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Short Communication

# CCR5 deficiency predisposes to fatal outcome in influenza virus infection

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Influenza epidemics affect all age groups, although children, the elderly and those with underlying medical conditions are the most severely affected. Whereas co-morbidities are present in 50 % of fatal cases, 25–50 % of deaths are in apparently healthy individuals. This suggests underlying genetic determinants that govern infection severity. Although some viral factors that contribute to influenza disease are known, the role of host genetic factors remains undetermined. Data for small cohorts of influenza-infected patients are contradictory regarding the potential role of chemokine receptor 5 deficiency (*CCR5-Δ32* mutation, a 32 bp deletion in the *CCR5* gene) in the outcome of influenza virus infection. We tested 171 respiratory samples from influenza patients (2009 pandemic) for *CCR5-Δ32* and evaluated its correlation with patient mortality. *CCR5-Δ32* patients (17.4 %) showed a higher mortality rate than WT individuals (4.7 %; $P=0.021$), which indicates that *CCR5-Δ32* patients are at higher risk than the normal population of a fatal outcome in influenza infection.

Influenza A viruses are a major source of acute respiratory infections and continue to be an important cause of acute illness and death worldwide. They cause annual epidemics and occasional pandemics with potentially fatal outcome. The mean global burden of seasonal influenza is more than 600 million cases, with 3 million cases of severe illness and almost 500 000 deaths per year worldwide (http://www.who.int/en/). A new H1N1 subtype influenza A virus emerged in 2009 [A(H1N1)pdm09], which was highly transmissible with relatively low virulence and caused the first pandemic of the 21st century (Neumann *et al.*, 2009).

Differences in disease severity can be due to pre-existing health conditions, predisposing host genetic factors, differences in the virulence of circulating viruses or a combination of these factors. The co-morbid conditions for A(H1N1)pdm09 include chronic metabolic disease, primarily diabetes mellitus and renal disease, chronic lung and cardiac disease, immunosuppressive conditions, neoplasms, obesity and pregnancy (Falagas *et al.*, 2011; Louie *et al.*, 2011; Singanayagam *et al.*, 2011).

Although co-morbidities are present in half of fatal cases, one-third of fatal cases have no co-morbid conditions (http://www.cdc.gov/h1n1flu/estimates_2009_h1n1.htm), suggesting that host genetic variation accounts for the distinct disease severity of A(H1N1)pdm09 infection. Several potential genetic determinants associated with A(H1N1)pdm09 infection have been described, including TNF (Antonopoulou *et al.*, 2012), IFN-inducible transmembrane (Everitt *et al.*, 2012), killer-cell immunoglobulin-like receptor (Aranda-Romo *et al.*, 2012), complement regulatory protein CD55 (Zhou *et al.*, 2012) and Toll-like receptor 3 (Esposito *et al.*, 2012). Data relating to the role of chemokine receptor 5 (CCR5) in severe A(H1N1)pdm09-infected patients are contradictory and have been debated (Keynan *et al.*, 2010; Rodriguez *et al.*, 2013; Sironi *et al.*, 2014).

CCR5 regulates various aspects of the adaptive immune response, and a non-functional allele resulting from a 32 bp deletion (*CCR5-Δ32*), which determines a loss of expression of functional CCR5 receptor, has been detected in homo- and heterozygosity (Benkirane *et al.*, 1997). The

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One supplementary figure and one table are available with the online Supplementary Material.

global distribution of *CCR5-Δ32* heterozygous individuals depends on race/ethnic group, with mean values ranging from 9.9 to 15 %; it is more frequent in Caucasians, irregular in native Amerindians, and rare or absent in other major ethnic groups such as native Africans and Asians (Libert *et al.*, 1998; Rodríguez-Rodríguez *et al.*, 2011; Zimmerman *et al.*, 1997). Detection of *CCR5-Δ32* homozygous individuals is rare, with a mean value of <1 % (Downer *et al.*, 2002; Rodríguez-Rodríguez *et al.*, 2011).

The *CCR5-Δ32* allele reduces susceptibility to human immunodeficiency virus infection (Dean *et al.*, 1996; Liu *et al.*, 1996; Samson *et al.*, 1996). Homozygosity for the *CCR5-Δ32* allele is a strong risk factor for symptomatic West Nile virus infection (Lim *et al.*, 2008) and correlates with disease severity after tick-borne encephalitis virus infection (Kindberg *et al.*, 2008). A function for CCR5 in influenza virus replication is shown by the increased mortality rate of *CCR5* knockout mice after influenza virus infection (Dawson *et al.*, 2000). Recent data regarding the *CCR5-Δ32* allele in severe A(H1N1)pdm09 virus-infected patients have provoked controversy. In one survey, increased *CCR5-Δ32* allele heterozygosity was found in A(H1N1)pdm2009 virus-infected patients with critical illness; this study was limited to a sample of 20 patients, nine of whom were white and none of whom was homozygous for the mutation (Keynan *et al.*, 2010). In contrast, a recent study of 29 A(H1N1)pdm09 virus-infected southern European patients indicated no *CCR5-Δ32* allele association with disease severity, as the *CCR5-Δ32* allele was found in only one individual who developed mild disease (Sironi *et al.*, 2014). We previously reported this mutation in homozygosis in a deceased patient infected with A(H1N1)pdm09 virus (Rodriguez *et al.*, 2013). Recent controversy regarding the CCR5 contribution to severe outcome in influenza infection prompted us to design a study with strong statistical power to analyse *CCR5-Δ32* allele association with the mortality of A(H1N1)pdm09 influenza-infected patients.

