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Evolução das quasiespécies da proteína NS5A doVírus da Hepatite C genótipo 3a

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Tese apresentada para obtenção do Título de Doutora em Genética

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Dedico este trabalho ...

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HCV	*Hepatitis C Vírus* (Vírus da Hepatite C)
HCC	Hepatocellular carcinoma (Carcinoma Hepatocelular)
DNA	Ácido desoxiribonucleico
RNA	Ácido ribonucléico
kb	Quilobases
C	Proteína Core
E1	Proteína do envelope 1
E2	Proteína do envelope 2
p7	Proteína p7
NS2	Proteína não estrutural 2
NS3	Proteína não estrutural 3
NS4A	Proteína não estrutural 4A
NS4B	Proteína não estrutural 4B
NS5A	Proteína não estrutural 5A
NS5B	Proteína não estrutural 5B
UTR	*Untranslatedregion* – Região não traduzida
IRES	*Internalribossomalentry site* (Sítio interno de entrada ribossomal)
IFN	Interferon
IFN-α	Interferon alfa

PEG-IFN	Interferon modificado por polietilenoglicol
RVS	Resposta virológica sustentada
FDA	US Foods and drugs administration
TVR	Telaprevir
BOC	Boceprovir
DAA	*Direct-acting antiviral drug*s(Drogas antivirais de ação direta)
CRS	*Cytoplasmicretentionsignal* (Sinal de retenção citoplasmático)
NLS	*Nuclear localizationsignal*(Sinal de localização nuclear)
PKR	*Proteinkinase RNA activated*(Quinase indutora de interferon ativada por RNA dupla fita)
ISDR	*Interferonsensitivitydeterminingregion* (Região Determinante de Sensibilidade ao Interferon)
IL8	Interleucina 8
V3	Regiãovariável 3
w	weeks
d	days
m	months
ω	Selective pressure estimation
dN	Number of non-synonymous substitutions per non-synonymous site
dS	Number of synonymous substitutions per synonymous site
BT	Before treatment

NR	Non responder
SVR	Sustained virological response
ETR	End of treatment response
Gln	Glutamin
Trp	Tryptophan
Lys	Lysin
HIV	Human Immunodeficiency Virus
cDNA	Complementary DNA
PCR	Polymerase chain reaction
µl	Microliter
$MgCl^2$	Magnesium Chloride
DMSO	Dimethyl sulfoxide
mM	Millimolar
pmol	Picomole
°C	Degrees Celsius
HKY	Hasegawa, Kishino and Yano 1985 model
G	Gamma distribution

RESUMO

RESUMO

A Hepatite C é uma doença presente em todo o mundo. O vírus da Hepatite C (HCV), o agente etiológico dessa doença, é um vírus de RNA de fita simples positiva. Seu genoma codifica uma única poliproteína precursora que após processamento origina dez proteínas virais. A NS5A, uma das proteínas virais não estruturais, esta associada com a resposta ao tratamento baseado em Interferon, tratamento aprovado para Hepatite C no Brazil.O HCV tem uma alta taxa de mutação levando a uma alta variabilidade, fator importante para a evasão da resposta imune e a resposta ao tratamento. O objetivo deste trabalho foi analisar a evolução das quasiespécies antes, durante e após o tratamento em pacientes infectados com HCV genótipo 3a que apresentaram diferentes respostas ao tratamento. O RNA viral foi extraído, o cDNA sintetizado, a região NS5A amplificada e clonada e 15 clones de cada ponto de coleta foram seqüenciados. As sequências foram analisadas com relação a história evolutiva, diversidade genética e seleção. Nossas análises mostram que a população viral que persiste após o tratamento na maioria dos pacientes não respondedores está presente em amostras pré-tratamento sugerindo uma aptidão para evadir o tratamento. Ainda a maioria das amostras pré-tratamento de pacientes respondedores ao final do tratamento ou não apresentou a população encontrada nas amostras pós-tratamento ou apresentou em menor freqüência. As exceções ilustram a característica única do processo evolutivo e conseqüentemente o processo de resistência ao tratamento em cada paciente. A evolução do vírus da Hepatite C ao longo do tratamento aparenta ser o resultado de uma relação evolutiva única entre as cepas virais e cada hospedeiro humano, levando a persistência do vírus ou a resposta ao tratamento.

ABSTRACT

ABSTRACT

Hepatitis C is a disease spread throughout the world. Hepatitis C virus (HCV), the etiological agent of this disease, is a single-stranded positive RNA virus. Its genome encodes a single precursor protein that yields ten proteins after processing. NS5A, one of the non-structural viral proteins, is most associated with interferon-based therapy response, the approved treatment for hepatitis C in Brazil. HCV has a high mutation rate and therefore high variability, which may be important for evading the immune system and response to therapy. The aim of this study was to analyze the evolution of NS5A quasispecies before, during, and after treatment in patients infected with HCV genotype 3a who presented different therapy responses.Viral RNA was extracted, cDNA was synthesized, the NS5A region was amplified and cloned, and 15 clones from each time-point were sequenced. The sequences were analyzed for evolutionary history, genetic diversity and selection. Our analysis shows that the viral population that persists after treatment for most non-response patients are is present in before-treatment samples, suggesting it is fitted to evasion of treatment. Accordingly, most before-treatment samples from end-of-treatment response patients either did not show the population found after the relapse or showed it in low abundance. The exceptions illustrate the uniqueness of the evolutionary process, and therefore the treatment resistance process, in each patient.Hepatitis C virus evolution throughout treatment appears to be the result of a unique evolutionary relationship between viral strains and each human host, leading to either persistence or clearance.

INTRODUÇÃO

1.INTRODUÇÃO

1.1 Histórico e Epidemiologia da Hepatite C

Originalmente acreditava-se que as Hepatites virais consistiam em dois tipos, Hepatite A e Hepatite B. Na ausência de testes diagnósticos que pudessem distinguir os agentes causadores, o diagnóstico ocorria pela identificação da via de transmissão e pelo tempo de incubação da doença(Krugman, Giles *et al.*, 1967). Com o isolamento dosvírus das Hepatites A e B amostras de sangue estocadas foram analisadas evidenciando que algumas amostras eram negativas para ambos os vírus identificados. Este fato levou a utilização do termo hepatite não-A não-B para os casos não identificados (Feinstone, Kapikian *et al.*, 1975). Em 1989 Chooet al isolou parte do genoma deste vírus desconhecido,a partir do plasma de um chimpanzé cronicamente infectado e este ficou conhecido como Vírus da Hepatite C (HCV), (Choo, Kuo *et al.*, 1989).

O HCV é um vírus de distribuição mundial sendo aprevalência global da infecção aproximadamente 2 a 3%, correspondendo a 130 a 170 milhões de pessoas soropositivas no mundo (Lavanchy, ; Shepard, Finelli *et al.*, 2005; Alter, 2007).

1.2 Fatores de Risco e Transmissão do vírus da Hepatite C

O HCV é transmitido pelo contato percutâneo com sangue contaminado. Os fatores de risco associados à infecção incluem transfusão de sangue e seus derivados, transplante de órgãos, usuários de drogas injetáveis, injeções terapêuticas não seguras, procedimentos percutâneos como piercing, tatuagem e circuncisão(com material inadequadamente esterilizado),exposição ocupacional a sangue, perinatal, e sexual (Who, 2000; Alter, 2007).A transmissão sexual é assunto ainda controversodevido à dificuldade de determinar se ocorreu por relações sexuais ou algum outro tipo de

contato próximo, sendo alvo de diversos estudos (Squadrito, Orlando *et al.*, 1999; Nakayama, Sugai *et al.*, 2005; Tahan, Karaca *et al.*, 2005; Mello, 2006; Urbanus, Van De Laar *et al.*, 2009; Nakamura, Tanaka *et al.*, 2011).

Antes da identificação do vírus causador da Hepatite C, a transfusão de sangue era a principal forma de transmissão da doença. Após a implementação dos testes-anti HCV em bancos de sangue a transmissão via transfusão foi praticamente eliminada nos países que adotamesse procedimento(Busch, Glynn *et al.*, 2005). Contudo, em países que não possuem recursos para testar os doadores essa ainda é uma importante via de transmissão juntamente com injeções terapêuticas não seguras(Hauri, Armstrong *et al.*, 2004; Hladik, Kataaha *et al.*, 2006; Alter, 2011).

Outra importante forma de transmissão do HCV é o uso de drogas injetáveis, sendo juntamente com a transfusão de sangue contaminado os dois principais fatores de risco. Usuários de drogas injetáveis constituem o grupo de risco com maior prevalência de infecção por HCV (Alter, 2011). Usuários com mais de trinta anos de idade que utilizam drogas há mais de cinco anos apresentam prevalência de 75% a 90%. Em usuários mais novos (abaixo de 30 anos) a prevalência nos primeiros 2 a 3 anos de uso reduziu de 80% no fim da década de 1980 para 30%desde o fim da década de 1990(Alter, 2011).

1.3 Sintomatologia e Prognóstico

O período de infecção, antes do aparecimento de sintomas clínicos, é de 15 a 150 dias. Na infecção aguda os sintomas mais comuns são fadiga e icterícia, porém a maioria dos casos, de 60% a 70%, é assintomática, mesmo quando evoluem para infecção crônica (Who, 2000).

Estima-se que a resolução espontânea da infecção ocorra em 14% a 29% dos casos (Alter, Purcell *et al.*, 1989; Aach, Stevens *et al.*, 1991; Alter, Conry-Cantilena *et al.*, 1997; Locasciulli, Testa *et al.*, 1997; Thomas, Astemborski *et al.*, 2000). Contudo uma taxa de resolução espontânea mais alta foi relatada em pessoas jovens variando de 42% a 45%(Kenny-Walsh, 1999; Vogt, Lang *et al.*, 1999; Casiraghi, De Paschale *et al.*, 2004; Seeff, 2009).

As pessoas infectadas que evoluem para a fase crônica possuem um risco de apresentar doença do fígado progressiva caracterizada pela inflamação continua levando a fibrose, cirrose e o carcinoma hepatocelular (HCC)(Seeff, 2000; , 2009). Dos indivíduos com infecção persistente estima-se que 20% irão desenvolver cirrose hepática e o carcinoma hepatocelular ocorre em até 2,5% dos casos. (Bowen e Walker, 2005). A princípio acreditava-se que a grande incidência de HCC em pacientes infectados por HCV era uma consequência indireta da infecção viral. A infecção pelo vírus da Hepatite C leva em muitos casos a cirrose hepática, fator que predispõe o desenvolvimento de câncer (Liang, Ye *et al.*, 2007). Outro importante fator indireto é a alta taxa de inflamação seguida de regeneração decorrente da infecção, combinado ao potencial de quebra de DNA dupla fita causada por estresse oxidativo(Nakamoto, Guidotti *et al.*, 1998; Okuda, Li *et al.*, 2002; Bartsch e Nair, 2004). Contudo hoje se acredita as proteínas virais possuam um papel direto na hepatocarcinogênese. As proteínas virais que vem sendo relacionadas com a hepatocarcinogênese são Core, E2, NS3 e NS5A (Levrero, 2006; Tsai e Chung, 2010).

1.4 O Vírus

O vírus da Hepatite C (HCV) é um vírus de RNA de fita simples, com polaridade positiva, pertencente à família Flaviviridae. O genoma do HCVpossui 9,6kb

e codifica uma grande poliproteína precursora de aproximadamente 3000 resíduos de aminoácidos, sendo essa clivada pelas proteases virais e do hospedeiro para gerar, pelo menos, 10 proteínas na seguinte ordem, iniciando pela extremidade amino terminal: core (C), envelope (E1; E2) e p7, não estruturais (NS2, NS3, NS4A, NS4B, NS5A, NS5B) (Kato, 2001). (Figura 1)

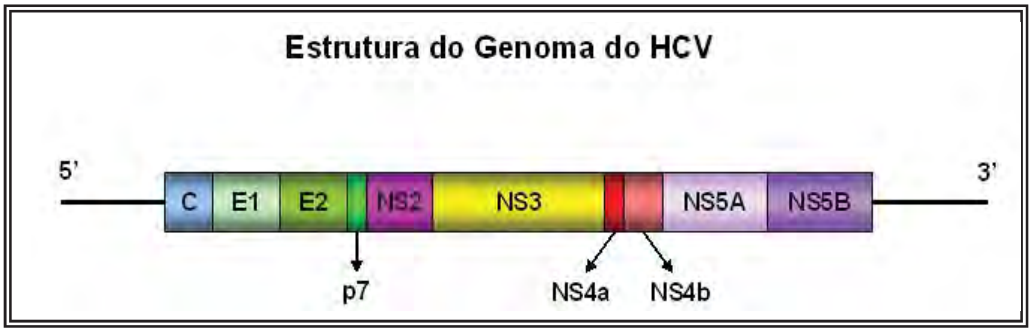

Figura 1: Representação esquemática da estrutura do genoma do vírus da Hepatite C (HCV). Proteínas estruturais: C – Core; E1 – Envelope 1; E2 – Envelope 2; p7; Não Estruturais:; NS2; NS3; NS4A; NS4B; NS5A; NS5B.

A região 5' não-traduzida(5´UTR) é altamente conservada e contém um sítio de entrada ribossomal (IRES) essencial para a tradução do RNA viral(Lyra, Fan *et al.*, 2004). A região 3' UTR é composta por uma pequena região variável, uma cauda poli (U/UC) e uma região altamente conservada de 98 nucleotídeos essenciais para a replicação viral (Moradpour e Blum, 2004).

A proteína Core,a primeira da poliproteína, é uma proteína de ligação a RNA e está envolvida na formação do nucleocapsídeo. Ela é removida da poliproteína por peptidases de sinal do hospedeiro que clivam na região C-terminal e liberam a forma imatura desta proteína (Santolini, Migliaccio *et al.*, 1994). Posteriormente o peptídeo de sinal presente na região C-terminal da proteína Core é processado por peptidases do hospedeiro resultando na proteína madura(Mclauchlan, 2000).

As proteínas de envelope E1 e E2 são glicoproteínas liberadas da poliproteína viral por peptidases de sinal do hospedeiro(Dubuisson, Penin *et al.*, 2002). Essas proteínas são essências para a entrada do vírus na célula e para a montagem das partículas infecciosas (Wakita, Pietschmann *et al.*, 2005; Bartosch e Cosset, 2006; Cocquerel, Voisset *et al.*, 2006).Por estarem envolvidas na entrada do vírus na célula constituem importante alvo no desenvolvimento de moléculas antivirais que bloqueiem a entrada do HCV (Helle, Wychowski *et al.*, 2006).

O polipeptídeop7 é localizado na poliproteína entre as proteínas estruturais e não estruturais, sendo liberada da poliproteína por peptidases do hospedeiro(Lin, Lindenbach *et al.*, 1994; Mizushima, Hijikata *et al.*, 1994; Dubuisson, Penin *et al.*, 2002). É uma pequena proteína de membrana (7KDa) composta por dois domínios transmembrânicos tendo as extremidades amino- e carboxi- terminal orientada em direção ao lúmen do retículo endoplasmático, contudo sua localização celular ainda não esta clara(Carrere-Kremer, Montpellier*et al.*, 2004). Foi demonstrado que este polipeptídeo pode atuar como canal de íons e que sua presença é essencial para a infectividade em chimpanzés(Griffin, Beales *et al.*, 2003; Pavlovic, Neville *et al.*, 2003; Sakai, Claire *et al.*, 2003; Griffin, Harvey *et al.*, 2004).

