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ORIGINAL ARTICLE

HIV infection and frequency of micronucleus in human peripheral blood cells

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Keywords

HIV infection • Micronucleus • Biomarker • DNA damage • Genetic instability • Cancer risk

Summary

Purpose. *People living with HIV have higher rates of malignancies than the general population in the era of active antiretroviral therapy (ART). Genotoxic effects of HIV infection and/or ART that can induce neoplastic development are not yet well known. A prospective cohort study to investigate DNA damage measured through the micronuclei (MN) frequency in HIV-patients has been performed.*
Methods. *Peripheral blood mononuclear cells (PBMC) were isolated from 52 HIV-patients treated with ART and 55 healthy controls.*
Results. *By the comparison of MN frequency, a significant difference between HIV-patients (15.5 ± 9.8) and controls (6.0 ± 3.6) ($p < 0.001$) has been revealed. In univariate linear regression analysis, HCV infection ($r = 0.31$; $p < 0.001$), HIV-RNA ($r = 0.29$; $p < 0.03$) and duration of infection ($r = – 0.16$; $p < 0.25$) were associated with MN frequency; while only viral load (VL) significantly correlates ($r = 0.29$; $p < 0.05$) in a multiple regression model.*
Conclusions. *The association of VL with MN frequency supports a genotoxic effect of HIV infection.*

Introduction

The Human Immunodeficiency Virus (HIV) infects cells of the host immune system, which is gradually destroyed; monocytes/macrophages and CD4+ T lymphocytes (CD4+) are the main targets of infection. Active viral replication leads to a progressive decline of CD4+ with gradual immunosuppression of host and increased susceptibility to opportunistic infections [1].
In HIV patients, CD4+ count and HIV-RNA (viral load, VL) are the markers of clinical progression used to manage and to monitor the infection. A large number of HIV patients are also coinfected with hepatitis C virus (HCV) [2, 3]; the co-infection is associated with an increased risk of progression to AIDS [4] and poor CD4 T cell recovery even after years of active antiretroviral therapy (ART) [5].
The introduction of ART in 1996 has significantly improved immune response and life expectancy of HIV infected individuals with consequent significant decline in the incidence of virus-related AIDS-defining malignancies (ADMs), as Kaposi's sarcoma, non-Hodgkin lymphoma and invasive cervical cancer [6], representing an index of clinically remarkable immunosuppression. On the other hand, several non-AIDS defining malignancies (NADMs), such as hepatocellular carcinoma (HCC), Hodgkin's lymphoma (HL), anal cancer, lung cancer, colorectal cancer (CRC), gastrointestinal cancer (GI), breast cancer, cardiovascular diseases, liver diseases, kidney and neurodegenerative diseases have been observed [7-9].
Nevertheless, long-term use of ART exposes the patients to an increased risk of metabolic disorders and oxidative stress (OS), all factors that can contribute to the onset of NADMs [10].
HIV patients show reduction of antioxidative activity [11], excessive production of reactive oxygen species (ROS) [12], reduced glutathione (GSH) levels and glutathione/oxidized glutathione (GSH/GSSG) ratio, that seem to contribute to an increase in DNA damage [13].
The incidence of NADMs is elevated in HIV infected patients compared with the general population and it is associated with smoking use, alcohol consumption, overweight/obesity and oncogenic virus infection [human papillomavirus (HPV), HCV and hepatitis B virus (HBV)] [14].
The levels of OS markers are generally higher in HIV/HCV co-infected than in HIV mono-infected patients [15, 16]. To date, numerous studies have shown that ART triggers further the OS [17, 18]. High incidence of malignant tumours, epidemiologically associated with HIV infection, can be attributable to genotoxic effect of HIV that leads to double-strand breaks of chromosomal DNA [19, 20].
Some studies report that *Vpr*, an accessory gene of HIV which induces abnormality of cell cycle causing the arrest in the G2-M phase, leads to a genomic instability including formation of micronuclei (MN) [21, 22].

MN, small additional nuclei originating from chromosome fragments or whole chromosomes during nuclear division not included in the daughter nuclei in telophase [23], are used as sensitive biomarker of chromosomal damage, genome instability and intermediate endpoint in carcinogenesis [24-26].
The aim of the study is to determine MN frequency in a cohort of HIV patients compared with healthy control-group and to evaluate the relationship between demographic and clinical data and markers of DNA damage.

