muscle cells.[16] PDGF is involved in fibrogenesis, angiogenesis and tumorigenesis.[17,18] PDGF expression is upregulated in the early stages of chronic hepatitis, suggesting its association with the development of fibrosis in chronic hepatitis C.[19] From a therapeutic point of view, inhibition of these targets has been shown to diminish the vascularity of tumors in preclinical studies.

Several intracellular signaling pathways are involved in HCC pathogenesis; the most studied are the PI3K/AKT/mTOR and RAS/RAF/MEK/ERK pathways. The PI3K/AKT/mTOR axis is involved in multiple cellular processes, including survival and proliferation.[20] This pathway mediates its effects through activation of various tyrosine kinase receptors, such as VEGFR, EGFR and PDGFR, which in turn recruit and activate PI3K. The activation of PI3K will lead to a cascade of activation of downstream effectors, leading to activation of mTOR (Fig. 1). The activation of the mTOR pathway in HCC is associated with aggressive tumor behavior and decreased survival, which supports the efforts to target this pathway for therapeutic interventions.[21] RAS/RAF/MEK/ERK signaling regulates many important cellular processes, such as proliferation, differentiation, angiogenesis, survival and cell adhesion.[22] Importantly, the RAS/RAF/MEK/ERK pathway is constitutively activated in HCC.[23]

Apart from these major signal pathways in the pathogenesis of HCC, the hepatocyte growth factor (HGF)/c-mesenchymal-epithelial transition factor-1 (c-MET) pathway is involved in tumor growth, invasion and angiogenesis in various types of cancer.[24] c-MET is a tyrosine kinase receptor, with its ligand, HGF.[25] HGF-induced activation of c-MET ultimately leads to the activation of downstream effector molecules, including PI3K and ERK (Fig. 1).[26] Expression of the c-MET receptor protein is present in human HCC samples[27–29] and has been shown to be a poor prognostic factor in patients with HCC. Therefore, therapeutics aimed at the c-MET receptor is a rational approach for HCC.

RESULTS OF PHASE III STUDIES

STUDIES ARE INVESTIGATING various agents for HCC, most of which target the previously described VEGF axis, FGF, PDGF, RAS/RAF/ERK and mTOR signaling pathways (Fig. 1). We describe these molecularly targeted agents and completed phase III trials. We also provide information on why phase III pivotal consecutive randomized controlled trials (RCT) in HCC did not meet the primary end-points (Table 1). Seven phase III trials reported negative results for first-line therapy (e.g. with sunitinib, brivanib, linifanib or erlotinib) and second-line therapy (e.g. with brivanib, everolimus or ramucirumab). Five of these studies were designed to test for superiority (i.e. study of SUN 1170, SEARCH, BRISK-PS, EVOLVE-1, REACH), and two of these studies were designed to test for non-inferiority (i.e. study of BRISK-FL, 0100953) with a primary end-point of OS.

Sorafenib

Sorafenib is a multikinase inhibitor that inhibits serine/threonine kinases (BRaf and CRaf and VEGFR-1, -2 and -3), PDGFR-α and -β, and the stem cell factor receptor, c-kit. In the Sorafenib HCC Assessment Randomized Protocol (SHARP) study,[5] a double-blind RCT with a primary end-point of OS, sorafenib significantly increased survival times of patients with HCC from 7.9 to 10.7 months (hazard ratio [HR], 0.69; 95% confidence interval [CI], 0.55–0.87; $P = 0.001$). Among the enrolled patients, the proportion of patients with Child–Pugh liver function class A and B disease was 97% and 3%, respectively, while that with BCLC stage B and C disease was 17% and 83%, respectively. Sorafenib was the first systemic therapy to demonstrate a significant improvement in OS in patients with advanced HCC, and its subsequent approval represented a major breakthrough in the treatment of advanced HCC. A parallel phase III study was conducted in the Asia–Pacific region. Median OS was 6.5 months in the sorafenib arm and 4.2 months in the placebo arm (HR, 0.68; 95% CI, 0.50–0.93; $P = 0.014$).[6] Among the enrolled patients, the proportion of patients with Child–Pugh liver function class A and B disease was 97% and 3%, respectively, while that with BCLC stage B and C disease was 5% and 95%, respectively. Similar toxicity profiles were seen in both studies; sorafenib treatment was associated with increased rates of diarrhea, weight loss, hand–foot skin reaction and hypophosphatemia. Sorafenib is the first and only agent to demonstrate an OS benefit and to be approved by regulators globally in patients with advanced HCC.

Sunitinib

Sunitinib is another multikinase inhibitor with broad activity, inhibiting all VEGFR and PDGFR, c-kit, Fms-like tyrosine receptor kinase (Flt)3 and rearranged during transfection (RET). Sunitinib was evaluated against sorafenib in a large phase III trial.[30] All patients had Child–Pugh liver function class A disease, and the proportion of patients with BCLC stage B and C disease was 15% and 85%, respectively. Median time to progression (TTP) for sunitinib and sorafenib was 4.1 and 3.8

Table 1 Results of completed phase III trials of molecularly targeted therapies in HCC

Drug	Main target	Design (trial)	TTP/PFS (months), HR, 95% CI	OS (months), HR, 95% CI
First-line advanced HCC				
Sorafenib	RAF, VEGFR, PDGFR, c-KIT	Sorafenib vs placebo (SHARP)	4.9 vs 4.1; $P = 0.77$; HR, 0.58; 95% CI, 0.45–0.74	10.7 vs 7.9; $P < 0.001$; HR, 0.69; 95% CI, 0.55–0.87
		Sorafenib vs placebo (Asia–Pacific)	2.8 vs 1.4; $P < 0.001$; HR, 0.57; 95% CI, 0.42–0.79	6.5 vs 4.2; $P = 0.014$; HR, 0.68; 95% CI, 0.50–0.93
Sunitinib	VEGFR, PDGFR, KIT, RET, Flt-3	Sunitinib vs sorafenib (SUN 1170)	4.1 vs 3.8; $P = 0.169$; HR, 1.13; 95% CI, 0.98–1.31	7.9 vs 10.2; $P = 0.0019$; HR, 1.30; 95% CI, 1.13–1.50
Brivanib	FGFR, VEGFR	Brivanib vs sorafenib (BRISK-FL)	4.2 vs 4.1; $P = 0.853$; HR, 1.01; 95% CI, 0.88–1.16	9.5 vs 9.9; $P = 0.373$; HR, 1.06; 95% CI, 0.93–1.22
Linifanib	VEGFR, PDGFR	Linifanib vs sorafenib (0100953)	5.4 vs 4.0; $P = 0.001$; HR, 0.76; 95% CI, 0.64–0.90	9.1 vs 9.8; $P =$ NS; HR, 1.05; 95% CI, 0.90–1.22
Erlotinib	EGFR, HER-1	Erlotinib + sorafenib vs placebo + sorafenib (SEARCH)	3.2 vs 4.0; $P = 0.91$; HR, 1.13; 95% CI, 0.94–1.36	9.5 vs 8.5; $P = 0.2$; HR, 0.92; 95% CI, 0.78–1.1
Second-line advanced HCC				
Brivanib	FGFR, VEGFR	Brivanib vs placebo (BRISK-PS)	4.2 vs 2.7; $P < 0.001$ HR, 0.56; 95% CI, 0.42–0.78	9.4 vs 8.2; $P = 0.331$; HR, 0.89; 95% CI, 0.69–1.15
Everolimus	mTOR	Everolimus vs placebo (EVOLVE-1)	3.0 vs 2.6; HR, 0.93; 95% CI, 0.75–1.15	7.6 vs 7.3; $P = 0.68$; HR, 1.27; 95% CI, 0.86–1.27
Ramucirumab	VEGFR	Ramucirumab vs placebo (REACH)	2.8 vs 2.1; $P < 0.001$; HR, 0.63; 95% CI, 0.52–0.75†	9.2 vs 7.6; $P = 0.14$; HR, 0.87; 95% CI, 0.72–1.05

†Progression-free survival.

