RESEARCH COMMUNICATION

Evaluation of Genetic Alterations in Inhabitants of a Naturally High Level Background Radiation and Kudankulam Nuclear Power Project Site in India

Vellingiri Balachandar[1], Ramesh Kumar Mithun Kumar[2], Varsha Prakash[1], Subramaniam Mohana Devi[1], Balasubramanian Lakshman Kumar[1,3], Pappusamy Manikantan[1], Keshavarao Sasikala[1*], Jeyapandian Malathi[4,5], Muruganandam Brahmanandhan[4,6], D Khanna[3,7], S Selvasekarapandian[3,8]

Abstract

Evaluation of genetic alterations in inhabitants of an area of Tamil Nadu, India, chronically exposed to high background radiation (HBRA), was the major purpose of the present study. A total of 216 samples (exposed inhabitants, 108; control subjects, 108) were selected based on the confirmation of radiation dose level using thermoluminescence dosimetry (TLD). After signing a consent form, volunteers provided blood samples (5 ml each) to establish cell cultures at 52 h. One hundred complete metaphase cells from each subject were evaluated for karyotyping. The frequencies of chromosomal alterations (CA) were found to be higher in the exposed groups and the aberrations predominately observed were of chromatid-type. Smoking was found to have considerable effect on the frequency of CA in exposed subjects. With the comet assay for DNA damage, a significant increase in comet tail frequency was also observed in exposed subjects compared to controls. At present there are no radioepidemiological data regarding the cytogenetic studies in these areas. Furthermore, the Kudankulam nuclear power plant nuclear power plant is being constructed in the same area. The study gives potentially important information on the general health effects due to radiation exposure and increases people's understanding of the hazardous nature of chronic low level natural radiation exposure. However, we may conclude that the HBRA by itself does not pose any significant risk of genetic damage as measured by conventional cytogenetic analysis.

Keywords: High background radiation area - DNA damage - nuclear power plant - cytogenetics

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Introduction

Natural radiation is a major component of radiation exposure for the general population. In our natural environment, we are chronically exposed to low doses of ionizing radiation. In some situations, this environmental exposure can reach significant doses, such as in the case of some inhabited areas where the soil displays abnormally high amounts of radionuclides. There are many high level natural radiation areas (HLNRAs) throughout the world like those in Brazil, China, India and Iran. The people who live in the HLNRAs of the world are of considerable interest because they have been exposed to abnormally high radiation levels over many generations (Mortazavi et al., 2005).

From the earlier reports of background radiation levels in India, it is well known that the Agastheeswaram taluk (sub-district) and its adjoining taluk, namely Thovalai of the Kanyakumari district, is one of the high background radiation areas (HBRA) and known for its high thoron concentrations (Khanna et al., 2005; Malathi et al., 2005; 2008). In addition, a 6000 MWe nuclear power plant is now under construction at Kudankulam, situated in the Radhapuram Taluk of Tirunelveli District (southern part of Indian sub-continent) by the Indian Government with the help of Russian Federation. The activity concentrations of ^{232}Th, ^{238}U and ^{40}K in Radhapuram taluk have been evaluated from the soil samples collected at various locations. The activity concentration of ^{232}Th in soil has been found to be 6 times higher than the world average (30 Bq.kg–1) and the activity concentration of ^{40}K has been found to be 0.7 times less than the world average (400 Bq.kg–1). In sand sample activity concentration of ^{232}Th is found to be 41 times higher than the world average

*[1]Human Molecular Genetics Laboratory, School of Life Sciences, Bharathiar University, Tamil Nadu, India, [2]PSG Medical College, Coimbatore, [3]Department of Biotechnology, Kongunadu college, Coimbatore, India, [4]Solid State and Radiation Physics Laboratory, Department of Physics, Bharathiar University, Tamil Nadu, India, [5]Universita degli studi di Padova, Department of Chemical Science, Padova, Italy, [6]Center for Frontier Life Sciences, Nagasaki University, Japan, [7]Department of Physics, Karunya University, Coimbatore, [8]Department of Physics, Kalasalingam University, Sivakasi, India. *For correspondence: drk_sasi@yahoo.com*

