Location on the evolutionary trees of the non-structural protein (NS) and neuraminidase (NA) genes of late human influenza A (H2N2) viruses: parental viruses of the NS and NA genes of Hong Kong influenza A (H3N2) viruses

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The nucleotide sequences of the non-structural protein (NS) and neuraminidase (NA) genes of human influenza A (H2N2) viruses isolated in 1967 and 1968 in Europe, Asia and North and South America were located on evolutionary trees in order to identify the parental virus of Hong Kong influenza A (H3N2) viruses, which appeared in the human population in 1968. From the evolutionary trees, the H2N2 viruses isolated during the 1967 to 1968 period were divided into two groups. Group I includes the A/Tokyo/3/67, A/Hachioji/1/67, A/Perg/1/68, A/Cordoba/522/67, A/Texas/2/68 and A/Berkeley/1/68 viruses, whereas group II includes the A/Georgia/1/67, A/England/10/67 and A/Poland/6/67 viruses. The NS and NA genes of Hong Kong H3N2 viruses isolated in 1968 were genetically closer to those of group II viruses and closest to those of A/Poland/6/67.

It has been generally accepted that Hong Kong influenza A (H3N2) virus was generated by a reassortment event between an avian influenza A virus carrying the H3 haemagglutinin (HA) gene and a human influenza A (H2N2) virus prevalent at that time. We analysed the genes of human influenza A (H2N2) and (H3N2) viruses by oligonucleotide fingerprinting previously (Nakajima et al., 1982). Our results showed that all genes except the HA gene of the A/Aichi/2/68 (H3N2) virus were similar to those of the A/Montevideo/333/67 (H2N2) virus, but considerably different from those of the A/Kumamoto/1/65 (H2N2) virus. We estimated that the reassortment event that generated the Hong Kong influenza A (H3N2) virus occurred after 1965, just prior to the pandemic outbreak in the human population. This estimation suggested that the new H3N2 virus caused a pandemic soon after its appearance. The outbreak of Hong Kong influenza first occurred in mainland China, where the reassortment event was thought to occur (Shortridge & Stuart-Harris, 1982). The phylogenetic or evolutionary trees of the three polymerase (PB1, PB2, PA) (Kawaoka et al., 1989; Gorman et al., 1990a; Okazaki et al., 1989), HA (Air, 1981; Bean et al., 1992), nucleoprotein (Altmüller et al., 1989; Gammelin et al., 1990; Gorman et al., 1990b), neuraminidase (NA) (Blok & Air, 1982), membrane protein (Ito et al., 1991) and non-structural protein (NS) (Buonagurio et al., 1986; Nakajima et al., 1990; Ludwig et al., 1991) genes have been constructed and, except for HA and PB2, the genes of the Hong Kong influenza A (H3N2) viruses were shown to be derived from those of an Asian influenza A (H2N2) virus. However, these studies were focused mainly on the relationships between the genes of human and animal influenza viruses.

Evolutionary trees of the same type or subtype of human influenza viruses were constructed mainly for the HA genes of A (H1N1) (Raymond et al., 1986; Cox et al., 1989; Nakajima et al., 1991), A (H3N2) (Both et al., 1983; Nakajima et al., 1988) and B (Yamashita et al., 1988; Kanegae et al., 1990; Rota et al., 1990, 1992; Nakajima et al., 1992) viruses. Buonagurio et al. (1986) sequenced 15 NS genes of the H1N1, H2N2 and H3N2 subtypes of human influenza A viruses and found that the NS genes of human influenza A viruses changed linearly from 1933 to 1985 at a constant rate. In their study they used the NS sequences of the A/Berkeley/1/68 virus as a late influenza A (H2N2) strain and the A/Udorn/72 virus as an early influenza A (H3N2) strain. Recently the NS sequence of the A/Aichi/2/68 (H3N2) virus was published (Odagiri & Tobita, 1990), but it differed considerably from that of the A/Berkeley/1/68 (H2N2) virus which was isolated in the same influenza season. This finding may mean either that the strains isolated in different parts of the world in
chronological order do not necessarily represent a direct evolutionary lineage or that the reassortment event occurred earlier than we thought.