CI, confidence interval; FGFR, fibroblast growth factor receptor; HCC, hepatocellular carcinoma; HER-1, human epidermal growth factor receptor-1; HR, hazard ratio; mTOR, mammalian target of rapamycin; NS, not significant; OS, overall survival; PDGFR, platelet-derived growth factor receptor; PFS, progression-free survival; RET, rearranged during transfection, Flt-3, Fms-like tyrosine receptor kinase-3; TTP, time to progression; VEGFR, vascular endothelial growth factor receptor.

months, respectively ($P = 0.169$); however, median OS for sunitinib and sorafenib was 7.9 and 10.2 months (HR, 1.30; 95% CI, 1.13–1.50; $P = 0.0019$), respectively. The decision was based on a higher incidence of significant toxicities (including grade 3/4 thrombocytopenia [30%], neutropenia [25%] and hemorrhagic events [12%]) in the sunitinib arm and the futility of showing either superiority or non-inferiority in OS when compared with sorafenib. This trial was stopped prematurely after inferior outcomes were noted in the sunitinib arm.

Brivanib

Brivanib is a dual inhibitor of VEGFR and FGFR, both of which are implicated in the pathogenesis of HCC.[31] Two randomized phase III clinical trials were conducted to assess the use of brivanib in the first-line (BRISK-FL) and second-line (BRISK-PS) settings. BRISK-FL was a head-to-head randomized phase III clinical trial comparing brivanib with sorafenib as the first-line therapy in patients with unresectable HCC. Among the enrolled patients, the proportion of patients with Child–Pugh liver function class A and B disease was 92% and 8%, respectively, while that with BCLC stage B and C disease was 22% and 78%, respectively. The brivanib arm failed to achieve a non-inferior median OS, with 9.5 months for brivanib and 9.9 months for sorafenib (HR, 1.06; 95% CI, 0.93–1.22; $P = 0.373$). There was also no difference in TTP between brivanib and sorafenib (4.2 vs 4.1 months; HR, 1.01; 95% CI, 0.88–1.16; $P = 0.853$).[31] The study did not meet its primary OS objective based upon a non-inferiority statistical design. In the second-line setting, BRISK-PS compared brivanib with placebo in patients who were refractory or intolerant to first-line treatment with sorafenib. Although TTP was significantly longer in the brivanib arm than with placebo (4.2 vs 2.7 months; HR, 0.56; 95% CI, 0.42–0.78; $P < 0.001$), the primary end-point of the study was not met, with a median OS for brivanib and placebo of 9.4 and 8.2 months, respectively (HR, 0.89; 95% CI, 0.69–1.15; $P = 0.331$).[32] The most common grade 3/4 adverse events (AE) were hypertension (19%), hyponatremia (18%), fatigue (15%) and decreased appetite (12%).

Linifanib

Linifanib is an oral tyrosine kinase inhibitor (TKI) with selective activity against VEGFR and PDGFR. Linifanib was compared with sorafenib as first-line therapy in a non-inferiority phase III trial.[33] Enrolled patients were those with a histological and cytological diagnosis of unresectable HCC and Child–Pugh liver function class A. TTP with linifanib was significantly improved when compared with sorafenib (5.4 vs 4.0 months; HR, 0.76; 95% CI, 0.64–0.90; $P = 0.001$). However, median OS was 9.1 months with linifanib and 9.8 months with sorafenib (HR, 1.05; 95% CI, 0.90–1.22). Linifanib was less well tolerated than sorafenib, with significantly increased discontinuations and dose reductions/interruptions because of AE.

Erlotinib

Erlotinib is an orally active, potent selective inhibitor of the EGFR/human epidermal growth factor receptor-1-related tyrosine kinase enzyme. In the phase III SEARCH trial, advanced HCC patients were randomized to sorafenib plus either erlotinib or placebo.[34] Inclusion criteria were a histological and cytological diagnosis of unresectable HCC and Child–Pugh liver function class A. Median OS was 9.5 months with sorafenib plus erlotinib and 8.5 months with sorafenib (HR, 0.92; 95% CI, 0.78–1.1; $P = 0.2$). This result failed the prespecified boundaries for non-inferiority. TTP was 3.2 months with sorafenib plus erlotinib and 4.0 months with sorafenib (HR, 1.13; 95% CI, 0.94–1.36; $P = 0.91$).

Everolimus

The mTOR inhibitor, everolimus, has demonstrated antitumor activity in several malignancies. A phase III study comparing everolimus with placebo (EVOLVE-1) in patients who have failed or become intolerant to sorafenib has recently been completed. All patients had Child–Pugh liver function class A, and the proportion of patients with BCLC stage B and C disease was 14% and 86%, respectively. There were no significant difference in TTP between everolimus (3.0 months) and placebo (2.6 months) (HR, 0.93; 95% CI, 0.75–1.15). Furthermore, no significant difference in OS was seen between everolimus (7.6 months) and placebo (7.3 months) (HR, 1.05; 95% CI, 0.86–1.27; $P = 0.68$). The most common grade 3/4 AE for everolimus were anemia (7.8%), asthenia (7.8%) and decreased appetite (6.1%). No patients experienced hepatitis C viral flare. The EVOLVE-1 study failed to reach its primary end-point of extending OS with everolimus.[35]

Ramucirumab

Ramucirumab is a recombinant humanized antibody that specifically targets the extracellular domain of VEGFR-2. A phase II study of 42 patients with advanced HCC and primarily well-preserved liver function showed that first-line ramucirumab monotherapy produced a disease control rate of 69%. The median progression-free survival (PFS) was 4.0 months and

median OS was 12.0 months, respectively. Grade 3/4 toxicities included gastrointestinal bleeding (7%), hypertension (12%) and fatigue (10%). These findings prompted the initiation of the phase III RCT (REACH) comparing ramucirumab versus placebo in patients who failed or were intolerant to sorafenib (NCT01140347).[36] Eligible patients had advanced HCC, stage BCLC C or B disease that was refractory or not amenable to locoregional therapy, and Child–Pugh liver function class A. However, according to the preliminary results released at European Society for Medical Oncology Congress in 2014, ramucirumab failed to demonstrate superiority in terms of OS when compared with placebo. The OS HR was 0.866 (95% CI, 0.717–1.046; $P = 0.1391$); median OS was 9.2 months for ramucirumab versus 7.6 months for placebo. Median PFS with ramucirumab and placebo was 2.8 and 2.1 months, respectively (HR, 0.63, 95% CI, 0.52–0.75; $P < 0.0001$).[37]

ONGOING PHASE III CLINICAL TRIALS

IN ADDITION TO the antiangiogenic multi-targeted TKI, there is a growing number of biologics that target different molecular pathways, such as c-MET. Some of these treatments act on elements of intracellular signaling pathways. A number of agents have shown promising preliminary data for HCC. We also comment on ongoing phase III pivotal trials (Table 2). The inclusion criterion of all four phase III studies was Child–Pugh liver function class A disease.

Lenvatinib

Lenvatinib is an oral multi-tyrosine kinase inhibitor that targets VEGFR-1–3, FGFR-1–3, RET, mast/stem cell growth factor receptor kit and PDGFR.[38] A phase I/II trial of lenvatinib in patients with advanced HCC and Child–Pugh score A liver function status showed a median OS of 18.7 months (95% CI, 12.8–25.1) and a median TTP of 7.4 months (95% CI, 5.5–9.4). Based on these results, a phase III trial was designed to compare the safety and efficacy of lenvatinib versus sorafenib in patients with unresectable or advanced HCC and Child–Pugh A liver status (NCT01761266).[39] Subjects were categorized as stage B (not applicable for transarterial chemoembolization [TACE]) or stage C based on the BCLC staging system.