Methods

Study population

The study involved the enrollment of HIV-infected patients and healthy controls afferent to the Infectious Diseases Division of Santa Caterina Novella's Hospital (Galatina, Italy) and to the Department of Immunohematology and Transfusion Medicine of Vito Fazzi Hospital (Lecce, Italy), respectively.
Patients with documented HIV infection treated with ART and older than 18 years of age were included in the study. Sex and age-matched, healthy HIV-uninfected individuals were enrolled as controls. Instead, HIV patients and healthy subjects under 18 years old, pregnant and/or exposed to risk factors associated with genetic damage (such as occupational or medical exposure to ionizing radiation) were excluded.
The study was approved by the local ethics committee and all patients approached for the study gave written consent to participate (Resolution n. 811; May 3, 2012).
HIV-related clinical information (mode of transmission, duration of infection, duration of ART, HCV co-infection and AIDS) including laboratory data (VL, CD4 counts and CD4 nadir), were collected from HIV-infected patients at the time of study enrollment.
Relevant data including age and sex, as well as risk factors like diabetes, obesity and smoking status were available for both HIV and controls. The patients' information and blood samples were collected at the enrollment time and the MN test was immediately carried out.

Micronucleus assay

Peripheral blood samples were collected by venipuncture into vacutainer blood tubes with lithium heparin anticoagulant.
Cellular cultures from each subject were set up by mixing 300 µl of whole blood with 4.7 ml of karyotyping medium. All cultures were incubated at 37°C for 44 h in a humidified atmosphere containing 5% CO_2. For evaluation of MN frequency, cells were blocked in cytokinesis by adding cytochalasin B after 44 h. Cell cultures were then harvested after 28 h and fixed for slide preparation. Therefore, the fixed cells were dropped onto clean iced slides, air dried and stained by the Giemsa technique [27].
Only binucleated lymphocytes are scored for DNA damage biomarkers which include MN. For each sample, 1000 binucleated cells were calculated blindly under the optical microscope for MN analysis, following the criteria for MN acceptance listed by Fenech [23]. We have evaluated the MN frequency as the number of micronucleated-binucleated lymphocytes, containing one or more MN per 1000 cells.

Statistical analysis

Continuous variables were reported as the mean ± standard deviation (SD) and categorical factors as percentages. The Levene's test was used to verify the normality of the distribution of continuous variables. Differences between the means of the two continuous variables were evaluated by 2-tailed unpaired Student t test. Differences in non-continuous variables were tested by Chi-square test analysis or by Fisher's exact test, as necessary.
The association between demographic and clinical variables and MN frequency was assessed by univariate linear regression analysis followed by multiple linear regression analysis per variables with $p < 0.05$. Statistical calculations were performed with MedCalc software, version 11.4.1.0. A p-value < 0.05 was considered to be statistically significant.

Results

52 HIV infected patients and 55 healthy controls admitted at the Infectious Diseases Division of Santa Caterina Novella's Hospital (Galatina, Italy) and at the Department of Immunohematology and Transfusion Medicine of Vito Fazzi Hospital (Lecce, Italy) from January 2013 to January 2015 were recruited in this study. Demographic and clinical characteristics of participants are illustrated in Table I.
No significant differences were observed between HIV patients and healthy controls in age ($p = 0.9658$), sex ($p = 0.8727$), smoking use ($p = 0.1053$) and diabetes ($p = 1.000$). A highly significant difference was found only for HCV infection ($p < 0.0001$).
In the cohort of HIV patients, the average duration of HIV infection was of 95 months, the mean CD4 cell count was of 517 ± 314 cells/mm^3 (range 5-1,305) and HIV-RNA copies/ml of 58,183 ± 151,553 (range 19-875, 716).
By the comparison of the MN frequency in peripheral blood mononuclear cells (PBMC), a significant difference between HIV patients (15.5 ± 9.8) and controls (6.0 ± 3.6) ($p < 0.001$) was revealed (Fig. 1). Table II shows the results of the univariate and multivariate linear regression analyses, demonstrating the relationships between MN and other variables. Two risk factors were strongly associated with increased MN frequency in HIV patients upon univariate analysis: HCV infection ($r = 0.31$; $p < 0.001$) and HIV-RNA ($r = 0.29$; $p < 0.05$). However, only VL significantly correlates ($r = 0.29$; $p < 0.05$) with the MN frequency in a multiple regression model, where variables with p value < 0.05 in the univariate analysis were included as independent (Tab. II).

