our experimental conditions (Fig. 5C). Of importance is the finding that sorafenib-induced apoptosis was markedly enhanced in *let-7c* miRNA-transfected cells. In addition, sorafenib treatment significantly reduced the viability of Huh7 cells and this decrease was markedly enhanced in cells transfected with *let-7c* miRNA (Fig. 5D). This finding implies that *let-7* miRNA transfection potentiates sorafenib-induced apoptosis and toxicity in hepatoma cells.

Discussion

Anti-apoptotic members of the Bcl-2 family, which consists of five members, Bcl-2, Bcl-xL, Mcl-1, Bcl-w, and Bfl-1, are critically involved in the mitochondrial pathway of apoptosis [28]. Cancer cells frequently over-express one or more members of this family to acquire a survival advantage [29]. These proteins are over-expressed in a variety of ways, including genetic translocation, particularly in the case of Bcl-2, and transcriptional regulation. Unlike the case of the *bcl-2* gene, mutations or amplification of the *bcl-x* gene have not been demonstrated in tumour cells. With regard to miRNA regulation, previous research clearly demonstrated that Bcl-2 is a direct target of *miR-15* and *miR-16*. The expression levels of *miR-15* and *miR-16* inversely correlate with Bcl-2 expression in chronic lymphocytic leukaemia [30]. More recently, Mcl-1 was reported to be suppressed by *miR-29* [31]. Our present study is the first demonstration of miRNA-mediated regulation of Bcl-xL expression. Since Bcl-xL is over-expressed not only in HCC but also in other tumours, the present findings may shed light on the mechanisms of Bcl-xL over-expression in other malignancies.

While more than 500 human miRNAs have been identified, *let-7* is a prototype of human miRNA and was first identified in 2001 [32]. *let-7* miRNAs are downregulated in several malignancies. A highly characterised example is non-small cell lung cancer in which downregulation of *let-7* miRNAs is well correlated with poor prognosis in patients [33]. In HCC, some reports showed downregulation of *let-7*, while others did not [7]. In the present study, *let-7c* miRNA was under-expressed at less than 40% of the normal level in approximately half of the HCC tissues. Further study is needed to determine the clinical significance of *let-7* miRNA in HCC. Several target genes have been identified for *let-7* miRNA, including Ras [34], Myc [35], HMGA2 [36], CDC25A, and CDK6 [37]. The major function of this miRNA is to promote cell proliferation. Since these proteins could act as oncogenes in tumour cells, *let-7* miRNA is believed to serve as a tumour suppressor [38]. In the present study, we have demonstrated that *bcl-xl* is a direct target for *let-7* miRNA, implying that this well-known tumour suppressor miRNA directly regulates apoptosis, another important process in malignancy.

Sorafenib is a recent FDA-approved anti-cancer drug for HCC [21]. It functions as a multi-kinase inhibitor and can induce

apoptosis at least in part by downregulating Mcl-1 in tumour cells [24]. Like Bcl-xL, several reports have identified Mcl-1 as being over-expressed in some HCCs [25–27]. Since Bcl-xL and Mcl-1 share a similar structure and functions, we reasoned that downregulation of both proteins would efficiently kill hepatoma cells. To verify this hypothesis, we treated hepatoma cells with sorafenib and *let-7* miRNA. As expected, sorafenib treatment downregulated Mcl-1 expression as early as 2 h post-treatment; however, it did not efficiently induce apoptosis. Transfection of *let-7* miRNA itself was not capable of inducing apoptosis of hepatoma cells despite a clear reduction in Bcl-xL expression. Importantly, *let-7* miRNA substantially increased sensitivity to sorafenib. Since both *let-7* miRNA and sorafenib may have pleiotropic effects on gene expression and cellular processes, downregulation of Bcl-xL and Mcl-1 may not be a single mechanism for killing hepatoma cells. However, our study revealed that Bcl-xL-targeting miRNA, *let-7*, controls resistance of hepatoma cells to this novel class of anti-HCC drug.

In conclusion, we have demonstrated that *let-7* miRNA negatively regulates Bcl-xL expression in HCCs. Reconstitution of *let-7* miRNA may reduce apoptosis resistance to anti-cancer drugs targeting Mcl-1 in HCC. Further study is needed to examine the significance of *let-7* miRNA expression for predicting responses to sorafenib therapy in patients with HCC.

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Disclosures

All authors have nothing to disclose.

Conflicts of interest

All authors have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhep.2009.12.024.

References

[1] Filipowicz W, Bhattacharyya SN, Sonenberg N. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? Nat Rev Genet 2008;9:102–114.
[2] Esquela-Kerscher A, Slack FJ. Oncomirs – microRNAs with a role in cancer. Nat Rev Cancer 2006;6:259–269.
[3] Zhang B, Pan X, Cobb GP, Anderson TA. MicroRNAs as oncogenes and tumor suppressors. Dev Biol 2007;302:1–12.
[4] Murakami Y, Yasuda T, Saigo K, Urashima T, Toyoda H, Okanoue T, et al. Comprehensive analysis of microRNA expression patterns in hepatocellular carcinoma and non-tumorous tissues. Oncogene 2006;25:2537–2545.
[5] Varnholt H, Drebber U, Schulze F, Wedemeyer I, Schirmacher P, Dienes HP, et al. MicroRNA gene expression profile of hepatitis C virus-associated hepatocellular carcinoma. Hepatology 2008;47:1223–1232.
[6] Wong QW, Lung RW, Law PT, Lai PB, Chan KY, To KF, et al. MicroRNA-223 is commonly repressed in hepatocellular carcinoma and potentiates expression of Stathmin1. Gastroenterology 2008;135:257–269.
[7] Gramantieri L, Ferracin M, Fornari F, Veronese A, Sabbioni S, Liu CG, et al. Cyclin G1 is a target of miR-122a, a microRNA frequently downregulated in human hepatocellular carcinoma. Cancer Res 2007;67:6092–6099.
[8] Meng F, Henson R, Wehbe-Janek H, Ghoshal K, Jacob ST, Patel T. MicroRNA-21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer. Gastroenterology 2007;133:647–658.
[9] Fornari F, Gramantieri L, Ferracin M, Veronese A, Sabbioni S, Calin GA, et al. MiR-221 controls CDKN1C/p57 and CDKN1B/p27 expression in human hepatocellular carcinoma. Oncogene 2008;27:5651–5661.
[10] Wang Y, Lee AT, Ma JZ, Wang J, Ren J, Yang Y, et al. Profiling microRNA expression in hepatocellular carcinoma reveals microRNA-224 up-regulation and apoptosis inhibitor-5 as a microRNA-224-specific target. J Biol Chem 2008;283:13205–13215.
[11] Budhu A, Jia HL, Forgues M, Liu CG, Goldstein D, Lam A, et al. Identification of metastasis-related microRNAs in hepatocellular carcinoma. Hepatology 2008;47:897–907.
[12] Ladeiro Y, Couchy G, Balabaud C, Bioulac-Sage P, Pelletier L, Rebouissou S, et al. MicroRNA profiling in hepatocellular tumors is associated with clinical features and oncogene/tumor suppressor gene mutations. Hepatology 2008;47:1955–1963.
[13] Takehara T, Liu X, Fujimoto J, Friedman SL, Takahashi H. Expression and role of Bcl-xL in human hepatocellular carcinomas. Hepatology 2001;34:55–61.
[14] Watanabe J, Kushihata F, Honda K, Sugita A, Tateishi N, Mominoki K, et al. Prognostic significance of Bcl-xL in human hepatocellular carcinoma. Surgery 2004;135:604–612.
[15] Ding ZB, Shi YH, Zhou J, Qiu SJ, Xu Y, Dai Z, et al. Association of autophagy defect with a malignant phenotype and poor prognosis of hepatocellular carcinoma. Cancer Res 2008;68:9167–9175.
[16] Chiao PJ, Na R, Niu J, Sclabas GM, Dong Q, Curley SA. Role of Rel/NF-kappaB transcription factors in apoptosis of human hepatocellular carcinoma cells. Cancer 2002;95:1696–1705.
[17] Selvendiran K, Koga H, Ueno T, Yoshida T, Maeyama M, Torimura T, et al. Luteolin promotes degradation in signal transducer and activator of transcription 3 in human hepatoma cells: an implication for the antitumor potential of flavonoids. Cancer Res 2006;66:4826–4834.
[18] Otsuka M, Kato N, Taniguchi H, Yoshida H, Goto T, Shiratori Y, et al. Hepatitis C virus core protein inhibits apoptosis via enhanced Bcl-xL expression. Virology 2002;296:84–93.
[19] Sarcar B, Ghosh AK, Steele R, Ray R, Ray RB. Hepatitis C virus NS5A mediated STAT3 activation requires co-operation of Jak1 kinase. Virology 2004;322:51–60.
[20] Pasquinelli AE, Reinhart BJ, Slack F, Martindale MQ, Kuroda MI, Maller B, et al. Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA. Nature 2000;408:86–89.
[21] Llovet JM, Bruix J. Molecular targeted therapies in hepatocellular carcinoma. Hepatology 2008;48:1312–1327.
[22] Takehara T, Tatsumi T, Suzuki T, Rucker 3rd EB, Hennighausen L, Jinushi M, et al. Hepatocyte-specific disruption of Bcl-xL leads to continuous hepatocyte apoptosis and liver fibrotic responses. Gastroenterology 2004;127:1189–1197.
[23] Takehara T, Takahashi H. Suppression of Bcl-xL deamidation in human hepatocellular carcinomas. Cancer Res 2003;63:3054–3057.
[24] Rahmani M, Davis EM, Bauer C, Dent P, Grant S. Apoptosis induced by the kinase inhibitor BAY 43-9006 in human leukemia cells involves down-regulation of Mcl-1 through inhibition of translation. J Biol Chem 2005;280:35217–35227.
[25] Sieghart W, Losert D, Strommer S, Cejka D, Schmid K, Rasoul-Rockenschaub S, et al. Mcl-1 overexpression in hepatocellular carcinoma: a potential target for antisense therapy. J Hepatol 2006;44:151–157.
[26] Fleischer B, Schulze-Bergkamen H, Schuchmann M, Weber A, Biesterfeld S, Müller M, et al. Mcl-1 is an anti-apoptotic factor for human hepatocellular carcinoma. Int J Oncol 2006;28:25–32.
[27] Schulze-Bergkamen H, Fleischer B, Schuchmann M, Weber A, Weinmann A, Krammer PH, et al. Suppression of Mcl-1 via RNA interference sensitizes human hepatocellular carcinoma cells towards apoptosis induction. BMC Cancer 2006;6:232.
[28] Youle RJ, Strasser A. The BCL-2 protein family: opposing activities that mediate cell death. Nat Rev Mol Cell Biol 2008;9:47–59.