A proteína NS2 é uma proteína de membrana não essencial a formação do complexo replicativo viral (Lohmann, Korner*et al.*, 1999; Blight, Kolykhalov *et al.*, 2000). A função da NS2 em sua forma madura é desconhecida, contudo no contexto da poliproteína, ela participa da clivagem entre NS2-NS3 (Dubuisson, 2007). Estudos sugerem que esteja relacionada a expressão e liberação da partícula viral (Lin, Lindenbach *et al.*, 1994; Kalinina, Norder *et al.*, 2002; Pietschmann, Kaul *et al.*, 2006).Embora a proteína NS2 não seja comumente relacionada à hepatocarcinogênese,

ela foi reportada como anti-apoptótica (via CIDE-B) e tendo um possível envolvimento na inibição da transcrição de genes celulares

A NS3 é uma proteína multifuncional, que possui um domínio aminoterminalserina-protease e um domínio carboxiterminalhelicase. A NS3 forma um complexo não covalente com a NS4A que é um polipeptídeo ancorado na membrana. A NS4A age como um co-fator da NS3 e também a estabiliza(Kim, Morgenstern *et al.*, 1996; Love, Parge *et al.*, 1996). A proteína NS3 interage com diversas proteínas virais e pode estar envolvida na hepatocarcinogênese(Love, Parge *et al.*, 1996; Deng, Nagano-Fujii *et al.*, 2006).

A proteína NS4B, não está ainda muito bem caracterizada, porém estudos sugerem que esta proteína provoca alterações específicas na membrana produzindo um ambiente favorável à replicação viral(Egger, Wolk *et al.*, 2002).

A proteína NS5A é uma fosfoproteína que tem atraído atenção considerável por seu potencial papel de modulação da resposta ao interferon (IFN) e em outros processos da célula do hospedeiro(Le Guillou-Guillemette, Vallet *et al.*, 2007).

A NS5B é uma proteína enzimática com função de RNA polimerase dependente de RNA, sendo, portanto responsável pela replicação viral(Bartenschlager, Frese *et al.*, 2004).

A figura 2 mostra uma representação esquemática da organização do genoma viral (topo) e a localização das proteínas na membrana do reticulo endoplasmático(Dubuisson, 2007).

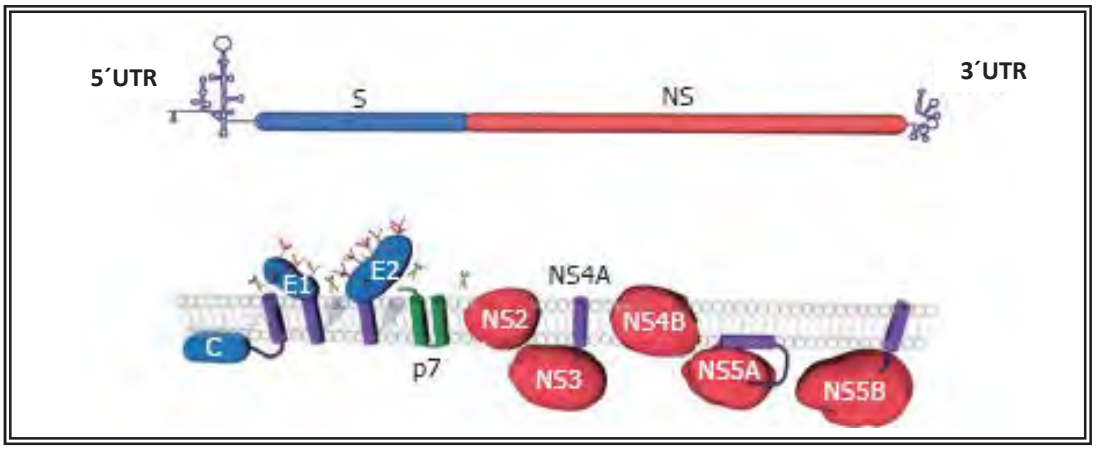

Figura 2: Representação esquemática da organização do genoma viral (topo) e a localização das proteínas na membrana do reticulo endoplasmático. (Extraída de Dubuisson, 2007)

1.5 Variabilidade Genética

Os vírus de RNA são organismos que apresentam altas taxas de mutação. Isso ocorre devido aos erros acumulados durante sua replicação, em decorrência da falta de atividade corretiva da RNA polimerase dependente de RNA viral(Domingo, Escarmis *et al.*, 1996).

A comparação de sequências de nucleotídeos de HCV de variantes coletadas de diferentes indivíduos e regiões geográficas, revelou a existência de pelo menos 7 grandes genótipos. Numerados de 1 a 7,muitos contém variantes mais relacionadas, os subtipos, classificados alfabeticamente (1a, 1b, 3a, 3e, etc)(Simmonds, Bukh*et al.*, 2005; Murphy, Chamberland *et al.*, 2007). Em média, os genótipos diferem, no genoma completo, de 30% a 35% com relação aos nucleotídeos enquanto os subtipos diferem de 20% a 25% (Simmonds, Holmes *et al.*, 1993; Murphy, Chamberland *et al.*, 2007).

Os diferentes genótipos influem na evolução da doença e seu tratamento. Estudos mostram relação entre paciente infectados com genótipo 3 e o desenvolvimento de esteatose, tendo sido identificada uma sequência de aminoácidos específica na proteína Core deste genótipo relacionada a esteatose(Mihm, Fayyazi*et al.*, 1997; Rubbia-Brandt, Quadri *et al.*, 2000; Rubbia-Brandt, Leandro *et al.*, 2001; Sharma, Balan *et al.*, 2004; Hissar, Goyal *et al.*, 2006; Hourioux, Patient *et al.*, 2007; Qiang, Yang *et al.*, 2009). A resposta ao tratamento também esta relacionada ao genótipo sendo o genótipo 1 mais resistente ao tratamento com IFN que os demais genótipos (Kanai, Kako *et al.*, 1992; Yoshioka, Kakumu *et al.*, 1992; Chayama, Tsubota *et al.*, 1993).

Os genótipos 1, 2 e 3 e seus subtipos têm distribuição mundial(Kershenobich, Razavi *et al.*, ; Bukh, Purcell *et al.*, 1993; Simmonds, Holmes *et al.*, 1993; Smith, Pathirana *et al.*, 1997; Cornberg, Razavi *et al.*, 2011; Sievert, Altraif *et al.*, 2011). Já os genótipos 4 e 5 estão presentes na África e o genótipo 6, principalmente na Ásia (Sievert, Altraif*et al.*, 2011).

No Brasil, um estudo, que analisou 1688 amostras de todo o país, mostrou a presença dos genótipos 1, 2, 3, 4 e 5. As freqüências gerais encontradas foram de 64,9% para o genótipo 1, 4,6% para o genótipo 2, 30,2% para o genótipo 3, 0,2% para o genótipo 4 e 0,1% para o genótipo 5 (Campiotto, Pinho *et al.*, 2005). Em outro estudo realizado no interior do estado de São Paulo, para um total de 1018 amostras foram encontrados em 62,9% HCV genótipo 1, 2,1% genótipo 2, 34,5% genótipo 3 e 0,2% genótipo 5(Corvino, Henriques *et al.*, 2006).

1.5.1 Quasiespécies

O HCV, assim como outros vírus de RNA, devido às mutações aleatórias decorrentes da falta de atividade corretiva da RNA polimerase viral, a proteína NS5B,

circula no hospedeiro como um conjunto de variantes virais geneticamente semelhantes chamadas de quasispecies (Martell, Esteban *et al.*, 1992). Essa característica possibilita a existência de um conjunto de variantes possivelmente benéficas ao vírus quando consideradas as populações. Essas variantes podem contribuir para a rápida evolução viral, sendo as populações de vírus capazes de mudar em um curto período de tempo. As mutações aleatórias decorrentes de erros durante a replicação, ocorrem ao acaso maspodem conferir um melhor *fitness* ao organismo, permitindo que o gene mutado persista e eventualmente seja predominante na população onde ele confere alguma vantagem adaptativa (Simmonds, 2004).A característica do HCV de circular como quasiespécies confere ao vírus um maior potencial de se adaptara cada novo hospedeiro e aos desafios da infecção(Eigen e Biebricher, 1988; Eigen, 1993; Coffin, 1995; Domingo e Holland, 1997).

Os impactos biológicos da variabilidade incluem o aparecimento de mutações de escape, alteração do tropismo celular e mudanças na virulência e na abrangência de hospedeiros. O acúmulo de mutações também é importante no desenvolvimento de resistência a agentes anti-virais(Forns e Bukh, 1999).

Contudo a seleção “Darwiniana” ou seleção natural não é o único processo que guia a evolução do vírus da Hepatite C. Na verdade, as trocas de nucleotídeos neutras são responsáveis pela variabilidade genéticanão só do HCV, mas dos genomas em geral (Kimura, 1968). Trocas de nucleotídeos que possuem pouco ou nenhum efeito no *fitness* do organismo podem ou não ser fixadas por acaso e a freqüência de fixação de trocas neutras em um gene ao longo do tempo é relativamente constante(Kimura, 1983). Evidências de ambas, evolução neutra e Darwiniana podem ser encontradas no genoma do HCV. Regiões como E2 apresentam rápida evolução em sua região hipervariavel para evitar o reconhecimento por anticorpos (seleção natural), enquanto a variação

apresentada entre os genótipos, da ordem de 30%, não confere alteração de fenótipo viral (evolução neutra) (Simmonds, 2004). Sendo assimo genoma evolui em diferentes taxas variando de acordo com a região e a pressão a que ela esta submetida. Regiões como a 5'UTR e core são altamente conservadas, as proteínas NS2, NS3, NS5B e 3'UTR são relativamente variáveis, enquanto as proteínas de envelope E1 e E2, NS4 e NS5A exibem a maior diversidade de sequência(Simmonds, 2004; Le Guillou-Guillemette, Vallet*et al.*, 2007).

1.6Tratamento

O primeiro tratamento para a Hepatite C surgiu antes mesmo de sua descoberta. Em 1986, Hoofnagaleet al, reportou que IFN-α era efetivo no tratamento da Hepatite não-A, não-B(Hoofnagle, Mullen *et al.*, 1986). A efetividade doIFN-α, no entanto era limitada correspondendo de 20% a 30% dos casos. Outros estudos levaram à utilização do IFN-α juntamente com a ribavirina que produzia resposta virológica sustentada (RVS) em aproximadamente 40% dos casos, variando de 30% para genótipo 1 a 60% para os genótipos 2 e 3 (Kato, 2001). Mais recentemente a utilização de uma forma de interferon-alfa modificado por polietilenoglicol (PEG-IFN) em combinação com ribavirinaaumentou em 40% a resposta virológica sustentada (Manns, Mchutchison *et al.*, 2001; Fried, Shiffman *et al.*, 2002).

A decisão de tratar os pacientes com Hepatite C crônica depende de diversos parâmetros, incluindo uma avaliação da severidade da doença e de seu prognóstico, a presença de contra-indicações à terapia e à disponibilidade do paciente em ser tratado. A genotipagem viral deve ser realizada antes do inicio por ser determinante da duração do tratamento e do procedimento de monitoramento virológico(Chevaliez e Pawlotsky, 2007).

No Brasil, atualmente o tratamento adotado para a Hepatite C é a combinação de interferon-alfa (IFN-α) com ribavirinapara genótipos 2 e 3 e uma forma de interferon-alfa modificado por polietilenoglicol (PEG-IFN) juntamente com ribavirina para genótipo 1(Brasil, 2011).

O ano de 2011 inaugurou um novo capítulo na história do tratamento da Hepatite C.A FDA (U.S. FoodandDrugAdministration), agência que regulamenta o uso de novas drogas, aprovou o uso de duas drogas antivirais de ação direta (DAA – *direct-acting antiviral dru*gs). As primeiras DAAs aprovadas são os inibidores de protease Telaprevir (TVR) e Boceprovir (BOC)(Poordad, 2010; Vachon e Dieterich, 2011).As estratégias para o tratamento com esses novos medicamentos incluem a combinação com PEG-IFN e Ribavirina(Poordad, 2010).

1.7 NS5A

A proteína NS5A é a proteína mais relacionada à resistência ao IFN. É uma proteína pleoitrópica envolvida na replicação viral e em muitas interações com vias de sinalização celular (Le Guillou-Guillemette, Vallet *et al.*, 2007). Esta proteína parece ter um importante papel, apesar de incerto, como modulador direto da atividade da RNA polimerase dependente de RNA (Shirota, Luo *et al.*, 2002) e interfere nas vias celulares induzidas por IFN (Polyak, 2003).

Em sua região N-terminal, os primeiros 27 aminoácidos constituem o sinal de retenção citoplasmático (CRS – *Cytoplasmicretentionsignal*) que é capaz de manter a proteína NS5A no citoplasma (Reyes, 2002). A NS5A completa parece estar localizada exclusivamente no citoplasma, contudo esta proteína possui um sinal de localização nuclear (NLS – *Nuclear localizationsignal*) e formas com a região N-terminal deletadas

foram encontradas no núcleo sugerindo que tais formas atuariam como potentes ativadores transcricionais(Pawlotsky e Germanidis, 1999).

A resistência ao IFN seria explicada pela habilidade da NS5A ligar-se a quinase indutora de interferon ativada por RNA dupla fita (PKR – *Proteinkinase RNA activated*), levando a sua inibição (Gale, Korth *et al.*, 1997). A região responsável pela resistência ao IFN, uma seqüência conservada de 40 aminoácidos dentro da PKR, localiza-se no centro da NS5A, e foi denominada região determinante de resistência ao IFN (ISDR – *Interferonsensitivitydeterminingregion*). (Figura 3) Em 1995 Enomotoet al descreveram uma correlação entre um maior número de mutações presentes nesta região e a resposta virológica sustentada, em pacientes infectados com genótipo 1 (Enomoto, Sakuma *et al.*, 1995). Outros estudos de grupos japoneses encontraram resultados semelhantes também para genótipo 1b (Enomoto, Sakuma *et al.*, 1996; Chayama, Tsubota *et al.*, 1997; Kurosaki, Enomoto *et al.*, 1997; Arase, Ikeda *et al.*, 1999; Nakano, Fukuda *et al.*, 1999). Contudo estudos europeus e americanos não corroboraram estes resultados (Zeuzem, Lee *et al.*, 1997; Duverlie, Khorsi *et al.*, 1998; Chung, Monto *et al.*, 1999; Squadrito, Orlando *et al.*, 1999; Berg, Mas Marques *et al.*, 2000; Nousbaum, Polyak *et al.*, 2000; Sarrazin, Berg *et al.*, 2000; Murphy, Rosen *et al.*, 2002). Em 2004 um trabalho de Pascu e colaboradores (2004) estudaram seqüências de ISDR de genótipo 1b de pacientes japoneses e europeus, onde foi confirmada a relevância da ISDR para predizer a resposta ao tratamento. Este estudo também encontrou maior probabilidade das mutações na ISDR levarem a RVS em pacientes japoneses quando comparados aos europeus, sugerindo uma diferença geográfica (Pascu, Martus *et al.*, 2004).

Ainda, alguns estudos também com genótipo 1 mostram evidências de efeitos da NS5A na atividade do IFN, independentes da PKR. Um exemplo é a regulação positiva,

pela NS5A, da interleucina8 (IL8), que tem a propriedade de atenuar as propriedades antivirais do IFN, sugerindo que o produção endógena de IL8 possa facilitar a infecção viral (Polyak, Khabar*et al.*, 2001; Girard, Shalhoub *et al.*, 2002).

Na região C-terminal, o domínio V3 com 27 aminoácidos, tem alta taxa de variabilidade. Duverlieet al observaram relação entre o nível de mutações em V3 e a resistência à terapia de interferon. Cepas virais resistentes são mais conservadas em comparação às sensíveis(Duverlie, Khorsi *et al.*, 1998).

