Molecular genetic insights into Varicella zoster virus (VZV), the vOka vaccine strain and the pathogenesis of latency and reactivation

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Summary

This review describes how molecular genetic tools have been used to understand the molecular epidemiology, evolution and pathogenesis of VZV and its live attenuated vOka vaccine strain.

Key Words

VZV molecular epidemiology, vOka vaccine, Genetics

Footnote page

Conflicts of Interest

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Background: Genetic tools for molecular typing of VZV have been used to understand the spread of virus, to differentiate wild type and vaccine strains and to understand the natural history of VZV infection in its cognate host.

Results: Molecular genetics has identified seven clades of VZV (1-7) with two more mooted. Differences between the vOka vaccine strain and wild type VZV viruses have been used to distinguish the cause of post-immunisation events and to provide insight into the natural history of VZV infections. Importantly molecular genetics has shown that reinfection with establishment of latency by the re-infecting strain is common, that dual infections with different viruses can occur, and reactivation of the superinfecting genotype can both occur. Whole genome sequencing of the vOka vaccine has been used to show that vesicles form from a single virion, that latency is established within a few days of inoculation and that all vaccine strains are capable of establishing latency and reactivating. Novel molecular tools have characterised the transcripts expressed during latent infection in vitro

Conclusions: molecular genetic tools have provided novel insights into the spead and pathogenesis of VZV and its vaccine particularly in its natural host.

Background

Varicella zoster virus (VZV) with a genome of approximately 125,000 base pairs and 71 open reading frames is the smallest of the human herpesviruses and the first to be fully sequenced [1]. The virus is known to encode at least seven clades, designated 1-5 at an international meeting in 2010[2] with a further two confirmed[3-5] and two more proposed but awaiting confirmation[5]. Early analyses using restriction enzyme analysis noted that variation between unrelated VZV strains was less than between Herpes Simplex virus (HSV) strains [6]. In 1995 Takayama and colleagues used long PCR to amplify fragments of DNA from 6.8 to 11.4kb from 40 Japanese viruses [7]. Restriction digests of the fragments which covered ORFs 12-16, 38-43 and 54-60 were generated with 10 enzymes yielding 12 variable

restriction sites. Comparison with the only full length sequence then available, the Dumas strain showed a total of 28 substitutions in 65,000 nucleotide residues analysed, giving an estimated inter-strain variation of 0.043% or one base in 2,300[8]. Our data from heteroduplex mobility assays of 37 fragments across 10 VZV genomes found 92 nucleotide substitutions compared with the Dumas, in 15059 nucleotides giving a variation of 0.061% or 1 in every 1637 nucleotides [8]. Both of these estimates were considerably lower than those for herpes simplex 0.32-0.81%, CMV 2.5%, HHV8 1.5-2% and PRV 2-3% [8].

Several groups working in this area used single nucleotide polymorphisms (SNPs) and whole genome sequencing to identify distinct VZV clades [8-10] and in 2010 an international meeting agreed a common terminology for these[2]. Clades 1-5 were defined from the start while single representatives of two putative new clades were also identified and designated by the Roman numerals VI and VII until further examples of these clades could be found. Since then whole genome sequencing has confirmed Clade 6[3], uncovered two further clades VIII and IX[5] and most recently confirmed clade 9 [4].These clades can be distinguished by a new SNP typing schema [4] which replaces the original first proposed in 2010[2]. However, the recent development of high throughput next generation sequencing methods that can generate whole VZV genomes from the low amounts of DNA found in clinical samples, are proving as cheap and tractable for VZV genotyping, especially if the variable regions are not required[11] . With whole genome sequencing a maximum variation of 0.2% (1 in 1000 bases), excluding variable regions, between Clades 5 and 2 [12] has been observed in one study, while in another study Clades 1 and 2 were the most divergent [12, 13]. In addition intra-clade diversity has been calculated to be 0.03-0.07% or 1 in 1429-1 in 3333 bases, using sequences from 8 clade 1 viruses[13]. Whole genome sequencing also confirms early experiments showing recombination between different strains[3, 14]