[29] Lessene G, Czabotar PE, Colman PM. BCL-2 family antagonists for cancer therapy. Nat Rev Drug Discov 2008;7:989–1000.
[30] Cimmino A, Calin GA, Fabbri M, Iorio MV, Ferracin M, Shimizu M, et al. MiR-15 and miR-16 induce apoptosis by targeting BCL2. Proc Natl Acad Sci USA 2005;102:13944–13949.
[31] Mott JL, Kobayashi S, Bronk SF, Gores GJ. Mir-29 regulates Mcl-1 protein expression and apoptosis. Oncogene 2007;26:6133–6140.
[32] Lagos-Quintana M, Rauhut R, Lendeckel W, Tuschl T. Identification of novel genes coding for small expressed RNAs. Science 2001;294:853–858.
[33] Takamizawa J, Konishi H, Yanagisawa K, Tomida S, Osada H, Endoh H, et al. Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoperative survival. Cancer Res 2004;64: 3753–3756.
[34] Johnson SM, Grosshans H, Shingara J, Byrom M, Jarvis R, Cheng A, et al. RAS is regulated by let-7 microRNA family. Cell 2005;120:635–647.
[35] Sampson VB, Rong NH, Han J, Yang Q, Aris V, Soteropoulos P, et al. MicroRNA let-7a down-regulates MYC and reverts MYC-induced growth in Burkitt lymphoma cells. Cancer Res 2007;67:9762–9770.
[36] Lee YS, Dutta A. The tumor suppressor microRNA let-7 represses the HMGA2 oncogenes. Genes Dev 2007;21:1025–1030.
[37] Johnson CD, Esquela-Kerscher A, Stefani G, Byrom M, Kelnar K, Ovcharenko D, et al. The let-7 microRNA represses cell proliferation pathways in human cells. Cancer Res 2007;67:7713–7722.
[38] Büssing I, Slack FJ, Grosshans H. let-7 microRNAs in development, stem cells and cancer. Trend Mol Med 2008;14:400–409.

EXPERT REVIEWS

Inhibitor of MEK1/2, selumetinib, for biliary tract cancer

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Evaluation of: Bekaii-Saab T, Phelps MA, Li X *et al.* Multi-institutional Phase II study of selumetinib in patients with metastatic biliary cancers. *J. Clin. Oncol.* 29, 2357–2363 (2011).

It is necessary to establish effective chemotherapy to improve the survival of patients with biliary tract cancer. Although the useful of some molecular-targeted agents as first-line therapies has been investigated, none have been found to exert satisfactory efficacy. In this article, we report the results of a Phase II study of selumetinib in patients with metastatic biliary cancer. Selumetinib is an inhibitor of MEK1/2 targeting the RAS/RAF/MEK/extracellular signal-related kinase pathway. Three out of 28 patients showed a confirmed partial response, representing a response rate of 12%. The median progression-free survival was 3.7 months and the median overall survival was 9.8 months. The most common toxicities were rash, xerostomia and nausea. Most toxicities were grade 1 or 2, and the most common grade 3/4 toxicities were diarrhea and nausea. All toxicities were manageable and reversible. The results warrant further evaluation of the use of selumetinib in patients with metastatic biliary cancer.

Keywords: biliary tract cancer • mitogen-activated protein kinase kinase • molecular targeted therapy • Phase II study • selumetinib

Bile duct cancer is subdivided according to the anatomic location of origin into intrahepatic cholangiocarcinoma, gallbladder cancer, extrahepatic cholangiocarcinoma or cancer of the ampulla of Vater. Although surgery currently remains the only potentially curative treatment for each of the aforementioned diseases, many patients are diagnosed at an unresectable advanced stage of the disease. Chemotherapy has been recognized as a recommended therapy for unresectable biliary tract cancer based on the results of comparative studies between chemotherapy and best supportive care.

Despite the numerous Phase II studies conducted on treatments for advanced biliary tract cancer, no accepted standard treatment for this tumor type has been established as yet owing to the low incidence of this cancer, the small number of patients studied and the lack of adequately powered randomized controlled trials. Recently, randomized controlled trials comparing the combination of cisplatin plus gemcitabine with gemcitabine alone have shown the survival benefit of the former regimen [1,2]. Thus, the combination of gemcitabine plus a platinum agent (cisplatin or oxaliplatin) has come to be recognized as standard therapy for unresectable biliary tract cancer.

One of the next issues that needs to be addressed is whether molecular-targeted agents might also be effective against biliary tract cancer. To date, although clinical trials of molecular-targeted therapies as monotherapy or in combination with gemcitabine-based regimens have been conducted, no molecular-targeted agent has been confirmed to be of clinical benefit for biliary tract cancer.

Methods & results

This study was a Phase II study of selumetinib monotherapy in patients with unresectable biliary tract cancer, including intra- or extra-hepatic cholangiocarcinoma and gallbladder cancer [3]. Selumetinib is an inhibitor of MEK1/2 targeting the RAS/RAF/MEK/extracellular signal-related kinase (ERK) pathway, which plays a central role in the regulation of cellular processes, including proliferation, apoptosis and metabolism.

The primary objective of this study was to determine the overall response rate, as defined by the Response Evaluation Criteria In Solid

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Tumors, and the secondary objectives included evaluation of toxicity, overall survival, progression-free survival, assessment of *BRAF* and *KRAS* mutations, and measurement of phosphorylated (p) ERK and pAKT as indicators of activation of the relevant pathways.

With regard to the starting dose and dosing schedule of selumetinib, the drug was administered orally at 100 mg twice daily in 28-day cycles without interruption. Two levels of dose reductions were planned (50 mg twice daily and 50 mg once daily), with patients taken off the study in the case of a need for any additional dose reductions.

A total of 29 patients were enrolled between December 2007 and January 2009. Three patients showed a confirmed partial response, representing a response rate of 12%. In total, 17 patients (68%) showed stable disease. The majority of patients (52%) showed a decrease in the size of the target lesion. The median progression-free survival was 3.7 months (95% CI: 3.5–4.9) and the median overall survival was 9.8 months (95% CI: 5.97–not available).

The most common toxicities were rash (90%), xerostomia (54%) and nausea (51%). Although most toxicities were grade 1 or 2, the most common grade 3/4 toxicities were diarrhea, nausea and fatigue; in particular, grade 4 fatigue was observed in 4% of the patients. All toxicities were manageable and reversible.

Analyses of biologic markers revealed no *BRAF* V600E mutations. Two patients with short-lived stable disease had *KRAS* mutations. Absence of pERK staining was associated with lack of response and positive immunostaining for pERK was associated with improved overall survival.

Expert commentary

Some growth factors, including EGF receptor (EGFR) and VEGF receptor (VEGFR), and various signal transduction pathways that play important roles in the progression, proliferation and metastasis of various cancers, have been identified. Some studies have demonstrated overexpression of EGFR and VEGFR or mutations of their signaling pathways in biliary tract cancer [4]. Furthermore, biliary tract cancer includes various types of cancers, each with different molecular biological characteristics. For example, overexpression of EGFR has been reported to be observed in 10.7, 5.1, 12.4 and 0% of cases of intrahepatic cholangiocarcinoma, extrahepatic cholangiocarcinoma, gallbladder cancer and cancer of the ampulla of Vater, respectively [5]. Relationships between the presence/absence of various genetic mutations and the efficacy of molecular-targeted agents have been identified in various cancers; for example, the efficacy of anti-EGFR antibodies was limited to colorectal cancer patients with wild-type KRAS expression in the tumor. MEK inhibitors, including selumetinib, may be expected to exhibit activity, even against tumors with KRAS mutation. There is as yet, however, no consensus on the molecular–biologic characteristics of biliary tract cancer.

Recently, combined gemcitabine plus cisplatin or oxaliplatin therapy has been established as the standard first-line treatment for biliary tract cancer. Usage of molecular-targeted agents has been focused on as the next step. There are two directions in which molecular-targeted agents can be expected to be applied: one is in combination with standard chemotherapy regimens as first-line therapy, and the other is as monotherapy in second-line chemotherapy. In many patients with progressive disease receiving first-line chemotherapy with the relatively toxic regimen of cisplatin plus gemcitabine or gemcitabine plus oxaliplatin, the general condition is poor and serious cholangitis can easily develop. Less toxic therapy, such as monotherapy with a targeted agent, may be useful in such patients.