Regorafenib

Regorafenib is a multikinase inhibitor that targets kinases involved in angiogenesis (e.g. VEGFR-1–3), oncogenesis (e.g. c-kit, RET and BRAF) and the tumor microenvironment (e.g. PDGFR and FGFR).[40] Regorafenib (160 mg/day) was tested in an uncontrolled phase II study in patients with advanced HCC after failure of prior sorafenib therapy (RESORCE).[41] Median TTP was 4.3 months and median OS was 13.8 months. The most common grade 3/4 AE included fatigue (17%), hand–foot skin reaction (14%) and diarrhea (6%). Based on this data, a phase III RCT in the second-line setting is under development (NCT01774344). Inclusion criteria were BCLC stage B or C disease, and failure to receive prior treatment with sorafenib.

Tivantinib

Tivantinib is a selective inhibitor of c-MET.[42] In a randomized phase II trial comparing the use of tivantinib

Table 2 List of ongoing phase III trials of novel targeted therapy for HCC

Drug	Main target	Design (trial)	Status	NCT number
1st line				
Lenvatinib	VEGFR, PDGFR, FGFR, RET, SCFR	Lenvatinib vs sorafenib (E7080)	Recruiting	NCT01761266
2nd line				
Regorafenib	VEGFR, PDGFR, BRAF, FGFR, KIT, RET	Regorafenib vs placebo (RESORCE)	Recruiting	NCT01774344
Tivantinib	c-MET	Tivantinib vs placebo in subjects with c-MET overexpressing (JET-HCC)	Recruiting	NCT01755767
Cabozantinib	c-MET, VEGFR, RET	Cabozantinib vs placebo (CELESTIAL)	Recruiting	NCT01908426

c-MET, c-mesenchymal-epithelial transition factor-1; FGFR, fibroblast growth factor receptor; HCC, hepatocellular carcinoma; PDGFR, platelet-derived growth factor receptor; RET, rearranged during transfection; SCFR, stem cell growth factor receptor kit; VEGFR, vascular endothelial growth factor receptor.

Table 3 Results of completed phase III trials of molecularly targeted therapy in combination with TACE for HCC

Drug	Main target	Design	TTP (months, HR, 95% CI)	OS (months)
Sorafenib	RAF, VEGFR, PDGFR, c-KIT	TACE + sorafenib vs TACE + placebo	5.4 vs 3.7; $P = 0.252$; HR, 0.87; 95% CI, 0.70–1.09	29.7 vs NE; $P = 0.790$; HR, 1.06; 95% CI, 0.69–1.64
Brivanib	FGFR, VEGFR	TACE + brivanib vs TACE + placebo	12.0 vs 10.9; $P = 0.62$; HR, 0.94; 95% CI, 0.72–1.22	26.4 vs 26.1; $P = 0.53$; HR, 0.90; 95% CI, 0.66–1.23
Orantinib	VEGFR, PDGFR, FGFR	TACE + orantinib vs TACE + placebo	†	†

†Full data have not yet been reported at November 2014.
CI, confidence interval; HCC, hepatocellular carcinoma; FGFR, fibroblast growth factor receptor; HR, hazard ratio; NE, not estimable due to immaturity of data; OS, overall survival; PDGFR, platelet-derived growth factor receptor; TTP, time to progression; VEGFR, vascular endothelial growth factor receptor.

versus placebo as second-line treatment, the overall analysis showed a marginal but significant improvement in TTP in tivantinib over placebo (1.6 vs 1.4 months; HR, 0.64; 95% CI, 0.43–0.94; $P = 0.04$). A preplanned analysis of patients whose tumors demonstrated overexpression of MET by immunohistochemistry revealed a more notable improvement in TTP, with 2.7 months in the MET-high tivantinib subset versus 1.4 months in the MET-high placebo subset (HR, 0.43; 95% CI, 0.19–0.97; $P = 0.03$). Median OS was 7.2 months for patients with MET-high tumors who received tivantinib versus 3.8 months for MET-high patients who received placebo (HR, 0.38, 95% CI, 0.18–0.81; $P = 0.01$).[43] The most common grade 3/4 AE in the tivantinib group were neutropenia and anemia; severe neutropenia rates were higher prior to mandated dose reduction. Currently, a phase III study is underway to compare tivantinib versus placebo in subjects with c-MET-overexpressing HCC who have failed one prior systemic therapy (NCT01755767).

Cabozantinib

Cabozantinib, a multikinase inhibitor that inhibits MET, VEGFR-2 and RET, was studied in a phase II trial of HCC patients who had received at most one prior systemic therapy.[44] Impressive efficacy was observed; the PFS was 4.4 months while the median OS was 15.1 months in the cabozantinib arm.[45] A phase III clinical trial testing the efficacy of cabozantinib in the second-line setting is planned (NCT01908426).

Combination therapy

With regard to molecularly targeted agents combined with other treatments, surgical resection and local ablation are curative therapies for BCLC stage A, whereas TACE is used for the management of patients of BCLC stage B. Hepatic arterial infusion chemotherapy (HAIC) is used for the management of patients of BCLC stage B to C. In this article, we focused mainly BCLC stage B to C. Tables 3 and 4 summarizes data regarding the use of molecularly targeted agents combined with TACE or HAIC.

The high rate of HCC recurrence after TACE may be due to its enhancement of angiogenesis and upregulation of VEGF and PDGFR expression, resulting in the formation of rich vascular beds in residual tumors.[46] Administration of an antiangiogenic agents with TACE may block angiogenesis and may therefore lengthen time to recurrence and improve survival.

A phase III study of sorafenib in combination with TACE versus TACE alone performed in Japan and Korea likewise did not demonstrate any benefit with the combination (TTP; sorafenib vs placebo [5.4 vs 3.7 months, HR, 0.87; 95% CI, 0.70–1.09; $P = 0.252$]; OS sorafenib vs placebo; 29.7 months vs not estimable due to immaturity of data [HR, 1.06; 95% CI, 0.69–1.64;

Table 4 List of ongoing phase III trials of therapy in combination with TACE or HAIC for HCC

Drug	Design (trial)	Status	NCT number
Sorafenib	TACE + sorafenib vs TACE + placebo	Recruiting	NCT01004978
Sorafenib	TACE + sorafenib vs TACE + placebo	Recruiting	NCT01324076
Sunitinib	TACE + sunitinib vs TACE + placebo	Recruiting	NCT01164202
Sorafenib	HAIC + sorafenib vs sorafenib	Recruiting	NCT01214343

HAIC, hepatic arterial infusion chemotherapy; HCC, hepatocellular carcinoma; TACE, transcatheter arterial chemoembolization.

$P = 0.79$]).[47] Two other phase III, randomized, placebo-controlled trials evaluating the efficacy of sorafenib in combination with conventional TACE are ongoing (NCT01004978 and NCT01324076).

Other phase III RCT exploring the combinations of TACE and orantinib (ORIENTAL trial, NCT01465464) and brivanib (BRISK-TA trial) have been completed, and sunitinib (TURNE trial, NCT01164202) are ongoing.

In the BRISK-TA trial, although brivanib improved time to radiographic progression (brivanib vs placebo; 8.4 vs 4.9 months; HR, 0.61; 95% CI, 0.48–0.77; $P < 0.0001$), brivanib did not improve TTP (brivanib vs placebo; 12.0 vs 10.9 months; HR, 0.94; 95% CI, 0.72–1.22; $P = 0.62$) or OS (brivanib vs placebo; 26.4 vs 26.1 months; HR, 0.90; 95% CI, 0.66–1.23; $P = 0.53$).[48]

Orantinib is an oral small molecule inhibitor of VEGFR, PDGFR and FGFR.[49] A recent press release announced that a phase III trial comparing TACE plus orantinib versus TACE plus placebo did not meet the primary end-point, but the full dataset has not yet been reported.