Molecular Epidemiology of VZV

Before the advent of routine whole genome sequencing SNP markers that distinguished between clades fuelled extensive studies of VZV molecular epidemiology. Much of this work was driven by the need to distinguish between the vOka vaccine strain and circulating wild type viruses. Two restriction sites a *Bgl*1 site in ORF 54 and a *Pst 1* site present in ORF 38 can be used to distinguish Japanese derived vaccine and wild type strains from US wild type strains [15] Adams et al. 1989; [16], although these clade based markers will not distinguish wildtype clade 2 from vaccine strains which are also clade 2. We used the SNPs delineating these sites to survey over 240 strains of varicella and zoster collected from our local population [17]. In common with the findings by La Russa that 3 of 20 wild type US strains were *Bgl*1 positive[16], we found 20 of 244 (8%) viruses circulating in London to be *Bgl*1 positive[16, 17]. All were *Pst* 1 positive. Two clear messages emerged from this. First that UK wild type strains, could be distinguished in the same way as US viruses from the Japanese vOka vaccine, a finding of public health utility should the vaccine be introduced into the UK [18]. The development of more extensive SNP based assays enabled us and others to demonstrate the geographical segregation of clades. Clades 1 and 3 predominated in Europe and in countries which had experienced significant European immigration, clade 5 predominated in Africa and in countries with had African migration, clade 2 predominated in Japan and the far east and clade 4 was present in Asia. Clade 6 has since been found in Europe [3]. Making use of the finding that clades 1 and 3 were negative for the *Bgl 1* restriction site in ORF 54 while clades while clades 2, 4 and 5 were *Bgl1* positive, we carried out several studies of VZV epidemiology. On the premise that the virus isolated from a patient with zoster is identical to the original infecting strain of varicella we showed that among the cases of zoster *Bgl*1 positive viruses were significantly more common ($p<0.05$) in subjects who had immigrated from the Africa, India, Asia and the Far East than in subjects

who had grown up in the UK [17]. In contrast we found no strong association of *Bgl* 1 positive varicella viruses with immigrants. Instead there was a significant rise ($p<0.001$)from 5 to 40% over 25 years in the prevalence of *Bgl1* positive varicella cases, suggesting that *Bgl* 1positive viruses had been introduced with immigration and were now spreading in the UK[17] . Although limited in its scope, this study did provide the first indication that *Bgl* 1 and *Pst1* genotyping, especially in view of its simplicity and low cost, were useful for investigating questions about VZV strain distribution, spread and possibly even pathogenesis. Further analysis of viruses from around the world confirmed the *Bgl*1 positive genotype as being present in 100% of 100 viruses collected from different countries in Africa, the Far East and the Indian subcontinent, but accounting for fewer than 20% of viruses collected in Europe, the USA and other countries settled by Europeans[19]. A prospective study of over 400 patients presenting with clinical zoster, allowed us to type VZV in 200 white British born subjects aged from 5 to 98 years [19, 20]. Again assuming their zoster virus was identical to the virus which first caused varicella and that, having grown up in the UK, their varicella occurred aged ≤ 10 years of age, we showed that *Bgl* negative viruses (Clades 1 and 3) accounted for 80-90% of viruses circulating in London over the past 100 years in London [20]. From this we concluded that the data obtained from opportunistic sampling of varicella and zoster viruses, which is the basis of most molecular epidemiological studies carried out to date provides an reasonably accurate picture of VZV strain prevalence across the world [20]. Moreover, out data confirms that Bgl 1 genotyping can be used to distinguish strains of European (*Bgl* 1 negative) and non-European origin (*Bgl* 1 positive) and thus provides an easy tool for molecular epidemiology [20, 21]. These simple tools, now used by many, support the geographical distribution of VZV clades and provide further confirmation that *Bgl1* positive African and Asian Clade 4 and 5 strains are spreading in countries with European populations [17, 20, 22-24].

In contrast very few European Clade 1 and 3 viruses and no Japanese Clade 2 viruses have been found in surveys of over 100 strains from African countries (Guinea Bissau, Zambia, Sudan, Chad, and Democratic Republic of Congo) [10, 19, 25]. Two cases of Clade 1 viruses which were genotyped as part of an outbreak in Guinea Bissau, were in fact imported by children returning from Europe [26]. Despite the presence of many household members who later acquired a local African strain of VZV, both the imported European strains spread only to a single household contact. A few European Clade 3 viruses causing atypical palmar and plantar lesions, which were confused clinically with monkeypox, have also been found in the Congo [27] and one Clade 3 virus has been opportunistically identified in Sudan, again as part of genotyping of a monkeypox outbreak [28] . Nonetheless, the impression is that non-clade 5 viruses have not spread in Africa. Molecular genotyping of viruses in an outbreak occurring in Guinea Bissau, has confirmed that VZV transmission generally is impaired compared to outbreaks in temperate climates but can be overcome to some extent by high density living and larger family size[26]. While environmental factors are thought to be of most importance, the possibility that strain differences are responsible cannot be excluded Further studies would be informative, for example in areas of large scale European settlement such as South Africa.