In this study, although 11 patients (39%) had a previous history of exposure to prior chemotherapy, there were three objective responses, representing a response rate of 12%, and another 14 patients (68%) showed stable disease [3]. In addition, both the progression-free survival and overall survival compare favorably with published historical controls. These results of selumetinib seem to suggest the promising activity of the drug against biliary tract cancer. Validation is required to confirm the efficacy of MEK inhibitors against biliary tract cancer according to the tumor site or the biological characteristics of the tumor.

Few preclinical studies of molecular-targeted agents for biliary tract cancer have been reported. In an examination conducted using human cholangiocarcinoma cell lines, ZD6474, an inhibitor of VEGFR and EGFR signaling, showed promising anticancer activity [6]. This study revealed that the absence of KRAS mutation and presence of EGFR amplification may be potentially predictive molecular markers of the sensitivity of cholangiocarcinoma to EGFR-targeted therapy [6]. Thus, therapeutically beneficial effects of molecular-targeted agents, including MEK inhibitors, may be expected against tumors with KRAS mutations and further investigations are warranted to confirm the efficacy.

Five-year view

Molecular-targeted therapy should be established based on the biologic features, and it is important to identify the characteristic biologic features of each of the aforementioned types of cancer of the biliary tract. Furthermore, efficient development of targeted therapy should be advanced based on the identification of appropriate biological markers.

Biliary tract cancer is still a difficult disease to treat. Development of new molecular-targeted agents will hopefully allow for improvement of the survival rates in patients with biliary tract cancer, and individualized therapy using targeted agents can be established according to the tumor's biological features.

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Key issues

- Selumitinib is an inhibitor of MEK1/2, and a Phase II study of selumetinib showed promising activity against biliary tract cancer.
- The most common toxicities were rash, xerostomia and nausea, and all toxicities were manageable and reversible.
- Analyses of biologic markers suggested the existence of a relationship between the KRAS and BRAF status, and the efficacy of selumitinib.
- The results warrant further evaluation of the use of selumetinib in patients with metastatic biliary cancer.

References

Papers of special note have been highlighted as:
- • of interest
- •• of considerable interest

1 Valle J, Wasan H, Palmer DH *et al.* Cisplatin plus gemcitabine versus gemcitabine for biliary tract cancer. *N. Engl. J. Med.* 362, 1273–1281 (2010).

•• **Large randomized controlled trial that demonstrated the survival benefits of gemcitabine and cisplatin in patients with advanced biliary tract cancer.**

2 Okusaka T, Nakachi K, Fukutomi A *et al.* Gemcitabine alone or in combination with cisplatin in patients with biliary tract cancer: a comparative multicentre study in Japan. *Br. J. Cancer* 103, 469–474 (2010).

• **Randomized clinical trial in Japan using the same regimen of gemcitabine and cisplatin as the UK trial.**

3 Bekaii-Saab T, Phelps MA, Li X *et al.* Multi-institutional Phase II study of selumetinib in patients with metastatic biliary cancers. *J. Clin. Oncol.* 29, 2357–2363 (2011).

•• **First Phase II study of selumetinib for metastatic biliary cancers.**

4 Thomas MB. Biological characteristics of cancers in the gallbladder and biliary tract and targeted therapy. *Crit. Rev. Oncol. Hematol.* 61, 44–51 (2007).

5 Nakazawa K, Dobashi Y, Suzuki S *et al.* Amplification and overexpression of c-erbB-2, epidermal growth factor receptor, and c-met in biliary tract cancers. *J. Pathol.* 206, 356–365 (2005).

6 Yoshikawa D, Ojima H, Kokubu A *et al.* Vandetanib (ZD6474), an inhibitor of VEGFR and EGFR signalling, as a novel molecular-targeted therapy against cholangiocarcinoma. *Br. J. Cancer* 100, 1257–1266 (2009).

• **Preclinical examination of vandetanib in cholangiocarcinoma.**

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Phase III study of sorafenib after transarterial chemoembolisation in Japanese and Korean patients with unresectable hepatocellular carcinoma ☆

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ABSTRACT

Background: In Japan and South Korea, transarterial chemoembolisation (TACE) is an important locoregional treatment for patients with unresectable hepatocellular carcinoma (HCC). Sorafenib, a multikinase inhibitor, has been shown effective and safe in patients with advanced HCC. This phase III trial assessed the efficacy and safety of sorafenib in Japanese and Korean patients with unresectable HCC who responded to TACE.

Methods: Patients ($n = 458$) with unresectable HCC, Child-Pugh class A cirrhosis and ⩾25% tumour necrosis/shrinkage 1–3 months after 1 or 2 TACE sessions were randomised 1:1 to

☆ Results from this trial were presented at the American Society of Clinical Oncology Gastrointestinal Cancers Symposium, Orlando, Florida, USA, 22–24 January 2010.

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Randomised Controlled trial

sorafenib 400 mg bid or placebo and treated until progression/recurrence or unacceptable toxicity. Primary end-point was time to progression/recurrence (TTP). Secondary end-point was overall survival (OS).

Findings: Baseline characteristics in the two groups were similar; >50% of patients started sorafenib >9 weeks after TACE. Median TTP in the sorafenib and placebo groups was 5.4 and 3.7 months, respectively (hazard ratio (HR), 0.87; 95% confidence interval (CI), 0.70–1.09; $P = 0.252$). HR (sorafenib/placebo) for OS was 1.06 (95% CI, 0.69–1.64; $P = 0.790$). Median daily dose of sorafenib was 386 mg, with 73% of patients having dose reductions and 91% having dose interruptions. Median administration of sorafenib and placebo was 17.1 and 20.1 weeks, respectively. No unexpected adverse events were observed.

Interpretation: This trial, conducted prior to the reporting of registrational phase III trials, found that sorafenib did not significantly prolong TTP in patients who responded to TACE. This may have been due to delays in starting sorafenib after TACE and/or low daily sorafenib doses.

1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide, the third most common cause of cancer deaths in men and the sixth most common in women.[1] It has been estimated that 650,000 people per year die from HCC, about three-quarters in East Asian countries.[2,3] Aetiologic factors vary by geographic region; ~70% of HCC patients in the Asia-Pacific (AP) region have chronic hepatitis B virus (HBV) infection, except in Japan, where ~75% of HCC patients have chronic hepatitis C virus (HCV) infection.[2,3]

Many patients with HCC are not diagnosed until the disease is unresectable, such that only non-curative treatment options are available.[4,5] The most frequent locoregional treatment for unresectable HCC is transarterial chemoembolisation (TACE), which concentrates chemotherapeutic agents at the tumour site while blocking the primary artery feeding the tumour.[6,7] Compared with symptomatic treatment alone, TACE has been found to enhance survival in patients with unresectable HCC.[8,9] A meta-analysis of seven randomised trials of arterial embolisation in 545 patients showed that chemoembolisation with cisplatin or doxorubicin showed a significant 2-year survival benefit compared with control, whereas embolisation alone showed no benefit.[10] A subsequent meta-analysis of randomised trials showed that TACE improves patient survival compared with untreated patients, but not when compared with patients treated with arterial embolisation alone.[11] Furthermore, no chemotherapeutic agent was found superior to any other, and there was no evidence that lipiodol had any benefit.[11]

Although TACE effectively delays HCC progression or prevents recurrence within 6 months, it is less effective over longer periods,[12] with 2-year survival rates of 24–63%.[13] Recent trials in Asian patients have found that 2-year overall survival (OS) rates following TACE with a suspension of a fine powder formulation of cisplatin in lipiodol, an emulsion of doxorubicin in lipiodol, and epirubicin-loaded superabsorbent polymer microspheres were 76%, 46% and 59%, respectively.[14,15] Although multiple courses of TACE may improve local tumour control,[11] it may also worsen liver function, both because TACE itself damages the hepatic arterial system[16] and because many patients have poor underlying liver function due to cirrhosis.[17] New and effective treatment strategies for patients with unresectable HCC are therefore needed, including the optimisation of TACE and its combination with other treatment modalities.

The high rate of HCC recurrence after TACE may be due to its enhancement of angiogenesis and upregulation of vascular endothelial growth factor (VEGF) expression, resulting in the formation of rich vascular beds in residual tumours.[18–20] Post-TACE treatment with systemic multikinase inhibitors that are both antiproliferative and antiangiogenic may therefore lengthen time to recurrence, improve survival, and target lesions distal to the TACE site.

Sorafenib is a multikinase inhibitor with antiangiogenic and antiproliferative properties, targeting multiple pathways.[21–23] Two large randomised phase III studies, the Sorafenib Health Assessment Randomised Protocol (SHARP)[24] and Sorafenib Asia-Pacific (AP)[25] trials, demonstrated that sorafenib significantly improves OS in patients with advanced HCC, leading to its approval for the treatment of HCC in more than 90 countries. To date, sorafenib remains the only available systemic therapy proven to extend survival in these patients.

In patients with unresectable HCC, sorafenib after TACE may prolong time to recurrence/progression and/or minimise loss of liver function associated with repeated courses of TACE. This double-blind, placebo-controlled, phase III trial, designed before the results of the SHARP and Sorafenib AP trials were reported, assessed the efficacy and safety of sorafenib in patients in Japan and South Korea with unresectable HCC who responded to TACE.

