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EPSTEIN-BARR VIRUS IN HEALTHY INDIVIDUALS FROM PORTUGAL

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SUMMARY

Introduction: The Epstein-Barr virus (EBV) persists for long periods in latent state inside B--lymphocytes after primary infections, and reactivation usually occurs associated to immunosuppression conditions of the host. Recently, the detection of EBV DNA in circulation has been suggested as a predictor marker for the development of EBV related malignancies.

Aim of the study: The aim of our study was to characterize the frequency of circulating EBV in healthy individuals (n=508) from the North region of Portugal, using peripheral blood samples. Detection was performed by Nested-PCR which amplifies a fragment from the BamHIW region of the EBV genome.

Results: Our results revealed an overall frequency of 37.2% positive cases for EBV in circulation, with distinct distribution according to genre (39.7% in male individuals and 33.2% in females). We also found that EBV is more frequent in individuals with more than 56 years old compared to individuals with less than 56 years old (p=0.032; RR=1.41), mainly in the male group (p=0.024; OR=1.51).

Conclusion: This is the first study which characterizes the frequency of EBV in circulation in healthy donors from the Northern Region of Portugal, revealing increased frequency of EBV in circulation in healthy individuals with differences depending on gender or age. Further studies are required to analyze the role of circulating EBV in the definition of susceptibilities to EBV associated diseases.

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RESUMO

CARACTERIZAÇÃO DA FREQUÊNCIA DO VÍRUS DE EPSTEIN-BARR EM DADORES SAUDÁVEIS DE PORTUGAL

Introdução: O Vírus de Epstein-Barr (EBV) é um vírus latente capaz de permanecer longos períodos de tempo inactive dentro dos Linfócitos B, sendo que as reactivações acontecem frequentemente apenas em situações de imunosupressão do hospedeiro. Estudos recentes demonstraram que a presença de DNA viral em circulação no sangue pode ser considerado um factor preditivo para o desenvolvimento de doenças associadas ao EBV.

Objectivos: O objectivo deste estudo é caracterizar a frequência de EBV em sangue periférico de indivíduos saudáveis dadores de sangue na região Norte de Portugal. A detecção foi efectuada por Nested-PCR com amplificação de um fragmento da região BamHIW do genoma do EBV.

Resultados: Os nossos resultados demonstraram uma frequência de DNA de EBV em indivíduos saudáveis de 37,2%, com uma distribuição distinta considerando o género (39,7% em homens e 33,2% em mulheres). Revelaram ainda que a frequência de DNA de EBV é maior em indivíduos com mais de 56 anos de idade comparada com indivíduos com menos

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(p=0,032; RR=1,41), principalmente no grupo dos homens (p=0,024; RR=1,51). Conclusão: Este estudo é o primeiro a caracterizar a frequência de EBV em indivíduos saudáveis na Região Norte de Portugal, revelando uma frequência elevada com características diferentes relativamente ao género e à idade. Contudo, mais estudos são necessários para avaliar o papel do EBV na definição de susceptibilidade para o desenvolvimento de várias doenças associadas, tendo em conta a diferente distribuição nas várias populações

INTRODUCTION

The Epstein-Barr virus (EBV) is an ubiquitous virus with a wide distribution worldwide, mainly due to its *ability* to be spread through the saliva, especially during childhood between members of the family. It infects the majority of the adult human population, being more than 90% of the population seropositive[1-3].

Primary infection with EBV usually occurs during the first few years of life and is often asymptomatic, although, after primary infection the individual remains a lifelong carrier of the virus[4-8]. EBV remains in latent state inside B-lymphocytes of infected individuals for long periods[3]. Ocasionally however, in some occasions it reactivates the lytic phase and spreads in the tissue, infecting new cells that if receptive will allow EBV to reactivate its ability to transform cells, leading to the development of neoplasic cells[9]. This event is usually frequent in the presence of immunosuppressant conditions of the host, and therefore it will be more susceptible to develop a neoplasic disease[3,10,11].

The great majority of published studies regarding identification of EBV infection in healthy individuals refer to serological data. Nevertheless, recently it has been suggested that the detection of circulating EBV DNA can improve the accuracy of the level of infection that the individual is actually experiencing[12-16].

Therefore, we aimed to develop a hospital-based case study to characterize the frequency of healthy individuals from the North region of Portugal which have actually circulating EBV DNA, contributing for the improvement of medical care prevention measures.

MATERIALS AND METHODS

Population

This study was performed as a retrospective hospital-based case study with 508 randomly selected clinically evaluated healthy blood donors, including 314 males and 194 females, with median age of 40 years old (y.o.) collected at the Portuguese Institute of Oncology of Porto. All samples were obtained after informed consent, according to the Declaration of Helsinki.

Procedures

Sample Collection and DNA extraction

Samples were obtained from peripheral blood samples (8-10mL), collected using standard venipuncture technique in EDTA-containing tubes. The white blood cell (WBC) fraction was pelleted and DNA extraction was performed using a Salting Out protocol[17]. These samples belong to a DNA bank from a study of oncogenic viruses' prevalence in healthy individuals, and were previously tested for the presence of genomic DNA with PCR protocol for housekeeping genes.