which shows the abnormal levels of natural radiation in these sea shores (UNSCEAR, 2000; Brahmanandhan et al., 2005 ; Brahmanandhan et al., 2007). Biological dosimetry based on chromosomal damage to peripheral blood lymphocyte culture (PBLC) after accidental overexposure to radiation was first performed in 1962 on victims of the Recuplex criticality accident in Hanford (Bender et al., 1966). Today, chromosomal aberrations (CA) in human peripheral lymphocytes are recognized as a valuable biomarker of effect, probably the only one which has been internationally standardized and validated (Albertini et al., 2000; Russell, 2002). Nowadays it is generally accepted that a high frequency of CA in PBLC is predictive of an increased risk of cancer (Bonassi et al., 2000; Hagmar et al., 2004; Norppa et al., 2004; Rossner et al., 2005; Celik et al., 2007; Balachandar et al., 2007; 2008; 2010). Comet assay, or single cell gel electrophoresis (SCGE) is a rapid, simple, and sensitive technique for measuring and analyzing DNA breakage in individual cells (Tice et al., 2000; Sellappa et al., 2010) and is widely applied in genetic toxicology, environmental biomonitoring and clinical investigations (Kumaravel et al., 2007; Fracasso et al., 2009). It is being extensively used in studies involving genotoxicity of various chemicals, DNA damage and repair and human biomonitoring (Betti et al., 1994; 1995; Piperakis et al., 1998; Sram et al., 2000; Dhawan et al., 2001; Bajpayee et al., 2002; Lam et al., 2002). Previously, studies have been undertaken to examine the effects of low level exposure to ionizing radiation (IR) and HBRA in human lymphocytes (Evans et al.,1979; Bauchinger et al., 1980; Lloyd et al., 1980; Stephen et al., 1981; Bigatti et al ., 1988; Edwards et al., 1989; Jha et al., 1991; Pohl-Ruling et al., 1991; Kubelka et al., 1992; Sabti et al., 1992; Barquinero et al., 1993; Hayata, 2000; Jiang et al., 2000; Touil et al., 2000; Amundson et al., 2001; Cardoso et al., 2001; Tsai et al., 2001; Hayata et al., 2002; Maffei et al., 2002; Zhang et al., 2003; Zhang et al., 2004; Dias et al., 2007).

The strengths and the novelty of the present study is the investigation of biological markers of effect and susceptibility on the same population exposed chronically to IR. In addition, as already mentioned, a nuclear power plant is being constructed at Kudankulam, situated very near to Kanyakumari which is a HBRA. Rationally, it is necessary to screen the indigenous population for genetic monitoring due to HBRA and dispel the unnecessary myths and fear about nuclear power plant. Kanyakumari, Tirunelveli and Thoothukudi districts have been chosen not only for the reason of these regions being HBRAs, but also with the view that these six taluks lie within the 30km radius from the planned Kudankulam nuclear power plant. Biomarkers usage in the field of environmental and occupational health is increasing as a consequence of escalating demands on information about health risks from unfavorable exposures. Considering the known health risks from IR, the main objective of this study was focused on the potential impact of this exposure on the health of local residents as well as the detection of any changes that could be attributed to the effects of high level natural radiation. Hence, the focal aim of the present study was to identify the genetic effects in the inhabitants living in 30-killometer radius in Kudankulam surrounding areas by using relevant methods of biomonitoring such as CA and SCGE. Furthermore, the present study also aimed to correlate the external dose measured by Thermo luminescence dosimetry (TLD) and internal dose level by CA and SCGE.

Materials and Methods

Study Area and Subjects (see Figure 1)

The Kanyakumari district lies between 77°15´ E and 77°36´ E longitudes and 8°03´ N and 8°35´ N latitudes. This district has two taluks namely Agastheeswaram, Thovalai and it is situated at the foot of the Western Ghats. In the above taluk totally 27 samples (Group I n= 12 (44.44%) [Male n= 1 (3.70%); Female n=11 (40.74%)]; Group II n= 15 (55.6%) [Male n= 6 (22.22%); Female n=9 (33.3%)] were selected.