2. Patients and methods

We screened patients ⩾18 years of age with unresectable HCC and Child-Pugh A cirrhosis who sustained a response 1–3 months after TACE, defined using the then-prevailing criteria in Japan as ⩾25% tumour necrosis and/or shrinkage.[26,27] Additional inclusion criteria were life expectancy ⩾12 weeks; maximum target lesion size of 70 mm; ⩽10 target lesions; Eastern Cooperative Oncology Group (ECOG) performance status (PS) 0 or 1; and adequate bone marrow (absolute

neutrophil count $\geqslant 1000/mm^3$; platelet count $\geqslant 50 \times 10^9/L$; prothrombin time [PT] – international normalised ratio $\leqslant 2.3$ or PT $\leqslant 6$ s above control), liver (total bilirubin $\leqslant 3$ mg/dL; alanine aminotransferase and aspartate aminotransferase $\leqslant 5 \times$ upper limit of normal [ULN]), and renal (serum creatinine $\leqslant 1.5 \times$ ULN; amylase and lipase $\leqslant 2 \times$ ULN) function.

Patients were excluded if they had macroscopic vascular invasion, renal failure, history of cardiac disease, active clinically serious infection, history of human immunodeficiency virus infection, symptomatic metastatic brain or meningeal tumour, extrahepatic metastasis, seizure disorder requiring medication, prior use of systemic agents for advanced HCC (although prior use of interferon, retinoid and/or vitamin K_2 as adjuvant treatment after curative local treatment was allowed), use of hematopoietic growth factors within 3 weeks before start of study drug, concomitant treatment with cytokines after the last course of TACE, history of organ allograft, documented history of substance abuse, or were pregnant or breast-feeding.

All patients provided written informed consent. The study was approved by the appropriate ethics committees and institutional review boards at each centre, and complied with Good Clinical Practice Guidelines, the Declaration of Helsinki, and local laws and regulations. Ongoing safety and efficacy were assessed independently by the Data Monitoring Committee. This study was registered at Clinicaltrials.gov as trial number NCT00494299.

2.1. *Procedures*

TACE was performed by injecting gelatin foam plus lipiodol in all cases. The chemotherapeutic agents used concurrently were epirubicin, cisplatin, doxorubicin and mitomycin. Eligible patients were stratified by response to TACE (complete response [CR], defined as 100% tumour necrosis or shrinkage versus non-complete response [non-CR], defined as $\geqslant 25\%$ but <100% tumour necrosis or shrinkage),[26] by ECOG PS (0 versus 1), and by number of courses of TACE (one versus two). Patients were blindly randomised 1:1 to 400 mg (two 200-mg tablets) sorafenib (Bayer Schering Pharma; Leverkusen, Germany) or matching placebo twice daily.

Treatment interruptions and dose reductions (first 400 mg qd, then 400 mg qod) were allowed for drug-related toxicity. Patients were monitored for adverse events (AEs) using the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI-CTCAE) version 3.0, except that the hand-foot skin reaction (HFSR) was classified and managed by a protocol-defined scale. Treatment continued until radiologic progression or recurrence of HCC, unacceptable toxicity associated with study drug, or withdrawal of consent.

The trial was divided into 28-day cycles. Patients were evaluated for safety and compliance every 2 weeks during cycles 1–3, and every 4 weeks thereafter. Tumours were evaluated, centrally at an image registration centre, $\leqslant 28$ days before the first dose of study drug and every 8 weeks thereafter, or when evaluating recurrence or progression. Throughout treatment, lesions were evaluated by dynamic computed tomography (CT), preferably by the same investigator or radiologist as at screening.

The primary study end-point was time to progression (TTP) by central review, defined as time to recurrence in patients with CR and TTP in those with non-CR at study entry. Progression was defined as a $\geqslant 25\%$ increase in tumour size or development of a new lesion. The secondary end-point was OS, defined as time from randomisation to death from any cause. Exploratory analyses included TTP by investigator assessment and subgroup analyses of TTP by central review, based on aetiology (HBV versus HCV), response to TACE (CR versus non-CR), number of lesions ($\leqslant 3$ versus >3), number of prior courses of TACE (1 versus 2), age (<65 versus $\geqslant 65$ years), sex, treatment lag ($\leqslant 9$ versus >9 weeks), country of enrolment (Japan versus South Korea), and ECOG PS (0 versus 1).

2.2. *Statistical analysis*

Patient sample size was estimated based on TTP. If 30% and 70% of patients achieved CR and non-CR, respectively, in response to TACE, the median TTP for the placebo group in the mixed population would be 5.7 months. Clinically meaningful improvement was defined as median TTP 50% higher in the sorafenib than in the placebo group. Assuming one formal interim and one final analysis performed using an O'Brien-Fleming-type alpha spending function with a two-sided alpha of 0.05, 318 events would be required to achieve a statistical power of 95%. Accrual of 372 patients (186 in each group) within 18 months would be expected to result in 318 events after 30 months; if 10% of patients were lost to follow-up, 414 patients would have to be randomised to observe 318 events.

Efficacy was assessed in the intention-to-treat (ITT) population, defined as all randomised patients. The safety population included all patients who received at least one dose of study medication. TTP and OS in the two treatment arms were calculated by the Kaplan–Meier method and compared by the log-rank test, as were subgroups stratified by response to TACE (CR versus non-CR), ECOG PS (0 versus 1) and number of prior courses of TACE (1 versus 2). Hazard ratios (HRs) for sorafenib versus placebo and 95% confidence intervals (CI) were estimated by Cox proportional hazards models.

2.3. *Role of the funding source*

The study sponsors were involved in the design of the study; the collection, analysis and interpretation of data; the writing of the report; and the decision to submit the paper for publication.

3. Results

3.1. *Patients*

From 27th April 2006 to 10th July 2009, 552 patients were screened at 69 centres in Japan and seven centres in South Korea. Of these, 458 patients (387 at 67 centres in Japan and 71 at six centres in South Korea) met the eligibility criteria and were randomised, 229 each to the sorafenib and placebo groups. All were included in the ITT analysis (Fig. 1), whereas

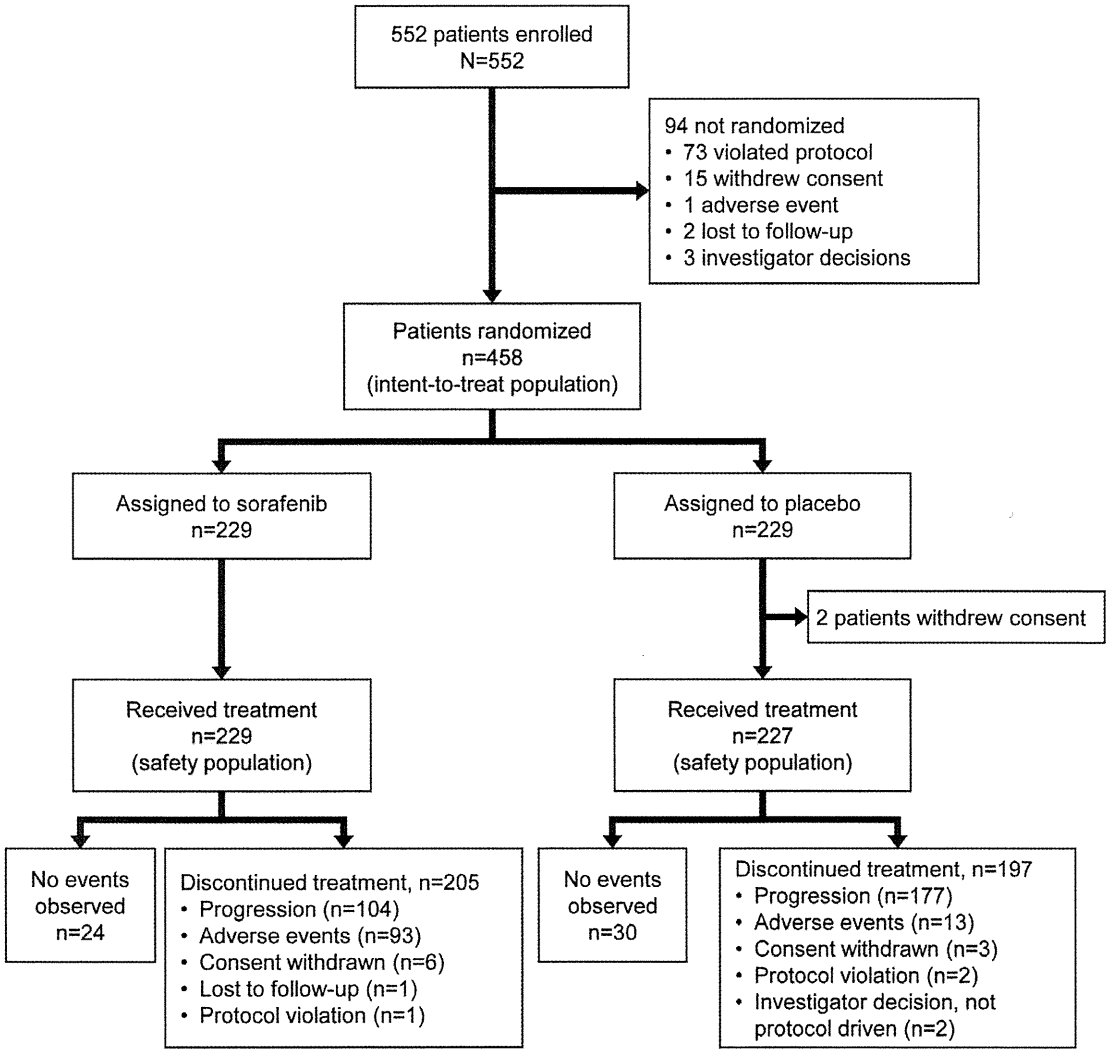

Fig. 1 – Enrolment and outcomes.

the 456 who received at least one dose of study drug were included in the safety analysis.