A phase III study of sorafenib plus low-dose cisplatin/fluorouracil HAIC versus sorafenib in patients with advanced HCC is ongoing (NCT01214343).

Biomarkers

Studies have investigated whether several biomarker can predict the response to sorafenib. Tissue markers, such as FGF3/FGF4,[50] αB-crystallin,[51] c-Jun N-terminal kinase,[52] VEGF-A[53] and pERK,[54] serum marker and angiogenesis-related cytokine have been reported.[55] Conventional tumor markers for the diagnosis of HCC, namely, des-γ-carboxyprothrombin and α-fetoprotein, have been reported to show contrasting behavior after administration of sorafenib.[56–60] However, no definitive biomarker for sorafenib has been identified. Lovelt *et al.* reported that no biomarker was significantly associated with the response to sorafenib within the SHARP study, which was the largest study of sorafenib.[61] The difficulty in identifying a specific biomarker in sorafenib therapy for HCC may be due to the presence of multiple molecular targets.

FUTURE DIRECTIONS

NINE PHASE III clinical trials (i.e. SHARP, Asia–Pacific, SUN 1170, BRISK-FL, 0100953, SEARCH, BRISK-PS, EVOLVE-1, REACH) of patients with advanced HCC have been completed, and four phase III clinical trials (i.e. E7080, RESORCE, JET-HCC, CELESTIAL) are ongoing. No targeted agent or regimens other than sorafenib significantly improve OS in patients with advanced HCC, according to phase III trials in the first- or second-line setting. Three phase III clinical trials did not demonstrate any benefit with combination therapy.

Potential reasons for negative results include heterogeneous patient population and the lack of understanding of critical drivers of tumor progression/dissemination. Other reasons include liver toxicity, flaws in trial design or marginal antitumoral efficacy of the agents. When dissecting the results of recent trials,[30–34] we can speculate that the main shortcomings for sunitinib are liver toxicity and issues with trial design.[30] Other shortcomings include lack of efficacy for erlotinib,[34] toxicity for linifanib[33] and lack of efficacy and issues with trial design for brivanib.[31,32]

Hepatocellular carcinoma is a heterogeneous disease, both in regard to its clinical manifestations with underlying liver disease, and its complex pathogenesis involving aberrant signaling in several molecular pathways. Advances in targeted therapy for HCC require a better understanding of various molecular events driving the progression of HCC as well as identification of biomarkers to predict treatment response to targeted agents. Due to the complexity of the mechanisms involved in progression of HCC, the establishment of personalized therapy will require the identification of tissue biomarkers in HCC.

Regarding patient selection, recommendations emphasized the need for standardization of inclusion criteria based on stage, such as the BCLC classification. It is evident that the population of patients with unresectable HCC consists of a highly heterogeneous group of patients with a wide spectrum of survival, ranging from a few months to longer than 2 years.[62,63] Therefore, it is difficult to precisely estimate the survival of patients during the design of clinical trials that encompass a heterogeneous population. As a result, the staging system is suboptimal in identifying a homogeneous group of patients in terms of prognosis and disease behavior.

In summary, success in the development of targeted agents for HCC relies on concerted efforts of testing of novel agents in clinical trials, advancement of knowledge of the molecular events of HCC, discovery of biomarkers to guide personalized treatment and improvements in patient selection.

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Original Article

Serum granulysin levels as a predictor of serious telaprevir-induced dermatological reactions

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Aim: Telaprevir-based therapy for chronic hepatitis C patients is effective; however, the high prevalence of dermatological reactions is an outstanding issue. The mechanism and characteristics of such adverse reactions are unclear; moreover, predictive factors remain unknown. Granulysin was recently reported to be upregulated in the blisters of patients with Stevens–Johnson syndrome (SJS). Therefore, we investigated the risk factors for severe telaprevir-induced dermatological reactions as well as the association between serum granulysin levels and the severity of such reactions.

Methods: A total of 89 patients who received telaprevir-based therapy and had complete clinical information were analyzed. We analyzed the associations between dermatological reactions and clinical factors. Next, we investigated the time-dependent changes in serum granulysin levels in five and 14 patients with grade 3 and non-grade 3 dermatological reactions, respectively.

Results: Of the 89 patients, 57 patients had dermatological reactions, including nine patients with grade 3. Univariate analysis revealed that grade 3 dermatological reactions were significantly associated with male sex. Moreover, serum granulysin levels were significantly associated with the severity of dermatological reactions. Three patients with grade 3 dermatological reaction had severe systemic manifestations including SJS, drug-induced hypersensitivity syndrome, and systemic lymphoid swelling and high-grade fever; all were hospitalized. Importantly, among the three patients, two patients' serum granulysin levels exceeded 8 ng/mL at onset and symptoms deteriorated within 6 days.

Conclusion: Male patients are at high risk for severe telaprevir-induced dermatological reactions. Moreover, serum granulysin levels are significantly associated with the severity of dermatological reactions and may be a predictive factor in patients treated with telaprevir-based therapy.

Key words: drug-induced hypersensitivity syndrome, granulysin, hepatitis C virus, telaprevir, toxic epidermal necrolysis

INTRODUCTION

HEPATITIS C IS a major pathogen causing liver cirrhosis and hepatocellular carcinoma worldwide. Until recently, standard therapies for chronic hepatitis C virus (HCV) genotype 1 infection were based on the combination of pegylated interferon (PEG IFN) and ribavirin (RBV); these combination therapies yield a sustained virological response (SVR) rate of approximately 50%.[1] Several classes of novel direct-acting antivirals

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(DAA) were recently developed and tested in clinical trials. Two first-generation HCV NS3/4A protease inhibitors, boceprevir[2,3] and telaprevir,[4-6] have been approved for the treatment of genotype 1 HCV infection. The inclusion of these agents in HCV treatment regimens has led to large improvements in treatment success rates.

Telaprevir, the first DAA, is administrated in combination with PEG IFN and RBV for 24 weeks, resulting in SVR rates up to 70–80%.[4,6-8] Although the telaprevir combination regimen is highly effective, the high frequency and severity of adverse events are outstanding issues limiting its use. Dermatological reactions are particularly prevalent, developing in 56–84.6% of patients treated with telaprevir, PEG IFN and RBV combination therapy.[9,10] Moreover, the prevalence of severe dermatological reactions including Stevens–Johnson syndrome/toxic epidermal necrolysis (SJS/TEN) and drug-induced hypersensitivity syndrome (DIHS) are substantially higher in patients treated with telaprevir-based therapy than PEG IFN and RBV combination therapy.[8,10] McHutchison *et al.* reported that 7% of patients treated with telaprevir, PEG IFN and RBV combination therapy discontinue therapy because of rash or pruritus in contrast to only 1% of patients treated with PEG IFN and RBV.[8] In some patients, serious skin reactions persist even after stopping all drugs.[10] However, the pathogenesis and clinical predictors of these adverse reactions are poorly understood.

Granulysin is a 15-kDa cationic cytolytic protein released by cytotoxic T lymphocytes and natural killer cells that induces apoptosis in target cells and has antimicrobial activities.[11] Serum levels of granulysin are elevated in primary virus infections including Epstein–Barr virus and parvovirus B19.[12] It was recently reported that serum granulysin levels are significantly elevated in patients with several types of severe dermatological lesions including SJS/TEN, which is the characteristic serious adverse event in telaprevir-containing regimens.[13,14]

Accordingly, the present study determined the risk factors for severe dermatological reactions in patients receiving telaprevir, PEG IFN and RBV combination therapy as well as the association between serum levels of granulysin and severe dermatological reactions.