In order to gain more information about the evolutionary origin of new influenza A (H3N2) viruses, we determined the sequences of the NS and NA genes of influenza A (H2N2) viruses isolated during 1967 and 1968 in Europe, Asia and North and South America, together with those of two influenza A (H3N2) viruses isolated in 1968, and located them on the evolutionary trees. We used A/Aichi/2/68 virus as a prototype new Hong Kong H3N2 influenza virus. The Hong Kong influenza outbreak occurred around 10 July 1968. The swab sample of the A/Aichi/2/68 virus was obtained on 25 July from a sailor who travelled from Hong Kong to Nagoya port in the Aichi prefecture.

In the present study, the NS genes of the following influenza virus strains were sequenced: A/Kumamoto/1/65 (Kumamoto/65) (Japan), A/Georgia/1/67 (Georgia/67) (U.S.A.), A/Poland/6/67 (Poland/67) (Poland), A/Cordoba/522/67 (Cordoba/67) (Argentina), A/England/10/67 (England/67) (U.K.), A/Hachioji/1/67 (Hachioji/67) (Japan), A/Perg/1/68 (Perg/68) (Argentina) and A/Texas/2/68 (Texas/68) (U.S.A.). The NA genes of the Georgia/67, England/67, Poland/67, Cordoba/67 and Perg/68 H2N2 strains and the A/Aichi/2/68 (Japan) H3N2 strain were sequenced. The NS-specific primers GGGTGACAAAGCTTAATTTGCGAAAGCTTA, corresponding to nucleotide sequences 1 to 19 and 900 to 879, and 879 to 900 and 900 to 879, were used for cDNA synthesis. The cDNA was amplified by the PCR. The amplified cDNAs were inserted into the pUC19 plasmid vector at the HincII site. The genes were sequenced by an autosequencer (Applied Biosystems) with two or three clones for each sample to avoid erroneous readings caused by artificial mutations during the amplification process.

Fig. 1 shows the nucleotide differences in the NS genes of 10 human influenza A strains isolated between 1965 and 1968 from that of the A/Ann Arbor/6/60 (AA/60) virus isolated in 1960 (Cox et al., 1988). The nucleotide changes occurred in at least 68 positions in the 11 NS genes. These changes were classified either as mainstream changes inherited by most subsequent strains, or strain-specific changes occurring in only one or at most a few strains. An evolutionary tree of the NS gene is shown in Fig. 2. This was constructed by giving priority to mainstream changes over strain-specific changes, minimizing the chance that a strain-specific change shared by two viruses occurred independently. The NS gene of Kumamoto/65 was located between those of the 1960 and 1967 viruses. The NS genes of the H2N2 viruses isolated during the 1967 to 1968 period had seven to 20 base differences between each other, indicating that a number of genetically different influenza A (H2N2) viruses cocirculated in the world during this period. The NS genes of the influenza A viruses isolated during the 1967 to 1968 period were located on five branches derived from the stem. The NS gene of Cordoba/67 formed one branch, Hachioji/67, Perg/68, Texas/68 and
Fig. 2. The evolutionary tree for the NS genes of the influenza A virus strains isolated between 1960 and 1968. Numbers refer to the mainstream nucleotide changes that have become fixed in most of the subsequent strains (horizontal line), or to strain-specific nucleotide changes on the side branches.

Berkeley/68 formed the second branch, Georgia/67 and England/67 formed the third and the fourth branches, and Poland/67 and Aichi/68 (H3N2) the fifth branch. Therefore, among the H2N2 viruses isolated in the 1967 to 1968 period, the NS gene of Poland/67 was the closest to that of the 1968 Hong Kong H3N2 virus. As shown in Fig. 1, the NS gene of Berkeley/68 had one of the largest number of base changes from that of Aichi/68 (17 changes), while that of Poland/67 had the smallest number (seven changes) among the 1967 to 1968 H2N2 viruses.

The evolutionary tree of the NS gene indicates that the parent of the NS gene of 1968 Hong Kong H3N2 viruses was closest to the NS gene of the Poland/67 virus. Buonagurio et al. (1986) found that base changes in the NS gene of human influenza A viruses were about 1.73 nucleotide substitutions per year. Ludwig et al. (1991) indicated that the rate of base changes of the NS gene of H2N2 and H3N2 viruses is faster than that of the H1N1 viruses and calculated this to be 2.23 nucleotide substitutions per year for the H2N2 and H3N2 viruses. As reported previously (Nakajima et al., 1991), when we compared the HA genes of influenza viruses isolated in a short period, the rate of nucleotide substitution was different for each virus. The same concept can be applied.