Tirunelveli district is lying with in 8º08”-9º25” north latitude and 77º09”-77º59” of east longitude. Radhapuram Taluk is one of the taluks in India which has “Red garnet sands” and “teri structures”. In the above area totally 32 samples (Group I n= 13 (40.62%) [Male n= 8 (25%); Female n=5 (15.62%)]; Group II n= 19 (59.37%) [Male n= 10 (31.25%); female n=9 (28.12%)] were selected. The Thoothukudi district lies in 8.72° N latitudes and 78.12° E longitudes. In Thoothukudi district, Sattankulam and Tiruchendur taluk totally 49 exposed samples (Group I n= 27 (55.10%) [Male n= 13 (26.53%); Female n=14 (28.57%)]; Group II n= 22 (44.89%) [Male n= 9 (18.36%); Female n=13 (26.53%)] were selected.

Subject recruitment and sample collection

In the present study, totally 216 samples including exposed inhabitants (n=108) and controls subjects (n=108) were selected by confirming the radiation dose level by using the TLD with no immediate history of viral disease during the past 3-4 months which were categorized into Group I (40 years and below of age) and Group II (above 41 years of age). The control group was matched according to the gender, smoking status and age (±2 years relaxed). In exposed inhabitants the total radiation dose level range was between 144-171 nGyh1 to 603-702 nGy-1, and the

Figure 1. Tamilnadu (India) Map (Kudankulam Nuclear Power Plant Surrounding Area - 30 km Radius)

radiation dose level of controls was 46-54 nGy.h-1 to 71-90 nGy.h-1. Written informed consent was obtained from all donors. A heparinized blood sample was taken at the occasion of the periodical medical examination. The volunteers were asked to fill in a questionnaire to obtain the information necessary for the study which includes age, smoking or non-smoking, previous exposures to diagnostic X-rays as patient and nuclear medical examinations. A questionnaire survey was conducted to collect information on lifestyles of all the residents in the study area (Akiba et al., 2002; Binua et al., 2005).

Chromosomal aberration (CA) assay

Cytogenetic analyses was done on cultured peripheral blood lymphocytes, stimulated with phytohemagglutinin M, using standard techniques (Moorhead et al.,1960). The karyotype of each subject was determined by G-banding using trypsin and Giemsa (GTG) (Wuu and Jan 1978). As recommended by the International standard for chromosomal nomenclature (ISCN), 100 well spread metaphases were scored for each subject under a microscope (100x) to identify numerical and structural CA.

Comet assay (SCGE)

The cover slips were gently removed after placing the slides on ice for 5 min. Slides were immersed in a jar containing a freshly prepared cold lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10) to which 1% Triton X-100 and 10% DMSO were added just before use. Lysis was done at 4°C for 1 h in the dark. Initial DNA damage was determined by alkaline comet assay, as described (Singh et al., 1988). With minor modifications. About 104 cells either in 5μL or 10μL blood samples were mixed with 75μL or 80μL of warm low-melting-point agarose (Gibco) at 0.75%, 37°C, in a microfuge tube and spread over a fully frosted microscopic slide pre-coated with 200 μ of 0.1% agarose by layering a cover slip.

Electrophoresis and staining

The slides were then removed and placed on a horizontal gel electrophoresis unit and the unit was filled with freshly prepared alkaline buffer (1mM EDTA and 300mM NaOH, pH > 13) to around 0.25 cm above the slides. The cells were exposed to the alkaline conditions for 20min to allow DNA unwinding, and expression of single-strand breaks and alkali-labile sites. Following this, electrophoresis was conducted for 20min by applying an electric current of 0.7V/cm (25V/300mA). All of these steps were conducted sheltered from the daylight to prevent the occurrence of additional DNA damage. After electrophoresis, the slides were neutralized with 0.4M Tris (pH 7.5) and the DNA was exposed for 5 mins to absolute ethanol in order to preserve all the comet assay slides. Subsequently, the slides were air-dried and then stored at room temperature until scored for DNA migration. Just prior to scoring, the DNA was stained using propidium iodide (20μg/ml distilled water; 25μl per slide).

Image analysis

Slides were examined at 400× magnification using a fluorescent microscope (Leica Microscopy, Germany) equipped with an excitation filter of 515-560 nm and a barrier filter of 590 nm, connected through a gated CCD camera to Comet Image Analysis System, version 4.0 software. Images of 100 randomly selected cells were analyzed from each investigated slide.