METHODS

Patients and methods

IN THIS RETROSPECTIVE case–control study, at Hokkaido University Hospital and associated hospitals in the NORTE Study Group, between December 2011 and November 2013, a total of 123 patients positive for HCV genotype 1 with high serum HCV RNA titer (>5 log IU/mL) received PEG IFN, RBV and telaprevir combination therapy. Patients were excluded if they required hemodialysis or had a positive test result for serum hepatitis B surface antigen, co-infection with other HCV genotypes or HIV, evidence of autoimmune hepatitis or alcoholic hepatitis, or malignancy. Serum granulysin levels were analyzed in five healthy volunteers with no HCV, HIV or hepatitis B virus infection or any inflammatory diseases.

Written informed consent according to the process approved by the hospital's ethics committee was obtained from each patient. The study protocol conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the ethics committee of each participating hospital.

Study design and treatment regimen

Telaprevir 500 or 750 mg was typically administrated every 8 h after meals for 12 weeks. PEG IFN-α-2b (Peg-Intron; MSD, Tokyo, Japan) 1.5 IU/kg was administrated s.c. once per week for 24 weeks. RBV (Rebetol; MSD) was administrated for 24 weeks in two divided daily doses according to bodyweight: 600, 800 and 1000 mg for patients with bodyweights of less than 60, 60–80 and more than 80 kg, respectively. The doses of PEG IFN-α-2b, RBV and telaprevir were reduced at the attending physician's discretion on the basis of hemoglobin levels, decreased white blood cell or platelet counts, or adverse events.

During treatment, patients were assessed as outpatients at weeks 1, 2, 4, 6 and 8, and then every 4 weeks thereafter for the duration of treatment. Physical examinations and blood tests were performed at all time points.

Outcomes

The primary end-point was SVR, which was defined as undetectable serum HCV RNA at 24 weeks after the end of treatment. The secondary end-points were end-of-treatment virological responses (HCV RNA undetectable in serum) and rapid virological response (RVR), which was defined as undetectable serum HCV RNA at 4 weeks after the start of treatment. Dermatological reactions were classified according to severity in the same manner as in phase III trials in Japan.[10]

Serum granulysin measurement

To evaluate serum granulysin levels in chronic hepatitis C, we first measured serum granulysin levels in five

healthy volunteers and compared them with those of 20 chronic hepatitis C patients before treatment. Serum granulysin levels were measured at the onset of dermatological reactions (within 3 days of onset); if the symptoms worsened, the time when worsening occurred was adopted. Meanwhile, in patients with no dermatological reactions, the highest serum granulysin level during treatment was adopted.

Serum granulysin levels were measured by a sandwich enzyme-linked immunosorbent assay as described previously.[12,14,15] Briefly, plates coated with 5 mg/mL mouse antibody against human granulysin, RB1 antibody, were washed with phosphate-buffered saline containing 0.1% Tween-20. Next, they were blocked with 10% fetal bovine serum in washing buffer at room temperature for 2 h. The samples and standards (Recombinant Granulysin; R&D Systems, Minneapolis, MN, USA) were incubated for 2 h at room temperature. Next, they were reacted with 0.1 mg/mL biotinylated mouse antibody against human granulysin, RC8 antibody. The plates were subsequently treated with horseradish peroxidase-conjugated streptavidin (Roche Diagnostics, Basel, Switzerland). The plates were then incubated with tetramethyl-benzidine substrate (Sigma, St Louis, MO, USA), and 1 M sulfuric acid was then added. The optical density was measured at 450 nm using a microplate reader.

Diagnosis of dermatological reactions

Dermatological reactions were investigated throughout the 24-week administration period in the telaprevir-based combination therapy. Dermatological reactions were classified according to severity as follows. Grade 1 was defined as involvement of less than 50% of the body surface and no evidence of systemic symptoms. Grade 2 was defined as involvement of less than 50% of the body surface but with multiple or diffuse lesions or rashes with characteristic mild systemic symptoms or mucous membrane involvement with no ulceration/erosion. Grade 3 was defined as a generalized rash involving 50% or more of the body surface or a rash with any new significant systemic symptoms and considered to be related to the onset and/or progression of the rash. Life-threatening reactions included SJS, TEN, drug rash with eosinophilia and systemic symptoms (DRESS)/DIHS, erythema multiforme and other life-threatening symptoms, or patients presenting with features of serious disease.

When adverse skin reactions were detected, the attending physician classified the degree of severity and referred the patients to a dermatologist as needed. In principal, when grade 3 dermatological reactions occurred, the attending physician referred the patient to a dermatologist and discontinued telaprevir. When severe dermatological reactions including SJS/TEN and DRESS/DIHS were suspected, all drugs were discontinued immediately. SJS/TEN and DIHS were diagnosed by skin biopsy and according to disease criteria, respectively.

Statistical analysis

Categorical and continuous variables were analyzed by the χ^2-test and the unpaired Mann–Whitney *U*-test, respectively. All *P*-values were two-tailed, and the level of significance was set at $P < 0.05$. Multivariate logistic regression analysis with stepwise forward selection included variables showing $P < 0.05$ in univariate analyses.

The association between dermatological reactions and serum granulysin levels were evaluated by one-way ANOVA followed by Tukey's honestly significant difference test. All statistical analyses were performed using SPSS version 21.0 (IBM Japan, Tokyo, Japan).

RESULTS

Patients

WE INCLUDED 123 chronic hepatitis C patients who received telaprevir-based triple therapy. Of these, 89 patients who had proper information of dermatological adverse events were included. The baseline characteristics of patients are shown in Table 1.

Of these 89 patients, time-dependent changes of serum granulysin concentrations were measured in 20 who had had conserved serum, at least, at the pretreatment point, 1 and 2 weeks after commencement of therapy, 1 and 2 months after commencement of therapy, the onset point of dermatological adverse reaction and the worsening point if symptoms became worse.

Among the 89 patients, 64% (57/89) developed dermatological reactions, including nine with grade 3 reactions (Table 2). The characteristics of dermatological reactions by grade are shown in Table 2. Non-grade 3 dermatological reactions tended to occur early during treatment compared to grade 3 dermatological reactions.

Association between dermatological reactions and treatment outcomes

First, we determined whether dermatological reactions were associated with final treatment outcomes.

Table 1 Baseline characteristics of the participating patients

Total number	89
HCV genotype 1b (1b/others)	89/0
Age (years)†	60.0 (19–73)
Sex (male/female)	48/41
Bodyweight (kg)†	63.0 (32–97)
Baseline white blood cell count (/μL)†	4800 (1500–9800)
Baseline hemoglobin level (g/dL)†	13.5 (9.9–16.7)
Baseline platelet count ($\times 10^3$)†	15.9 (6.6–86)
Baseline ALT level (IU/L)†	40 (15–300)
Baseline HCV RNA level ($\log^{10}$ IU/mL)†	6.5 (3.2–7.6)
Initial telaprevir dose (1500/2250 mg)	20/89
Initial PEG IFN dose (1.5/<1.5 μg/kg)	775/14
Initial RBV dose (mg/kg)†	9.8 (2.2–15.5)
IL28B gene (rs8099917) (TT/non-TT/ ND)	51/22/16
HCV 70 core mutation (wild/mutant/ND)	43/24/22
Previous treatment (naïve/relapse/NVR)	40/38/11

†Data are shown as median (range) values.
ALT, alanine transaminase; HCV, hepatitis C virus; IL28B, interleukin 28B; ND, not done; PEG IFN, pegylated interferon; RBV, ribavirin.