Fig. 3. Nucleotide sequences of the NA genes of seven influenza A (H2N2) and two influenza A (H3N2) virus strains isolated between 1957 and 1968. The nucleotide bases are numbered according to the coding region of the NA gene of the RI/57 (Elleman et al., 1982). Only positions that drifted from the NA gene of RI/57 are shown.
in the present case to the NS gene. The numbers of differences in the NS gene between 1967 and 1968 viruses and the AA/60 virus were about 21 to 31. The average rate of mutation of the NS gene of H2N2 viruses was calculated to be 3-9 nucleotide substitutions per year. If these base changes occurred in the NS gene of each H2N2 virus, Poland/67 (H2N2) and Aichi/68 (H3N2) must have branched off after 1966.

Fig. 3 shows nucleotide differences of the NA gene of six influenza A (H2N2) viruses and two influenza A (H3N2) viruses isolated during the 1967 to 1968 period from that of the A/RJ/5/57 (RJ/57) virus (Elleman et al., 1982). The similarity of the NA proteins of the H2N2 and the H3N2 viruses was shown by comparing the polypeptide sequences of the A/Tokyo/3/67 (Tokyo/67) (H2N2) virus and the A/Udorn/72 (H3N2) virus [or the Aichi/68 or A/NT/60/68 (NT/68) virus] (Air et al., 1983; Azad et al., 1983). As shown in Fig. 3, among the NA genes of the H2N2 viruses investigated in this study, that of Poland/67 was again genetically closest to that of the 1968 Hong Kong H3N2 viruses, Aichi/68 and NT/68. The number of differences in base sequences between Aichi/68 and NT/68 was five, and Aichi/68 was genetically closer to the H2N2 viruses. The number of differences in base sequences between Aichi/68 and Tokyo/67 or Poland/67 was 21 and 10, respectively. There are only three amino acid differences between the NA proteins of Poland/67 and Aichi/68 (amino acid residues 40, 78 and 332).

An evolutionary tree of the NA gene was constructed by the same method as used for the NS gene (Fig. 4). The evolutionary trees of the NS and the NA genes of human influenza A viruses during antigenic shift from subtype H2N2 to H3N2 are similar except for the locations of Cordoba/67 and Perg/68. Cordoba/67 and Perg/68 were located on side branches derived from the same main branch in the NA tree, but were located on different branches in the NS tree. However, the NS genes of these viruses shared one base change (base 51). From the evolutionary trees of the NS and NA genes, human influenza A (H2N2) viruses during the 1967 to 1968 period were divided into two groups. Group I includes the Asian (Japanese), South American and some North American strains, i.e. Tokyo/67, Hachioji/67, Cordoba/67, Perg/68, Berkeley/68 and Texas/68. Group II includes one North American strain, Georgia/67, and the European strains England/67 and Poland/67. Among the virus strains studied the closest to the new H3N2 viruses was Poland/67.

Bean et al. (1992) investigated the nucleotide sequences of the H3 HA genes of avian isolates and found that the H3 HA gene of human H3N2 viruses belonged to a Eurasian avian type. Their results suggest that the reassortment event occurred in Europe or Asia. The
creation of a new Hong Kong H3N2 influenza virus is thought to have occurred in mainland China (Shortridge & Stuart-Harris, 1982). Unfortunately, the H2N2 or H3N2 viruses prevalent during 1967 to 1968 in mainland China are not available. In our present study, we used two Japanese strains, Tokyo/67 for the NA gene and Hachioji/67 for the NS gene, as Asian isolates. However, unexpectedly, the NS and NA genes of these viruses were located a far distance away from those of Hong Kong H3N2 strains Aichi/68 or NT/68 in the evolutionary trees.

In the present study, we tried to determine the time of the reassortment event, and found that the NS and the NA genes of Poland/67 were much closer to those of 1968 Hong Kong H3N2 viruses than reported before. This does not mean, however, that the reassortment event occurred in Europe. Rather, our results indicate the possibility that the reassortment between an animal influenza A virus carrying the H3 gene and the human influenza A (H2N2) virus occurred just before the outbreak caused by new Hong Kong H3N2 influenza viruses.

References


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