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MAJOR ARTICLE

Pre-existence and Persistence of Resistant Minority Hepatitis C Virus Variants in Genotype 1-Infected Patients Treated With Simeprevir/Peginterferon/Ribavirin

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Background. The pre-existence of minority hepatitis C virus (HCV) variants and their impact on treatment outcome, as well as the persistence of emerging resistant variants posttreatment in patients failing treatment with simeprevir/peginterferon/ribavirin (SMV/PR), were assessed by deep sequencing (DS).

Methods. Population sequencing (PS) and Illumina DS were performed on HCV genotype 1 isolates from patients treated with SMV/PR in Phase 2b (PILLAR [NCT00882908] and ASPIRE [NCT00980330]) and Phase 3 (QUEST-1 [NCT01289782], QUEST-2 [NCT01290679], and PROMISE [NCT01281839]) trials.

Results. Minority polymorphisms (ie, detected pretreatment by DS only) reducing SMV activity in vitro were uncommon (3.6%, 19 of 534 patients). These SMV-resistant minority polymorphisms were detected in similar proportions of patients achieving (3.7%) and not achieving (3.3%) sustained virologic response with SMV/PR and generally did not emerge as major variants at time of failure. SMV-resistant variants emerging at time of failure were no longer detected at end of study in 69.3% and 52.0% of the patients by PS and DS, respectively.

Conclusions. Minority polymorphisms did not impact outcome of SMV/PR treatment. The majority of emerging variants that became undetectable at end of study by PS were also undetectable by DS. These results suggest no added value of DS for clinical usage of SMV.

Keywords. deep sequencing; HCV; minority (viral) variants; resistance; simeprevir.

Simeprevir ([SMV] TMC435) is a once-daily hepatitis C virus (HCV) NS3/4A protease inhibitor approved with peginterferon/ribavirin (PegIFN/RBV) for chronic HCV genotype 1 infection in the United States and genotype 1 and genotype 4 infection in the European Union (EU). SMV is also approved as part of an IFN-free combination with sofosbuvir for HCV genotype 1 infection in the United States and genotype 1 and genotype 4 infection in the EU. SMV with PegIFN/RBV has been shown to significantly increase sustained virologic response (SVR) rates and enable a shorter treatment duration, ie, 24-week overall, compared with PegIFN/RBV alone [1–5].

Resistance analyses in clinical trials are typically performed by standard population sequencing using the Sanger technique, which can detect viral variants with a sensitivity of approximately 20%–25%. In the SMV Phase 2b/3 trials, as assessed by standard population sequencing, pre-existing baseline polymorphisms associated with reduced SMV activity in vitro were generally uncommon (1.3%) among HCV genotype 1-infected patients, with the exception of the NS3 Q80K polymorphism. The prevalence of Q80K pretreatment was 14% in the overall trial population and 30% in HCV genotype 1a-infected patients, with the efficacy of SMV plus PegIFN/RBV substantially reduced in this latter group of patients [6, 7]. Given the quasi-species nature of HCV, resistant minority viral variants might pre-exist at a frequency undetectable by population sequencing and may influence treatment outcome.

Most patients (91%) treated with SMV/PegIFN/RBV and not achieving SVR in the SMV Phase 2b/3 trials carried emerging viral variants with mutations at NS3 positions 80, 122, 155, and/or 168 at time of failure; mostly R155K in genotype 1a and D168V in genotype 1b. In half of the patients with emerging mutations at time of failure, these variants were no longer detected by population sequencing within a median follow-up time of 28 weeks [7]. However, these resistant viral variants might remain enriched in the viral quasi-species population at levels undetectable by population sequencing, potentially limiting future treatment options.

Next-generation sequencing technologies enable detection of viral variants at a higher sensitivity than that of standard

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population sequencing [8–10]. In this study, Illumina deep sequencing was performed retrospectively on HCV clinical isolates obtained from patients treated with SMV plus PegIFN/RBV in Phase 2b (PILLAR and ASPIRE) and Phase 3 (QUEST-1, QUEST-2, and PROMISE) clinical trials [1–5]. The objective of the analyses was to identify pre-existing minority viral variants associated with SMV in vitro resistance, which are not detected by population sequencing, and to assess their impact on treatment outcome. In addition, the posttreatment persistence of viral variants that emerged in patients failing treatment with SMV/PegIFN/RBV was assessed.

METHODS

Samples

Illumina deep sequencing data were generated for a total of 1058 plasma isolates with HCV ribonucleic acid (RNA) ≥ 10 000 IU/mL collected from 543 HCV genotype 1-infected patients: 308 genotype 1a and 235 genotype 1b, treated with SMV and PegIFN/RBV in the PILLAR (NCT00882908) and ASPIRE (NCT00980330) Phase 2b and the QUEST-1 (NCT01289782), QUEST-2 (NCT01290679), and PROMISE (NCT01281839) Phase 3 clinical studies (Supplementary Table 1) [1–5]. All patients were naive to treatment with HCV protease inhibitors, and the majority (62.8%; 341 of 543) were naive to prior PegIFN/RBV therapy. Treatment-experienced patients included relapsers (11.2%; 61 of 543) and partial (11.8%; 64 of 543) and null responders (14.2%; 77 of 543) to prior PegIFN/RBV therapy.