Tail parameters

The olive tail moment (OTM) was used to evaluate DNA damage. The tail length between the edge of comet head and the end of the comet tail was measured. A major advantage of using the OTM as an index of DNA damage is that, both the amount of damaged DNA and the distance of migration of the genetic material in the tail are represented by (Andersen and Krishnan, 1994).

Statistical analysis

All statistical analysis were performed using the software SPSS for Windows, version 13 to assess the Group statistics in experimentals for mean age, smoking status, radiation dose level and CA, mean ± SD. The level of statistical significance was set at $P < 0.05$ level. An analysis of linear regression was used to estimate the effect of the duration of occupational exposure of antineoplastic drugs on the DNA damage. Analysis of variance was used for interpretation of results regarding the frequencies of CA. The level of statistical significance was set at the $p < 0.05$ level.

Results

In the present study a total of 216 (n=108 exposed inhabitants; n=108 controls) samples were selected. They were grouped as follows; Group I 40 years and below of age (n=52) and Group II above 41 years of age (n=56). In kanyakumari district (Agastheeswaram and Thovalai taluks) totally 27 samples (Group I n= 12(44.44%); Group II n= 15 (55.55%) were selected. In group I and II, the total radiation dose level range was 313.87 ± 90.57 to 342.67 ± 90.39 and 308.51 ± 81.70 to 328.53 ± 78.67. In group I totally 9 chromatid-type aberrations (CTAs) and 2 chromosomal-type aberrations (CSAs) were observed whereas in group II 31 CTAs; 11 CSAs were noted. In Nanguneri and Radhapuram taluks totally 32 exposed subjects (Group I n= 13 (40.62%); Group II n= 19 (59.37%) were selected. The total radiation dose level range in group I was 298.76 ± 27.81 to 312.23 ± 24.53 and group II was 298.40 ± 49.39 to 311.95 ± 46.82. In group I totally 9 CTAs and 4 CSAs were observed whereas in group II 33 CTAs; 16 CSAs were displayed.In Sattankulam and Tiruchendur taluk totally 49 exposed subjects (Group I n= 27 (55.10%); Group II n= 22 (44.89%) were selected. The total radiation dose level range in group I and II was 214.2 ± 7.42 to 241.9 ± 121.98 and 280.2 ± 135.33 to 247.91 ± 114.14. In Thoothukudi district also group-II (41-CTAs and 13 CSAs) showed higher numbers of CA when compared to group- I (16-CTAs and 6 CSAs). Dicentric chromosomes were observed in both groups, but group II exhibits more number of dicentrics when compared to group I. In controls there were no dicentric chromosomes.

Table 1. Frequency of Sister Chromatid Exchange (SCE) in Controls

Subject area (s)		Total number of samples	Age	Dose- Level	Smokers/ Alcohol users	Chromosomal – type aberration		Chromatid – type aberration		% of aberrant cells	Total aberration
						Mean±SD	P value	Mean±SD	P value		
Kanyakumari district	Group I	12	31.3±6.03	313.87±90.57 to 342.61±90.39	1	0.17±0.39	0.1522	0.75±0.45	0.0002	0.91	0.92±0.51
	Group II	15	52.9±9.19	308.51±81.79 to 328.53±78.67	3	0.81±0.68	0.0173	2±0.85*	8.479e-07	2.8	2.8±1.26*
Tirunelveli district	Group I	13	30.1±6.17	298.76±27.81 to 312.23±24.53	2	0.23±0.44	0.2957	0.92±0.49	5.644e-07	1	1.15±0.69
	Group II	19	53.6±8.78	298.40±49.39 to 311.95±46.82	7	0.74±0.65	0.006	1.74±0.56*	9.598e-07	2.47	2.47±0.90*
Thoothukudi district	Group I	27	24.7±7.42	214.22±105.65 to 241.93±121.98	5	0.22±0.42	0.282	0.59±0.75	0.0378	0.81	0.81±0.96
	Group II	22	52.1±7.40	247.91±114.14 to 280.18±135.33	3	0.59±0.50	0.0243	1.86±0.83*	2.705e-11	2.45	2.45±0.86*

