Neurofibromatosis type 1 (NF1) is an autosomal dominant disorder which displays considerable inter- and intra-familial variability in phenotypic expression. NF1 is characterized particularly by café-au-lait spots and fibromatosous tumors of the skin. In this study, the comet assay was used to evaluate levels of basal single strand breaks, H₂O₂ oxidation-induced DNA damage, and repair capacity in lymphocytes of NF1 patients compared to healthy control subjects. No significant differences in DNA damage were observed between controls and patients (p > 0.05), but DNA repair capacity was significantly slower in NF1 patients (p < 0.05). It suggests less efficient DNA repair capacity may be associated with NF1 disease. Using this assay we could identify individuals with poor repair capacity who would be good candidates for intensive follow-up and screening.

Keywords: DNA repair, neurofibromatosis, peripheral blood lymphocytes, comet assay, oxidative damage

Introduction

Neurofibromatosis type 1 (NF1, OMIM# 162200) is a common autosomal dominant disorder affecting one in 3500 individuals. It is caused by deletion or point mutation of NF1, a tumor suppressor gene mapping to the chromosomal region 17q11.2. About half of the NF1 cases are caused by de novo mutations [1-3].

The main clinical features of the disease are café-au-lait spots, freckling of the axillary or inguinal region, iris Lisch nodules, and cutaneous neurofibromas, au-lait spots, freckling of the axillary or inguinal region, iris Lisch nodules, and cutaneous neurofibromas, and CNS tumors. The NF1 gene, located on chromosome 17q11.2, encodes a protein, neurofibromin, which regulates the Ras signaling pathway. Mutations in NF1 lead to the development of neurofibromas and other tumors, as well as to the development of café-au-lait spots and freckling of the axillary or inguinal region, iris Lisch nodules, and plexiform neurofibromas.

The ability to repair DNA damage is strongly associated with risk of cancer and other diseases such as neurodegenerative inflammatory disorders. Repair of DNA damage plays an essential role in cell survival and the maintenance of genomic stability [9]. Altered DNA repair pathways can alter an individual’s ability to repair DNA damage, resulting in increased sensitivity to exogenous and endogenous agents and greater susceptibility to mutations and genetic instability [9].

Consequently, this would center attention on factors involved in DNA repair as possible modifiers of the NF1 phenotype, with detection of such phenotypic modifiers having potential prognostic value.
Particularly, DNA damage induced by reactive oxygen species (ROS) may lead to single- or double-strand breaks, point and frame-shift mutations and larger-scale chromosomal abnormalities [11]. Molecular oxygen reaction products induce point mutations, deletions and gene amplification and rearrangement in mammalian cells, which may result in proto-oncogene activation and/or tumor suppressor gene inactivation [12]. Among more than 30 different products of modified DNA by oxidative stress, 8-oxo-7,8-dihydropyrimidine (8-oxoGuo) is the most studied mutagenic lesion. This lesion induces an increased frequency of spontaneous G:C or T:A transversion mutations. The oxidative DNA lesion 8-oxoguanine is recognized by the specialized repair enzyme 8-oxoguanine DNA glycosylase (hOGG1). This enzyme can be used as specific tool for identification of oxidized guanine bases, as it reveals these lesions as single strand breaks that can be detected using the single cell gel electrophoresis or comet assay [13-15].

Various biomarkers have been used to determine cellular DNA damage in NF1; cytogenetic measurements include chromosomal aberrations, micronuclei and sister chromatid exchanges [16, 17]. Additionally, the comet assay technique is recognized among the most rapid, simple and sensitive methods available for measuring DNA strand breaks with a small number of cells [18, 19]. The alkaline comet assay resolves break frequencies up to a few thousand per cell, so the distances between breaks are in the order of 106 Da. To examine 8-OddG levels by this technique, DNAs can be incubated with hOGG1, a commercial endonuclease that generates additional breaks at sites containing 8-oxo-dGua, and by comparing the DNA migration in enzyme-treated and untreated slides, quantitation can easily be made [20, 21].

The aim of this work was to study the probable differences between NF1 patients and healthy controls regarding the levels of endogenous and induced DNA damage and the repair capacity of peripheral blood lymphocytes. We hypothesized that NF1 patients would have higher levels of pre-existing strand breaks, more oxidation-induced lesions in DNA, lower resistance to oxidant challenge, and poorer DNA repair when compared to healthy controls. For that purpose, DNA damage was quantified by measuring single strand DNA breaks and abasic sites of untreated and treated peripheral blood lymphocytes, using the alkaline comet assay. To the best of our knowledge, this is the first study using the Comet assay to evaluate DNA damage and repair capacity in lymphocytes of NF1 patients.