All studies were conducted in full compliance with the Declaration of Helsinki and Good Clinical Practice guidelines. All patients provided written, informed consent before participating in any study-related activity.

Study Assessments

HCV geno/subtype were determined pretreatment by sequencing a 329-base pair region within NS5B followed by basic local alignment search tool (BLAST) analysis. The results of the NS5B-based assay or, if missing, the results from the VERSANT HCV Genotype 2.0 Assay (LiPA) (Siemens Healthcare Diagnostics, Erlangen, Germany) or TRUGENE assay (Bayer HealthCare, Montville, NJ) were used.

HCV NS3/4A population sequencing using the conventional Sanger technique, as previously described, was performed pretreatment for all patients and postbaseline for patients treated with SMV/PegIFN/RBV who did not achieve SVR for any reason [7].

In vitro activity of SMV was assessed using genotype 1a or 1b replicons carrying site-directed mutants in a transient replicon assay, and cutoff values were used to differentiate between full susceptibility to SMV (≤2.0-fold reduction in SMV activity) and low-level versus high-level resistance (≥50-fold reduction in SMV activity) [6, 11].

Resistance analyses considered 18 NS3 amino acid positions (36, 41, 43, 54, 55, 80, 107, 122, 132, 138, 155, 156, 158, 168, 169, 170, 174, and 175) associated with resistance to SMV or other HCV NS3/4A protease inhibitors, or that were considered to be of interest based on in vitro or in vivo observations in studies with SMV [7].

HCV NS3/4A Deep Sequencing

Illumina deep sequencing was performed on amplicons encompassing HCV NS3/4A as described earlier [9, 12]. Using Illumina technology, viral variants can be reliably detected with a sensitivity of approximately 1% [9, 12].

In brief, RNA was isolated from plasma and reverse transcribed using random hexamer primers, followed by a subtype-specific polymerase chain reaction. After fragmentation of amplicons and ligation of sequencing adaptors, the barcoded isolates were pooled at equimolar amounts and loaded on a Genome Analyzer IIx (Illumina, San Diego, CA) running 147 cycles of paired-end sequencing. Obtained images were analyzed and base-called using Genome Analyzer IIx pipeline software, version 1.8 (Illumina, San Diego, CA).

Consensus mapping of the individual sequence reads per sample was performed using the CLCBio Workbench software (QIAGEN, Hilden, Germany). The relative frequencies of codon variants versus the respective H77 (GenBank accession number AF009606; HCV genotype 1a) or Con1 (GenBank accession number AJ238799; HCV genotype 1b) references were calculated per amino acid position. Additional quality value-based filtering was performed, and only the lowest frequency, either observed in the forward or reverse sequencing direction, was reported as described earlier [9]. For the current analyses, minority variants were defined as those detected by deep sequencing only, at a read frequency ≥1%.

RESULTS

Amino Acid Substitutions Detected by Population Sequencing and/or Deep Sequencing

A total of 1058 isolates from 543 HCV genotype 1-infected patients were analyzed by Illumina deep sequencing, with an average read coverage of 28 533 reads per amino acid position. This implies that an amino acid substitution present in viral variants at a frequency of 1% was observed, on average, in 286 independent reads.

Most (99.0%) amino acid substitutions at the NS3 positions of interest previously detected by population sequencing were also observed by deep sequencing, with 94.6% of those at a read frequency ≥25%. Of the amino acid substitutions detected by deep sequencing only, 93.0% had a read frequency <25% (Supplementary Table 2).

Prevalence of Pre-existing Minority SMV-Resistant Baseline Polymorphisms

Pretreatment isolates from 534 patients treated with SMV plus PegIFN/RBV and with HCV NS3 population sequencing data

available in the Phase 2b/3 clinical trials were retrospectively analyzed using Illumina deep sequencing, to determine the frequency of minority NS3 polymorphisms (ie, amino acid substitutions present pretreatment in viral variants at the 18 NS3 positions of interest and detected by deep sequencing only). These minority NS3 polymorphisms were observed in 150 patients (28.1%), overall, and in 31.1% and 24.0% of the genotype 1a and 1b patients, respectively (Figure 1).