As implicações da variabilidade na resistência aos agentes antivirais têm sido discutidas, contudo poucos estudos focam a proteína NS5A inteira com relação às quasiespécies (Le Guillou-Guillemette, Vallet *et al.*, 2007).

OBJETIVOS

2. OBJETIVOS

2.1 Geral

Estudar a evolução de quasiespécies da região NS5A de HCV genótipo 3 em amostras de pacientes Não-respondedores ao Tratamento e Respondedores ao Final do Tratamento com IFN e Ribavirina, durante e após o tratamento.

2.2 Específicos

1. Clonar e Sequenciar a região codificante da proteína NS5A do Vírus da Hepatite C genótipo 3 de amostras coletadas durante e após o tratamento de pacientes Não-respondedores ao Tratamento e Respondedores ao Final do Tratamento.

2. Analisar a evolução do perfil de quasiespécies durante e após o tratamento.

3. Comparar o perfil de quasiespécies encontrado nos pontos analisados com o perfil observado nas amostras pré-tratamento.

PARTE II

Artigo Científico

Title: On Hepatitis C virus evolution: the interaction between virus and host towards treatment outcome.

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On Hepatitis C virus evolution: the interaction between virus and host towards treatment outcome

Abstract

Background: Hepatitis C is a disease spread throughout the world. Hepatitis C virus (HCV), the etiological agent of this disease, is a single-stranded positive RNA virus. Its genome encodes a single precursor protein that yields ten proteins after processing. NS5A, one of the non-structural viral proteins, is most associated with interferon-based therapy response, the approved treatment for hepatitis C in Brazil. HCV has a high mutation rate and therefore high variability, which may be important for evading the immune system and response to therapy. The aim of this study was to analyze the evolution of NS5A quasispecies before, during, and after treatment in patients infected with HCV genotype 3a who presented different therapy responses.

Results: Viral RNA was extracted, cDNA was synthesized, the NS5A region was amplified and cloned, and 15 clones from each time-point were sequenced. The sequences were analyzed for evolutionary history, genetic diversity and selection. Our analysis shows that the viral population that persists after treatment for most non-response patients are is present in before-treatment samples, suggesting it is fitted to evasion of treatment. Accordingly, most before-treatment samples from end-of-treatment response patients either did not show the population found after the relapse or showed it in low abundance. The exceptions illustrate the uniqueness of the evolutionary process, and therefore the treatment resistance process, in each patient.

Conclusion: Hepatitis C virus evolution throughout treatment appears to be the result of a unique evolutionary relationship between viral strains and each human host, leading to either persistence or clearance.

Key words: Hepatitis C virus, NS5A, evolution

Introduction

Hepatitis C is a world-wide disease. The World Health Organization estimate is that 3% of the world's population has been infected with the hepatitis C virus (HCV), which is the etiological agent of this disease [1].

HCV is a single-stranded positive RNA virus member of the Flaviviridae family. Its genome is 9.6Kb long and encodes for a single precursor protein with approximately 3,000 amino acid residues. This polypeptide is processed by viral and host proteins, resulting in 10 individual proteins: core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B[2].

NS5A, one of the viral non-structural proteins, is the protein most associated with interferon-based therapy, the accepted treatment for hepatitis C. NS5A is multifunctional, and although not all of its functions are clearly established yet, it is known to be involved in viral replication and interactions with cell signaling pathways [3-5].

Some studies have identified regions in NS5A that have specific functions. At the amino terminal end there is a 27 amino acid cytoplasmic retention signal (CRS) responsible for keeping NS5A in the cytoplasm [6]. Another region, called the PKR-binding region, is responsible for binding to cellular protein kinase R (PKR), inhibiting it, and ultimately resulting in the suppression of interferon (IFN) antiviral activity [7]. Within the PKR-binding region there is an IFN sensitivity-determining region (ISDR), which some studies correlated the accumulation of mutations to therapy response [8, 9]. Despite the CRS, NS5A also contains a nuclear localization signal (NLS) that, in the absence of CRS, results in the translocation of NS5A to the nucleus [6, 10]. In the C-terminal portion of NS5A the genetic variability of a region called V3 is also associated with IFN therapy response [11, 12].

Owing to the lack of proof-reading activity of the viral RNA-dependent RNA polymerase (NS5B protein), HCV has a high mutation rate and therefore high variability. This variation happens at different levels, including genotypes (30% to 35% difference), subtypes (20% to 25% difference) and different but closely related genomes called quasispecies [13-15]. Although mutations can be prejudicial for the virus when they result in non-viable strains, having a pool of slightly different strains with

mutations that are initially neutral or quasi-neutral can increase fitness after changes in the initial condition, and be useful for evading the immune response and treatment [16-19].

The aim of this study was to analyze the evolution of NS5A quasispecies before, during, and after treatment of patients infected with HCV genotype 3a, which presents different therapy responses. This is the first work to analyze the evolutionary history, genetic diversity and selection of full-length NS5A quasispecies from HCV genotype 3a along treatment.

Results

Sequences

This study generated 435 sequences of full length NS5A (1,356 bp) from samples collected during and after interferon plus ribavirin treatment from 8 patients. The sequences were submitted to GenBank; their accession numbers are **JN689511-JN689930**.

Quasispecies Analysis

We analyzed the sequences to address quasispecies variability at each time point in each patient. Figure 1 shows a graphic representation of the results; each tile represents a different amino acid sequence and colored tiles represent a persistent sequence through time points. The viral quasispecies analysis from non-response patient samples shows that amino acid sequences that were circulating before treatment were also found during and after treatment in several patients, including patient P75 at 24 weeks (w) of treatment and in patient P145 at 28 days (d) after treatment (Figure 1). Patients P07, P60 and P75 had sequences that were sampled at more than one time-point after treatment. Patient P07 presented two strains that were sampled at the 28 day and 5 month (m) time-points, patient P60 presented one sequence that was found at both 2 months and 5 months, and patient P75 had the same sequence at the 21 day and 2 month time-points. Patient P145 presented one sequence that was first sampled at 12 weeks during the treatment, then later sampled at 5 months.

In contrast, most end-of-treatment response patients had a different profile. No quasispecies from before treatment were found during or after treatment. However, it was shown that a quasispecies that arises at the relapse time-point, or close to the relapse point, such as in patient P109 (2m), is sampled until the end of the follow-up, with the exception of patient P82. In two patients, P109 and P119, this sequence increased in frequency with increasing time (Figure 1).

Genetic distance

Genetic distance data show that the non-responder patients presented a more homogeneous population of quasispecies during treatment than the end-of-treatment response patients, except for patient P60 which the genetic distance was 0.0081 before treatment, and then rose through follow-up to 0.0205 at 5 months (Figure 1). Quasispecies in the end-of-treatment response patients showed variable genetic distances and different behaviors depending upon the patient. Patient P20, for example, although presenting quasispecies with similar distances in before-treatment (0.0289) and 5 month time-points (0.0296), showed much lower distances at 3 and 4 months (0.0046 and 0.0069, respectively) (for statistics see Table ST-1A to ST-1H, supplementary material). In patient P82, the genetic distance rose from 0.0097 to 0.0207, stabilizing at 3 and 5 months; in contrast, in patient P119 it decreased from 0.0144 to 0.0034. Patient P109 also showed a decrease in genetic distance from the before-treatment (0.0211) to 5 month (0.0047) time-points. However, at 21 days after treatment (relapse point) the distance was higher (0.0261) than before treatment; at 2 months it started falling (0.0170) and remained constant until 4 months, then it showed a drastic decrease at the 5 month time-point (0.0047).

Global and site-by-site selective pressure (ω) estimation

The estimates of the overall selective pressure (ω=dN/dS) of each of the response groups showed that non-responder patients have a similar ω (0.2828) to the end-of-treatment responders (0.2234). This analysis was also carried out for the sequences from the sustained virological response group (**EU826189 to EU826218; EU826249 to EU826263; EU826294 to EU826307**), which presented lower ω values (0.1494) (Figure 2), although the differences were not statistically significant.

When ω was analyzed by patient, the values varied from 0.20 to 0.46, suggesting that NS5A is under a relaxed purifying selection in the quasispecies circulating in the patients. However, by analyzing each time-point separately, variations could be detected. Patient P145 was the only individual who showed evidence of positive selection at 2 months, with ω=1.63, but by 5 months this had decreased to 0.7. Evidence of neutral evolution was found in time-points from two patients, P82 – BT (Before treatment) and P07 – 2m, with ω values of 1.03 and 1.01 respectively (Figure 3).

The estimation of the dN/dS rates for each site showed negatively selected sites in the NS5A sequences of all patients, in agreement with the above results. Figure 4 indicates the locations of these sites in the sequence and their percentages in each patient. However, evidence for positive selection was found in two amino acids, 122 and 408 of the quasispecies from patient P109. Negatively selected sites were identified in all patients. The results are shown in Figure 4. End-of-treatment response patients had more negatively selected sites (n-163; 9.02%) than non-response patients (n-101; 5.59%), and the difference was statistically significant ($p<0.001$). Some sites were negatively selected in more than one sequence.

Stop codons

During the analysis of all the sequences generated in this study and considered together with those obtained by Bittar *et al* (2010), 23 stop codons were detected [20]. The majority of them, 73.9% (17/23), were found in NR sequences, and no stop codon was identified in SVR. Some of these non-sense mutations were found at the same site in different patients and, in one case (P145), through different time-points. The sites where the stop codons occurred are shown in Figure 5. Most of the stop codons (15) were TAG, followed by TGA (5) and TAA (3). The amino acids that were most associated with mutating to a stop codon were glutamine (Gln) at four sites, and tryptophan (Trp) and lysine (Lys) at three sites each; these mutations correspond to 20 of the 23 mutations found. All of the sites where these codons were found were highly conserved. Some stop codons at the same site in different patient sequences were encoded by different codons (Figure 5).

Relative genetic diversity

The relative genetic diversity analyses can be seen in the skyride plots shown in Figure 6. In these plots we can see that the quasispecies diversity in most patients shows an exponential growth before the beginning of the treatment, except for patient P119, who seems to be constant, and patient P60, who shows variation with time. Around the beginning of the treatment (represented by the red line for the approximate BT time-point), all patients show a major oscillation in quasispecies diversity, with the exception of patient P82 samples, which remained almost constant with only a slight variation. Most patients presented a decrease in genetic diversity after the beginning of the treatment (P07, P75, P20, P109 and P119). Patient P145 showed an initial increase in diversity followed by a fluctuation, which is in accordance with the distance data for this patient.

Phylogenetic relationships and correlation with other data

A phylogenetic tree was constructed with the sequences from this study together with before treatment sequences from the previous study [20] (Accession numbers: EU826174 to EU826233 and from EU826249 to EU826352) and the reference sequence for genotype 3a NZL1 (Accession number: D17763) (Figure 7). All sequences from the same patient grouped on a monophyletic branch with significant supporting values (supplementary material - figures SR-1 to SR-9), showing there was no cross-contamination among patients. Sequences from two patients, P109 and P119, were grouped in a monophyletic branch with a high bootstrap (100) (Figure SR-9, supplementary material). These indicate a closer relationship between the viruses from these patients, suggesting an epidemiological link. From the epidemiological questionnaire, which patients filled in when they enrolled in the study, we could not establish any direct link between the patients and their routes of transmission. The only inference that can be made is that their viruses had the same origin.

Sequences from P60 and P82 from before the treatment clustered together, away from the other time-points, in a monophyletic branch with bootstrap of 98 and 86 respectively (Figures SR-3 and SR-5, supplementary material).

From the phylogenetic tree for patient P20 (Figure SR-2, supplementary material), the viral population at 5 months seems to be derived from two separate

before-treatment populations. Interestingly, one of these populations, which was the most prevalent before treatment, is not present in any of the 3 and 4 months time-point samples. This data supports the genetic distance data that show a major decrease in 3 and 4 month time-point sequences when only one population was sampled. At 5 months, the second viral population reappears, which increases the genetic distance. The ω values at 5 months can be explained not by an increase in selective pressure, but is probably a result of an increase in the synonymous changes due to the presence of two different populations. Ultimately patient P20 viral population changed converging towards a previously existing, but not predominant, population and, after evading treatment, both populations were able to reestablish themselves.

The phylogenetic tree also demonstrated some interesting evolutionary behaviors of the viral population of patient P60 (Figure SR-3, supplementary material). After-treatment sequences from patient P60 were grouped on two different branches with significant support. The first group comprises one sequence from the 28 day time-point, one at 2 months, and nine sequences from the 5 month time-point. The second group, which contains all other P60 after-treatment sequences time-points together with the six remaining sequences from the 5 month time point, grouped on a terminal monophyletic branch along with three sequences from the 28 day time-point and eight sequences from 2 months. The first group had a low prevalence in the first two time-points after treatment, having only one quasispecies in each, but became the most prevalent at the last time point. This scenario corroborates the genetic distance results where P60 showed an increase at 5 months, probably due to the existence of quasispecies that evolved from two different lineages.

The phylogenetic tree for patient P07 shows all of the sequences from the 2 month time-point clustering as a monophyletic group, with a bootstrap of 93, indicating a recent common ancestor with a before-treatment strain (Figure SR-1, supplementary material). All samples from the 28 day time-points are clustered in two different branches together with some 5 month strains. The remaining strains from the 5 month time-point are grouped on a third outer monophyletic branch separated from all other after-treatment time-points that share a common ancestor with a before-treatment strain. These data explain why the genetic distance rises in the fifth month (Figure 1).

Phylogenetic data on patient P109 show the sequences clustered on two major branches (Figure SR-6, supplementary material). This indicates that the majority of the after-treatment population is derived from a population that is not sampled in a before-treatment time-point. Conversely, all after-treatment strains from patient P119 are derived from the before-treatment samples.

Discussion

Our study characterizes hepatitis C evolution, based on NS5A protein, throughout treatment in patients infected with the HCV genotype 3a who show different treatment outcomes.

The high mutation rate of RNA viruses, such as HCV, provides a pool of closely related variants, the quasispecies, which provide the virus with many possibilities for evading the immune system and therapy. It is interesting that in two of the non-response patients, the same quasispecies that were sampled before the treatment were also sampled during (P75) or after treatment (P145). This is unlikely to be since these samples were collected more than six months apart, and HCV is estimated to produce 10^{12} copies of its genome per day [21] with a high mutation rate, suggesting that these strains provided some advantage to the virus enabling it to survive the treatment, and were therefore selected.

In contrast, the virus circulating in end-of-treatment response patients was not initially successful in surviving the treatment, so the virus was not detected using the PCR technique. Presumably, in order to reestablish the infection, the virus replicates at low rates and at some point strains present with a better fitness to the new condition, and because they are therefore capable of evading the treatment, are selected, resulting in the relapse. Relapsing indicates that the virus managed to survive the battle against treatment and to evade the immune response, and the relapse point is the closest representation of this scenario. After the best-fitted pool of strains for the new condition has been selected, the different sequences would be expected to converge to the most favorable genetic composition for survival. This is the case for patients P109 and P119. In the distance data we observe that at the time-point where a predominant quasispecies arises, the genetic divergence between the sequences falls, even though it still has a low

frequency. It is also expected that through time the virus population would recover its variability.

The analysis of selective pressure by site showed some sites that are negatively selected in more than one patient, suggesting a functional constraint of the protein. However, as this does not apply for all patients, and one site, site 408, was both positively and negatively selected in two different patients (P109 and P20, respectively), it seems that the evolutionary processes are unique in each patient and are guided by the interaction of virus and host. Viral samples from patients who had an end-of-treatment response have more sites undergoing negative selection than samples found in non-response patients, and this difference is statistically significant. Since this group passed through a recent bottleneck due to a strong selective event (Interferon/Ribavirin administration), it probably conserved the amino acids that proved most effective in evading both treatment and the immune system, thus allowing the infection to be reestablished. It is interesting, however, that the ETR group, despite having more negatively selected sites, presented an overall ω close to that of the NR group. A possible explanation for this is that the remaining sites have more relaxed constraints.