Demographic and baseline disease characteristics were similar in the sorafenib and placebo groups (Table 1). Of the 458 patients, 342 (74.7%) were male and 306 (66.8%) were ⩾65 years. Median age was 69 years (range, 29–86 years). At baseline, 403 patients (88.0%) had an ECOG PS of 0, 287 (62.7%) had HCV infection, and 336 (73.4%) had ⩽3 tumours. TACE consisted of gelatin foam plus lipiodol in all 458 patients, 60 for palliative intent and 398 for curative intent. Of these 458 patients, 355 received TACE monotherapy, including epirubicin ($n = 219$), cisplatin ($n = 89$), doxorubicin ($n = 49$) and mitomycin ($n = 1$); and 103 received combination treatments, including epirubicin + mitomycin ($n = 57$), cisplatin + epirubicin ($n = 16$), cisplatin + doxorubicin + mitomycin ($n = 13$), mitomycin + mitoxantrone ($n = 8$), doxorubicin + mitomycin ($n = 5$) and doxorubicin + iodixanol ($n = 4$). The median time from last TACE to randomisation was 9.3 weeks (range, 5.6–13.3 weeks), and the median time from initial diagnosis to study entry was 9.8 months (range, 1.6–144.3 months). Ten patients (2.2%) had received prior systemic anticancer therapy, consisting of prior adjuvant treatment with interferon, retinoid and/or vitamin K2 treatment after curative local treatment, and 219 (47.8%) had previously undergone some type of locoregional treatment, including radiofrequency ablation alone (10.7%), surgery alone (9.6%), percutaneous ethanol injection alone (5.9%), microwave coagulation therapy alone (0.2%) and other procedures (0.2%), with 21.2% having undergone multiple procedures (Table 1).

3.2. *Primary efficacy analysis*

By the cutoff date of 10th July 2009, 324 progression events (137 in the sorafenib and 187 in the placebo group) were confirmed by the Response Evaluation Committee. Median TTP by central review was 5.4 months (95% CI, 3.8–7.2 months) in the sorafenib group and 3.7 months (95% CI, 3.5–4.0 months) in the placebo group (HR [sorafenib/placebo], 0.87; 95% CI, 0.70–1.09; $P = 0.252$; Fig. 2). The 3-month progression-free rates in the sorafenib and placebo groups were 65.0% and 58.7%, respectively, and their 6-month progression-free rates were 45.7% and 33.5%, respectively.

Table 1 – Demographic and baseline characteristics of randomised patients (ITT population).

Variable	All patients			Japanese patients			Korean patients		
	Sorafenib + placebo (n = 458)	Sorafenib (n = 229)	Placebo (n = 229)	Sorafenib + placebo (n = 387)	Sorafenib (n = 196)	Placebo (n = 191)	Sorafenib + placebo (n = 71)	Sorafenib (n = 33)	Placebo (n = 38)
Median age (years)	69	69	70	71	70	71	60	61	59
Male (%)	74.7	76.0	73.4	72.9	74.0	71.7	84.5	87.9	81.6
ECOG PS[a] (%)									
0	88.0	87.8	88.2	91.5	91.3	91.6	69.0	66.7	71.1
1	12.0	12.2	11.8	8.5	8.7	8.4	31.0	33.3	28.9
Number of lesions (%)									
⩽3	73.4	72.9	73.8	70.8	69.9	71.7	87.3	90.9	84.2
>3	26.6	27.1	26.2	29.2	30.1	28.3	12.7	9.1	15.8
Aetiology (%)									
Alcohol	6.8	8.3	5.2	6.5	7.7	5.2	8.5	12.1	5.3
HBV	21.1	20.5	22.7	12.7	12.2	13.1	70.4	69.7	71.1
HCV	62.7	60.7	64.6	71.3	68.4	74.3	15.5	15.2	15.8
Other	5.9	7.0	4.8	7.0	8.2	5.8	0	0	0
Liver cirrhosis[b] (%)	68.3	69.4	67.2	66.7	67.3	66.0	77.5	81.8	73.7
Number of prior TACE[a] (%)									
1	64.4	64.2	64.6	66.7	66.3	67.0	52.1	51.5	52.6
2	35.6	35.8	35.4	33.3	33.7	33.0	47.9	48.5	47.4
Response to prior TACE[a,c] (%)									
CR	62.0	62.0	62.0	58.1	58.7	57.6	83.1	81.8	84.2
Non-CR	38.0	38.0	38.0	41.9	41.3	42.4	16.9	18.2	15.8
Prior local therapy (%)									
RFA	10.7	11.8	9.6	10.3	11.7	8.9	12.7	12.1	13.2
Surgery	9.6	7.0	12.2	10.3	8.2	12.6	5.6	0	10.5
PEI	5.9	4.8	7.0	6.5	5.1	7.9	2.8	3.0	2.6
MCT	0.2	0.4	0	0.3	0.5	0	0	0	0
Others	0.2	0.4	0	0	0	0	1.4	3.0	0
Multiple	21.2	20.5	21.8	24.0	23.0	25.1	5.6	6.1	5.3
Prior systemic therapy (%)	2.2	3.1	1.3	2.6	3.6	1.6	0	0	0

ITT = intention-to-treat; ECOG PS = Eastern Cooperative Oncology Group performance status; HBV = hepatitis B virus; HCV = hepatitis C virus; TACE = transarterial chemoembolisation; CR = complete response; non-CR = non-complete response; RFA = radiofrequency ablation; PEI = percutaneous ethanol injection; MCT = microwave coagulation therapy.

[a] Protocol-defined stratification factor.

[b] Clinically and/or histologically confirmed liver cirrhosis.

[c] Complete response was defined in the study protocol as 100% tumour shrinkage or necrosis.

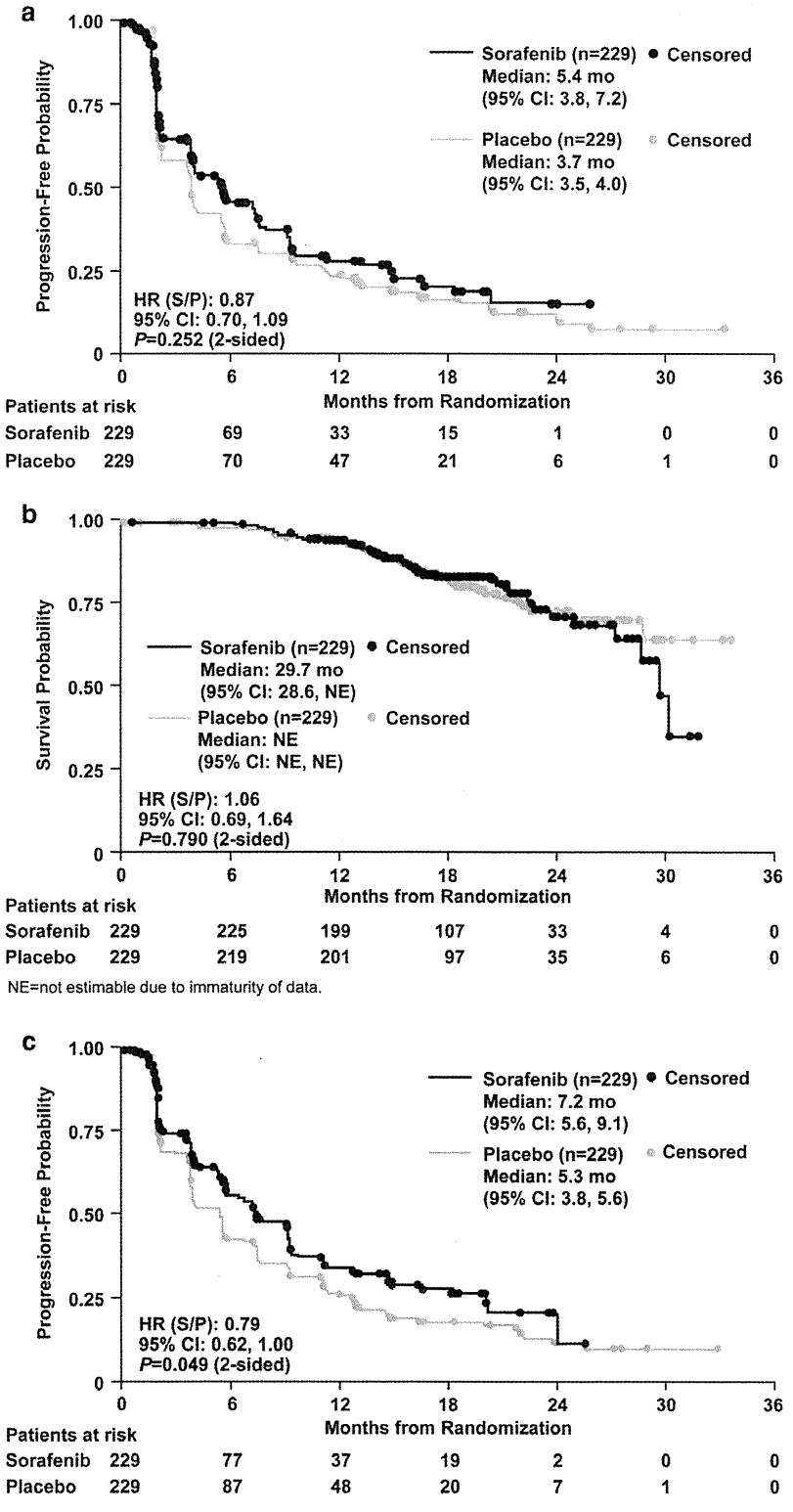

Fig. 2 – Kaplan–Meier analysis of time to progression (TTP) and overall survival (OS). (a) TTP by central review (primary intention-to-treat (ITT) analysis); (b) OS (secondary ITT analysis) and (c) TTP by investigator assessment (exploratory ITT analysis).

