International Journal of Occupational Medicine and Environmental Health 2013;26(3):324–336
DOI 10.2478/s13382-013-0110-6

A RETROSPECTIVE REVIEW OF CYTOGENETIC STUDIES ON METHYL ISOCYANATE WITH SPECIAL REFERENCE TO THE BHOPAL GAS TRAGEDY: IS THE NEXT GENERATION ALSO AT RISK?

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Abstract
The world's worst industrial disaster, at Union Carbide, Bhopal, India, took place on 2–3 December 1984, leading to the leakage of poisonous methyl-isocyanate into the environment, causing thousands of deaths, pregnancy loss and for some, incapacitation for life. More than a quarter of a century later, the Indian Council of Medical Research undertook to redefine the abysmal consequences of the toxic gas exposure on the exposed population. This invigorated the interest of scientific community in the evaluation of the long-term effects, with reference to cytogenetic parameters. The thrust area was identified in terms of genetic disorders, low birth weight, developmental/growth disorders and congenital malformations. Also the impact on epigenetic factors, which may have contributed to variations in the functional expression of genes, was not negated, stimulating intense scientific research on *in utero* exposure and the progeny of the exposed population. To accomplish this mammoth task, molecular cytogenetic investigations must be undertaken in conjunction with conventional cytogenetics, using techniques such as FISH, Immuno-FISH, SKY and SNP analysis, to build up a cytogenetic database of the surviving population.

Key words:
Bhopal gas tragedy, Methyl isocyanate (MIC), Cytogenetic studies, Molecular cytogenetic tools, Cytogenetic database

INTRODUCTION

During the midnight of 2nd December and dawn of 3rd December 1984, Bhopal was witness to the worst industrial disaster that occurred in history due to an explosion at the Union Carbide India pesticide plant, releasing more than 40 tones of methyl isocyanate (MIC) and its reaction products over the city [1]. The estimated mortality of this accident was believed to be between 2500 and 5000 people, with up to 200 000 injured [2–5]. The main component of the toxic release, MIC, caused havoc not only within human life but also within the flora and fauna. The immediate effects of MIC on plants in the vicinity included defoliation, burnt patches and the drying of most aerial parts. Maximum damage was observed in succulent and xerophytic plants in which stomata usually remain open during the night. Newly-formed leaves suffered from chlorosis, necrosis or white patches appearing in mosaic patterns [6]. The same group also documented that aberrations in root

Received: July 30, 2012. Accepted: April 25, 2013.
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cells of *Solanum surattense* varied depending on the exposure to the gas [7]. However, in some plants such as *Artocarpus integrifolia*, *Casuarina equisetifolia*, *Ficus bengalensis* and *Mangifera indica* [8], striking findings of resistance to MIC were documented.

Immediately after the disaster, the Indian Council of Medical Research (ICMR) took up the task of monitoring the health effects of the disaster. On the basis of mortality figures, the ICMR estimated that over 520 000 exposed persons had toxic adducts circulating in their bloodstream, which resulted in various degrees of damage to almost all the organ systems [9]. The ICMR published the findings of 24 research studies involving over 80 000 survivors in the form of 3 reports available at http://www.icmr.nic.in [10]. However, the social stratification and the weather conditions at the accident site created a distinct division of the population into individuals mildly, moderately or severely exposed to the toxins, and exhibiting an equally explicit response. Detailed studies on various disorders including neuromuscular dysfunction of muscle and brain cells in cell lines, as well as *in vivo* studies on animals and the exposed individuals demonstrate the wide spectrum of toxic effects of MIC [11–15]. However, a significant number of published studies evaluating the toxicity of MIC are largely restricted to histopathological findings, especially of lungs and eyes, with small cross sectional studies delineating symptomatology and clinical morbidity in the MIC exposed survivors. As time passed, health status evaluation and rehabilitation of gas victims declined [9].

Clinical studies established that the survivors experienced a higher incidence of health problems including febrile illnesses and respiratory, neurological, psychiatric and ophthalmic symptoms [16,17]. An important study was conducted in 1990 to establish a genetic link between cancer patterns among victims of the gas tragedy with MIC exposure [18]. The population-based cancer register of the ICMR revealed higher incidence rates (crude and age-adjusted) of gallbladder carcinoma (GBC) in the surviving population of the Bhopal gas tragedy [19,20]. A hospital-based registration study of 1261 cancer sufferers who had been exposed to MIC and their offspring, conducted by Senthilkumar et al. [21], reported a gradual increase of cancers in different locations, along with some new cancer cases previously not reported in their population.