Materials and methods

Study subjects

Thirty NF1 patients were enrolled (15 men and 15 women; age as mean ± SD: 24.9 ± 8.2 years). All the subjects were diagnosed to have NF1 based on standard diagnostic criteria at the Juan Manuel Márquez Pediatric Hospital and Hermanos Ameijeiras Clinical Hospital, both in Havana, Cuba. Medical histories of clinical and physical examinations were performed to all the NF1 individuals enrolled. The control group comprised 30 healthy subjects (10 men and 20 women; age: 35.2 ± 8.8 years) from Havana. Exclusion criteria for all subjects were chemotherapy or radiotherapy, infections, and blood transusion in the previous month. After agreement and signing the informed consent, all participants donated 5 mL of venous blood and completed a questionnaire that provided detailed information on occupational exposure, family history of cancer, medications, reproductive history, and past treatments for noncancer conditions. There were no age and gender restrictions for study eligibility. All the controls and patients were non-smokers.

The laboratory and questionnaire data were coded, entered and verified; neither the laboratory nor the data entry personnel had knowledge of the subjects’ case-control status. Written informed and educated consent was obtained from each patient or healthy volunteers and from parents of all children before entering into this patient-control study. This study was conducted according to the guidelines laid down in the Declaration of Helsinki [22] and approved by the ethics committee of the National Centre of Medical Genetics, Havana, Cuba.

Peripheral blood lymphocyte isolation

Heparinized blood samples from the NF1 patients and control subjects were protected from light, put on ice, and processed within 4 h of collection in the Oxidative Stress Laboratory at the National Centre of Medical Genetics, Havana, Cuba. Lymphocytes were isolated using standard Ficoll-Histopaque method. Briefly, 5 mL of whole blood from each subject was layered over 5 mL of Histopaque-1077 (Sigma Aldrich Co., St. Louis, MO) at 4 °C and centrifuged at 1500 rpm for 30 min. The mononuclear cells were removed from the interface, washed twice with cold (4 °C) phosphate buffered saline (PBS), pH 7.2, and centrifuged at 1500 rpm for 10-15 min. Cells were resuspended in 1 mL of cold PBS. Manual cell counts and the cell membrane integrity were determined by Trypan Blue solution 0.4 % and the cell suspension was adjusted with PBS to 1 × 106 cells/mL.

DNA damage assessment

Constitutive or endogenous DNA damage as pre-existing single strand breaks was assessed by the comet assay [20] with some modifications. Two slides per each patient and control and two gels per slide (i.e., four gels per patient and control) were prepared. Briefly, 50 µL of each cell suspension (estimated to contain approximately 1000 cells) were added to 75 µL of 1 % low melting point agarose solution made in PBS buffer at 37 °C, gently mixed, and the mixture was immobilized on a microscope slides which had previously received a layer of 0.5 % low melting point agarose. When the gel had set, the slides were placed in freshly prepared ice-cold lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl, and 1 % Triton X-100 with 10 % DMSO, pH 10) to remove cell proteins, leaving DNA as ‘nucleoids’. To allow for DNA denaturation and unwinding and the exposure of the alkali-label sites, slides were kept for 25 min in a horizontal electrophoresis chamber without power that was filled with freshly prepared alkaline buffer (0.3 M NaOH and 10 mM EDTA at pH > 13.0) at

4 °C. After the unwinding, DNA was electrophoresed at 0.8 V/cm and 300 mA for 25 min; all these steps were carried out in subdued light. Finally, the slides were washed three times in neutralizing buffer (0.4 M Tris, pH 7.5) to remove alkali and detergents, and were stained using a silver staining protocol [19]. Slides were: a) fixed for 10 min in a solution containing 15% trichloroacetic acid, 5% zinc sulphate heptahydrate, and 5% glycerol; b) washed three times with deionized water; c) placed back-to-back in a horizontal staining jar; d) stained for 35 min in dark conditions with shaker using 75 mL of freshly prepared stain solution composed by 34 mL of vigorously mixed stock solution B (0.1% ammonium nitrate, 0.1% silver nitrate, 2.5% tungstosilicic acid, 0.15% formaldehyde, v/v) and 66 mL of stock solution A (5% sodium carbonate); e) washed three times with deionized water; f) immersed 5 min in a stop solution (acetic acid 1%); and g) slides were air-dried.