Tab. I. Demographic, clinical characteristics and laboratory values of patient's HIV and control group.

	HIV-1+ (n = 52)	Control (n = 55)	P-value
Age, years (mean ± SD)	42.5 ± 9.6	42.6 ± 9.9	0.9658°
Gender, male, n (%)	40 (76.9)	42 (76.4)	0.8727^
Obesity, n (%)	1 (1.9)	1 (1.8)	1.000#
Smoke use, n (%)	25 (48.1)	17 (30.9)	0.1053^
HCV infection, n (%)	18 (34.6)	0 (0.0)	< 0.0001#
Diabetes, n (%)	1 (1.9)	1 (1.8)	1.000#
Mode of transmission			
• Heterosexual contact, n (%)	19 (36.5)	-	
• Male-to-male sexual contact, n (%)	21 (40.4)		
• Injection dug use, n (%)	11 (21.2)	-	
• Other, n (%)	1 (1.9)	-	
Duration HIV infection, months	95 (1-339)	-	
Duration HIV infection > 36 months, n (%)	33 (63.5)	-	
AIDS, n (%)	2 (3.8)	-	
HIV RNA (mean ± SD)*	126,042 ± 82,553	-	
Undetectable HIV viral load, n (%)	28 (53.8)	-	
CD4 cells/mm^3, mean ± SD (range)	517 ± 314 (5-1305)	-	
Nadir CD4 cells/mm^3, mean ± SD (range)	233 ± 166 (2-755)	-	
Duration of ART, months	61.1 (1-203)		
Duration of ART > 36 months, n (%)	30 (57.7)		

°: Independent samples t-test; ^: Chi-square test; #: Fisher's exact test; *: mean of viremic patients.

Discussion

The aim of this study was to evaluate the cytogenetic damage in PBMC of HIV patients, considering that DNA damage may develop ADMs and NADMs.

To this end, two groups have been enrolled: HIV patients undergoing ART treatment (study group) and subjects with no HIV-infection (control group). The HIV group included many recently diagnosed patients, which benefited from ART therapy for a limited time, with virus not yet suppressed. All the patients started therapy at the time of diagnosis.

The initial design of the study included a third group of HIV-infected subjects that did not receive therapy (naïve group). Anyhow, over the course of the study, very few new cases of HIV occurred. Therefore, it was not possible to select a sufficient sample size to make statistically significant comparison. The absence of a naïve group is a major weakness of this study because it has not allowed the dissociation of the damages induced by the infection and those induced by the therapy.

The introduction of ART has modified the natural history of HIV infection, leading to an increase of survival time and a reduced AIDS-related mortality, but also to an excess of neoplastic diseases that have becoming one of the most common cause of death among HIV patients [28, 29]. The treatment leads to viral suppression but does not completely restore the immune damage, so ineffective immune response could be the reason why HIV patients have an increased risk of developing different types of tumours [30, 31]. The role of HIV in the development of neoplastic pathologies can be linked to severe immunodeficiency with consequent impairment of immunological surveillance against infectious agents (with predisposition to the appearance of virus-associated tumours) and the cells with malignant transformation [32-34], although its role in the process of carcinogenesis has not yet been completely clarified.

Our study provides evidence that HIV infection may have an impact on the genetic damage. Indeed, MN frequency was significantly increased in the study group compared to the control group.