It must be noted that although a lot of scientific work has been published which summarize the health effects of MIC, some limitations of these studies have been highlighted. Many commentaries strongly emphasize the need for continued vigilance with regard to the long term adverse effects of MIC [22]. However, a limited number of reports is available on the conventional and molecular cytogenetic profiles of the individuals exposed to MIC, with reference to the genotoxic effects [23–27]. While these reports document the substantial linkage of MIC exposure to somatic mutagenesis, several shortcomings exist regarding their methodological approach. Hence, international groups advocated investigations on the toxicogenomic effects of MIC using cutting edge technologies [28–31]. The importance of experimental studies cannot be undermined, as alteration(s) at genomic and/or epigenetic level can have long-term health consequences that may range from immuno-compromised states to accelerated ageing, carcinogenesis, and more importantly, vertical transmission of genetic aberrations.

Recently, the ICMR has proposed incorporating any existing information into its MIC reports and seeking to decipher any persistent and subtle genotoxic effects of MIC in well-designed genome studies that might enhance the understanding of the extent and gravity of its long term effects [32]. Therefore, molecular cytogenetic investigations in conjunction with conventional cytogenetics, using the available cutting edge techniques such as FISH, Immuno-FISH, SKY and SNP analysis must be performed to build up a cytogenetic database of the surviving population.

METHYL ISOCYANATE AND CYTOGENETIC DAMAGE

Isocyanates (N=C=O) are low molecular weight organic, aromatic and aliphatic compounds, consisting of two double bonds, with strong chemical reactivity. Methyl isocyanate (MIC), toluene di-isocyanate (TDI), diphenyl methane di-isocyanate (MDI), hexamethylene di-isocyanate (HDI) and naphthalene di-isocyanate (NDI) are the most commonly used isocyanates for industrial purposes [33,34]. MIC (CH3N=C=O) is an intermediate chemical in the production of carbamate pesticides such as carbaryl, sevin, aldicarb, methomyl and carbofuran [35–37]. OEHHA (2001) guidelines have indexed MIC as a hazardous substance that during exposure targets the respiratory and reproductive systems [38].

Genetic information, encoded chemically in DNA, is maintained, replicated and transmitted to successive generations with high fidelity. Damage to DNA can occur through normal biological processes or as a result of interaction of DNA, either directly or indirectly, with chemical, physical or certain biological agents [39]. Genetic damage at the chromosomal level entails an alteration in either chromosome number or chromosome structure, and such alterations are measured in terms of chromosomal aberrations, micronucleus frequency and sister-chromatid exchanges. Hence, MIC, a well-known toxic industrial chemical found within the vicinity of a densely-populated area at UCIL, Bhopal, proved to be a potent disrupting agent of this biomolecule of life.

Cytogenetic biomonitoring of a population is an appropriate means of estimating the genetic risk from integrated exposure to complex mixtures of chemicals. Although a number of biomarkers are available to assess transient and permanent genotoxic responses, biomonitoring studies on human populations should essentially focus on more definitive cytogenetic end-points. New developments in the post-genomics era have provided tools for the assessment of environmental and occupational exposure to toxic substances. Identification and validation of various molecular cytogenetic biomarkers which confirm defined toxicogenomic effects and gene susceptibilities through polymorphism studies can substantiate our understanding of human occupational diseases.

Cytogenetic studies in cell lines and small animals with reference to MIC

The genotoxic potential of MIC in cultured cells after *in vitro* exposure to MIC has been evaluated in several studies (Table 1). Conner et al. [40] assessed sister chromatid exchange (SCE) levels and cell cycle kinetics in various murine tissues following MIC exposure, and discovered that despite its apparent cellular toxicity, MIC was not genotoxic as measured by SCE analysis. However, MIC was found to induce SCEs and chromosomal aberrations in CHO cells [41]. Shelby et al. [42] report that methyl isocyanate has the capacity to affect chromosome structure but not to induce gene mutations. In cultured peripheral blood lymphocytes, MIC was found to have a stimulatory effect on cell cycling rates, as measured by the replicative index, and it caused a significant reduction in mononuclear leucocyte counts and mitotic indices [43]. Meshram and Rao [44] report that MIC was mutagenic in its native form, or as its unknown metabolites, unlike its hydrolysis products [45]. Tamura et al. [46] demonstrated that the modification of M13mp9 RF DNA and SOS-dependent mutations in the beta-galactosidase locus in *E. coli* were MIC-dose dependent.