Induced DNA damage and repair

A modification of the basic alkaline comet assay was introduced to test the cells’ response and their capacity to repair after a controlled in vitro oxidative challenge. This was induced by exposure to 200 µmol/L hydrogen peroxide (H$_2$O$_2$, made up in PBS), for 5 min at 4 °C. Some of the challenged cells were washed and then embedded in agarose and run through the comet assay as described above, to measure its resistance to challenge, while some of the challenged cells were used to assess DNA repair. This last was done by re-suspending the washed cells in RPMI 1640 medium containing 20% fetal calf serum, and incubating the cells at 37 °C for 90 min, which were further placed on ice to stop DNA repair, and embedded in agarose and the comet assay run. The efficacy of DNA repair was taken as the relative difference between DNA damage immediately after challenge and after 90 min of repair.

DNA repair enzyme treatment

In order to examine basal levels of oxidation-induced damage in DNA, nucleoids embedded in agarose were incubated with 50 µL hOGG1 in buffer (20 mM Tris-HCl, 1 mM EDTA, 1 mM diethothreitol, 100 µg/mL bovine serum albumin) at 0.08 U per gel, for 45 min at 37 °C. hOGG1 is an endonuclease that recognizes lesions 8-oxo-dGuo and creates single strand breaks at 37 °C. The enzyme was expressed as the difference between the enzyme-treated gels and the buffer-treated gels.

Statistical analysis

The values of the comet assay were expressed as mean ± standard error of the mean. The statistical analyses were performed by the nonparametric Mann-Whitney U-test, since data showed no normal distribution. A p value lower than 0.05 was considered as significant. All the analyses were performed using the Statistica® software version 8 (StatSoft Inc.).

Results and discussion

Our study was performed in the course of a multicenter study in Havana about DNA damage in patients with genetic deficiency in tumors suppressor genes. In this study, DNA damage and repair testing was offered to children showing no severe clinical features characteristic of the disease who fulfilled the internationally established minimum clinical criteria in NF1 disease. NF1 group had approximately 25% higher baseline DNA damage than the control subjects, though this fell just short of statistical significance (p = 0.053), while the hOGG1-sensitive sites were very similar in the two groups. The controls had similar mean H$_2$O$_2$-induced DNA damage compared with cases (198.21 ± 9.94 versus 203.18 ± 10.90; p > 0.05) (Table). The DNA repair percentage of residual DNA damage among cases and controls are expressed as arbitrary units, which is related to the percent of DNA damage in the tail. Slides were analyzed under blind conditions.

To assess sensitivity to H$_2$O$_2$, the induced damage (IND) was calculated as the damage score in the H$_2$O$_2$-stressed cells and the damage score of cells from the same subject but without H$_2$O$_2$ exposure. The repair capacity was calculated as the percentage of H$_2$O$_2$-induced DNA damage remaining after 90 min repair time of stressed cells % RD in relation to the induced damage in cells immediately after exposure to H$_2$O$_2$ [24, 25].

Table. DNA damage in NF1 patients and controls according to the characteristics of the study

<table>
<thead>
<tr>
<th></th>
<th>Baseline damage</th>
<th>H$_2$O$_2$-induced damage</th>
<th>Repair capacity (%)</th>
<th>Oxidative damage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients (n=30)</td>
<td>45.69 ± 5.37</td>
<td>198.21 ± 9.94</td>
<td>44.90 ± 6.13</td>
<td>34.92 ± 6.90</td>
</tr>
<tr>
<td>Controls (n=30)</td>
<td>35.96 ± 3.88</td>
<td>203.18 ± 10.90</td>
<td>74 ± 0.27</td>
<td>34.35 ± 5.52</td>
</tr>
<tr>
<td>p</td>
<td>0.053</td>
<td>0.16</td>
<td>0.00041***</td>
<td>0.838</td>
</tr>
</tbody>
</table>

* Mean ± standard error of mean of the DNA fragmentation level, expressed in arbitrary units.

** Percentage of induced DNA remaining in challenged cells after 90 min of repair time.

*** Statistic comparison of the NF1 patients versus controls by the Mann-Whitney U test. *** p < 0.001.
In Nf1+/− mice, Gutmann and colleagues [32] found that a deficiency in MMR (Mhl1−/−) significantly accelerated myeloid leukemogenesis, with concomitant microsatellite instability and loss of neurofibromin expression in the tumors analyzed.

On the other hand, germline gene alterations play a significant role during malignant transformation of progenitor glial cells. In glioma patients have been demonstrated that germline p53 mutations are frequent in patients with multifocal glioma, gliomas and another primary malignancy [33]. Similarly, germline mutations in DNA repair genes BRCA-1 and BRCA-2 significantly increase the risk of developing multifocal glioblastoma [34]. Some studies suggest that reduced expression of MMR genes is frequent in human gliomas, and aberrant expression of more than one MMR gene may be associated with an increased risk of second primary malignancies in glioma patients [35]. Several studies that examined both spontaneous and induced chromosome instability in lymphocyte cultures suggested that chromosome instability can be detected in the peripheral blood lymphocytes of glioma patients and it may be a marker for identifying individuals at risk [36].