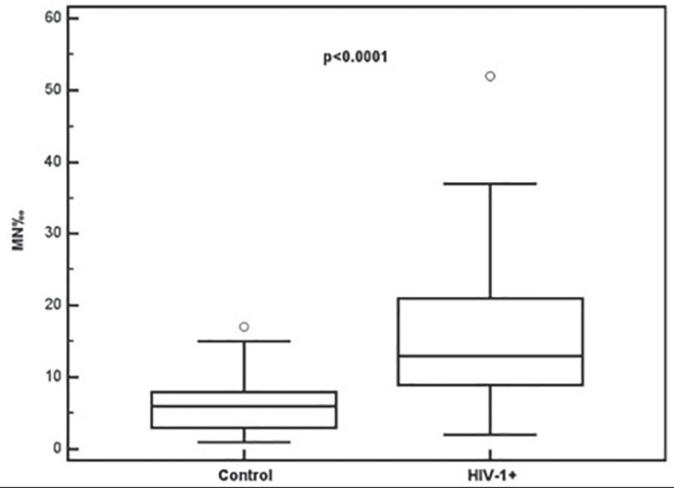
Our findings are in line with those of Lima and colleagues that show an increase in the frequency of multiple MN in oral mucosa cells in HIV patients compared to healthy controls [35].

The micronucleus cytome assay applied in buccal exfoliated cells is a complementary method for measuring DNA damage and cytotoxic effects caused by exposure to genotoxic agents, impact of nutrition, lifestyle factors and virus [25, 36].

Several studies, reporting the parallel application of the MN test in both PBMC and in buccal cells, have shown a positive correlation between the MN frequencies in the two surrogate tissues [37, 38], therefore the results of the two methods can be comparable. In addition, the strong correlation of MN frequency in buccal exfoliated cells with that in PBMC, implies that systemic genotoxic effects may also impact on and be detectable in buccal cells. Hence, the possible human health risks associated with high MN frequency in both tissues may also be comparable, including the association with cancer risk [24].

We have also analysed the association among the frequency of MN with baseline HIV-specific clinical variables and patient characteristics. The univariate analysis has showed a statistical significance with HCV infection and HIV-RNA, while the multivariate analysis has displayed significant association only between HIV-RNA and frequency of MN. The 54% of HIV-infected patients

Fig. 1. Frequency of micronuclei in peripheral blood lymphocytes in control group and HIV-1+ patients.



Tab. II. Univariate and multiple linear regression analysis of the relationship between micronucleus (MN) frequency and characteristics of HIV-1+ patients.

	Univariate		Multivariate	
	r	p-value	r	p-value
Age, years	– 0.07	0.605		
Gender	– 0.09	0.525		
Obesity	0.15	0.276		
Smoke use	– 0.11	0.440		
HCV infection	0.31	0.001	0.05	0.943
Diabetes	– 0.03	0.819		
Duration HIV infection	– 0.16	0.246		
HIV-RNA	0.29	0.032	0.29	0.022
CD4	0.05	0.701		
Nadir CD4	0.35	0.067		
Suboptimal ART	0.06	0.649		
Duration of ART	– 0.10	0.475		

enrolled, all in therapy with ART, were aviremic (with VL < 20 copies/ml). In these patients, the mean number of MN (13.2 ± 6.5) was significantly lower ($p < 0.002$) than in patients with uncontrolled viremia (17.8 ± 12.4). These data support the hypothesis that viremia plays a determinant role in the induction of chromosomal damage.

The current knowledge of genotoxic effects of infection and therapy are still limited. Only few studies have tried to explain the MN formation. Shimura M et al. and Tachiwana H et al. have linked this process to the action of the accessory gene Vpr of HIV, which induces double-strand breaks of chromosomal DNA [21, 22], while Lourenco ED and colleagues justify it with a clastogenic (chromosome breakage) and aneugenic (chromosome loss) action of the therapy [39].

Our work supports the hypothesis of cytogenetic damage induced by HIV infection, but further studies in naïve and in ART therapy patients and with different therapeutic programmes, are mandatory.

Moreover, our data show that exposure to the virus plays a key role in the development of cytogenetic damage, although the precise exposure date is not known. Finally, many questions regarding the relationship between HIV and AIDS related and non-AIDS related cancer are still left unanswered.

Future researches should be focused on identifying early and sensitive risk indicators for the development of cancer.

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Conflict of interest statement

None declared.

Authors' contributions

AZ, PG and MG conceived and designed the research. PG recruited patients and PN enrolled healthy controls. MRT and AB performed micronuclei assay. MG performed the statistical analyses. AZ, PG, MGA, ADD and MG evaluated the results. AZ, MRT and MG wrote the manuscript. All Authors revised the manuscript and gave their contribution to improve the paper. All authors read and approved the final manuscript.

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