The occurrence of stop codons at the same site in different patients, and in one case persisting through all time-points of the same patient, is quite interesting. One explanation could be that during translation the ribosome jumps or reads through the stop codon. However, this hypothesis does not explain why it occurs at the same site in a considerable number in different patients (12 out of 23). Since NS5A is a multifunctional protein and its functions are not fully understood, the proteins resulting from this RNA that encode stop codons could have a specific function during the infection, different from the one achieved by full-length NS5A. One of the known functions of NS5A is an important role in viral replication, and it has been shown that defective NS5A impairs HCV replication. Though it is also known that NS5A is the only HCV protein that can act in *trans* complementation, meaning it is acting on a viral RNA other than the one from which it has been translated [22, 23]. These characteristics make it possible that the defective genomes could persist by being encapsulated with the help of normal NS5A acting in *trans.* Other studies have shown that defective genomes circulate during HCV infection and also during other *Flavivirus* infections [24-26]. Another point to consider is that having NS5A sequences with stop codons do not seem to be disadvantageous for viral survival. No nonsense mutations were identified in

sequences from SVR patients, while 73.9% of them were found in sequences from non-responders [20]. These defective variants could give the virus some advantage and play an important role in evading the therapy response. Another issue that suggests some kind of viral fitness enhancement by these mutations is that most of the stop codons that were found at the same site in different patients were not encoded by the same codon. For example, site 111 (originally TGG) mutated into three different stop codons in three different patients, two of them changed to TGA and the other to TAG. In the case of site 166 (originally AAG), two clones from the same patient and same time-point presented two different stop codons (TAA and TGA), and in two other patients the nonsense mutation was due to TAG. This site (166) presented all three codons that stop translation. Site 386 had mutated from CAG to TAG in patient P60 at 5 months and from CAA to TAA in patient P119. These sites converged to nonsense mutations in different ways both in the same patient and in different patients, suggesting that stopping translation at these points provides some kind of evolutionary advantage to the virus.

Some insights on evolution

Viruses are microorganisms incapable of replicating, translating or assembling viral particles outside their hosts. In short, they cannot survive outside their host. This is one of the reasons why some do not consider the virus as a form of life. However, since this is a work on the evolution of a genomic region of the hepatitis C virus, and we understand that only living organisms are capable of evolving, we will use terms such as surviving, living and dying in relation to viruses in this section.

Well-adapted viruses are the ones capable of surviving in terms of population for a long time; not only surviving, but also having an efficient way of infecting, replicating and being transmitted from host to host. Viruses co-evolve with their hosts, with the virus trying to evade the host defenses at the same time as the host is trying to clear the infection. Well-adapted viruses do not kill their hosts, at least not in the short term, as by doing so they would ultimately be killing themselves and have less time to spread viral particles to other hosts. The Human Immunodeficiency Virus (HIV), for example, has adapted to infect, survive and replicate in humans, but it also can kill its host very quickly. As HIV is considered a virus of recent origin, thought to have been introduced into humans between 1884 and 1924 [27], it has probably had insufficient time to

establish the most fitted scenario through co-evolution. The hepatitis C virus, on the other hand, can be considered a successful virus in evolutionary terms. It causes a persistent infection and remains silent, presenting no clinical symptoms, for decades. Hepatitis C virus genotypes have been co-evolving with the human host for a long time; they are thought to have diverged 500 to 2000 years ago, leading to a better fitness [28].

The samples used in this study were obtained from chronically infected patients who had been through interferon and Ribavirin treatment and had not responded. These viruses not only evaded the host immune system, but also evaded therapy. What we can see in this study is that, although HCV is adapted to infect *Homo sapiens* in general, each individual specimen represents a different environment, leading to different evolutionary processes. In some cases the viral population from before treatment belongs to a separate monophyletic group from the other time-points (P60 and P82), or most samples from other time-points (P109). This means that persistence is achieved by strains that were not initially the best fitted for survival, and therefore were not sampled before the treatment started, but after a new selective environment – the treatment - was introduced the strain gained fitness. We can also find the opposite, where all after-treatment strains are derived from a unique before-treatment population (P119). In another case (P20) viral population changed by converging to a previously existing, but not predominant, population and, after evading treatment, both populations were reestablished. Finally, the after-treatment strains were all derived from before-treatment samples in some patients (P07, P75 and P145).

From this analysis we can notice that before-treatment samples from most of the non-response patients (except P60) presented a viral population that persisted after treatment, suggesting they were fitted to evade the treatment. Accordingly, most before -treatment samples from end-of-treatment response patients (except P119) either did not show or showed in a low frequency the population that was identified after the relapse. The exceptions illustrate the uniqueness of the evolutionary process, and therefore the treatment resistance process in each patient.

Conclusion

Hepatitis C virus is a highly variable virus, which confers a range of possibilities for it to evolve and adapt to new conditions. The evolution of this virus during and after treatment is linked to the environment to which it is subjected, that is the patient. Each patient has their own immune response and therefore the virus infecting each patient must find its own specific way to evade at the same time as the host is finding a way to clear the infection. The patients enrolled in this study were chronically infected with HCV, which means that the virus had already succeeded in the first step, evading the immune system, and was now facing the second one, the treatment. It seems that the viruses that resisted the treatment (non-responder samples) already had strains that could evade therapy before treatment started. No specific pattern could be found in viral strains that could determine therapy response. Finally, hepatitis C virus evolution throughout treatment appears to be the result of a unique evolutionary relationship between viral strains and each human host, leading either to persistence or to clearance.

Acknowledgements

This work was financially supported by FAPESP.

Materials and methods

Population and samples: Blood samples were collected from 12 HCV genotype 3-positive patients at the Blood Center of São José do Rio Preto, State of São Paulo, Brazil. All samples were collected before treatment, during (12 weeks or 24 weeks) and after treatment (21 days, 28 days, 2 months, 3 months, 4 months and 5 months) to provide treatment response data. Patients were classified into three groups according to treatment response: four patients were sustained virological responders (SVR), i.e. the virus was not detected after treatment or during the 6-months follow-up; four were non-responders (NR), since they showed no virological response; and four were end-of-treatment responders (ETR), i.e. a virological response was detected, but at the 6-months follow-up there was a relapse. Samples from the SVR group and before treatment samples from most patients enrolled in this study were analyzed in a previous

study and were used in this study for comparative analyses of the evolutionary behavior (**EU826189 to EU826218; EU826249 to EU826263; EU826294 to EU826307**) [20]. The exception was patient P82; before-treatment samples from patient P82 were analyzed in this work as they had not been analyzed in the previous study. All patients were infected with HCV genotype 3a. Treatment consisted of INF-α and Ribavirin administration for 24 weeks. For the non-response patients, one time-point sample was taken during treatment and three were taken after treatment. Owing to patient availability, some samples could not be collected. End-of-treatment response time-points were considered the relapse point, and all patients provided samples up to 5 months. Time-points for each patient were obtained as follows: NR (P07 – 28 days, 2 months and 5 months; P60 -28 days, 2 months and 5 months; P75 – 24 weeks, 21 days, 2 months and 5 months; P145 – 12 weeks, 28 days, 2 months and 5 months) ETR (P20 – 3 months, 4 months and 5 months; P82 – before treatment, 2 months, 3 months and 5 months; P109 - 21 days, 2 months, 3 months, 4 months and 5 months; P119 -3 months, 4 months and 5 months) (Supplementary Figure SM-1). Patients with a history of alcoholism or infection with another agent that could cause liver damage were excluded. This study was approved by the Ethics Committee of the Hospital de Base from São José do Rio Preto, and all participants signed an informed consent.

Extraction of HCV RNA and amplification of the NS5A region: Viral RNA was extracted from blood serum samples using a QIAamp Viral RNA Mini Kit (QIAgen), and cDNA was synthesized using a High-Capacity cDNA Archive Kit (Applied Biosystems) according to the manufacturer's instructions. For the PCR and NESTED-PCR reactions, three sets of forward and reverse primers for the NS5A region were designed (Table ST-2, supplementary material). The amplification mix for both PCR and NESTED-PCR reactions contained 1µl (5U) of a proofreading polymerase (Long PCR Enzyme Mix; Fermentas) together with 5µl of 10X Long PCR Buffer with $MgCl_2$, 4µl of DMSO, 1µl of dNTP (10mM), 1µl of each primer (20pmol), 10µl of synthesized cDNA for the PCR reaction and 5µl of PCR product for the NESTED-PCR reaction, plus nuclease-free water provided with the enzyme kit to a final volume of 50µl. The amplified products were analyzed on a 1% agarose gel.

Cloning and sequencing: Cloning was performed using a TOPO XL Cloning TM Kit (Invitrogen). Fragments of 15 clones at each time-point from each patient were purified

using a GeneJET Plasmid Purification Kit (Fermentas). The entire NS5A region was sequenced using eight primers: the vector primers *M13F* and *M13R*, and six inner primers: three sense and three anti-sense (Sense: *H.NS5AI-F1, H.NS5AI-F2* and *H.NS5AI-F3*, antisense: *H.NS5AI-R1*, *H.NS5AI-R2* and *H.NS5AI-R3*) [20]. The sequencing reaction was performed with BigDye Terminator (Applied Biosystems) and the products were sequenced in an ABI 3130XL sequencer (Applied Biosystems). The reaction mixture consisted of 2.2 µl of Milli-Q autoclaved water, 2µl of 5x Sequencing Buffer (Applied Biosystems), 0.8µl of primer (5pmol/µl), 2µl of Big ET Dye Terminator, plus 3µl of sample. Sequencing reactions consisted of a "hot start "step of 10 min at 95°C, followed by 96°C for 1 minute, then 25 cycles of 96°C for 15s, 50°C for 15s and 60°C for 4 min.

Sequence analysis: In order to analyze viral evolution over time, sequences from samples collected before treatment, and previously published (Accession numbers: EU826174 to EU826233 and from EU826249 to EU826352), were also used in the analyses [20]. The sequences obtained in this study were subjected to BioMol - Electropherogram quality analysis (http://adenina.biomol.unb.br/phph/) [29], a phred phrap [30, 31] analysis site, for quality checking and contig construction. The contigs obtained for each clone were aligned, along with the reference sequence NZL1 for genotype 3 (GenBank accession number D17763), using *Clustal W* software nested in the BioEdit 7.0.9.0 package [32, 33]. All sequences were edited on *Bio Edit* [32] to remove the vector fragments, leaving only the complete sequence of the NS5A region.

Evolutionary analysis

Quasispecies were analyzed using *LOCSPEQ 1.0* software [34]. The genetic distances were calculated using MEGA 5.0 software [35]. The overall dN/dS ratio (ω) and ω of each site were calculated with HyPhy [36]. Bayesian skyride plots were performed using the BEAST package [37]. Phylogenetic analysis was performed using *PhyML* [38]. A Maximum-Likelihood tree was constructed using the HKY85 substitution model including a Gamma distribution parameter (HKY+G). Bootstrap was performed with 500 replicates for the tree containing all the sequences and 1000 replicates for individual trees from each patient. Values above 70% were considered significant.

Statistics: Statistical analyses for the distance and ω data were performed by Mann-Whitney tests, and a Chi-square test was used for negatively selected sites. p-values lower than 0.005 were considered significant.

Author's Contributions

CB, ACGJ, IMVGCM , PR: contributed to the study design, carried out the molecular biology experiments, sequence alignment, evolutionary analysis and manuscript design.

LHTY: carried out the molecular biology experiments and statistical analysis.

CMAC: contributed with significant evolutionary knowledge on data analysis.

PL: carried out evolutionary analysis.

JRRP: contributed to the study design.

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Figures

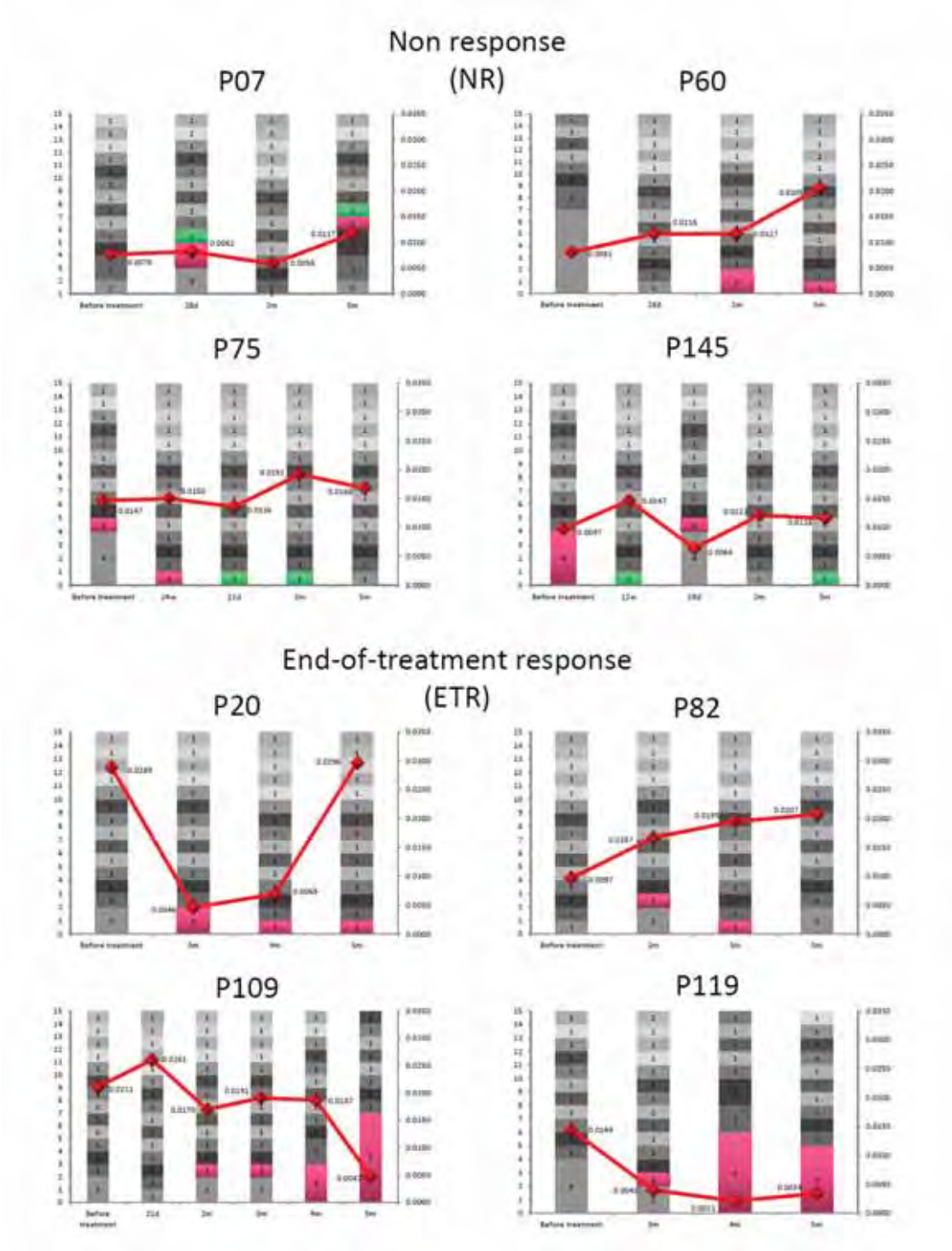


Figure 1: Schematic representation of distance versus quasispecies**.** Left Y axis - graphic representation of different amino acid quasispecies. Tiles in shades of gray represent sequences that were sampled in only one time-point. Colored tiles represent the same quasispecies in the same or different time-points. Right Y axis - genetic distance data. W-weeks; d- days; m-months