Minority NS3 polymorphisms reducing SMV activity in vitro (ie, SMV fold change in 50% effective concentration compared with wild-type HCV [SMV FC], assessed as site-directed mutant in transient replicon assay, >2.0) [11] were observed in 19 patients (3.6%) overall. In 7 of these 19 patients (7 of 534; 1.3% overall), ie, in 4 HCV genotype 1a and 3 HCV genotype 1b patients, minority polymorphism Q80K was detected pretreatment by deep sequencing only at a median (range) read frequency of 2.2% (1.5%–88.3%) (Figure 1; Supplementary Figures 1 and 2). Of note, in 1 of the HCV genotype 1b patients, polymorphism Q80K was detected by deep sequencing only at a read frequency of 88.3%, ie, well above the detection limit of population sequencing, which suggests it was missed during population sequencing analysis.

Deep sequencing confirmed the presence of the Q80K polymorphism in an additional 70 patients (13.1%) overall: in 69 HCV genotype 1a patients (22.6%) and 1 HCV genotype 1b patient (0.4%), at a median (range) read frequency of 99.4% (42.6%–99.9%) (Figure 2; Supplementary Figures 1 and 2).

Minority polymorphisms reducing SMV activity in vitro other than Q80K were observed in 12 patients (2.2% overall): Q41R (n = 1); Q80R (n = 3); I132V (n = 1); V132L (n = 1); R155K (n = 1); D168E (n = 1); I or V170T (n = 3); and S174F (n = 1). In addition, deep sequencing confirmed the presence of polymorphisms Q80R (n = 8), V132L (n = 2), R155K (n = 2), and D168E (n = 4) (Figure 2). Of note, with the exception of R155K, these polymorphisms all conferred low-level resistance to SMV in vitro (SMV FC >2 and <50) [11]. The most prevalent minority polymorphisms were observed at NS3 position 122, but none of these polymorphisms reduced SMV activity in vitro (SMV FC ≤2.0) [11].

Pre-existing SMV-Resistant Minority Polymorphisms and Treatment Outcome

To evaluate the impact of pre-existing minority baseline polymorphisms on treatment outcome of a SMV and PegIFN/RBV combination regimen, the presence of minority polymorphisms in patients achieving and not achieving SVR 12 weeks after the end of treatment (SVR12) was assessed.

Similar proportions of patients achieving (30.8%) and not achieving SVR12 (23.9%) had minority polymorphisms

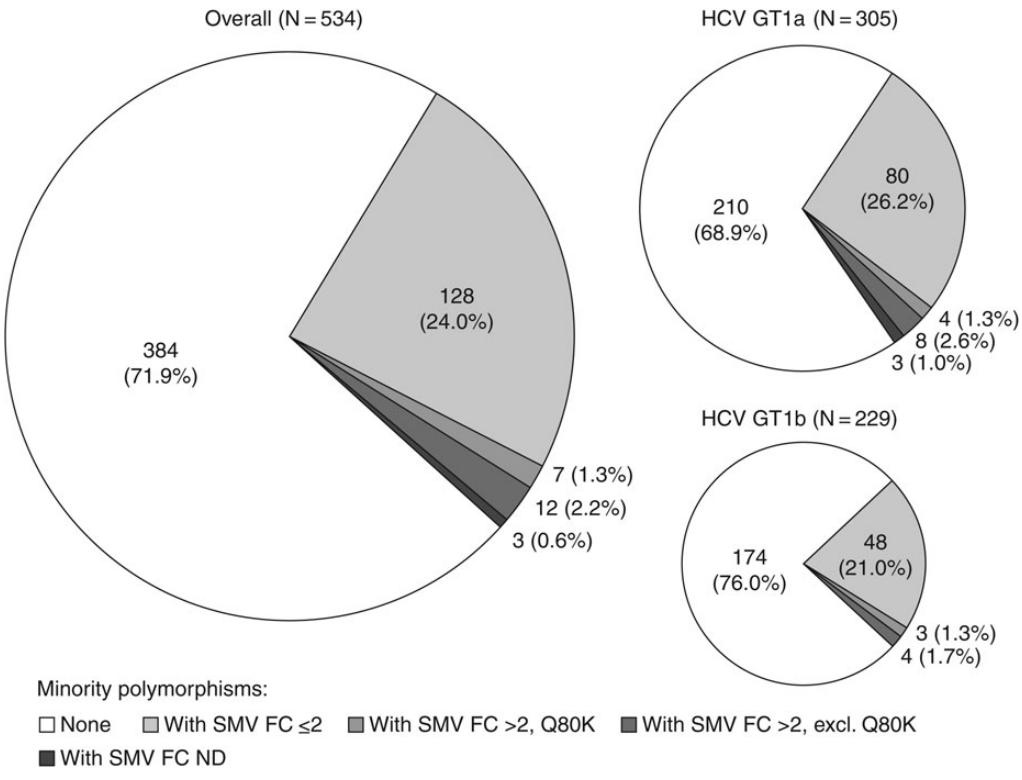

Figure 1. Prevalence of minority NS3 polymorphisms detected pretreatment by deep sequencing; overall and by hepatitis C virus (HCV) geno/subtype. Abbreviations: excl., excluding; FC, fold change in 50% effective concentration in vitro compared with wild-type HCV assessed as site-directed mutant in transient replicon assay; GT, genotype; N, number of patients; ND, not determined; SMV, simeprevir.