Detection of EBV by Nested-PCR

The detection and identification of Epstein-Barr virus was performed using a Nested-polymerase chain reaction (Nested-PCR) with the amplification of a fragment from the BamHI-W region. The first PCR reaction was made using the forward primer 5'-GCTAGGCCACCTTCTCAG-3´ and the reverse primer 5-GTCCAGGGCCTTCACTTC-3´and the second PCR was made with the forward and reverse primers 5'-TCTCCCCTAGGCTTGGAT-3', 5'-CAGCGCGTTTACGTAAG-3', respectively.

Both PCR reactions mixes were developed in a solution of 50 µl total volume with: 1x *Taq buffer*, 2.5 mM $MgCl_2$, 0.2 mM DNTPs, 0.25 µM from each *primer*, 1U de *Taq DNA Polymerase* and 100ng of each DNA sample. The amplification conditions for both reactions were: 95ºC for 5min, 30 cycles of 95ºC at 1min, annealing for 45sec at 51ºC, extension for 1min at 72ºC and a final extension step

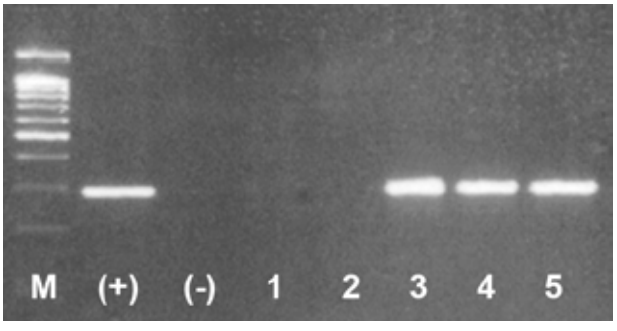

Fig. 1 – *Amplification product of the EBV BamHIW region from the second PCR in a 1.5% agarose gel. (M: 100 bp Ladder; (+), positive control; (–), negative control; 1 and 2, EBV negative cases; and 3 to 5, positive cases for EBV).*

at 72ºC for 7min. The amplified fragments (320bp in the first PCR and 280bp in the second PCR) were analyzed by electrophoresis in 1.5% agarose gels stained with ethidium bromide and visualized under UV light (Figure 1).

Negative controls were used, in a proportion of 1 per each 10 samples, in all reactions to avoid contamination and false positive results in the Nested-PCR protocol.

Statistical Analysis

Data analysis was performed using computer software Statistical Package for Social Sciences for Windows (Version 16.0; SPSS, Chicago, IL). A 5% level of significance was used in the Chi-square analysis to compare the categorical variables, and the relative risk (RR) with 95% Confidence Interval (CI) was used as a measure of the association.

RESULTS

The presence of circulating EBV DNA was evaluated in 508 healthy blood donors by amplification of a BamHI-W fragment from the EBV genome. The results showed that 37.2% were positive for EBV in circulation.

When discriminating by genre, we found a frequency of 39.7% positive cases with EBV DNA in circulation in males comparing to 33.2% in females (Figure 2). Despite there is no statistical significant association, there is an almost 21% increased probability of male individuals to have EBV DNA in circulation (p=0.122; RR=1.21; 95% CI 0.95-1.54) (Table 1).

Data was also analysed with stratification of individuals according to age groups: 1) ≤25 y.o. (n=72); 2) 26 to 40 y.o. (n=192); 3) 41 to 55 y.o. (n=185); and 4) ≥56 y.o. (n=58). The analysis revealed that there is a tendency to an increased prevalence of circulating EBV DNA in individuals older than ≥56 y.o. (p=0.032; RR=1.41; 95% IC 1.06–1.87) (Figure 3, Table 1). Moreover this tendency was observed with more significance in the male group (p=0.024; RR=1.51; 95% IC 1.10 – 2.06).

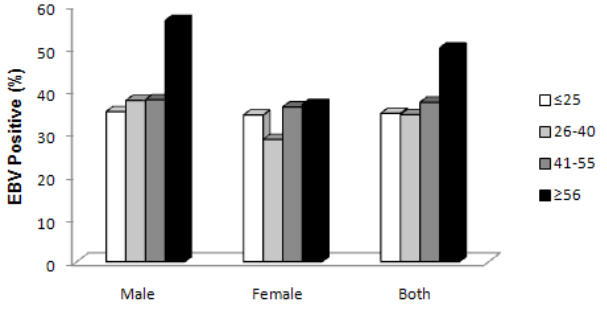

Fig. 2 – Distribution of the positive cases for circulating EBV DNA, according to age.