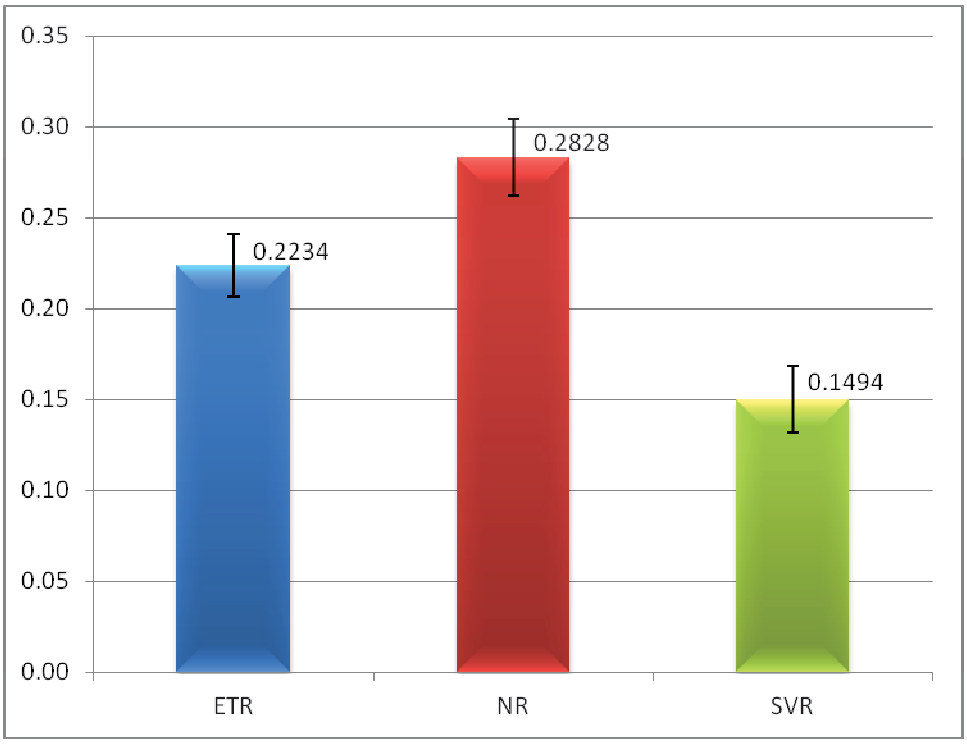


Figure 2: Overall ω of each response group. ETR: end-of-treatment response; NR: non-response; SVR: sustained virological response

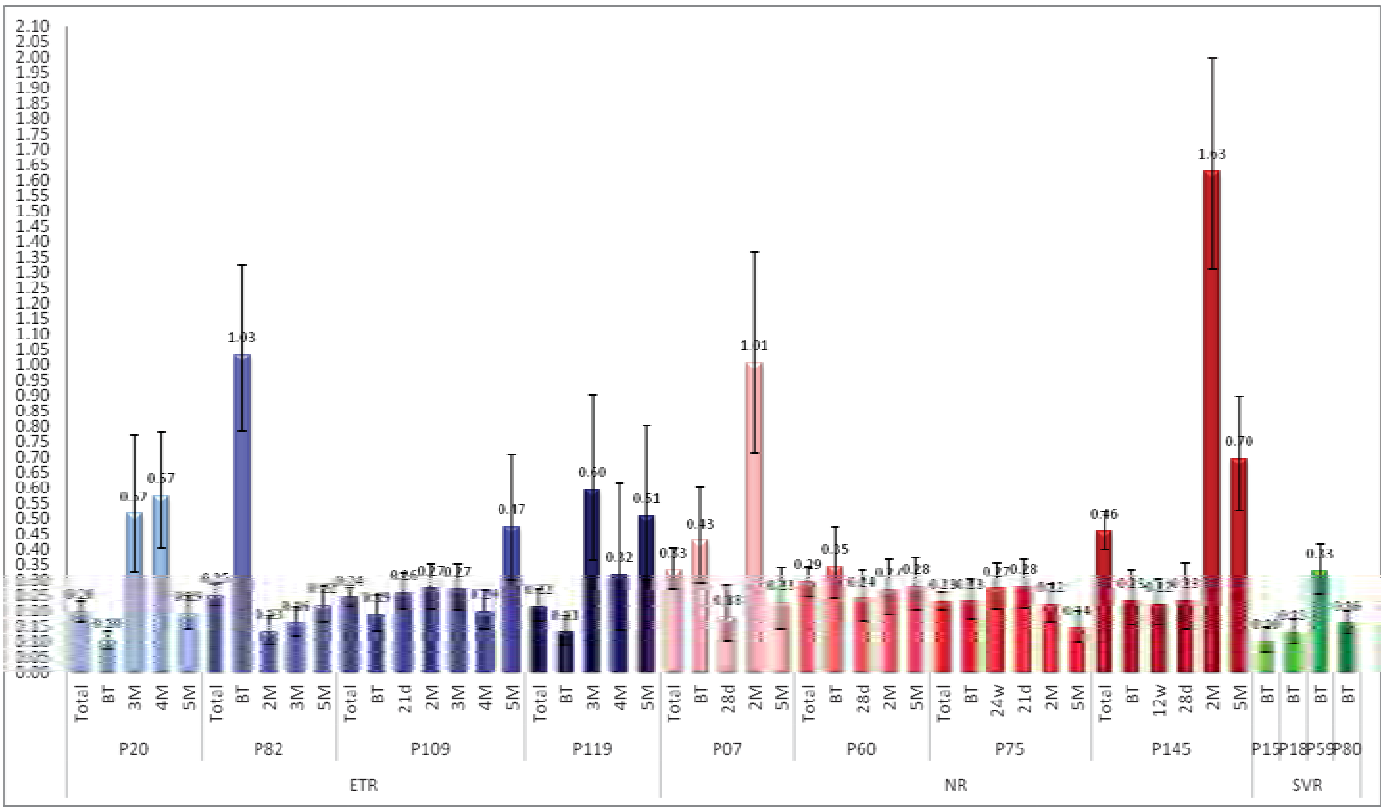
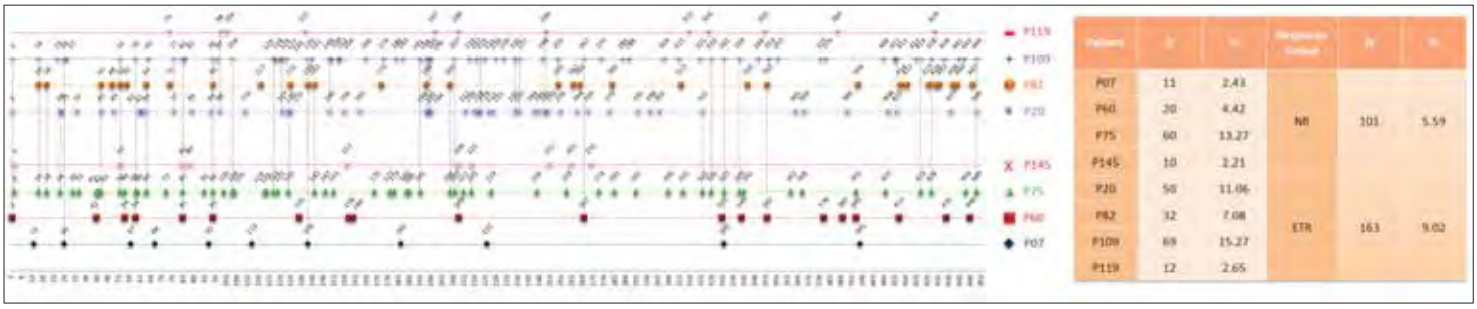
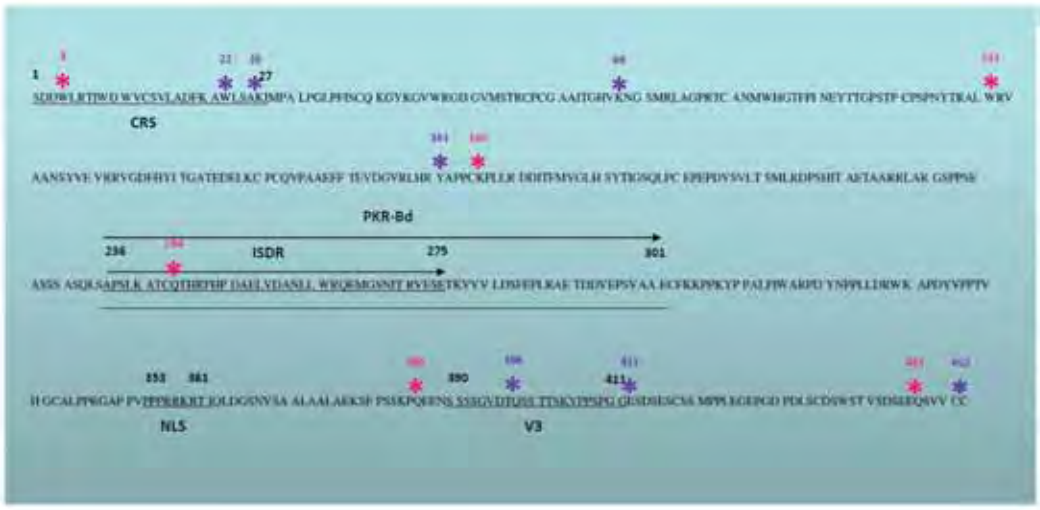


Figure 3: ω rates for each patient (Total) and for each time point. BT-Before treatment; w-weeks; d-days; m-months.



Patient	N	%	Response Cohort	N	%
P07	11	2.43			
P60	20	4.42	NR	101	5.59
P75	60	13.27			
P145	10	2.21			
P20	50	11.06			
P82	32	7.08	ETR	163	9.02
P109	69	15.27			
P119	12	2.65			

Figure 4: Sites under negative selection for each patient. Measured by ω rates considering p<0.1. Vertical lines link sites negatively selected in more than one patient.



4	P145	Before treatment	TGG/TGA
		12 weeks	TGG/TGA
22	P145	5 months	TGG/TAG
26	P145	2 months	AAG/TAG
98	P145	2 months	AAG/TAG
	P07	2 months	TGG/TGA
111	P60	5 months	TGG/TGA
	P109	21 days	TGG/TAG
181	P82	5 months	TAC/TAA
	P60	Before treatment (2 clones)	AAG/TAA AAG/TGA
186	P75	5 months	AAG/TAG
	P145	Before treatment	AAG/TAG
244	P82	2 months	CAG/TAG
309	P60	5 months	CAG/TAG
	P110	3 months	CAA/TAA
366	P75	24 weeks	CAG/TAG
412	P145	2 months	GAG/TAG
	P07	Before treatment	CAG/TAG
447	P75	2 months	CAG/TAG
	P109	Before treatment	CAG/TAG

Figure 5: Graphic representation of sites where stop codons were identified. Pink – more than one occurrence; purple-one occurrence. NZL1 (GenBank accession number D17763) – reference sequence for genotype 3 was used for the graphic representation. CRS – cytoplasmic retention site; PKR-Bd – PKR binding region; ISDR – IFN sensitivity determining region; NLS – nuclear localization signal; V3 – variable region 3.

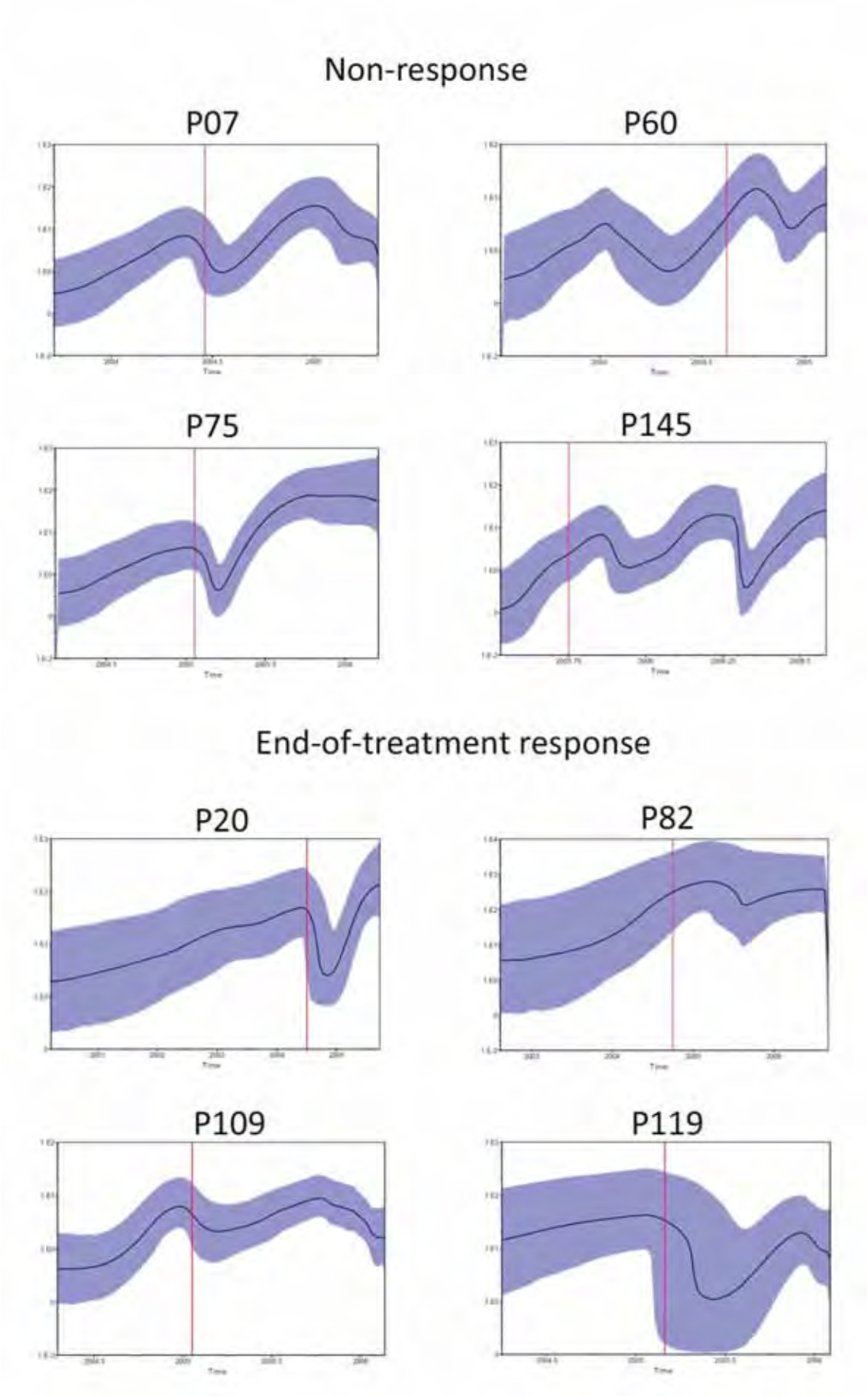


Figure 6: Bayesian Skyride plots showing the relative genetic diversity. Black solid lines represent the median posterior distribution, blue shaded areas are the 95% Bayesian credible intervals, vertical red lines represent the approximate time at which the treatment started (BT time-point).