3.3. *Secondary efficacy analysis*

At the same cutoff date, there were 84 deaths, 43 in the sorafenib and 41 in the placebo group; the remaining patients were censored on that date. Median OS was 29.7 months in the sorafenib group (95% CI, 28.6 months – not yet reached) but had not yet been reached in the placebo group (HR [sorafenib/placebo], 1.06; 95% CI, 0.69–1.64; P = 0.790). The

Table 2 – Exploratory subgroup analyses of TTP by central review based on demographic, baseline and prognostic characteristics (ITT population; subgroups that included at least 10% of patients).

Variable	Subgroup	*n*	Number of events	Number of patients censored	Median TTP (95% confidence interval [CI]) (months)		Hazard ratio [HR] (95% CI) for Sorafenib/placebo
					Sorafenib	Placebo	
Aetiology	HBV	99	56	43	9.1 (5.6–20.3)	5.6 (3.7–10.9)	0.84 (0.49–1.44)
	HCV	287	217	70	5.3 (3.7–7.1)	3.6 (2.0–3.7)	0.81 (0.62–1.07)
Response to TACE	CR	284	179	105	7.4 (5.6–9.2)	5.3 (3.7–7.4)	0.84 (0.63–1.14)
	Non-CR	174	145	29	2.1 (1.8–3.9)	1.9 (1.8–3.6)	0.85 (0.61–1.18)
Number of lesions	⩽3	336	219	117	7.1 (5.3–7.8)	3.8 (3.7–5.5)	0.83 (0.64–1.09)
	>3	122	105	17	3.7 (2.0–5.3)	2.0 (1.9–3.7)	0.87 (0.59–1.29)
Number of prior TACE	1	295	212	83	5.4 (3.8–7.4)	3.7 (3.5–5.5)	0.91 (0.70–1.20)
	2	163	112	51	5.3 (3.7–7.8)	3.7 (2.1–3.8)	0.76 (0.52–1.11)
Age group	<65 years	152	90	62	9.1 (5.6–18.2)	3.7 (3.5–7.2)	0.68 (0.44–1.03)
	⩾65 years	306	234	72	3.8 (3.5–5.4)	3.7 (2.1–3.9)	0.99 (0.76–1.28)
Sex	Male	342	241	101	5.4 (3.8–7.4)	3.7 (3.5–5.3)	0.78 (0.60–1.00)
	Female	116	83	33	5.3 (3.6–7.4)	3.7 (2.1–5.3)	1.16 (0.75–1.79)
Treatment lag[a]	⩽9 weeks	205	150	55	5.5 (3.9–9.1)	3.7 (3.5–5.3)	0.74 (0.53–1.03)
	>9 weeks	253	174	79	5.1 (3.7–7.2)	3.7 (2.0–5.3)	0.95 (0.71–1.29)
Country of enrolment	Japan	387	289	98	3.9 (3.7–5.5)	3.7 (2.1–3.8)	0.94 (0.75–1.19)
	South Korea	71	35	36	NE[b] (9.0–NE)	5.5 (3.7–11.0)	0.38 (0.18–0.81)
ECOG PS	0	403	286	117	5.4 (3.8–7.2)	3.7 (3.6–5.3)	0.88 (0.69–1.11)
	1	55	38	17	5.4 (1.8–16.6)	3.5 (1.8–5.5)	0.78 (0.40–1.51)

[a] Treatment lag was defined as time from the most recent TACE to randomisation.
[b] NE = not estimable due to censored data.

1-year survival rates in the sorafenib and placebo groups were 94.6% and 94.1%, respectively, and their 2-year survival rates were 72.1% and 73.8%, respectively.

3.4. *Exploratory analyses*

At the cutoff date, investigators had reported 304 progression events, 120 in the sorafenib and 184 in the placebo group. Median TTP by investigator assessment in the sorafenib and placebo groups were 7.2 months (95% CI, 5.6–9.1 months) and 5.3 months (95% CI, 3.8–5.6 months), respectively (HR [sorafenib/placebo], 0.79; 95% CI, 0.62–1.00; $P = 0.049$). Their 3-month progression-free rates were 74.1% and 67.9%, respectively, and their 6-month progression-free rates were 54.9% and 41.4%, respectively.

Exploratory analyses of TTP by central review were performed in subgroups containing ⩾10% of patients, including by aetiology (HBV versus HCV), response to TACE (CR versus non-CR), number of lesions (⩽3 versus >3), number of prior courses of TACE (1 versus 2), age (<65 versus ⩾65 years), sex, treatment lag (⩽9 versus >9 weeks), ECOG PS (0 versus 1) and country of enrolment. These analyses were performed to provide descriptive information only; the study was not powered to compare subgroup response to treatment, and no adjustments were made for multiple comparisons. Median TTP and the HR for TTP (sorafenib/placebo) in each subgroup are shown in Table 2, and Forest plots of HRs for TTP are shown in Fig. 3. Most HRs favored sorafenib. Differences were observed, however, between Japanese and Korean patients. The HR for TTP was 0.94 (95% CI, 0.75–1.19) for Japanese patients and 0.38 (95% CI, 0.18–0.81) for Korean patients (Fig. 4). Median TTP in sorafenib-treated patients in the

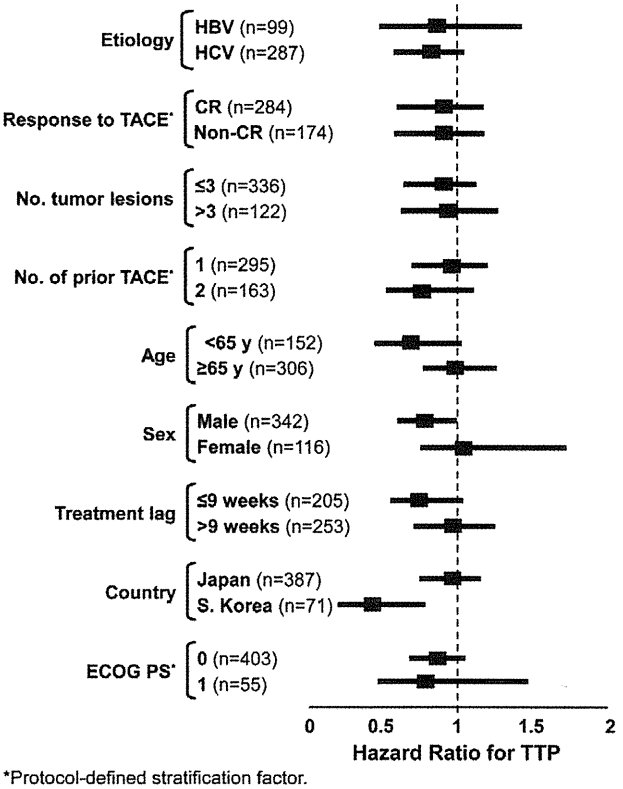

Fig. 3 – Subgroup analyses of TTP by central review (exploratory ITT analyses in subgroups that include at least 10% of patients): forest plot depicting hazard ratio (HR) for TTP (sorafenib over placebo) for each subgroup.

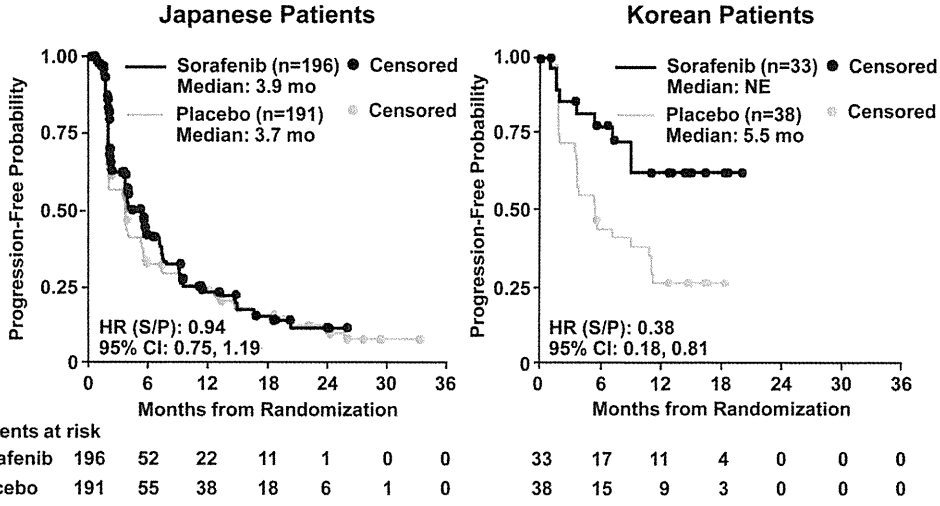

Fig. 4 – Kaplan–Meier analysis of TTP by central review, by country of enrolment (exploratory ITT analysis).

Korean subgroup could not be estimated since it was not attained by the study cutoff date.