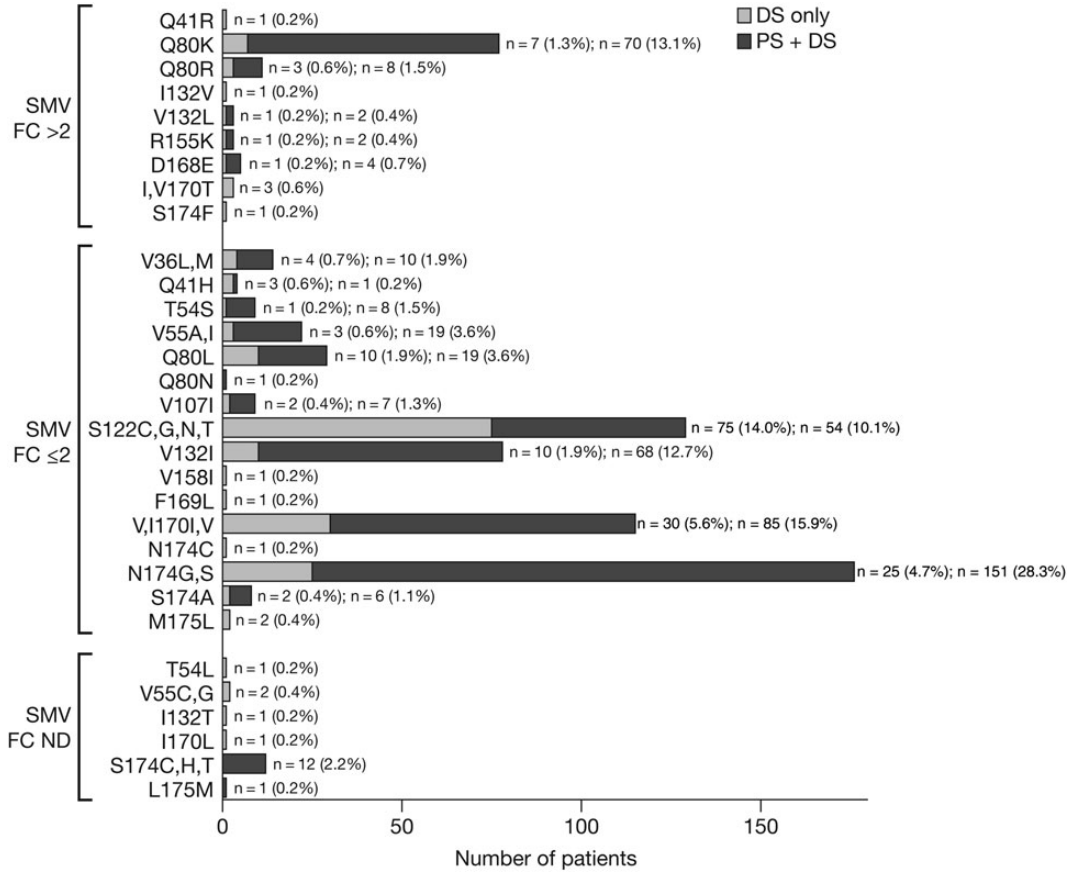

Figure 2. Proportions of patients with NS3 polymorphisms detected pretreatment by population sequencing (PS) and/or deep sequencing (DS) (N = 534). Note that a patient isolate can carry multiple polymorphisms. Abbreviations: FC, fold change in 50% effective concentration in vitro compared with wild-type hepatitis C virus assessed as site-directed mutant in transient replicon assay; n/N, number of patients; SMV, simeprevir.

pretreatment (Figure 3; Supplementary Table 3 for a listing of all 51 patients with minority polymorphisms not achieving SVR12). Furthermore, minority polymorphisms reducing SMV activity in vitro were also detected in similar proportions of patients achieving (3.7%) and not achieving (3.3%) SVR12 (Figure 3; Table 1 for a listing of the 7 patients with minority polymorphisms reducing SMV activity in vitro and not achieving SVR12).

Minority polymorphism Q80K was observed in 5 (1.6%) patients achieving SVR12 and 2 (0.9%) patients not achieving SVR12. These 2 patients included 1 patient (Patient 29; HCV genotype 1b) who discontinued treatment at week 2 for non-virologic reasons and 1 patient (Patient 45; HCV genotype 1a) who experienced viral breakthrough at week 36.