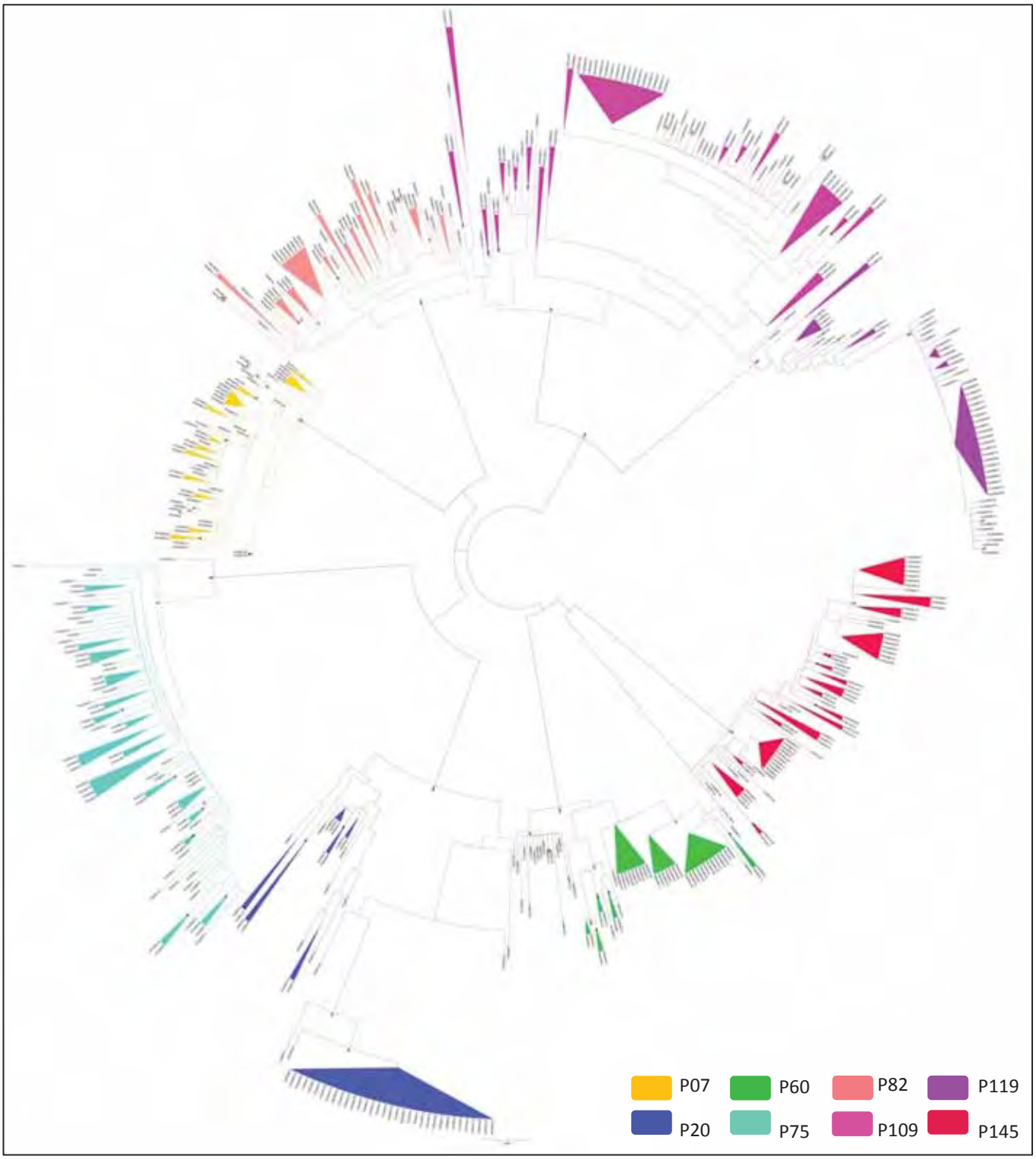


Figure 7: Unrooted Maximum-Likelihood phylogenetic tree was constructed with HKY85 substitution model including Gamma distribution parameter (HKY+G). Bootstrap was performed with 500 replicates. Values above 70% were considered significant.

Supplementary material

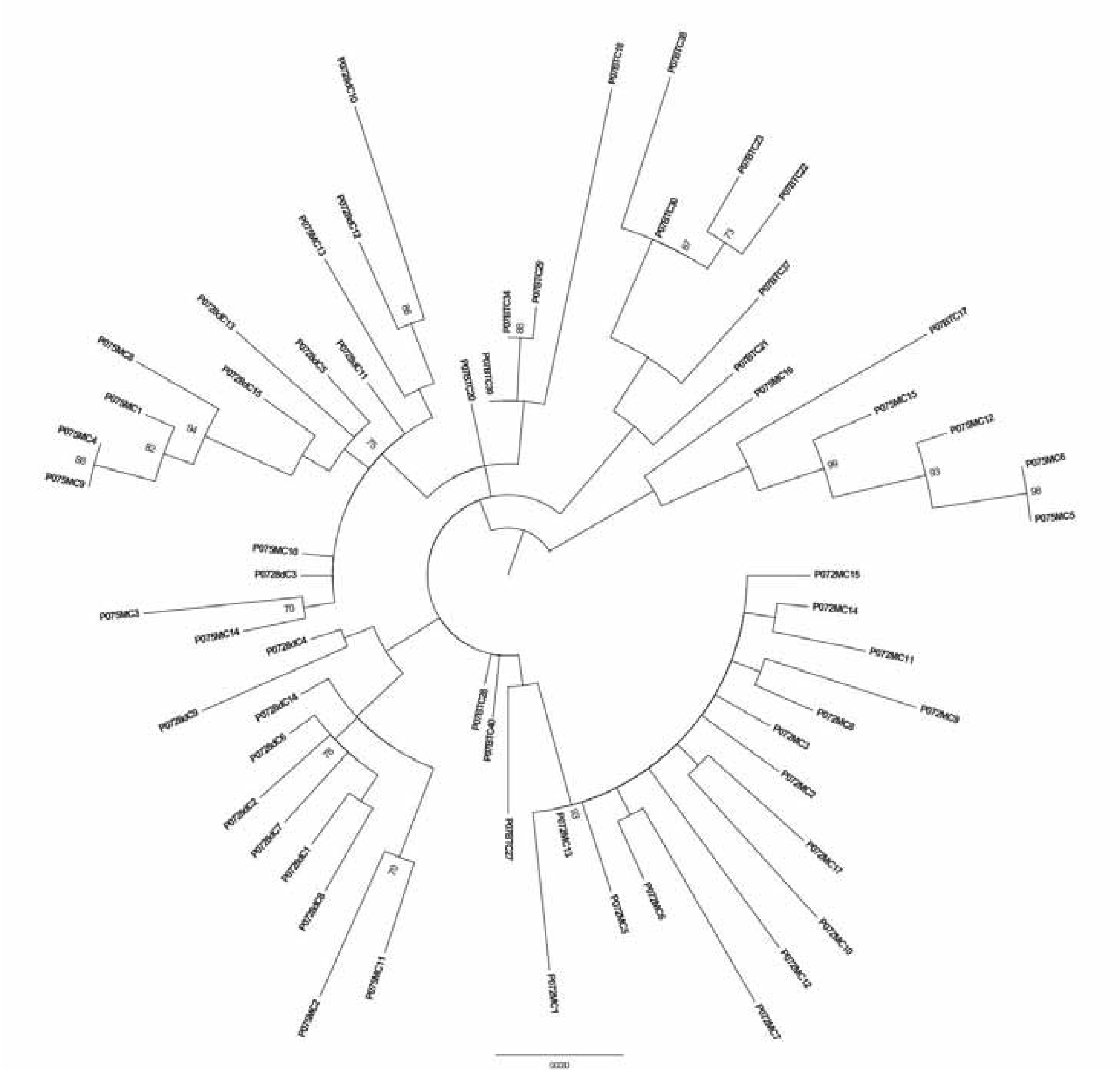


Figure SR-1: P07 Unrooted Maximum-Likelihood phylogenetic tree was constructed with HKY85 substitution model including Gamma distribution parameter (HKY+G). Bootstrap was performed with 1000 replicates. Values above 70% were considered significant. BT - Before treatment; w - weeks; d - days; m - months.

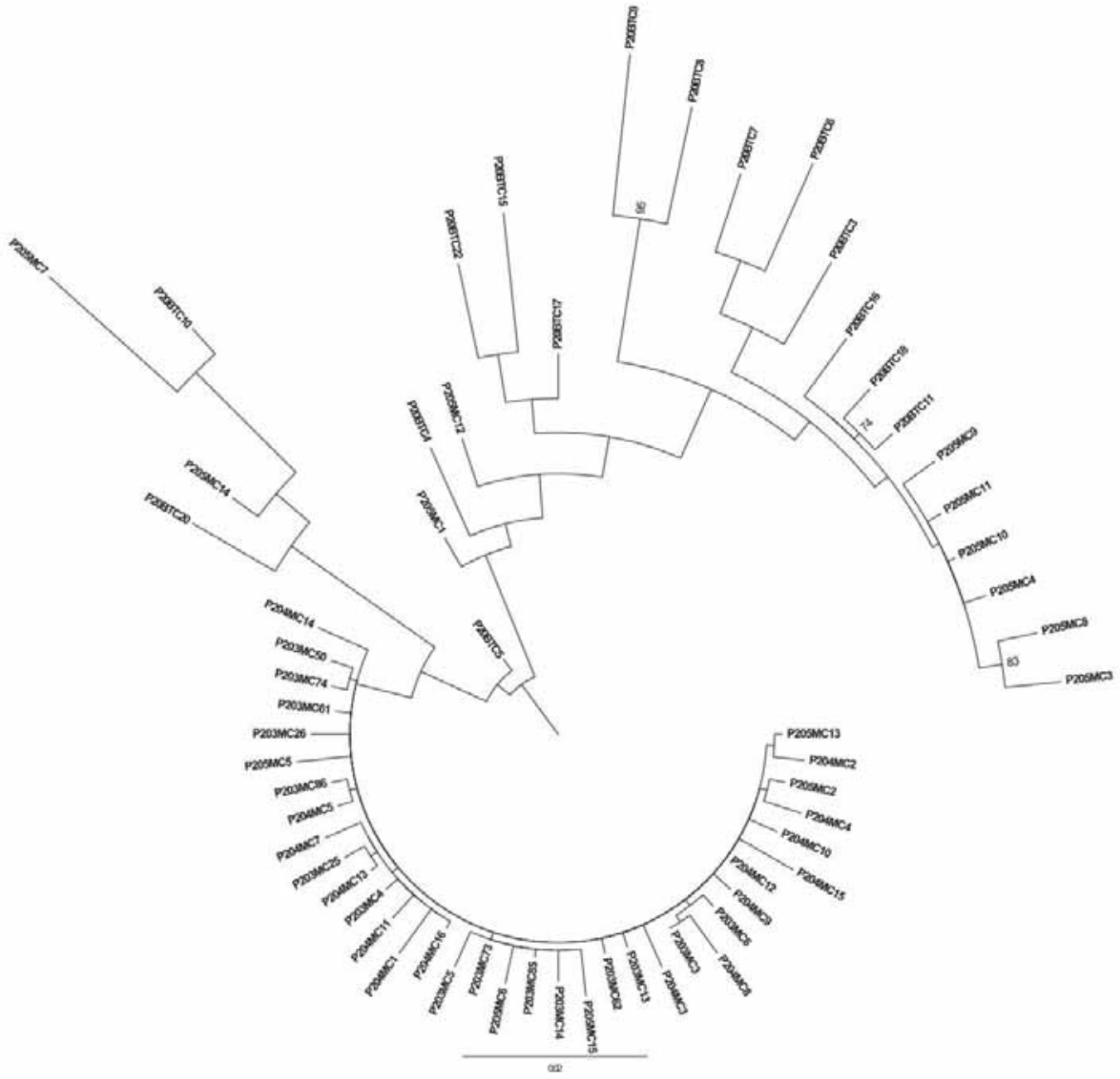


Figure SR-2: P20 Unrooted Maximum-Likelihood phylogenetic tree was constructed with HKY85 substitution model including Gamma distribution parameter (HKY+G). Bootstrap was performed with 1000 replicates. Values above 70% were considered significant. BT - Before treatment; w - weeks; d - days; m - months.

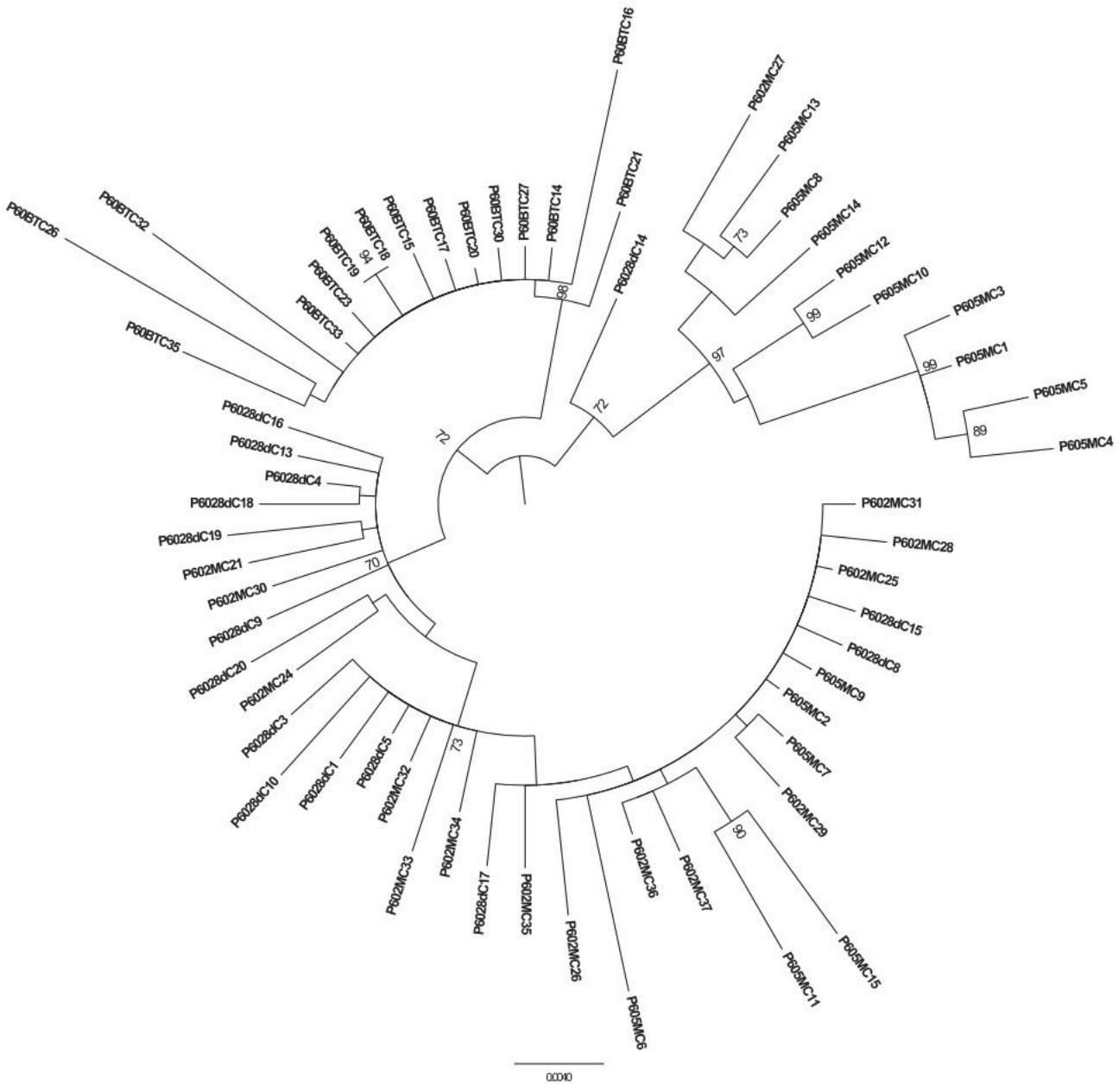


Figure SR-3: P60 Unrooted Maximum-Likelihood phylogenetic tree was constructed with HKY85 substitution model including Gamma distribution parameter (HKY+G). Bootstrap was performed with 1000 replicates. Values above 70% were considered significant. BT - Before treatment; w - weeks; d - days; m - months.