Molecular Genetics and VZV pathogenesis

Molecular epidemiological tools have also provided insights into VZV pathogenesis. Restriction enzyme analyses to genotype varicella and zoster viruses from the same patient proved Hope Simpson's hypothesis that zoster is due to reactivation of latent VZV originally acquired from chickenpox [29]. This finding was confirmed in a further three patients [30]. All these patients were immunosuppressed with primary varicella and zoster occurring close

together. In 2006 we made use of SNP genotyping to investigate a case of recurrent zoster in an immunocompetent man. This young man, who gave a history of one episode of varicella aged 5years old, presented as an adult with two episodes of zoster three years apart, the first time in the ocular division of the Left trigeminal nerve and the second in the left thoracic region [31]. Gentoyping showed the first strain to be a clade 5 and the second to be a clade 4 strain. Microsatellite typing of the human DNA proved the host to be the identical for both cases, thus ruling out laboratory contamination. This suggested that infection with two different viruses had occurred and that both had established latency and reactivated. The result overturned previous dogma that HZ is always caused by the strain of virus that caused primary varicella. Furthermore, the data corroborated findings from earlier studies which, using *Bgl* 1 genotyping, had shown that zoster due to *Bgl 1* negative viruses(Clades 1 and 3) occurred in 30% of adults who had immigrated to the UK from areas where *Bgl 1*positive (Clade 2/4/5)viruses circulate [19]. In none of these adults had there been a history of varicella in the UK. Together with studies of vOka vaccine immunised healthcare workers who developed proven wild type virus, having seroconverted to vaccine[32]. These confirm that asymptomatic reinfection of latently infected individuals is part of VZV natural history, with establishment of latency plus reactivation, at least in some cases, by the second strain. These findings have been further corroborated by studies showing that 86% of immunised children have wild type VZV within their enteric neurons without a history of varicella [33]. Importantly, most reinfections are asymptomatic with no evidence of skin rash, supporting the likelihood that haematogenous transfer of virus to ganglia is possible[34].

The evidence that VZV is highly recombinant provides conclusive evidence that co infections must be occurring although where in the body the process of viral recombination takes place remains a matter for conjecture [35] [3, 12]. Intriguingly genotyping of viruses from varicella outbreaks in the UK and Guinea Bissau has shown that multiple strains co circulate

within a single outbreak. In the first outbreak these are of different clades but in the latter there were distinct Clade 5 strains [36, 37]. We have also demonstrated co- infection with two different viruses within the same individual [38]. In that study, limiting dilution of PCR product allowed genotyping of single molecules, providing evidence that a swab of vesicle fluid from a child with chickenpox contained Clade 1and Clade 3viruses in a ratio of 3:1.

Molecular epidemiology of the vaccine

Where molecular genetic tools have been most extensively applied is in distinguishing the live attenuated vOka vaccine strain from circulating wild type strains. This is particularly important as 2-5% of vaccinees can develop rashes or other complications following vOka immunisation. The vOka vaccine was developed in Japan in 1974 to reduce severe or fatal complications of varicella among immunocompromised children [39]. The vaccine, named after the child from whom the original virus (known as parental or pOKa) had been obtained, was attenuated through serial passage of the isolate in guinea pig embryo cultures, with the attenuated virus then propagated in human diploid cells (WI-38)[40, 41]. vOka is attenuated for replication in skin but less so in other target tissues such as T cells and trigeminal ganglia[42, 43]. Live attenuated vOka (accession number AB097932) comprises a mixture of VZV genotypes[44] and by deep sequencing of multiple batches has been shown to differ from wild-type Oka strains by at least 224 SNPs [45]. Which of these is primarily responsible for attenuating the virulence of VZV in live vaccines remains unclear, but vaccine mutations at positions 106262, 107252 and 108111 in ORF62 are believed to play key roles[46]. Although highly prevalent in vOka vaccine and in rashes caused by the vaccine, these positions have been shown not to be fixed with up to 6% of clones from the vaccine testing positive for the wild type nucleotide at these positions[47]. A further 11 positions have been shown to be significantly more likely to be wild type in vaccine viruses recovered from post