3.5. *Safety*

The safety analysis included 229 sorafenib-treated and 227 placebo-treated patients; their incidence of drug-related AEs (DRAEs) were 100% and 61%, respectively. Most DRAEs were mild to moderate (Table 3), with the most frequent in the sorafenib and placebo groups being HFSR (82% versus 7%), elevated lipase (44% versus 8%), alopecia (41% versus 3%) and rash/desquamation (40% versus 11%). In the sorafenib group, 24% and 4% of patients experienced grades 3 and 4 elevated lipase, respectively, compared with 3% and <1%, respectively, in the placebo group. There was no radiographic or clinical evidence of pancreatitis in either group. The overall incidences of grade 3 HFSR (protocol-defined scale) in the sorafenib and placebo groups were 35% and 0%, respectively, and the overall incidence of serious DRAEs was 18% and 9%, respectively. There were no drug-related deaths.

The median durations of treatment in the sorafenib and placebo groups were 17.1 weeks (range, 1.0–112.1 weeks) and 20.1 weeks (range, 2.1–144.1 weeks), respectively (Table 4), and the median daily doses of sorafenib and placebo were 386.0 mg (range, 112.0–794.5 mg) and 785.8 mg (range, 276.1–810.3 mg), respectively. In the sorafenib group, 40 patients (17.5%) received >80% of the planned dose, compared with 206 (90.7%) in the placebo group. The most common reasons for discontinuing treatment in the sorafenib and placebo groups were disease progression (104/229 [45%] versus 177/229 [77%]) and adverse events (93/229 [41%] versus13/229 [6%]).

Doses were reduced in 166 of the 229 sorafenib-treated (72.5%) and in 33 of the 227 placebo-treated (14.5%) patients,

Table 3 – Treatment-emergent, drug-related adverse events occurring in ⩾20% of patients in either group.[a]

Adverse event	Sorafenib ($n = 229$) Grade (%)			Placebo ($n = 227$) Grade (%)		
	Any	3	4	Any	3	4
HFSR	82	35	–	7	0	–
Elevated lipase[b]	44	24	4	8	3	<1
Alopecia	41	–	–	3	–	–
Rash/desquamation	40	4	0	11	0	0
Other metabolic abnormality	32	8	1	4	2	<1
Diarrhoea	31	6	0	5	1	0
Hypertension	31	15	0	7	1	0
Hypophosphatemia	28	16	0	6	3	0
Thrombocytopenia	25	11	1	2	<1	0
Elevated AST	25	12	<1	5	3	0
Elevated ALT	21	8	<1	5	2	0
Elevated amylase	21	6	1	8	2	<1

HFSR = hand–foot skin reaction; AST = aspartate aminotransferase; ALT = alanine aminotransferase; NCI-CTCAE = National Cancer Institute Common Terminology Criteria for Adverse Events.

[a] Patients were monitored for adverse events using NCI-CTCAE v3.0, except for HFSR, which was classified according to a 3-grade, protocol-defined scale (grade 1, HFSR does not disrupt normal activities; grade 2, HFSR affects the activities of the patient; and grade 3, patient is unable to work or perform activities of daily living because of HFSR).

[b] There was no radiographic or clinical evidence of pancreatitis in either arm.

Table 4 – Summary of study drug administration.

Assessment	All patients		Japan		South Korea	
	Sorafenib (*n* = 229)	Placebo (*n* = 227)	Sorafenib (*n* = 196)	Placebo (*n* = 190)	Sorafenib (*n* = 33)	Placebo (*n* = 37)
Median duration of treatment (weeks)	17	20	16	20	31	33
Median daily dose (mg)	386	786	382	786	403	766
Patients with dose reduction (%)	73	14	71	11	82	32
Patients with dose interruption (%)	91	18	92	17	85	24
Patients with discontinuation (%)	90	87	93	88	70	78
Due to progression (%)	51	90	52	90	39	90
Due to adverse events (%)	45	7	44	7	57	3
HFSR	11	0	10	0	18	0
Thrombocytopenia	4	0	5	0	3	0
Hypophosphatemia	4	<1	4	1	3	0
Hypertension	4	0	5	0	0	0
Neutropenia	4	<1	4	1	0	0
Elevated AST	2	<1	2	1	3	0
Rash/desquamation	2	0	2	0	3	0
Elevated ALT	2	1	1	1	6	0
Diarrhoea	1	0	1	0	3	0
Other	11	4	19	3	18	3

HFSR = hand–foot skin reaction; AST = aspartate aminotransferase; and ALT = alanine aminotransferase.

due primarily to AEs (163 versus 27). Forest doses were interrupted temporarily in 208 of the 229 sorafenib-treated (90.8%) and 41 of the 227 placebo-treated (18.1%) patients, again due primarily to AEs (206 versus 38).

A total of 107 patients – 94 of the 229 (41.0%) in the sorafenib group and 13 of the 227 (5.7%) in the placebo group – permanently discontinued study drug due to AEs. The most common AEs leading to discontinuation of sorafenib were HFSR (11.4%), thrombocytopenia (4.4%), hypertension (3.9%), hypophosphatemia (3.9%) and neutropenia (3.5%); the most common AE leading to discontinuation of placebo was increased ALT (0.9%).

Death within 30 days of receiving study drug occurred in one patient (0.4%) in each group; neither was deemed drug-related.

4. Discussion

This phase III randomised, controlled trial, assessing the efficacy and safety of sorafenib after response to TACE in Japanese and Korean patients with unresectable HCC, employed a protocol consistent with the practice of TACE in these countries at that time.[28,29] Moreover, the protocol was designed before the combination or sequential use of TACE and sorafenib or their optimal timing had been adequately studied, and before the effect of TACE on susceptibility to sorafenib had been characterised. In this setting, sorafenib did not significantly prolong TTP or OS by central review in patients with unresectable HCC who responded to TACE. Exploratory secondary and subgroup analyses suggested, however, that post-TACE sorafenib had a positive impact on these patients. Median TTP by investigator review was approximately 2 months longer in the sorafenib than in the placebo group, and exploratory subgroup analyses suggested that TTP may have been affected by several factors, including age, number of prior TACE courses, treatment lag, treatment duration, total exposed dose and nationality.

Several factors may have contributed to these results. For example, unusually high percentages of sorafenib-treated patients required dose reductions (73%) and/or interruptions (91%), resulting in a much lower than planned median daily dose of sorafenib (386 mg). In comparison, 26% and 44% of sorafenib-treated patients in the SHARP trial, and 31% and 43% of those in the Sorafenib AP trial, required dose reductions and interruptions, respectively, due to AEs,[24,25] and median daily doses of sorafenib were higher in the SHARP (797 mg) and Sorafenib AP (795 mg) trials.

The better outcomes observed in Korean patients may have been due to their substantially longer median treatment duration (31 versus 16 weeks), resulting in a favourable HR in Koreans (0.38; 95% CI, 0.18–0.81). Moreover, the Korean and Japanese subgroups differed in baseline characteristics. Japanese patients were older and a higher percentage had ⩾3 lesions on enrolment. Moreover, Japanese patients were less likely to have received >1 TACE to achieve CR prior to sorafenib. Finally, these subgroups differed in principal aetiology of HCC, in that ~70% of Japanese patients had HCV and ~70% of Korean patients had HBV.

We found that the incidence of treatment-emergent adverse events in the sorafenib-treated patients in this trial was generally higher than that observed in previous trials of sorafenib in patients with HCC. We found that the rates of all grade HFSR, Grade 3 HFSR and discontinuation due to HFSR were higher in this trial than in the SHARP[24] and Sorafenib AP[25] trials. We also found that the rates of all grade alopecia; rash/desquamation; hypertension, including grade 3 hypertension; thrombocytopenia and elevated liver function enzymes were higher in this trial than in the two previous phase III trials of sorafenib in patients with HCC. These results were unexpected and may have been due to the combination of TACE with sorafenib treatment in this trial. These findings suggest that adjustments in sorafenib dose (e.g. starting at a lower dose after TACE) or the timing of sorafenib treatment with respect to TACE may be required for these two

modalities to be tolerated in combination and also have synergistic effects.

The timing of post-TACE sorafenib may also have contributed to the absence of a positive effect of sorafenib observed in this study. Local hypoxia resulting from TACE can induce angiogenesis[18] and enhance serum concentrations of VEGF,[19,20] suggesting that sorafenib may exert its greatest antiangiogenic effects when administered immediately after or even before TACE. Serum VEGF concentrations have also been found to correlate with impaired liver function, tumour size, tumour number, macroscopic vascular invasion,[30] and poor OS.[31] Of our sorafenib-treatment patients, 60% had a treatment lag >9 weeks prior to randomisation, due primarily to the need for central review of CT scans, and shorter lag time has been found associated with better outcomes.

Several ongoing phase II/III trials in patients with unresectable HCC may provide insight into the optimal combination treatment and the optimal timing of sorafenib relative to TACE. These include trials testing TACE with doxorubicin-eluting beads and sorafenib or placebo and alterations in timing of conventional TACE relative to sorafenib or placebo.[32–35]

5. Conclusion

Sorafenib did not significantly improve median TTP by central review in Japanese and Korean patients with unresectable HCC who responded to TACE, although exploratory analyses suggested that sorafenib may have clinical benefits in certain patient subsets, including males, patients <65 years of age, and those with a shorter treatment lag between TACE and sorafenib; and that longer treatment duration and greater total daily dose may be associated with clinical improvements. No new or unexpected AEs were observed. The results of these and other clinical investigations may help refine the use of sorafenib and TACE, and define their optimal combination, in patients with unresectable HCC.