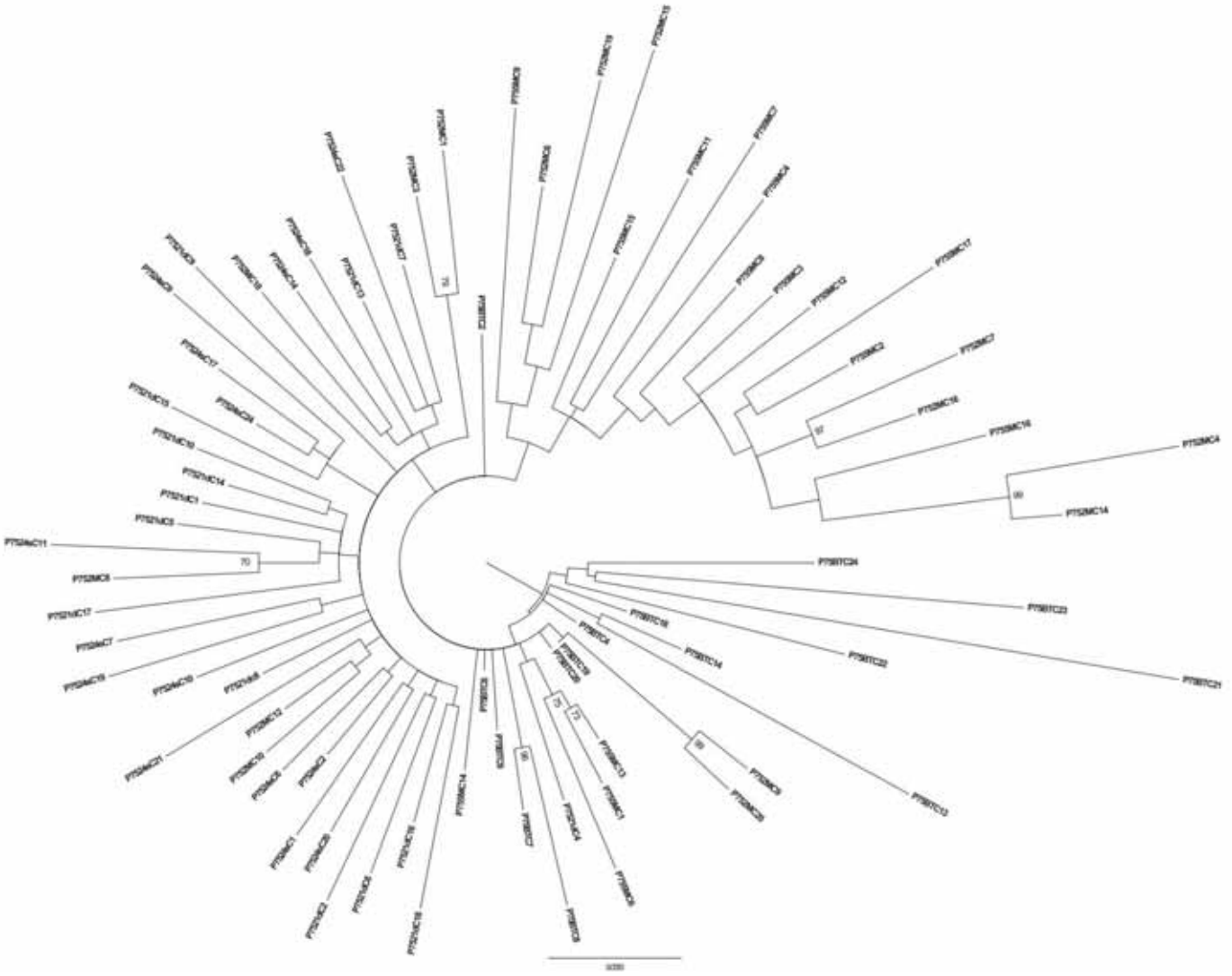


Figure SR-4: P75 Unrooted Maximum-Likelihood phylogenetic tree was constructed with HKY85 substitution model including Gamma distribution parameter (HKY+G). Bootstrap was performed with 1000 replicates. Values above 70% were considered significant. BT - Before treatment; w - weeks; d - days; m - months.

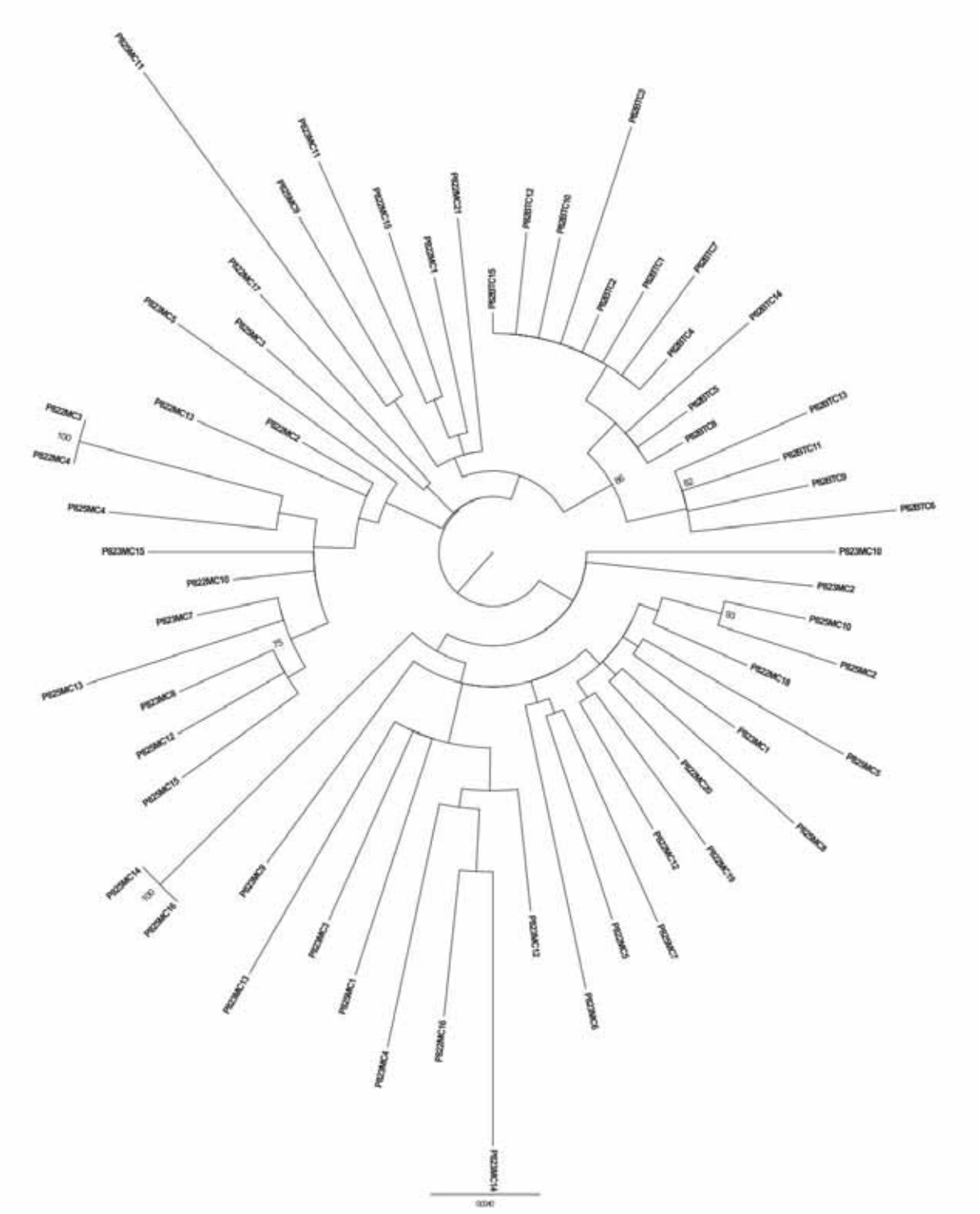


Figure SR-5: P82 Unrooted Maximum-Likelihood phylogenetic tree was constructed with HKY85 substitution model including Gamma distribution parameter (HKY+G). Bootstrap was performed with 1000 replicates. Values above 70% were considered significant. BT - Before treatment; w - weeks; d - days; m - months.

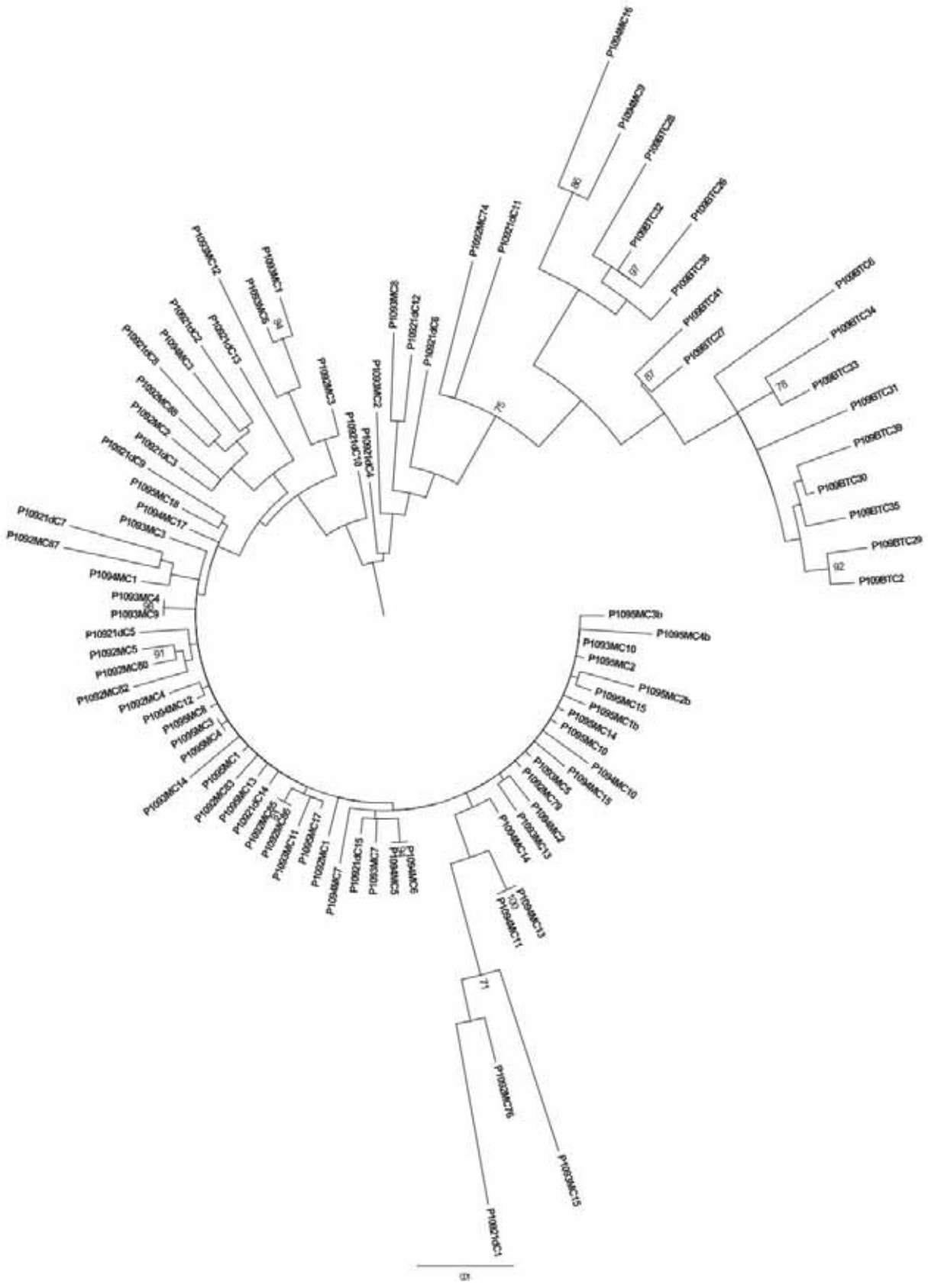


Figure SR-6 P109 Unrooted Maximum-Likelihood phylogenetic tree was constructed with HKY85 substitution model including Gamma distribution parameter (HKY+G). Bootstrap was performed with 1000 replicates. Values above 70% were considered significant. BT - Before treatment; w - weeks; d - days; m - months.

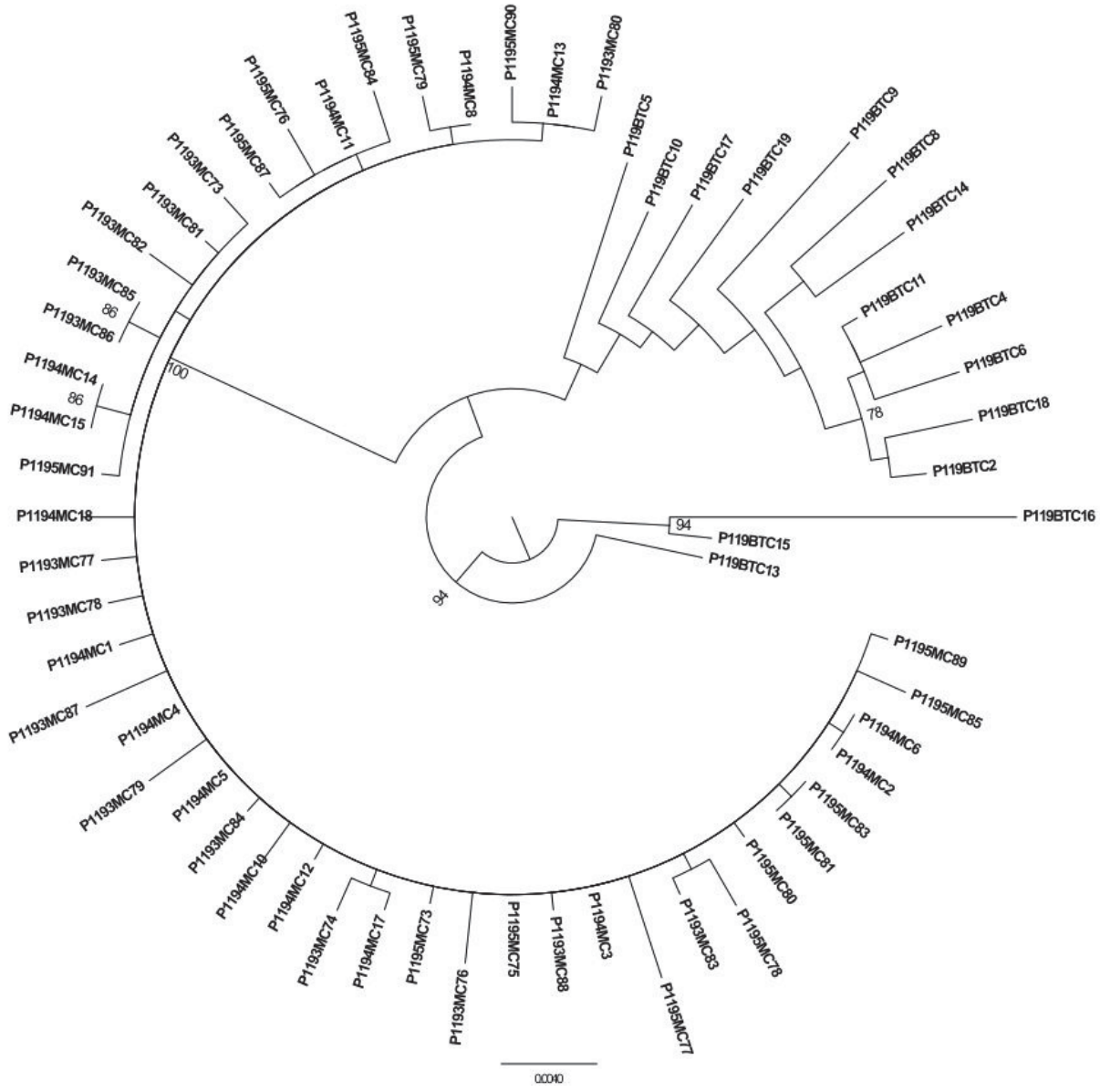


Figure SR-7: P119 Unrooted Maximum-Likelihood phylogenetic tree was constructed with HKY85 substitution model including Gamma distribution parameter (HKY+G). Bootstrap was performed with 1000 replicates. Values above 70% were considered significant. BT - Before treatment; w - weeks; d - days; m - months.