Cytogenetic studies in rodents and human lymphocytes with reference to MIC

Methyl isocyanate (MIC) has been assayed in a number of *in vivo* genetic toxicity tests to determine its ability to interact with DNA and to induce genetic damage (Table 2). Ennever and Rosenkranz [47] evaluated the genotoxic carcinogenicity of MIC and predict that it has significant potential for inducing cancer in rodents. Many groups of

Table 1. Summarized investigations on MIC induced damage in cell lines and small animals

In vitro studies	Cytogenetic and molecular parameters studied	References
Evaluation of genotoxic potential of MIC in cultured cells after *in vitro* exposure	Sister chromatid exchanges (SCEs) and chromosomal aberrations (CA) in Chinese hamster ovary cells, mouse lymphoma cells and cultured mammalian cells	[42]
	Peripheral blood and spleen lymphocytes cultured in the presence of BrdU *in vitro*	[40]
	Mononuclear leucocyte counts and mitotic indices	[43]
	MIC was assayed both in the presence and absence of Aroclor-1254-induced S9, using 5 tester strains of Salmonella typhimurium, TA97a, TA98, TA100, TA102 and TA104	[44]
	Ames Salmonella/microsome mutagenicity test	[45]
	Studied genotoxic response of MIC-modified DNA in *E. coli*	[46]

Table 2. Summarized investigations on *in vivo* cytogenetic studies in plants, animals and humans exposed to MIC

In vivo cytogenetic study	Cytogenetic parameters studied	References
Genotoxic potential of MIC in exposed plants	Chromosomal abnormalities at highly significant levels in cultivated plants such as *Solanum melongena, Lycopersicon esculentum, Raphanus sativus, Brassica campestris* and *Lagenaria vulgaris*, and wild plants *Solanum surattense, Datura alba* and *Argemone mexicana* from affected areas	[6]
	Chromosome aberrations in root cells, and growth retardation and chlorophyll mutation of seedlings, the frequencies of which varied from one locality to another in seeds of *Solanum surattense* Burm	[7]
	Striking findings of resistance were documented in plants like *Artocarpus integrifolia, Casuarina equisetifolia, Ficus bengalensis and Mangifera indica*	[8]
Genotoxic potential of MIC in exposed animal subjects	MIC has a significant potential for inducing cancer in rodents	[47]
	Sister chromatid exchanges (SCEs) and cell cycle kinetics in murine tissues	[40]
	Chromosomal aberrations and sister chromatid exchanges in bone marrow metaphase cells, induction of micronuclei in polychromatic erythrocytes and the inhibition of bone marrow cellular proliferation and erythropoiesis in mice	[48]
	In vivo micronucleus test, chromosomal aberrations, sister chromatid exchanges in bone marrow cells of rodent somatic cells	[49]
	In vivo micronucleus test and chromosomal analysis of bone marrow cells in mice	[50]
	Mutagenic and cytotoxic effects in mouse micronucleus test	[51]
Genotoxic potential of MIC in exposed human subjects	Increased sister chromatid exchanges and chromosomal breaks have been reported in exposed persons	[12,23–25]
	Illustrated chromosomal profile of 154 persons studied during 1986–1988	[26]
	Chromosomal abnormalities even 1114 days after exposure to the gas is suggestive of residual effect on T-cell precursors and possibility of higher susceptibility to chromosome damage of persons exposed to MIC	[27]
	Established a genetic link of cancer patterns among victims of gas tragedy with MIC exposure	[18]
	High pregnancy loss, increased first 5-year mortality and delayed development of male progeny	[57]
	The mean percentage of acrocentric associations in exposed population was significantly higher	[52,53]

workers have demonstrated that exposure to MIC by inhalation results in bone marrow damage, indicating that MIC or its reactive metabolites possess systemic genotoxic/cytotoxic activity [48–50]. MIC exposure was found to lead to inhibition of bone marrow cell proliferation by evaluation of the mutagenic and cytotoxic effects of MIC with a mouse micronucleus test [51].