Exposed subjects: Kanyakumari, Tirunelveli and Thoothukudi districts and surrounding inhabitants; Group I (40 years and below of age) and Group II (above 41 years of age); Dose level measured by thermoluminescence dosimetry (TLD); * significant at $p < 0.05$ level

Table 2. Tail Moment, Tail Length and Percentage of DNA in Tail Depending on the Dose Level in Kanyakumari, Tirunelveli and Thoothukudi Districts

Subject area(s)		Total number of samples	Dose- Level	T (%) (percentage DNA in tail) (mean ±SD)	TL μm (Tail length) (mean ±SD)	Tail Moment
Kanyakumari district	Group I	12	313.87 ± 90.57 to 342.61 ± 90.39	4.03 (3.78-11.98)	3.95 (2.85-11.65)	0.28
	Group II	15	308.51 ± 81.79 to 328.53 ± 78.67	4.23 (4.11-11.65)	4.07 (3.05-11.48)	0.36
Tirunelveli district	Group I	13	298.76 ± 27.81 to 312.23 ± 24.53	3.99 (3.81-10.95)	4.01 (2.61-10.93)	0.27
	Group II	19	298.40 ± 49.39 to 311.95 ± 46.82	4.18 (4.32-11.80)	4.04 (3.01-10.80)	0.34
Thoothukudi district	Group I	27	214.22 ± 105.65 to 241.93 ± 121.98	4.01 (3.81-12.08)	3.98 (2.85-11.39)	0.27
	Group II	22	247.91 ± 114.14 to 280.18 ± 135.33	4.11 (4.06-11.95)	4.01 (2.81-10.91)	0.32
Controls	Group I	52	64.13±13.41 to 79.75±6.56	3.19 (3.60-11.21)	3.01 (2.45-9.19)	0.14
	Group II	56	61.32±13.78 to 76.18±13.62	3.28 (3.91-11.78)	3.01 (2.68-9.42)	0.18

In the present study group II showed higher number of CA compared to group I (Table 1).

The SCGE (comet) assay was used to measure DNA damage see Table 2). DNA strand breaks were represented by the mean tail moment and tail intensity. A statistically significant increase in comet tail frequency was observed in exposed subjects compared to controls. Cigarette smoking significantly increased the tail length in both exposed subjects and controls. Group II exposed subjects and controls were signified by their mean tail moment and intensity which was higher than group I. Statistically significant results were obtained in exposed subjects compared to controls ($P < 0.05$) by using the ANOVA for CTAs and CSAs (exposed subjects & controls).

Discussion

The investigational area of present study is well known for its high level of natural radiation which has been confirmed doubtlessly due to the radiation level surveys that have been carried out previously in the Kanyakumari (Malathi et al., 2005; 2008). Tirunelveli (Brahmanandhan et al., 2005; 2007) and Thoothukudi districts which identified some of the places as encrusted HBRA apart from the fact of Kudankulam power plant (an upcoming nuclear power plant) was being constructed at Tirunelveli. At present there are no radio epidemiological data regarding cytogenetic studies in these areas. Hence, the aim of the present study was to identify the effect of CA by using G-banding technique and applying comet assay method to identify the DNA damage in the inhabitants of the Kudankulam nuclear power plant surrounding area. Analysis using G-banding allows the identification of rearrangements involved in any chromosome of the genome and also the location of breakpoints within each chromosome, even though it is a time consuming procedure. In our study, majority of the samples were collected from females, as housewives tend to stay in the dwelling most of the time, compared to their husbands. Besides this, most of the male samples studied in the present investigation were cigarette smokers which exhibit the effect of smoking also along with the

radiation cause whereas female samples (all samples were that of non-smokers) clearly highlighted the impact of the radiation only.

In the present study, higher degrees of doses were recorded in Kanyakumari compared to Tirunelveli and Thoothukudi. Extent level of CA frequency was identified in kanyakumari district (3.71%). An increase of CA in PBLC of persons living in an environment with elevated natural background radiation level has also been observed in India (Kochupillai et al., 1976; Cheriyan et al., 1999). Brazil (Marcello, 1975; Franca, 1997), China (Chen et al., 1985; Yao et al., 1985; Chen et al., 1991). The analysis for CA, notably the dicentric in cultured blood lymphocytes, is a long-established method for investigating radiation exposure and is routinely used as a biodosimeter (Pfeiffer et al., 2000; IAEA, 2001). In our study, dicentric chromosomes were observed in exposed subjects, where group II exhibited more number of dicentrics compared to group I.