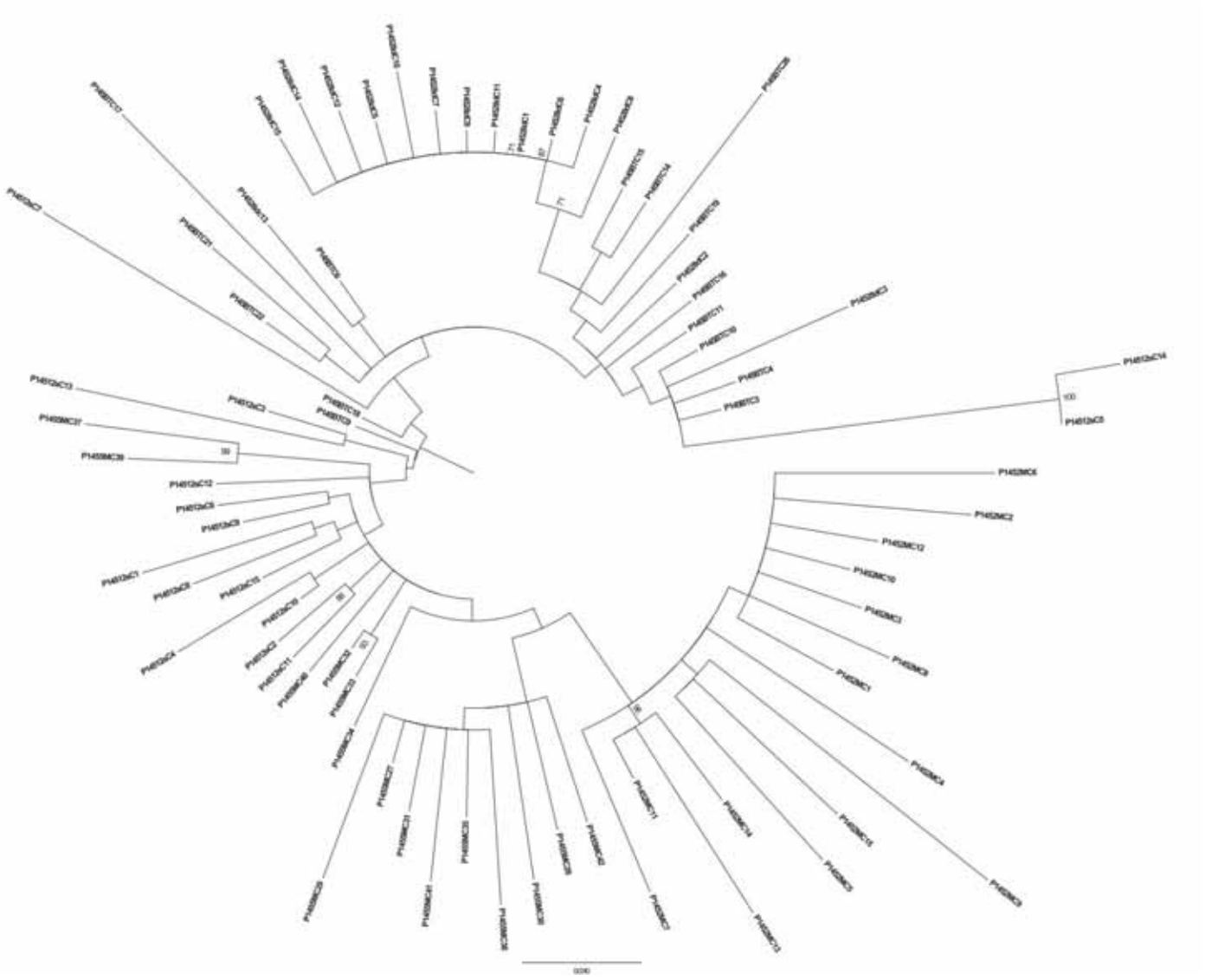


Figure SR-8: P145 Unrooted Maximum-Likelihood phylogenetic tree was constructed with HKY85 substitution model including Gamma distribution parameter (HKY+G). Bootstrap was performed with 1000 replicates. Values above 70% were considered significant. BT - Before treatment; w - weeks; d - days; m - months.

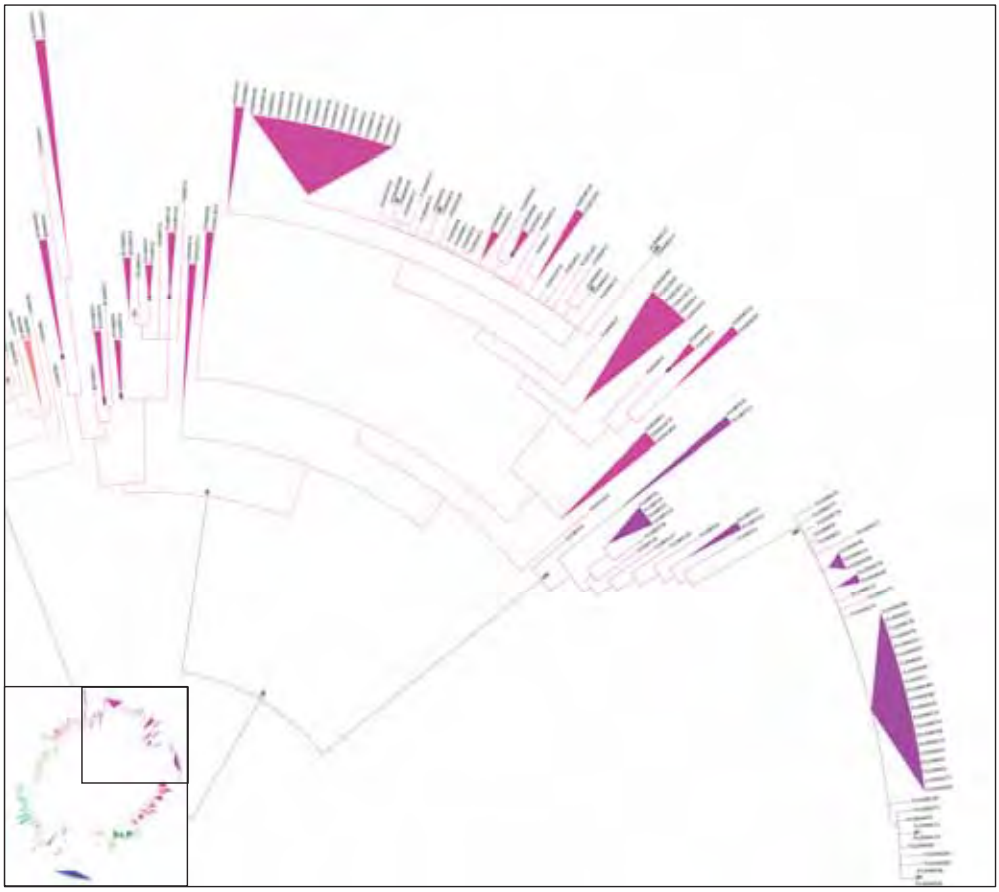


Figure SR-9: Amplification of the branch from patients P109 and P119 of the phylogenetic tree presented in Figure 8. Amplified region is represented on the left lower side.

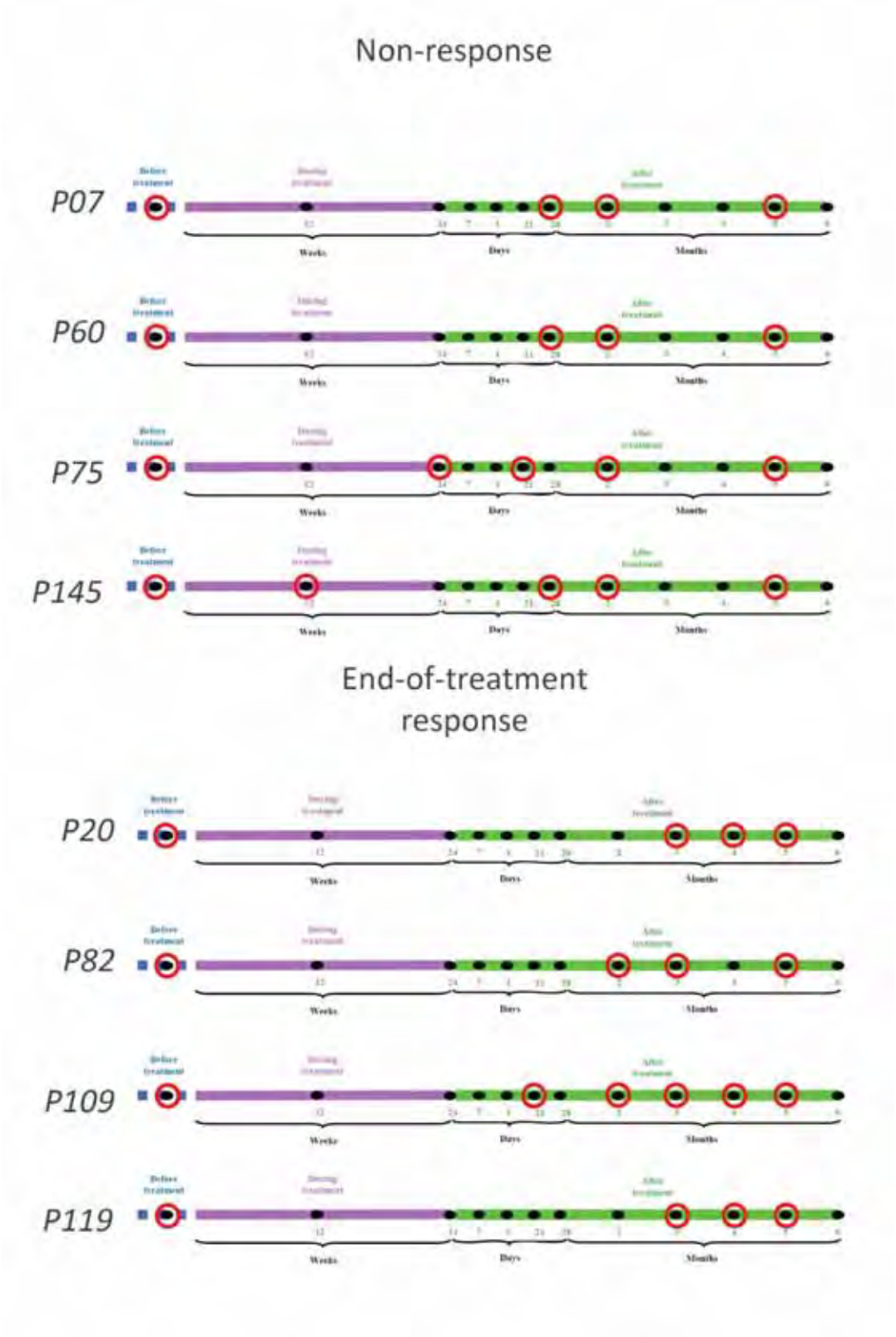


Figure SM-1: Graphic representation of the time-points used for treatment follow-up. The red circles represent the time-points when the sequences were used for the analysis from this study. Before treatment sequences from P07, P20, P60, P75, P109, P119, P145 were from a previous study detailed in Bittar *et al* (2010).

Non response (NR)

A.

P07		BT	28d	2M
	BT			
	28d	0.6628		
	2M	0.0000	0.0000	
	5M	0.0000	0.0000	0.0000

B.

P60		BT	28d	2M
	BT			
	28d	0.0000		
	2M	0.0000	0.3618	
	5M	0.0000	0.0000	0.0000

C.

P75		BT	24s	21d	2M
	BT				
	24s	0.1870			
	21d	0.6558	0.0000		
	2M	0.0000	0.0000	0.0000	
	5M	0.0068	0.0003	0.0000	0.0003

D.

P145		BT	12s	28d	2M
	BT				
	12s	0.0000			
	28d	0.0000	0.0000		
	2M	0.0000	0.0196	0.0000	
	5M	0.0005	0.0027	0.0000	0.2595

End-of-treatment response (ETR)

E.

P20		BT	3M	4M
	BT			
	3M	0.0000		
	4M	0.0000	0.0000	
	5M	0.4333	0.0000	0.0000

F.

P82		BT	2M	3M
	BT			
	2M	0.0000		
	3M	0.0000	0.0000	
	5M	0.0000	0.0000	0.0199

G.

P109		BT	21d	2M	3M	4M
	BT					
	21d	0.0001				
	2M	0.0025	0.0000			
	3M	0.3221	0.0001	0.1184		
	4M	0.0001	0.0000	0.5671	0.3030	
	5M	0.0000	0.0000	0.0000	0,0000	0.0000

H.

P119		BT	3M	4M
	BT			
	3M	0.0000		
	4M	0.0000	0.0000	
	5M	0.0000	0.0030	0.0000

Table ST-1: Mann-Whitney statistical test on distances between each time-point. A. P07; B. P60; C P75; D. P145; E. P20; F. P82; G. P109; H. P119. In red differences that were not significant (significance $p<0.005$).

Set	Reaction	Name	Sequence (5' – 3')	Nucleotide position in NZL1 (GenBank D17763G)
1*	PCR	*H.NS5AP-F*	GAGCGGTACAGTGGATGAAC	6089 to 6108
		H.NS5AP-R	CCTCCTTTAATGCAGTCTTG	7821 to 7840
	Nested-PCR	*H.NS5AN-F*	CGCATTGCTGAGTTCTCTAAC	6206 to 6226
		H.NS5AN-R	CAACAAGGAGTTGCTGAGTG	7703 to 7722
2*	PCR	*H.NS5AP-F2*	GGTACAGTGGATGAACAGG	6093 to 6111
		H.NS5AP-R2	ACGACGTTGAATAGACTAGG	7734 to 7753
	Nested-PCR	*H.NS5AN-F2*	CTCTAACTGTCACAAGTCTGC	6206 to 6226
		H.NS5AN-R2	CAGCACTACATGGTGTTATC	7659 to 7678
3	PCR	*H.NS5AP-F4*	TTGCCCGCCATACTATCT	6004 to 6021
		HNS5AP-R4	TTTACCTCCTKTAATRCA	7827 to 7844
	Nested-PCR	*H.NS5AN-F4*	GTVCAGTGGATGAACAG	6094 to 6110
		H.NS5AN-R4	AGGTAACCTTCYTCTGAC	7772 to 7789

Table ST-2: Primers used on PCR and NESTED-PCR reactions.

* published in Bittar *et al* (2010).

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ANEXO

1. COMPROVANTE DE SUBMISSÃO EM REVISTA CIENTÍFICA

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