Minority polymorphisms reducing SMV activity in vitro other than Q80K were observed in 7 (2.2%) patients achieving SVR12 and 5 (2.3%) patients not achieving SVR12. Among these latter 5 patients, minority polymorphism Q80R was present in 2 genotype 1a patients (Patients 5 and 23), and I132V, R155K, and Q41R were present in 1 genotype 1a patient each (Patients 30, 36, and 51, respectively). All 5 patients failed for virologic reasons. The 7 patients with minority polymorphisms

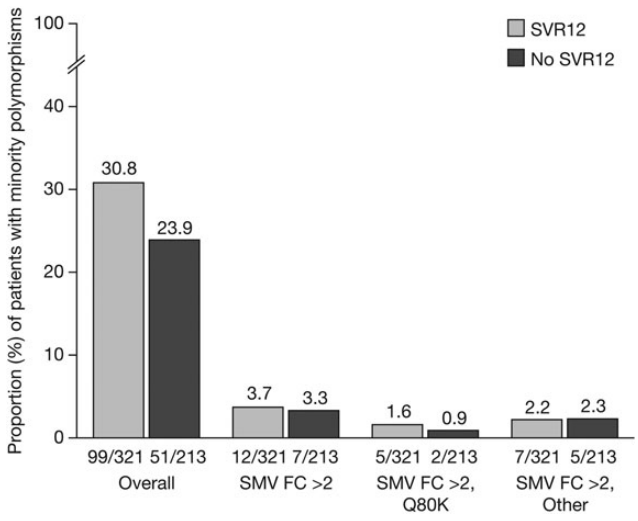

Figure 3. Proportion of patients with NS3 minority polymorphisms by treatment outcome (sustained virologic response 12 weeks after the end of treatment [SVR12]) of a simeprevir (SMV) and peginterferon (PegIFN)/ribavirin (RBV) combination regimen. Abbreviations: FC, fold change in 50% effective concentration in vitro compared with wild-type hepatitis C virus assessed as site-directed mutant in transient replicon assay.

Table 1. Patients Carrying Simeprevir-Resistant Minority Polymorphisms Pretreatment and Not Achieving SVR12 Upon Treatment With a Simeprevir and PegIFN/RBV Combination Regimen[a]

		Pretreatment		Time of Failure	
Pt	GT	Variants by PS	Variants by DS (Read Frequency, %)	Variants by PS	Variants by DS (Read Frequency, %)
5	1a	N174S	**Q80R (1.0%)** + **S122G (2.9%)** + N174S (98.1%)	D168V[b] + N174S	ND
23	1a	N174S	**Q80R (1.1%)** + **S122G (6.9%)** + **N174G (24.8%)** + N174S (68.5%)	V132I/L[b] + R155K[b] + D168E[b] + N174G[b]	ND
29	1b	S122T + V132I + V170I	**Q80K (2.4%)** + S122T (23.2%) + V132I (98.4%) + V170I (99.3%)	ND	ND
30	1a	Q80K	Q80K (99.8%) + **I132V (4.1%)** + **I170V (1.0%)**	Q80K + D168E[b]	ND
36	1a	T54S + V55I	T54S (72.2%) + V55I (58.6%) + R155K (10.2%)	T54S + R155K[b]	ND
45	1a	N174S	**Q80K (1.7%)** + N174S (99.5%)	Q80K[b] + R155K[b] + N174S	ND
51	1a	T54S + V55I + Q80K	**Q41R (1.7%)** + T54S (99.3%) + V55I (99.8%) + Q80K (99.8%)	T54S + V55I + Q80K + D168E[b]	T54S (99.7%) + V55I (99.8%) + Q80K (99.4%) + D168E[b] (99.8%)

Abbreviations: DS, deep sequencing; FC, fold change in 50% effective concentration in vitro compared with wild-type hepatitis C virus assessed as site-directed mutant in transient replicon assay; GT, genotype; ND, not determined; PegIFN, peginterferon; PS, population sequencing; Pt, patient; RBV, ribavirin; SVR12, sustained virologic response 12 weeks after the end of treatment.

[a] Variants detected by DS only (ie, minority variants) are shown in bold. Variants known to reduce simeprevir activity in vitro (simeprevir FC >2) are underlined.

[b] Variants emerging compared with pretreatment population sequence.

who achieved SVR12 carried Q80R (n = 1), V132L (n = 1), D168E (n = 1), I170T (n = 2), V170T (n = 1), and S174F (n = 1).