Author contributions

Drs. Masatoshi Kudo and Kiwamu Okita were involved with the study concept and design; acquisition of data; analysis and interpretation of data; drafting of the manuscript; critical revision of the manuscript for important intellectual content; statistical analysis; and study supervision.

Drs. Kazuho Imanaka, Nobuyuki Chida, Kohei Nakachi, Won-Young Tak, Tadatoshi Takayama, Jung-Hwan Yoon, Takeshi Hori, Hiromitsu Kumada, Norio Hayashi, Shuichi Kaneko, Hirohito Tsubouchi, Dong Jin Suh, Junji Furuse, Takuji Okusaka, Katsuaki Tanaka and Osamu Matsui were involved with the acquisition of data; analysis and interpretation of data; drafting of the manuscript; critical revision of the manuscript for important intellectual content; statistical analysis; and study supervision.

Drs. Michihiko Wada, Iku Yamaguchi, Toshio Ohya and Gerold Meinhardt were involved with the study concept and design; analysis and interpretation of data; drafting of the manuscript; critical revision of the manuscript for important intellectual content; statistical analysis; administrative and technical support; and study supervision.

Clinical trials

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Conflict of interest statement

Masatoshi Kudo received advisory and speaker fees and research and travel grants from Bayer. Won-Young Tak received advisory and speaker fees from Bayer, Junji Furuse received advisory fees from Bayer, Takuji Okusaka received advisory and speaker fees, research and travel grants from Bayer. Osamu Matsui received consulting and advisory fees and research grants from Bayer. Michihiko Wada, Iku Yamaguchi, Toshio Ohya and Gerold Meinhardt are employees of Bayer. Kiwamu Okita received consulting fees from Bayer. All other authors declared no conflicts of interest.

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REFERENCES

1. Kudo M. The 2008 Okuda lecture management of hepatocellular carcinoma: from surveillance to molecular targeted therapy. *J Gastroenterol Hepatol* 2010;**25**:439–52.
2. Yuen MF, Hou JL, Chutaputti A. Hepatocellular carcinoma in the Asia-Pacific region. *J Gastroenterol Hepatol* 2009;**24**:346–53.
3. Asia-Pacific working party on prevention of hepatocellular carcinoma. Prevention of hepatocellular carcinoma in the Asia-Pacific region: consensus statements. *J Gastroenterol Hepatol* 2010;**25**:657–63.
4. Llovet JM, Burroughs A, Bruix J. Hepatocellular carcinoma. *Lancet* 2003;**362**:1907–17.
5. Makuuchi M, Kokudo N. Clinical practice guidelines for hepatocellular carcinoma: the first evidence based guidelines from Japan. *World J Gastroenterol* 2006;**12**:828–9.
6. Bruix J, Sherman M. Management of hepatocellular carcinoma: an update. *Hepatology* 2011;**53**:1020–2.
7. Trevisani F, De Notariis S, Rossi C, Bernardi M. Randomized control trials on chemoembolization for hepatocellular carcinoma: is there room for new studies? *J Clin Gastroenterol* 2001;**32**:383–9.
8. Lo CM, Ngan H, Tso WK, et al. Randomized control trial of transarterial lipiodol chemoembolization for unresectable hepatocellular carcinoma. *Hepatology* 2002;**35**:1164–71.
9. Llovet JM, Real MI, Montaña X, et al. Arterial embolisation or chemoembolisation versus symptomatic treatment in patients with unresectable hepatocellular carcinoma: a randomised controlled trial. *Lancet* 2002;**359**:1734–9.
10. Llovet JM, Bruix J. Systematic review of randomized trials for unresectable hepatocellular carcinoma: chemoembolization improves survival. *Hepatology* 2003;**37**:429–42.
11. Marelli L, Stigliano R, Triantos C, et al. Transarterial therapy for hepatocellular carcinoma: which technique is more effective? A systematic review of cohort and randomized studies. *Cardiovasc Intervent Radiol* 2007;**30**:6–25.

12. Cheng H-Y, Wang X, Chen D, Xu A-M, Jia Y-C. The value and limitation of transcatheter arterial chemoembolization in preventing recurrence of resected hepatocellular carcinoma. *World J Gastroenterol* 2005;**11**:3644–6.
13. Llovet JM. Updated treatment approach to hepatocellular carcinoma. *J Gastroenterol* 2005;**40**:225–35.
14. Kasai K, Ushio A, Sawara K, et al. Transcatheter arterial embolizaiton with a fine-powder formulation of cisplatin for hepatocellular carcinoma. *World J Gastroenterol* 2010;**16**:3437–44.
15. Seki A, Hori S, Kobayashi K, Narumiya S. Transcatheter arterial embolization with epirubicin-loaded superabsorbent polymer microspheres for 135 hepatocellular carcinoma patients: single-center experience. *Cardiovasc Intervent* 2011;**34**:557–65.
16. Takayasu K, Arii S, Ikai I, et al. Prospective cohort study of transarterial chemoembolization for unresectable hepatocellular carcinoma in 8510 patients. *Gastroenterology* 2006;**131**:461–9.
17. Groupe d'Etude et de Traitement du Carcinome Hépatocellulaire. A comparison of lipiodol chemoembolization and conservative treatment for unresectable hepatocellular carcinoma. *N Engl J Med* 1995;**332**:1256–61.
18. Fernández M, Semela D, Bruix J, et al. Angiogenesis in liver disease. *J Hepatol* 2009;**50**:604–20.
19. Wang B, Xu H, Gao ZQ, et al. Increased expression of vascular endothelial growth factor in hepatocellular carcinoma after transcatheter arterial chemoembolization. *Acta Radiol* 2008;**49**:523–9.
20. Li X, Feng G-S, Zheng C-S, Zhuo C-K, Liu X. Expression of plasma vascular endothelial growth factor in patients with hepatocellular carcinoma and effect of transcatheter arterial chemoembolization therapy on plasma vascular endothelial growth factor level. *World J Gastroenterol* 2004;**10**:2878–82.
21. Wilhelm S, Chien DS. BAY 43-9006: preclinical data. *Curr Pharm Des* 2002;**8**:2255–7.
22. Wilhelm SM, Carter C, Tang L, et al. BAY 43-9006 exhibits broad spectrum oral antitumor activity and targets the RAF/MEK/ERK pathway and receptor tyrosine kinases involved in tumor progression and angiogenesis. *Cancer Res* 2004;**64**:7099–109.
23. Carlomagno F, Anaganti S, Guida T, et al. BAY 43-9006 inhibition of oncogenic RET mutants. *J Natl Cancer Inst* 2006;**98**:326–34.
24. Llovet JM, Ricci S, Mazzaferro V, et al. Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med* 2008;**359**:378–90.
25. Cheng A-L, Kang Y-K, Chen Z, et al. Efficacy and safety of sorafenib in patients in the Asia-Pacific region with advanced hepatocellular carcinoma: a phase III randomised, double-blind, placebo-controlled trial. *Lancet Oncol* 2009;**10**:25–34.
26. Liver Cancer Study Group of Japan. Criteria for the evaluation of direct efficacy in treatment of liver cancer. *Kanzo* 2004;**45**:380–5.
27. Takayasu K, Arii S, Matsuo N, et al. Comparison of CT findings with resected specimens after chemoembolization with iodized oil for hepatocellular carcinoma. *AJR Am J Roentgenol* 2000;**175**:699–704.
28. Yamada R, Nakatsuka H, Nakamura K, et al. Hepatic artery embolization in 32 patients with unresectable hepatoma. *Osaka City Med J* 1980;**26**:81–96.
29. Yamada R, Sato M, Kawabata M, et al. Hepatic artery embolization in 120 patients with unresectable hepatoma. *Radiology* 1983;**148**:397–401.
30. Shim JH, Park J-W, Kim JH, et al. Association between increment of serum VEGF level and prognosis after transcatheter arterial chemoembolization in hepatocellular carcinoma patients. *Cancer Sci* 2008;**99**:2037–44.
31. Schoenleber SJ, Kurtz DM, Talwalkar JA, Roberts LR, Gores GJ. Prognostic role of vascular endothelial growth factor in hepatocellular carcinoma: systematic review and meta-analysis. *Br J Cancer* 2009;**100**:1385–92.
32. Lencioni R, Zou J, Leberre M, et al. Sorafenib (SOR) or placebo (PL) in combination with transarterial chemoembolization (TACE) for intermediate-stage hepatocellular carcinoma (SPACE). *J Clin Oncol* 2010;**28**(15 Suppl.) [Abstract TPS178].
33. Thomas MB, Jaffe D, Choti MM, et al. Hepatocellular carcinoma: consensus recommendations of the National Cancer Institute Clinical Trials Planning Meeting. *J Clin Oncol* 2010;**28**:3994–4005.
34. Park J-W, Kim HY, Shim JH, et al. Interim analysis of a phase II study of the combination of TACE and sorafenib for unresectable hepatocellular carcinoma in National Cancer Center Korea (COTSUN Korea Trial). In: Proceedings of the fourth annual conference of the International Liver Cancer Association (ILCA), Montreal, Quebéc, Canada; 10–12 September 2010. p. 26 [Abstract O-027].
35. US national institutes of health. Transcatheter arterial chemoembolization therapy in combination with sorafenib (TACTICS), NCT01217034. Available from: http://www.clinicaltrials.gov/ct2/show/NCT01217034?term=sorafenib±AND±tactics&rank=1 [accessed 07.12.10].