Another factor identified as involved is poly (ADP-ribose) polymerase-1 (PARP-1), an enzyme involved in DNA repair regulation. PARP-1 interaction with NF-kB has been identified as a major factor regulating macrophage and microglial activation. PARP-1 gene deficiency prevents the morphological changes associated with microglial activation, and suppresses microglia release of proteases [37]. Thus, the inhibition of microglia activation is able to reduce optic glioma proliferation in NF1 patients and influences the clinical variability of NF1 phenotype.

In addition to genotypes, functional phenotypic assays which integrate the different pathways provide useful tools to explore the role of DNA repair in inter-individual variability clinic. Methodologies for measuring DNA damage differ between laboratories and depend upon the DNA-damaging agent used, DNA repair kinetics, the endpoint measured and ways to measure the endpoint (quantitatively or qualitatively). In this sense, the alkaline comet assay protocol used in this study was adequate to detect significant differences in single strand breaks between NF1 patients and controls.

The alkaline comet assay test was designed to provide the most comprehensive picture of the DNA damage induced, quantifying the cellular capacity to repair the observed lesions by showing the disappearance of damaged sites and the genome restoration. The assay was used to assess oxidative, baseline, H2O2-induced DNA damage and repair capacity of DNA and its related genetic instability in NF1 patients’ peripheral blood lymphocytes. H2O2 is a well established genotoxic factor that can be used to evaluate the efficiency of DNA repair pathways as well as being used to assess resistance of cells to oxidant challenge. Exposure to hydrogen peroxide may result in DNA base damage and/or single- and double-strand breaks (SSBs and DSBs, respectively) due to the decrotonation or generation of free radicals [20]. Base modification and SSBs are repaired primarily by base excision repair (BER) [21, 38]. The majority of DSBS
are repaired by nonhomologous end-joining (NHEJ) and homologous recombination repair (HRR) [39].

The use of peripheral blood lymphocytes was used based on the assumption that the DNA repair capacity of an individual is a genetic predisposition measurable in various cell types. Furthermore, this cellular population is easy to acquire from a blood draw and its measurements can serve as surrogates for other target tissues. This notion is supported by the results of studies on relatives and twins showing heritable repair phenotypes [40, 41].

Our main result was that the peripheral blood lymphocytes from case patients with NF1 showed decreased repair of damaged DNA than those from control subjects. We did not observe any difference between media baseline and oxidative endogenous level of DNA damage in lymphocytes of NF1 patients and subjects controls. These findings were consistent with other studies showing similar levels of constitutive DNA damage in the form of spontaneous chromosomal aberrations and sister chromatid exchanges (SCEs) in neurofibroma-derived cells and in normal skin fibroblasts, melanocytes, and peripheral blood lymphocytes among NF1 patients and controls [16, 17].

To the best of our knowledge, this is the first report on the assessment of endogenous damage, oxidized, induced, and unrepaird DNA damage in NF1 patients with the use of the comet assay. Further studies of genetic linkage and association are underway to identify the specific genetic variants associated with variable expression in NF1. Understanding the genetic mechanisms that control phenotypic expression in NF1 will provide further insight into the fundamental disease processes. All these raise the possibility that repair gene(s) playing a role in the pathogenesis of NF1 might be directly or indirectly implicated in pathways contributing to the control of genomic integrity.

Additionally, our results suggest that DNA repair kinetics measured by the comet assay may serve to identify the presence of genetic modifiers and would offer clues to the molecular pathogenesis of NF1. This hypothesis requires verification by long-term monitoring of the study patients and by correlates between DNA repair capacity and disease progression or severity. An altered expression of non-linked repair genes may eventually support more precise predictions of specific clinical features and complications of NF1 that could possibly lead to new therapeutic approaches.

In summary, no differences were found in the endogenous, oxidative and induced DNA damage by \( \text{H}_2\text{O}_2 \) between NF1 patients and healthy controls by using the comet assay. Nevertheless, there was a significant difference in repair kinetics in leukocytes of NF1 patients compared to the control group. Moreover, knowing that most of the patients involved in this study were children and that the frequency of more serious complications tends to increase with age, it would be important to carry on intensive follow-up and screening to all the patients, to see if they develop severe clinical events or not and to determine its relationship to their DNA repair capacity.

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