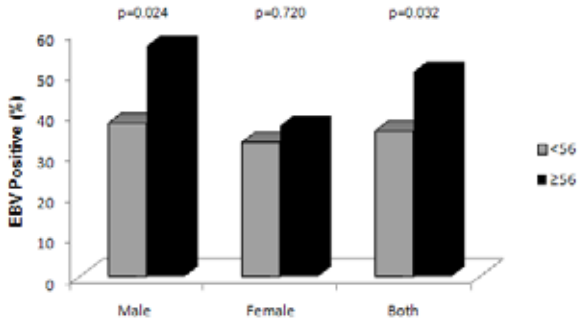

Fig. 3 – Percentage of positive samples for circulating EBV comparing individuals with less and more than 56 years old.

DISCUSSION

Nowadays, the diagnosis of EBV infection are often performed either by serology and/or *in situ* hybridization (ISH), nevertheless, several authors are concerned with the fact that both methodologies have limitations: 1) serology may identify the existence of antibodies against EBV and the individuals may remain EBV negative; 2) several other antibodies from other viruses have been referred to interfere with the titration of EBV antibodies; and 3) ISH requires a tissue sample to perform the analysis which is obtained through an invasive technique. Moreover, despite that when serological data point to a recent or active infection, we cannot assure that it will be enough for the development of an EBV associated disease.

Serological studies point to almost 90% of world population to be infected or to have been infected once on their life[18]. These values make difficult the analysis of the EBV active infection on worldwide population. Recent data point that the detection of EBV in peripheral blood samples might help to diagnose/predict acute infection, and despite the fact that the detection of EBV in the blood does not mean that the individual is developing an EBV associated disease, but it may help to predict the risk of[12-15]. There are not many studies regarding the frequency of

Table 1 – Frequency of circulating EBV found in healthy individuals.

	EBV Positive (%)
Controls (n=508)	189 (37.2)
Male (n=314)	125 (39.7)
Female (n=194)	64 (33.2)
Age	
<25 years (n=72)	25 (34.7)
26-40 years (n=192)	66 (34.4)
41-55 years (n=185)	69 (37.3)
>56 years (n=58)	29 (50.0)

circulating EBV in healthy individuals, and they might be important to evaluate the overall risk that populations have to develop EBV associated malignancies[19].

Since there was a gap on the information regarding the presence of circulating EBV in healthy individuals, we aimed to show the frequency of this event in the Portuguese Population. However, we are aware that this study may have some limitations: firstly, the use of a nested-PCR protocol has several different appreciations, and despite the increased probability to false positives, it increases the sensibility for UV visualization of agarose gels and also the specificity of the test; considering the possibility of contaminations and false positive results in Nested-PCR protocols, we have included 1 negative control per each 10 samples in the test and all came out as negative in both PCR reactions; and second, actually the detection of EBV as negative/positive is considered out of date since several quantification protocols are available in several publications. Nevertheless, we have DNA samples from a DNA bank and therefore we were not able to measure the amount of blood used for each DNA extraction and therefore we cannot make a correct approach to EBV quantification.

At the best of our knowledge, this is the first study in Portugal and Western Europe to show data concerning circulating EBV in Healthy individuals. Our study revealed the presence of circulating EBV in 37% of healthy individuals.

Our study also reveals curious findings that might be correlated with the differential individual behaviour. There is a higher frequency of EBV DNA in male individuals (39.7%), although it is not statistically different from the one found in females (33.2%). Despite not statistically significant, the relative risk (RR=1.21) reveals a significant increase in the probability of EBV infection of about 21% for male gender, nevertheless as the male/female ratio included in the study was not the same this data still to confirm if it is a fact or an artefact. Since there is no study in healthy individuals to be compared to our, we may only suggest that physiological differences between male and females might be important in the susceptibility or resistance to EBV infection.

Moreover, we found significant differences in the frequency of EBV according to age, revealing that the frequency of circulating EBV is higher in individuals with ≥56 years old (50.0%) than in younger individuals (35.5%). These results reveal that individuals with more than 56 years have 1.5-fold increased risk to have EBV in circulation comparing with less of 56 years. Furthermore, male individuals contribute more to this difference. The plausible explanation for this result may be correlated with the decrease of the host immunity in older individuals[20].

Despite the genetic and environmental differences our study showed similar results to the only published report considering healthy individuals which revealed that 39.5% of Japanese individuals have EBV in circulation[21]. This result was surprisingly at first time, however, there are a few malignancies, such as gastric and nasopharyngeal carcinoma correlated with EBV that shows similar patterns of incidence[19]. Therefore considering that there are several papers showing an association between EBV and gastric carcinoma and nasopharyngeal carcinoma development, we may theorize that there might be a connection with higher frequency of circulating EBV, which is still to be clarified.

This is the first study which characterizes the frequency of EBV in circulation in healthy donors from Portugal, revealing that there are differences on the frequency depending on the group analyzed according to gender or age. There is still a lack of information about EBV viremia in healthy individuals, and further studies should be carefully analyzed since many features, such as the methodologies and population characteristics, can interfere in the results[22]. Furthermore, it is necessary the development of more studies regarding EBV viremia, including genotyping and quantification, in other populations around the world that may reveal different frequencies and thus different susceptibilities to EBV associated diseases. These studies can be useful in the development of prevention strategies for the development of EBV associated diseases.

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180
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