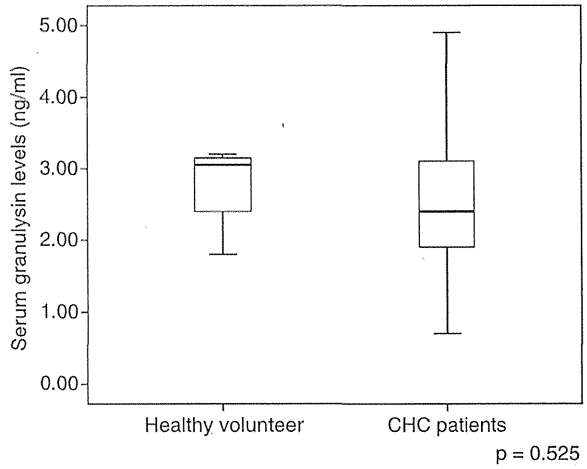

Figure 1 Serum granulysin levels of healthy volunteers and chronic hepatitis C patients. Serum granulysin levels were compared between five healthy volunteers and untreated 20 chronic hepatitis C patients. $P < 0.05$, Mann–Whitney *U*-test.

Univariate analyses identified baseline white blood cell and platelet counts, RVR, and non-grade 3 dermatological reactions significantly associated with SVR (Table 3). Among the nine patients with grade 3 dermatological reactions, three discontinued all treatment and six discontinued telaprevir administration; SVR was achieved in zero of the three (0%) and two of the six (33%), respectively.

Multivariate analysis showed that RVR and non-grade 3 dermatological reactions were significantly associated with SVR (Table 3).

Analysis of risk factors for telaprevir-induced dermatological reactions

Next, we analyzed the association between severe (i.e. grade 3) dermatological reactions and clinical parameters (Table 4). Univariate analysis showed that only sex was significantly associated with the grade 3 dermatological reactions ($P = 0.03$).

Serum granulysin levels in healthy subjects and chronic hepatitis C patients

As shown in Figure 1, serum granulysin levels did not differ significantly between healthy volunteers and chronic hepatitis C patients. Next, we evaluated the association between the severity of dermatological reactions and serum peak granulysin levels in 20 patients including five, four, five and six with grades 1, 2 and 3, and no dermatological events, respectively. One-way ANOVA showed that serum granulysin level was significantly associated with the severity of dermatological reactions ($P = 0.036$); in addition, Tukey's honestly significant difference test revealed that the serum

Table 2 Characteristics of the patients with each dermatological adverse event grade

	n	Age†	Sex (male/female)	Initial telaprevir dose (2250/1500)	Onset of DAR (days)
No DAR	32	61 (28–72)	15/17	26/6	
Grade 1	32	58 (19–73)	15/17	24/8	7 (3–50)
Grade 2	16	61 (44–73)	10/6	12/4	3.5 (1–56)
Grade 3	9	61 (48–65)	8/1	8/1	22 (1–60)

†Data are shown as median range) values.
DAR, dermatological adverse reaction

Table 3 Comparison of the clinical and laboratory characteristics of the patients with HCV infection based on therapeutic response

All patients	SVR	Non-SVR	Univariate analysis	Multivariate analysis		
$n = 89$	$n = 68$	$n = 21$	*P*	OR	95% CI	*P*
Age (years)†	60 (19–73)	62 (28–73)	0.402			
Sex (male/female)	37/31	11/10	0.870			
Bodyweight (kg)†	62 (39–97)	64 (32–87)	0.761			
Baseline white blood cells (/μL)†	5135 (1500–9800)	4200 (2490–7200)	0.048	0.492	(0.121–1.993)	0.320
Baseline hemoglobin level (g/dL)†	13.5 (10.5–16.7)	12.1 (9.9–15.4)	0.862			
Baseline platelet count ($\times 10^3$)†	16.7 (6.6–31.5)	12.8 (7.2–86)	0.025	0.388	(0.093–1.614)	0.193
Baseline ALT level (IU/L)†	37 (15–300)	53 (23–159)	0.070			
Baseline HCV RNA level ($\log^{10}$ IU/mL)†	6.7 (3.2–7.6)	6.4 (5.7–7.3)	0.812			
Baseline Cr level (mg/dL)	0.7 (0.5–1.3)	0.7 (0.5–0.9)	0.433			
Initial telaprevir dose (1500/2250 mg)	52/16	17/4	0.460			
Initial PEG IFN dose (1.5/<1.5 μg/kg)	58/10	17/4	0.430			
Initial RBV dose (mg/kg)†	9.9 (2.2–15.5)	9.5 (4.4–12.5)	0.546			
IL28B gene (rs8099917) (TT/non-TT/ND)	43/15/10	8/7/6	0.107			
Core 70 a.a. mutation (wild/mutant/ND)	36/16/16	7/8/6	0.108			
Previous treatment (naive/relapse/NVR)	34/28/6	6/10/5	0.095			
Rapid virological response (+/−)	60/8	10/11	<0.001	10.89	(2.838–41.83)	0.001
Grade 3 DAR (−/+)	66/2	14/7	<0.001	27.44	(3.718–202.5)	0.001

†Data are shown as median (range) values.
a.a., amino acid; ALT, alanine transaminase; CI, confidence interval; Cr, creatinine; DAR, dermatological adverse reaction; HCV, hepatitis C virus; IL28B, interleukin 28B; ND, not done; NVR, non-virological response; OR, odds ratio; PEG IFN, pegylated interferon; SVR, sustained virological response; RBV, ribavirin.

granulysin levels of patients with grade 3 dermatological reactions were significantly higher than those of patients with grade 1 or no dermatological reactions (both $P < 0.05$, Fig. 2).

Time-dependent changes in serum granulysin levels

We investigated the time-dependent changes in serum granulysin levels in five and 15 patients with grade 3 and non-grade 3 dermatological reactions, respectively (Fig. 3). Serum granulysin levels of patients with non-grade 3 dermatological reactions never exceeded 10 ng/ml. Of the five patients with grade 3 reactions, three had severe systemic manifestations that necessitated hospital admission: one each had SJS, DIHS, and systemic lymphoid swelling and high fever (>39 °C). All patients with grade 3 dermatological reactions with systemic manifestations had peak serum granulysin levels exceeding 10 ng/mL; importantly, the serum granulysin levels of two patients already exceeding 8 ng/mL at the onset of the reactions worsened within 6 days.

DISCUSSION

THE PRESENT STUDY demonstrates a significant association between telaprevir-induced dermatological reactions and elevated serum granulysin levels for the first time. Moreover, serum granulysin levels were significantly associated with the severity of dermatological reactions. Thus, the results indicate that serum granulysin level seems to be a useful predictor of telaprevir-induced dermatological reactions. Because the emergence of grade 3 dermatological reactions was significantly associated with non-SVR (Table 3), probably associated with high rate of treatment discontinuation, it is important to predict dermatological events in the early stage to achieve good treatment outcomes.