Pre-existing Minority Polymorphisms and Emergence of Resistance Mutations in Patients Not Achieving Sustained Virologic Response

To further assess whether the minority variants present pretreatment might have contributed to treatment failure, the emergence of these variants as majority at time of failure was assessed. Of the 7 patients not achieving SVR12 with pretreatment minority polymorphisms reducing SMV activity in vitro, 6 had population-sequencing data available at time of failure (Table 1). In 2 of these 6 patients, the SMV-resistant minority variant observed pretreatment was detected as major variant by population sequencing at time of failure: Patient 36 with high-level resistant R155K detected as minority polymorphism pretreatment at a read frequency of 10.2% had emerging R155K as major mutation at time of failure, and Patient 45 with minority Q80K at a read frequency of 1.7% pretreatment had Q80K in combination with R155K (which was not detected pretreatment by deep sequencing) emerging as major mutation at time of failure. None of the SMV-resistant minority polymorphisms detected pretreatment in the other 4 patients were found emerging at time of failure by population sequencing (nor by deep sequencing in the 1 patient with data available at time of failure), but, instead, other high-level SMV-resistant mutations were observed.

In addition, 44 patients who did not achieve SVR12 had minority polymorphisms not reducing or not known to reduce SMV activity in vitro. Population sequencing data were available at time of failure for 42 of these patients (Supplementary Table 3). In 38 of these 42 patients (90.5%), the minority polymorphisms detected pretreatment were not observed as major emerging mutations at time of failure by population sequencing, nor could they be detected by deep sequencing in the 15 patients with additional deep sequencing data available at time of failure. In 4 patients (Patients 6, 14, 38, and 42), the minority polymorphisms observed pretreatment were found emerging at time of failure by population sequencing. All of these patients had additional emerging mutations conferring high-level resistance to SMV (SMV FC ≥50) [11] at time of failure, which were not detected pretreatment, except for Patient 6. This patient had Q80K (SMV FC = 9) detected pretreatment by both population and deep sequencing; minority V36M (SMV FC <2) was detected pretreatment by deep sequencing only at a read frequency 48.5%; and, at time of failure, V36M was observed as single emerging mutation by population sequencing (SMV FC for double mutant Q80K + V36M = 22).

Persistence of Emerging SMV-Resistant Mutations in Patients Not Achieving Sustained Virologic Response

Mutations emerging at time of failure tend to disappear over time and are no longer detected by population sequencing at end of study in a substantial proportion of patients. However, these mutations might remain enriched at levels below the detection limit of population sequencing.

For 127 patients who did not achieve SVR12 and who had emerging SMV-resistant mutations at time of failure by population sequencing, additional population and deep sequencing data were available at end of study (Figure 4). The median follow-up time for these patients was 44.0 (range, 10.9–75.6)

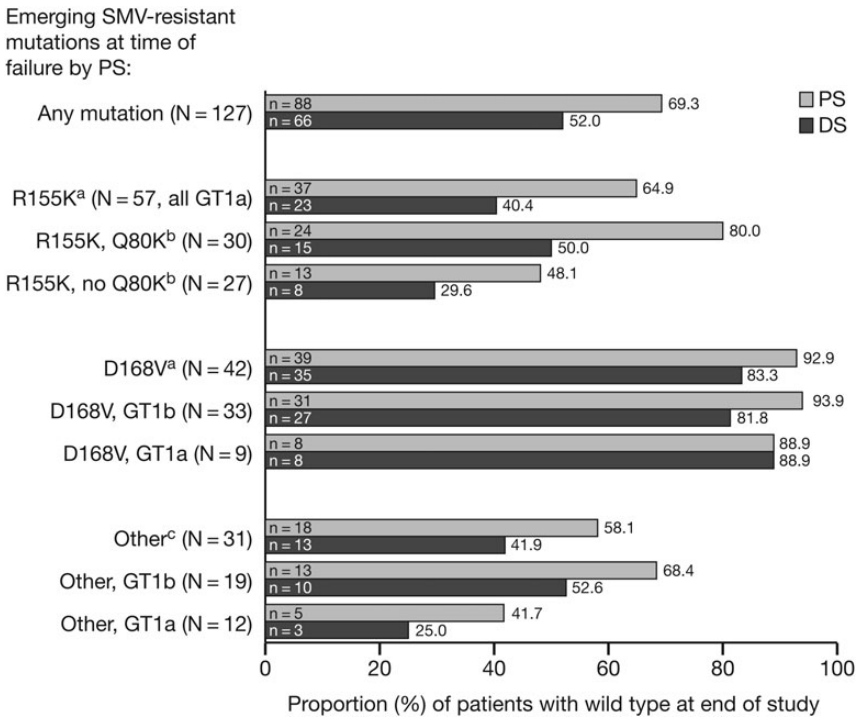

Figure 4. Return to wild type at end of study assessed by population sequencing (PS) and deep sequencing (DS) in patients not achieving sustained virologic response 12 weeks after end of treatment and with emerging simeprevir (SMV)-resistant mutations at time of failure by PS. The analysis considered return to the wild-type amino acid at the positions with amino acid substitutions reducing SMV activity in vitro observed at time of failure by PS. [a]Includes 3 patients with emerging R155K + D168V. [b]Presence or absence of polymorphism Q80K pretreatment as assessed by PS. [c]Includes patients with SMV-resistant emerging mutations other than R155K and/or D168V at time of failure by PS: emerging Q80R, S122R, D168A, E, F, H, T, or Q80K, R + D168E in genotype (GT) 1b and emerging D168A, E, H, T, or Q80R + D168E in GT 1a. Abbreviations: N, number of patients; n, number of observations.

weeks. The emerging SMV-resistant mutations detected at time of failure by population sequencing were no longer detected at end of study in 69.3% (88 of 127) of the patients by population sequencing, and in 52.0% (66 of 127) they were no longer detected by deep sequencing either, but instead wild type was observed at these positions.