immunisation rashes, encephalitis and retinitis, suggesting a role for the vaccine allele in attenuating replication in these tissues [45]. Of these, a stop codon at position 560 in ORF0 has been shown to reduce VZV replication in epithelial xenografts in SCID-hu mice[48], while a substitution of lysine for proline at position 446 in a transactivating region of ORF 62 is always wild type in rashes (p 7.25×10^{-11}) suggesting a critical role in recovery of replicative ability in skin[45, 49]. Both pOka and vOka are, like many Japanese viruses, of clade 2 origin and are positive for the *Bgl1* and *Pst1* restriction sites. Genotyping of viruses recovered following clinical events in patients who have been vaccinated with the vOka strain using the *Bgl 1, Pst1* and the vaccine SNPs 106262, 107252 and 108111 which are at near fixation, has been carried out for post surveillance studies of varicella and zoster vaccines. Using these data it has been shown that vaccine associated rashes occurring within the first 14 days are largely due to wild type virus while those occurring more than 14 days post vaccination are more likely to be caused by vOKa [50, 51]. An important finding from studies in leukaemic children has been that herpes zoster due to vOka occurs less frequently (~7 tiimes less often) than herpes zoster due to wild type virus, confirming that the vOka strain is impaired for reactivation. [52]. While vOka associated events, albeit mostly benign, occur in 2- 5% of children immunised against varicella, very few vOka associated events have been documented following use of the more concentrated vOka vaccine against herpes zoster. Three cases of vOka strain herpes zoster have been identified. One led to disseminated infection and death in an immunocompromised man. The third case resulted in retinitis; virus recovered from the vitreal fluid was sequenced and found to carry some of the 11 mutations associated with increased risk of vOka complications [53]. At least three commercial vOka preparations are available for prevention of varicella and one for prevention of zoster.

Molecular Genetics of the vaccine and VZV natural history

The unique opportunity posed by having a live vaccine that results in varicella- like and zoster rashes prompted us to compare the genome sequences of the infecting virus (vaccine) with that of the virus recovered from rashes. This provided a model for the natural history of VZV, a human-restricted virus for which there are no tractable animal models that accurately reproduce its natural history. Using statistical genetic analyses, we established that the live vaccine is highly stable from batch to batch[45, 54], that following inoculation, all viruses within the vaccine are capable of establishing latency and reactivating [45], that there were no neurotropic variants within the vaccine and no evidence of selection acting on any of the vOka variants[55]. Mathematical modelling of the results yielded three important findings. First, the vaccine virus evolves rapidly at a rate of ~ 10^{-3}/site/year. This is about 10 times faster than has previously been calculated from dated tips calculations for short term evolution for ds DNA viruses[55]. A rate of 10^{-5} to -^{6} subsitutions/site/year was calculated from circulating varicella transmitted in outbreaks [36]. Conversely the rate of 10^{-9} substitutions/site/year was based on a small subset of highly conserved genes and calculated over long timescales[56]. The new estimates of VZV mutation rates date the origins of the clades to between 20 and 50,000 years ago, ie earlier than hitherto supposed, a finding that fits with other estimates[35] and with the evidence for considerable rates of recombination [3] and of global spread of African and Asian strains associated with population migrations [3]. Secondly we were able to show that most individual VZV vesicles arise from a single virion with a few apparently originating from up to three virions[45]. Individual vesicles within the same zoster rash harboured different varicella strains. Finally we were able to show that mutation rates for viruses recovered from varicella rashes were several logs higher than for viruses recovered from zoster. In the former, the mutation rate remained constant with time while in the latter, the mutation rate declined over time [55]. The best explanation

for these findings is that viruses reacting to cause herpes zoster are not replicating during the time for which they are latent in neurons and therefore do not acquire mutations. When the mutation rates derived from replicating viruses that had caused varicella, were applied to the zoster viruses, we calculated that the zoster viruses had replicated for a mean of 13 days during the period of establishing latency and reactivating to cause the zoster rash[55]. These findings provide a unique insight into VZV natural history in its cognate host.

New Applications of molecular genetic technologies to transcriptomes

The studies of VZV vOka natural history were made possible by the development by our group of new technologies that allow recovery and enrichment for very low copy number viral genetic material from clinical samples for whole genome sequencing[11]. In the studies of VZV pathogenesis, these methods were applied to recovery of viral genomes. Applying these methods to transcriptomes recovered from VZV infected cells, it has been possible to examine the viral transcripts elaborated by wild type and vOka infected cells, even when these are at low level[57]. Analysis of the transcriptome of Induced pluripotent stem cell (IPSC) neurons infected with VZV, provided data on the behaviour of wildtype parental strain pOKa and vOka VZV in a new in vitro model of VZV latency and reactivation. The results suggest that low level transcription is occurring across the genome during both pOka and vOka latency in this model but that the level is lower in vOKa. Both pOka and vOka were able to establish latency but pOKa reactivated nearly five times more frequently[57]. Most recently the same methodology has been used to demonstrate the expression of a unique antisense VZV latency associated transcript (VLT) during viral latency in trigeminal ganglia neurons[58]. In summary, molecular tools have provided unique insights into the molecular epidemiology, transmission and pathogenesis of VZv and the live attenuated vOka vaccine.

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