Table 4 Comparison of the clinical and laboratory characteristics of the patients based on the presence or absence of at least a grade 3 dermatological adverse event

All patients $n = 89$	Non-grade 3 $n = 80$	Grade ≥3 $n = 9$	Univariate analysis P
Age (years)†	60 (19–73)	61 (48–65)	0.453
Sex (male/female)	40/40	8/1	0.027
Bodyweight (kg)†	62 (32–97)	64 (51–87)	0.593
Baseline white blood cell count (/μL)†	4900 (1500–9800)	4700 (3000–7000)	0.876
Baseline hemoglobin level (g/dL)†	13.5 (9.9–16.7)	14.4 (12.1–15.4)	0.196
Baseline platelet count ($\times 10^3$)†	16.0 (6.6–86.0)	13.5 (10.4–22.5)	0.605
Baseline ALT level (IU/L)†	40(15–300)	37 (23–87)	0.765
Baseline Cr level (mg/dL)	0.7 (0.5–1.3)	0.8 (0.6–0.9)	0.123
Baseline HCV RNA level ($\log^{10}$ IU/mL)†	6.6 (3.2–7.6)	6.4 (5.7–7.1)	0.465
Initial telaprevir dose (1500/2250 mg)	62/18	7/2	0.675
Initial telaprevir/bodyweight (mg/kg)	33.7 (20–71.4)	30.0 (23.6–44.1)	0.563
Initial PEG IFN dose (1.5/<1.5 μg/kg)	66/14	9/0	0.198
Initial RBV dose (mg/kg)†	9.7 (2.2–15.5)	10.7 (7.7–12.9)	0.161
IL28B gene (rs8099917) (TT/non-TT/ND)	47/19/14	4/3/2	0.353
Core 70 a.a. mutation (wild/mutant/ND)	38/22/20	5/2/2	0.511
Previous treatment (naïve/relapse/NVR)	35/36/9	5/2/2	0.972
Onset of dermatological AE (days)	5 (1–75)	22 (1–60)	0.352

†Data are shown as median (range) values.
a.a., amino acid; AE, adverse event; ALT, alanine transaminase; Cr, creatinine; HCV, hepatitis C virus; IL28B, interleukin 28B; NVR, non-virological response; PEG IFN, pegylated interferon; RBV, ribavirin.

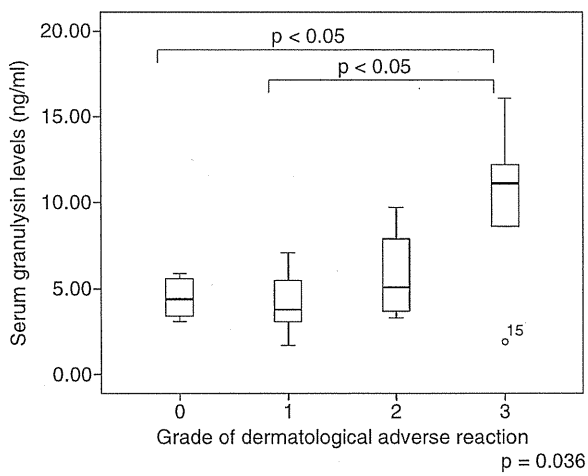

Figure 2 Association between dermatological adverse reaction severity and serum granulysin level. Serum granulysin levels were measured at the onset of dermatological reactions (i.e. within 3 days of onset); if the symptoms worsened, the time of worsening was adopted. In patients with no dermatological events, the highest serum granulysin level during treatment was adopted. $P < 0.05$, one-way ANOVA.

Recent genome-wide association studies have identified that genetic polymorphisms around the IL28B gene locus significantly associated with the outcome of PEG IFN and RBV combination therapy in HCV patients. Thus, PEG IFN and RBV combination therapy is ineffective in a subset of HCV-infected patients who have IL28B TG or GG genotypes, limiting the use of this therapy.[16] Therefore, novel drugs with different antiviral mechanisms were required. Accordingly, DAA were developed; they are mainly classified as NS3/4A protease inhibitors, or NS5B or NS5A inhibitors.[17] The NS3/4A serine protease inhibitor telaprevir, in combination with PEG IFN and RBV, has demonstrated the most promising results.[6–8] However, adverse events, especially severe dermatological reactions, develop more frequently in patients treated with telaprevir than those treated with only PEG IFN and RBV.

Little is known about the mechanisms of telaprevir-induced dermatological reactions. Reactions develop in patients treated with PEG IFN and RBV combination therapy[18,19] as well as telaprevir monotherapy.[20,21] It should be noted that the dermatological reactions in telaprevir monotherapy or PEG IFN and RBV therapy alone are generally mild.[7,8,20] However, dermatological

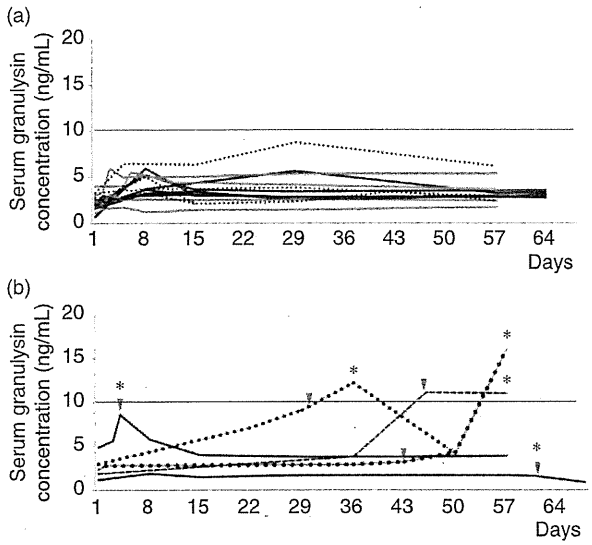

Figure 3 Association between time-dependent changes in serum granulysin levels and severe telaprevir-induced dermatological adverse reactions. (a) Time-dependent changes in serum granulysin levels patients with non-grade 3 dermatological reactions (three, five and six with grade 2, grade 1 and no reactions, respectively). The dashed line, gray line and black line indicate grade 2, grade 1 and no reaction, respectively. (b) Time-dependent changes in serum granulysin levels of five patients with grade 3 dermatological events. The dashed line indicates patients with severe systemic manifestations. Arrowheads indicate the onset of dermatological events and asterisks indicate the onset of grade 3 dermatological events.

reactions in telaprevir and PEG IFN/RBV combination therapy may be severe, indicating a synergistic effect. Severe dermatological events including SJS/TEN and DIHS have been reported in telaprevir-based triple therapy; these are life-threatening, and fatal cases have been reported.

The onset of grade 3 dermatological reactions tended to be later than non-grade 3 reactions, the same as in the study of Torii *et al.*[10] Taken together with the finding that male sex is a clinical risk factor, the results indicate that late-onset dermatological reactions in male patients treated with telaprevir-based triple therapy require more attention.

Roujeau *et al.* analyzed the risk factors for telaprevir-induced eczematous dermatitis and report that the incidence of telaprevir-related dermatitis was significantly higher age of more than 45 years, body mass index of less than 30 (kg/m^2), Caucasian ethnicity and treatment-naïve status.[9] While they analyzed the risk factors for telaprevir-induced eczematous dermatitis, the present study focused on the risk factors for severe telaprevir-induced dermatological reactions, because such reactions can affect treatment outcome (Table 2) and can be fatal. As mentioned above, male sex was significantly associated with grade 3 dermatological reactions. Sex is reported to be associated with the prevalence of some kinds of severe drug-induced dermatological events, although the underlying mechanism remains unknown.[22]

Fujita *et al.* report that serum granulysin levels are significantly elevated in SJS/TEN patients and thus may be a good predictive factor.[14] Therefore, we hypothesized that in telaprevir-based triple therapy for chronic hepatitis C patients, serum granulysin levels are associated with the severity of dermatological reactions and may thus be a predictive biomarker. However, Ogawa *et al.* report that serum granulysin levels also increase as a result of primary virus infections such as Epstein–Barr virus or parvovirus B19.[12] Thus, it remains unclear whether and how chronic viral infections, especially HCV, affect serum granulysin levels. In the present study, we compared serum granulysin levels between healthy volunteers and chronic hepatitis C patients; the results show that chronic HCV infection was not associated with serum granulysin levels (Fig. 1).