In 57 patients, an emerging R155K mutation was observed at time of failure by population sequencing (median follow-up time 48.9 [range, 10.9–75.6] weeks); all patients were infected with HCV genotype 1a, and in 30 of them the Q80K polymorphism was present pretreatment (Figure 4). In 37 of 57 (64.9%) patients the emerging R155K was no longer observed at end of study by population sequencing, nor was it observed in 23 of 57 (40.4%) patients at end of study by deep sequencing. In 14 patients, the R155K mutation remained detectable by deep sequencing at read frequencies ranging from 1.2% to 23.0%. The proportion of patients with the wild-type amino acid at position 155 at end of study by deep sequencing was higher among patients with pretreatment Q80K polymorphism (50.0%; 15 of 30) than in patients without this polymorphism (29.6%; 8 of 27).

In 42 patients, an emerging D168V mutation was observed at time of failure by population sequencing (median follow-up time 44.0 [range, 11.6–72.0] weeks); the majority (n = 33) were infected with HCV genotype 1b (Figure 4). The wild-type amino acid at position 168 was observed at end of study in 35 (83.3%) of these patients by deep sequencing. The emerging D168V mutation was still observed at end of study by population and deep sequencing in 2 genotype 1b patients and by deep sequencing only in an additional 3 genotype 1b patients at read frequencies ranging from 1.9% to 14.7%. The remaining 2 patients had a D168E mutation at end of study, which was detected by population and deep sequencing in a genotype 1a patient and by deep sequencing only at a read frequency of 2.0% in a genotype 1b patient.

Kaplan–Meier analyses were performed to evaluate the persistence of emerging mutations over time. The median time until mutations emerging at time of failure returned to wild type was shortest for the D168V mutation (20.7 and 32.6 weeks by population and deep sequencing, respectively) and shorter for an emerging R155K mutation in the presence of a pretreatment Q80K polymorphism (36.4 and 59.3 weeks by population and deep sequencing, respectively), compared with R155K in the absence of pretreatment Q80K polymorphism (69.1 and >76 weeks by population and deep sequencing, respectively) (Figure 5).

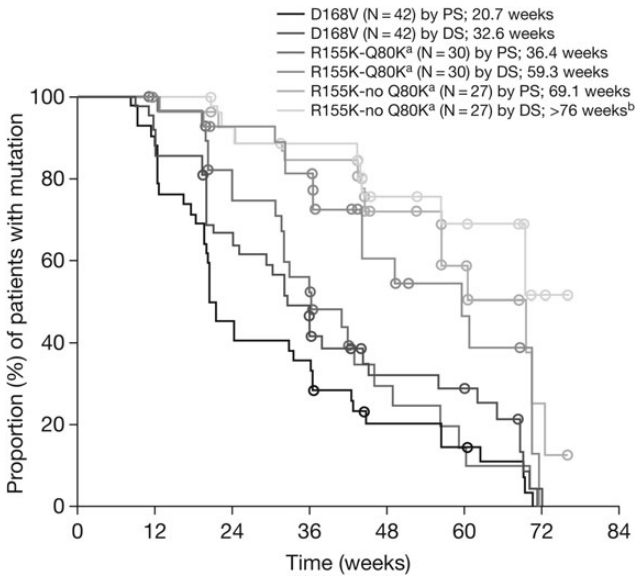

Figure 5. Time to return to wild type for simeprevir-resistant mutations D168V and R155K emerging at time of failure in patients not achieving sustained virologic response 12 weeks after the end of treatment; assessed by population sequencing (PS) and deep sequencing (DS). [a]Presence or absence of polymorphism Q80K pretreatment as assessed by PS. [b]Because the R155K variant was still detected in >50% of the patients at the last available visit, the median time to return to wild type is assumed to be longer than the maximum follow-up time of 76 weeks. Abbreviation: N, number of patients.

DISCUSSION

Viral variants carrying resistance to HCV direct-acting antiviral agents and present at levels undetectable by standard population sequencing might impact treatment outcome. The introduction of deep sequencing technologies has enabled the detection of viral variants with greater sensitivity and allows assessment of the impact of these minority variants on outcome of treatment with regimens containing direct-acting antiviral agents.