During the 2009–2010 and 2010–2011 influenza seasons, a total of 2046 respiratory samples were confirmed cases of influenza A(H1N1)pdm09 virus infection in respiratory samples received at the National Influenza Center in Madrid (Instituto de Salud Carlos III), included in the Spanish Influenza Surveillance System network. To analyse the role of CCR5 in A(H1N1)pdm09 mortality, we used the survey system software to calculate the necessary sample size for a 99 % confidence level and 10 % confidence interval (CI), which established a minimum sample size of 154 respiratory samples (http://www.surveysystem.com/sscalc.htm). We selected those samples in which an accurate record including the presence or absence of co-morbidities associated with severe influenza disease was available. We genotyped CCR5 in 171 samples, of which 11 were from patients who had died.

Respiratory samples were received from 13 regions of Spain (from 86 females and 85 males). The *CCR5* sequence that potentially comprises the Δ32 mutation was amplified by PCR using previously described primers (Keynan *et al.*, 2010), and the amplified products were visualized by electrophoresis in a 2.5 % agarose gel. We obtained three types of amplification products, a unique 197 bp product in the case of homozygous WT *CCR5*, a unique 165 bp product in the case of homozygous *CCR5-Δ32*, and a double 197 and 165 bp product in the case of heterozygosity (Fig. S1, available in the online Supplementary Material). Sequence analysis of selected amplification samples confirmed that they corresponded to the *CCR5* gene. *CCR5-Δ32* deletion was detected in 23 patients (13.5 %), of which 20 were heterozygous (11.7 %) and three were homozygous (1.8 %).

**Table 1.** Comparison of fatality rates between WT patients and those with the *CCR5-Δ32* mutation in heterozygosity or homozygosity

| Fatal case | WT (*n*=148) | +/Δ32 (*n*=20) | Δ32/Δ32 (*n*=3) | +/Δ32 and Δ32/Δ32 (*n*=23) |
|---|---|---|---|---|
| No | 141 | 17 | 2 | 19 |
| Yes | 7 (4.7 %) | 3 (15 %) | 1 (33.3 %) | 4 (17.4 %)* |

*Association between presence of the *CCR5-Δ32* allele and fatal cases was statistically significant: odds ratio 4.24 (95 % CI 15.85–1.13), $P=0.021$.

Distribution of *CCR5* WT and *CCR5-Δ32* alleles among the fatal cases is shown in Table 1. Patients bearing the *CCR5-Δ32* mutation showed a 17.4 % fatality rate, significantly higher than the 4.7 % fatality rate for WT individuals [odds ratio (OR) 4.24, 95 % CI 15.85–1.13), $P=0.021$]. This proportion increased to 33.3 % for *CCR5-Δ32* homozygous patients, although the small sample size did not provide adequate statistical power. The frequency of *CCR5-Δ32* heterozygous individuals was consistently higher (27.3 %; 3 of 11; Table 2) in the deceased patient cohort than it was in the general population (10–15 %) (Downer *et al.*, 2002; Rodríguez-Rodríguez *et al.*, 2011). *CCR5-Δ32* homozygote frequency was also higher in our

**Table 2.** *CCR5* genotype frequency in deceased patients with confirmed diagnosis of A(H1N1)pdm09 virus infection compared with the frequency in the general population

| CCR5 genotype | No. fatal cases | Frequency of allele in fatal influenza cases (%) | Frequency of allele in general population (%) |
|---|---|---|---|
| Total | 11 | – | – |
| WT | 7 | 63.6 | 84–90 |
| +/Δ32 | 3 | 27.3 | 10–15 |
| Δ32/Δ32 | 1 | 9.1 | <1 |
| +/Δ32+Δ32/Δ32 | 4 | 36.4 | 11–16 |

deceased patient group (9.1 %; 1 of 11) than in the general population (<1 %) (Downer *et al.*, 2002; Rodríguez-Rodríguez *et al.*, 2011), although the small sample size might not be representative. We thus found a higher prevalence of the *CCR5-Δ32* allele in homo- or heterozygosis patients (36.4 %; 4 of 11; Table 2) compared with that in the general population (11–16 %) (Downer *et al.*, 2002; Rodríguez-Rodríguez *et al.*, 2011). These results establish a clear correlation between the *CCR5* genotype and the fatality rate of A(H1N1)2009-infected patients.