In this study exposed subjects and controls were analysed for CTAs and CSAs, likely to be formed by different cellular mechanisms and different DNA repair mechanisms which might be probably involved in their formation (Pfeiffer et al., 2000). This finding provides new information on the effects of chronic radiation exposure and its relevance to human health which mainly lies in the fact that radiation in a dose range close to the level of natural radioactivity has the ability to induce a CTA or CSA. According to the Nordic and Italian cohorts, high frequencies of both CTAs and CSAs indicate increased cancer risk (Hagmar et al., 1994; 2004). In the present study, exposed subjects and controls were categorized based on the age wise manner (group I- 40 years and below & group II - 41 years and above) since the cases are chronically exposed to natural background radiation for many generations. In our study, higher degree of CA was observed in group II subjects compared to group I. Aging in humans appears to be associated with genetic instability. Consistent with these studies, our data showed that age was positively associated with CA frequency, strongly indicating that the studies on CA frequency in human PBLC must take into account, the potential confounding effect of age. An age-related increase in aneuploid cells in human lymphocytes has been reported in a number of studies (Nowinski et al., 1990; Wojda et al., 2003; Huang et al., 2009).

DNA damage was evaluated by the alkaline SCGE (comet) assay. The intensity of DNA damage was assessed by computing the tail moment. Three tail parameters (tail length, tail intensity, and tail moment) were considered to quantify DNA damage in the comet assay. These parameters were found to be closely correlated throughout the experiments. In our study, the comet assay was particularly suitable because initial DNA damage at the individual cell level can be analyzed rapidly, in addition to being a sensitive method allowing the study of doses as low as 1.02 mGy (Hagmar et al., 2004). In the present study some of the exposed subjects showed elevated level of tail movement which denotes the DNA damage in that specific individual. Particularly, some of the samples from kanyakumari district showed increased tail movement than the other exposed inhabitants (0.36). Since oxidative DNA damage is an age-related ongoing process, the DNA damage in leukocytes which was detected by the comet assay showed an increase with age. Interestingly, in the present study also group II showed higher degree of tail movement compared to group I. Summation of all these findings showed a decrease in the resistance of cells to DNA damage with increasing age of the organism in the presence of endogen and exogenous stressors.

Further more, the present study also aimed to corroborate the external exposure (TLD) by using the Biodosimetry (CA), for the reason according to (Kubelka et al., 1992). that an increased frequency of dicentric chromosomes in nuclear power plant workers even if the film dosimeter had not indicated that the maximum annual radiation dose was exceeded. The present study indicates that elevated levels of CA were observed in exposed subjects. However, the amount of increased CA is not significant when it is compared with the total amount of other mutagenic factors such as smoking (Balachandar et al., 2008). Chemicals and metabolic factors. The lack of dose–effect relationship between chromosome damage and chronic exposure to low level ionizing radiation could be attributed to various factors, which may, in combination, prevent the detection of exposure effects by cytogenetic biomarkers.

In conclusion, we have proved that the inhabitants of the HBRA have significant risk of genetic damage which has been confirmed by the conventional cytogenetic analysis. The comet assay is appreciably considered to be a powerful technique for investigating effects of environmental mutagens on cells, humans as it is simple and reliable, though labour-intensive. The development of higher throughput versions would certainly help its full potential to be realized. The overall conclusion of the survey was that, with the exception of smokers, the mean dicentric frequencies were not elevated in non smokers. As some of the findings are based on relatively small number of subjects and might also be due to chance, further studies are needed to confirm them and to obtain explanations for the suggested influence of DNA repair polymorphisms on chromosomal damage. It also remains to be examined whether these or other genetic polymorphisms could explain the observed cancer risk predictivity of high CA frequency. Considering the future, it is prudent to accumulate similar information on this relationship using initial biological damage (chromosomal aberrations) analyzed with respect to radiation dose. Nevertheless, some of the residents are still worried and remain fearful about the kudankulam nuclear power plant, not only for themselves, but also in concern of their offspring's welfare.

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