However, the sensitivity of these deep sequencing technologies is still bound by the number of errors introduced during sample preparation and sequencing. To reliably call viral variants in clinical isolates, the sensitivity for Illumina deep sequencing has been determined at 1% based on the sequencing of HCV plasmids [9, 12]. In addition, the sensitivity of deep sequencing is dependent on both the HCV RNA level in a patient's isolate and the number of sequencing reads obtained [9, 12].

In this study, patient isolates from a subset of HCV NS3/4A protease inhibitor treatment-naive HCV genotype 1-infected patients treated with SMV/PegIFN/RBV in Phase 2 and Phase 3 studies were retrospectively analyzed, using the Illumina deep sequencing technology. Previous studies showed that deep sequencing technologies can detect pre-existing minority viral variants carrying NS3 resistance, generally reporting a low prevalence of these resistant minority variants, whereas for NS5A resistance-associated variants a higher prevalence is reported, by both population and deep sequencing [12–15].

Pre-existing viral variants detected by deep sequencing and not by population sequencing, ie, minority polymorphisms, reducing SMV activity in vitro, were observed in 3.6% of the patients. Minority polymorphism Q80K was detected in 7 patients (1.3%; 4 HCV genotype 1a and 3 genotype 1b), and 5 of these patients achieved SVR12, whereas 1 patient failed for virologic reasons and another for nonvirologic reasons. Population sequencing analyses in the Phase 2 and Phase 3 studies showed a high (30%) prevalence of polymorphism Q80K in the HCV genotype 1a-infected population, whereas this polymorphism was hardly observed in HCV genotype 1b (0.5%) [7]. Thus, when a Q80K polymorphism is present pretreatment, it is generally observed as the major variant among HCV genotype 1a-infected patients and can be detected by population sequencing. Efficacy analyses in the Phase 2 and Phase 3 studies showed that the presence of polymorphism Q80K as major variant reduced SVR rates in HCV genotype 1a-infected patients treated with SMV/PegIFN/RBV [6, 7]. Only a few patients harbor polymorphism Q80K as a minority variant and, based on these limited data, this does not seem to affect response to treatment.

Overall, the presence of minority polymorphisms pretreatment did not influence the treatment outcome with SMV/PegIFN/RBV, because similar proportions of patients achieving and not achieving SVR12 had minority polymorphisms pretreatment. Furthermore, these minority polymorphisms observed pretreatment did not generally emerge at time of failure, but instead other high-level SMV-resistant mutations, not detected pretreatment, were observed at time of failure. These results are consistent with data reported for other HCV NS3/4A protease inhibitors [12, 16, 17].

Hence, it can be concluded that the presence of resistant minority polymorphisms, which are uncommonly detected pretreatment by deep sequencing, do not predict outcome with SMV/PegIFN/RBV, and deep sequencing of patient isolates pretreatment does not provide additional information beyond the data obtained by population sequencing for the clinical use of SMV with PegIFN/RBV.

In most patients in whom the emerging mutations conferring resistance to SMV were observed at time of failure and no longer detected at end of study by population sequencing, these SMV-resistant variants could also no longer be detected by deep sequencing at end of study. Viral variants carrying D168V appeared to be relatively unfit compared with variants carrying R155K, since a higher proportion of patients with emerging D168V at time of failure no longer had these variants detectable at the end of study by deep sequencing. This is consistent with earlier findings based on population sequencing [7]. As a result, the median time for an emerging D168V mutation to return to wild-type sequence was shorter when compared with an emerging R155K. In

addition, among the patients with an emerging R155K mutation, the presence of polymorphism Q80K pretreatment led to a faster return to the wild-type amino acid at position 155.

Limitations of this study include that the deep sequencing analyses were performed retrospectively for a subset of the patients and isolates. In addition, the 1% sensitivity of the Illumina deep sequencing assay implies that the presence of minority variants <1000 IU/mL cannot be discriminated from technical background noise in pretreatment isolates with HCV RNA levels >100 000 IU/mL [12]. Therefore, it cannot be excluded that resistant viral variants are present below the sensitivity of the sequencing assay and may affect treatment outcome.

CONCLUSIONS

In conclusion, pre-existing minority NS3 polymorphisms associated with SMV resistance in vitro, such as polymorphism Q80K, were uncommon, and their presence did not predict outcome of treatment with SMV/PegIFN/RBV. In most patients with emerging mutations at time of failure that became undetectable at end of study by population sequencing, deep sequencing could also no longer detect these mutations. These results suggest no added value of deep sequencing over population sequencing for the clinical use of SMV with PegIFN/RBV. Although this likely also applies to IFN-free regimens with SMV, further studies would be needed to verify this theory.

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Supplementary Data

Supplementary material is available online at Open Forum Infectious Diseases online (http://OpenForumInfectiousDiseases.oxfordjournals.org/).

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