Chung *et al.* have reported that granulysin is the most highly expressed cytotoxic molecule in blisters of SJS/TEN and that massive keratinocyte death was induced by granulysin.[11] Fujita *et al.* reported that serum granulysin levels increased in the early stage of SJS/TEN caused by drugs including carbamazepine, imatinib and phenytoin.[14] Taken together with our results, we speculate that granulysin may be involved in the pathogenesis of early stage telaprevir-mediated dermatological adverse reactions possibly through induction of keratinocyte death.

Of five patients with grade 3 reactions, two patients without severe systemic manifestations did not have elevated serum granulysin of more than 10 ng/mL or did not have elevated levels before symptoms worsened. On the contrary, three patients with severe systemic manifestations had peak serum granulysin levels exceeding 10 ng/mL, and the symptoms of two patients with serum granulysin levels already exceeding 8 ng/mL at onset and within 6 days worsened. Therefore, serum granulysin tests may predict grade 3 dermatological adverse reaction with systemic manifestations. Furthermore, if serum granulysin levels elevate more than 8 ng/mL, more attention should be paid.

In Western countries, the prevalence of dermatological reactions in patients treated with telaprevir-based and

PEG IFN/RBV therapy are reported to be approximately 55% and 33%, respectively;[9,23] meanwhile, in Japanese patients, the respective rates are 74.9% and 58.7%. Moreover, approximately 4% and 9% of patients in Western and Japanese patients develop grade 3 reactions, respectively;[10] this is almost the same as that in the present study (10%). The difference may be due to genetic or ethnic variation. Therefore, genome-wide association studies may have identified a gene locus associated with telaprevir-induced severe dermatological reactions.

A limitation of this study is that the number of patients with grade 3 dermatological reactions is relatively small. However, the serum granulysin levels of patients with grade 3 dermatological reactions were significantly higher than those of other patients. Also, in two of the three patients with severe dermatological reactions, the serum granulysin level elevated before symptoms worsened, which are novel findings. Further study is required.

Triple therapy with the second-generation protease inhibitor simeprevir is reported to result in a similar prevalence of adverse reactions as PEG IFN and RBV combination therapy.[24,25] However, simeprevir is not approved worldwide. Although simeprevir-based triple therapy is effective, only 36–53% of prior non-responders achieve SVR.[24] Shimada *et al.* recently reported that by extending PEG IFN and RBV therapy from 24 to 48 weeks, telaprevir-based triple therapy improves the SVR to up to 68% in prior null responders.[26] Thus, telaprevir is a therapeutic option for prior null responders.

In conclusion, the present study suggests that male sex is a significant risk factor for severe telaprevir-induced dermatological reactions. In addition, serum granulysin levels are significantly associated with the severity of dermatological reactions and thus may be a good predictor of severe dermatological reactions with systemic manifestations in patients treated with telaprevir-based triple therapy.

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RESEARCH ARTICLE

Guidelines for the Nomenclature of Genetic Elements in Tunicate Genomes

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Additional Supporting Information may be found in the online version of this article.

The latest version of these guidelines can be downloaded from the Tunicate Portal (http://www.tunicate-portal.org/). To better identify the latest version, the file name for the guidelines should follow the following syntax: Genetic_Guidelines_Tunicate_[year]_[month]_[day] (example, Genetic_Guidelines_Tunicate_2014_05_01).

International Code for Zoological nomenclature. http://iczn.org/code.

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Summary: Tunicates are invertebrate members of the chordate phylum, and are considered to be the sister group of vertebrates. Tunicates are composed of ascidians, thaliaceans, and appendicularians. With the advent of inexpensive high-throughput sequencing, the number of sequenced tunicate genomes is expected to rise sharply within the coming years. To facilitate comparative genomics within the tunicates, and between tunicates and vertebrates, standardized rules for the nomenclature of tunicate genetic elements need to be established. Here we propose a set of nomenclature rules, consensual within the community, for predicted genes, pseudogenes, transcripts, operons, transcriptional *cis*-regulatory regions, transposable elements, and transgenic constructs. In addition, the document proposes guidelines for naming transgenic and mutant lines. genesis 53:1–14, 2015. © 2014 Wiley Periodicals, Inc.

Key words: tunicates; genome annotation; gene; transposable element; *cis*-regulatory sequences

INTRODUCTION

Affordable high-throughput sequencing is leading to a paradigm shift in evolutionary developmental biology (aka, Evo-Devo) as an increasing number of near-complete genome sequences will soon be available for most taxa, including the tunicates. The genomes of two solitary species of the genus *Ciona*, *Ciona intestinalis* (Dehal *et al.*, 2002) and *Ciona savignyi* (Small *et al.*, 2007), were sequenced in the early 2000s, followed by the genome of the appendicularian *Oikopleura dioica* (Denoeud *et al.*, 2010). The first genome of a colonial ascidian, *Botryllus schlosseri*, was released in 2013 (Voskoboynik *et al.*, 2013), and the genomes of at least seven more species belonging to the genera *Phallusia*, *Halocynthia*, and *Molgula* (Stolfi *et al.*, 2014) are currently being sequenced and annotated. Comparison of these genomes to each other, and to the lancelet (*Amphioxus*) and vertebrate genomes, will shed light on the last common ancestor of tunicates and vertebrates, and help explain how ascidians could retain a particularly conserved embryonic development in spite of rapid genome divergence (Lemaire, 2011; Lemaire *et al.*, 2008).

The definition of precise naming rules for genetic elements, which would greatly facilitate tunicate comparative genomics, has so far not been attempted. This article proposes uniform guidelines for ascidians, thaliaceans, and appendicularian genetic elements. A central concern when designing these rules is that orthologous features across the subphylum receive the same name, and that this name is chosen, when possible, to reflect the orthology to features in the human genome, the most completely sequenced and annotated chordate genome.

Computational analyses are taking center stage in biology and a second concern was to make sure that the nomenclature rules would be compatible with the efficient parsing of large files, while remaining understandable to the bench biologist. This involved the avoidance of symbols and identifiers of characters that could be interpreted as separators in tabulated files. We also made sure that alphabetical or numerical symbols and identifiers have a constant syntax and number of characters.

The following sections will first define species abbreviations, then rules for coding and non-coding genetic features, before closing on the nomenclature for transgenic elements and lines of transgenic or mutant animals.

SPECIES SYMBOLS

Comparative analyses frequently necessitate distinguishing between orthologous elements in different species. In such cases, an abbreviated species symbol is used as a prefix to the feature name.

A species is identified by a binomial name composed of a generic epithet and a specific epithet, written in italics (International Code for Zoological nomenclature). The first epithet describes the genus, and its first letter is capitalized (e.g., *Ciona*). The second epithet describes the particular species within the genus and is written in lower case italics (e.g., *intestinalis*). A third epithet, usually written in lower case italics, can be added to describe subspecies, when applicable.

Such binomial species names can be abbreviated and used as prefix or suffix in the names of genetic elements. The following abbreviation rules are proposed, which were tested on the 3,018 Tunicate species listed in the World Registry of Marine Species (WoRMS) database at the time of writing. Current validated abbreviations are listed in the Supporting Information Table 1 and deposited with the WoRMS. Novel abbreviations should be registered with WoRMS.

A species symbol is a binomial abbreviation consisting of six letters. In most cases, the syntax is "2G4S." The first two letters are an abbreviation of the generic epithet (G), and the final four letters are an abbreviation of the specific epithet (S). In some cases, explained below, three letters are used to abbreviate generic epithets, in which case the binomial abbreviation is 3G3S.

Abbreviations of generic epithets should not be ambiguous. When possible, two-letter abbreviations should be used, built from the first two letters of the generic name (e.g., Py for *Pyura*). In case of ambiguity, (e.g., *Pycnoclavella* vs. *Pyura*), one of the conflicting genera receives an unambiguous abbreviation built from the first letter of the generic name, followed by a