The characteristics of patients included in the study according to *CCR5* allele composition and disease outcome are shown in Table 3. We found no significant difference in the frequency of WT *CCR5* (32 %) or *CCR5-Δ32* (31.5 %) in patients with non-fatal influenza who presented the co-morbidity factors described above. This percentage increased to 86 % in the deceased WT *CCR5* patients, compared with 50 % for deceased *CCR5-Δ32* patients (OR 0.072, 95 % CI 0.66–0.009, $P=0.003$). These data indicated that co-morbidities associated with influenza severity are more common in deceased WT *CCR5* than in *CCR5-Δ32* patients, which further supports a role for CCR5 in the outcome of this infection. Gender distribution and mean age were similar for WT and *CCR5-Δ32* patients. The data and clinical features of the deceased patients are shown in Table S1.

This study helps to clarify a controversy that has arisen regarding the relationship between CCR5 and fatal outcome in A(H1N1)pdm09 virus infection (Keynan *et al.*, 2010; Sironi *et al.*, 2014). The strong design of our study, with statistical power sufficient to test the hypothesis regarding genetic mutation and disease phenotype, allowed us to correlate CCR5-Δ32 with the increased fatality rate of A(H1N1)pdm09-infected patients, which reinforces a CCR5 effect on disease outcome in infections by this virus (Keynan *et al.*, 2010). These data concur with results from experimental studies in *CCR5* knockout mice (Dawson *et al.*, 2000). Data for West Nile virus-infected patients suggest a recessive model for CCR5 mode of action, as only homozygous individuals showed a high risk of severe infection outcome (Lim *et al.*, 2008). Our results here for A(H1N1)pdm09 virus-infected individuals showed a significant increase in fatality rate of heterozygous compared with WT CCR5 patients (Table 1), and a larger number of heterozygotes than predicted among deceased patients (Table 2). The presence of a single *CCR5-Δ32* allele was sufficient to increase the likelihood of a fatal outcome, which indicates a non-recessive model, although we cannot rule out a possible additive effect when both alleles are mutants.

With regard to the possible mechanism of action of CCR5, studies in influenza virus-infected mice showed a crucial role for CCR5 in accelerating the recruitment of memory $CD8^+$ T-cells to lung airways during virus challenge (Kohlmeier *et al.*, 2008). CCR5 deficiency led to decreased memory T-cell recruitment and accelerated macrophage accumulation in the lungs; this gave rise to an acute inflammatory response that impaired the control of virus replication and increased mortality rates associated with acute severe pneumonitis (Dawson *et al.*, 2000). Although it was not confirmed experimentally, the accelerated macrophage accumulation in $CCR5^{-/-}$ mice appears to be linked to enhanced expression of CCL2 and CCL5, the natural CCR5 ligands, and their binding to other intact chemokine receptors on the CCR5-deficient macrophages (Dawson *et al.*, 2000). These results highlight the importance of macrophages in generating an appropriate immune response to influenza infection and in the development of associated lung pathology, as well as the relevance of CCR5 in this response.

In summary, we established a statistically significant association between a host genetic determinant, the deletion of 32 bp in the CCR5 chemokine receptor, and fatalities associated with a pandemic influenza infection. Additional work is needed to address more critically the specific CCR5 function in human influenza virus infection. The

**Table 3.** Characteristics of patients according to *CCR5* allele composition and disease outcome

| Characteristic | *CCR5* WT ($n=141$) | *CCR5* WT ($n=7$) | *CCR5*+/Δ32 and Δ32/Δ32 ($n=19$) | *CCR5*+/Δ32 and Δ32/Δ32 ($n=4$) |
|---|---|---|---|---|
| Fatal outcome | No | Yes | No | Yes |
| Co-morbid factor* | 45 (32 %) | 6 (86 %)† | 6 (31.5 %) | 2 (50 %)‡ |
| Mean age ± SD (years) | 36.5 ± 21.6 | 46.7 ± 20.4 | 37.3 ± 25.6 | 55 ± 22.9 |
| Gender | | | | |
| Male | 72 (51 %) | 4 (57.1 %) | 7 (36.8 %) | 2 (50 %) |
| Female | 69 (49 %) | 3 (42.9 %) | 12 (63.1 %) | 2 (50 %) |

*Co-morbid factors include cardiopathy, diabetes, pregnancy, pulmonary disease, immunodeficiency, renal failure, obesity and cardiopulmonary disease.

†Association between the presence of co-morbid factor and WT *CCR5* fatal cases was statistically significant: OR 0.072 (95 % CI 0.66–0.009), $P=0.003$.

‡Association between the presence of co-morbid factor and *CCR5-Δ32* fatal cases was not significant.

identification of genetic factors that modulate influenza virus pathogenesis could aid in the definition of new risk groups, which would then be included in preventative protocols and vaccination programmes. In addition, defining such factors could also open up new possibilities for the prognosis of virus pathogenicity and the development of improved or alternative preventative and therapeutic strategies.

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