Increased sister chromatid exchanges and chromosomal breaks have also been reported in exposed persons [12,23–25]. A separate study illustrates the chromosomal profile of 154 persons studied over the years 1986–1988. The exposed subjects developed at least two categories of chromosomal aberrations, including a high number of Robertsonian translocations, most of which were in acrocentric chromosomes 13 and 21 [26]. Another investigation on 83 individuals directly exposed to MIC recorded chromosome breaks, gaps, dicentrics, rings, triradial and quadriradial configurations which were found to occur in statistically higher frequencies as compared to the non exposed group [27]. "The persistence of chromosomal abnormalities in the form of replicating minutes and exchange configurations, even 1114 days after exposure to the gas, indicates that MIC has a residual effect on T-cell precursors, and raises the possibility of persons exposed to MIC having greater susceptibility to chromosome damage" [27]. The presence of significantly more common structural aberrations in the MIC exposed population has been corroborated by Malla et al. [52,53], suggesting that the population is more vulnerable to genetic disorders and cancer.

In utero studies in humans with reference to MIC

As the impact of toxic exposure on epigenetic factors contributing to variation in the functional expression of genes has not fallen, a greater emphasis has been placed on researching the *in utero* exposure and progeny of the exposed population. Environmental signals during early life may lead to adverse long term effects independent of the obvious effects on fetal growth. Experimental data in rodents and recent observations in humans suggest that the epigenetic changes in the regulatory and growth-related genes play a significant role in foetal programming [54], whereby adverse long term effects reflect a mismatch between early (foetal and neonatal) and environmental conditions (Table 3).

Kapoor [55] documented, through a 5 year biodemographic study, that the fetal loss rate among the women affected by MIC gas was abnormally high (26.3%) as compared to a control group (7.8%). It has been suggested that MIC exposure *in utero* in the first trimester of pregnancy may induce a persistently hyper-responsive state of the immune system [56]. The non-targeted effects of toxic exposure such as bystander responses, genomic instability, gene induction, adaptive responses and low hypersensitivity may also be mirrored as trans-generational health effects. Sarangi et al. [57] report that exposure of pregnant

Table 3. Summarized investigations on *in utero* studies in humans exposed to MIC

In utero studies	Parameters studied	References
In utero exposed human subjects	The fetal loss rate among the gas-affected women was abnormally high (26.3%) compared to that of women in the control area (7.8%)	[55]
	MIC exposure *in utero* in the first trimester of pregnancy has caused a persistently hyper-responsive state of the immune system	[56]
	Stunted growth in males until puberty, followed by a period of accelerated growth. Results also suggest a post-puberty effect on head circumference of females exposed to gases *in utero*	[57]
	A high incidence spontaneous abortions and still-births in gas- exposed pregnant women and significantly higher perinatal and neonatal mortalities in the affected areas were reported	[58]

women to toxic gases in 1984 resulted in high pregnancy loss, increased 5-year mortality of the offspring of the exposed parents and delayed development of male progeny. They report that male, but not female, offspring exposed to gases *in utero* or born to exposed parents, were stunted in growth until puberty, which was followed by a period of accelerated growth. Their study results also suggest a post-puberty effect on head circumference of females exposed to gases *in utero*. There was a high incidence of spontaneous abortions and still-births in pregnant women exposed to the gas and significantly higher perinatal and neonatal mortalities in the affected areas [58]. Consequently, screening of MIC-induced trans-generational alterations in the germline genome of F0, F1 and F2 generations of Bhopal gas victims may address the less understood environmental influence of heritable or familial components of susceptibility to infectious and chronic non-communicable diseases [59].

Molecular studies with reference to MIC

There has been little progress over the last few years regarding understanding the genotoxic mechanisms of MIC within human cells *in vitro* (Table 4). It has been demonstrated that methyl isocyanate might promote cell cycle arrest and apoptosis in cultured mammalian cells suggestive of causing genetic alterations by negative regulation of the DNA damage response pathway [60]. The toxic response of cultured human colon epithelial-FHC cells to methyl isocyanate has been previously investigated with regard to genomic instability in colonocytes [61]. According to Raghuram et al. [62], ovarian epithelial cells manifest a persistent DNA damage response upon treatment with methyl isocyanate. Another study performed in order to determine the pathophysiological implications of isocyanate exposure on the male germ line GC-1 spg cell line indicated induced genomic instability with evidence of dysregulation of cell cycle progression [63]. Bose and Bathri [64] used ISSR-PCR to examine the effects of MIC exposure among a cross-section of current survivors suffering from COPD for microsatellite instability. Their study showed a weak association between microsatellite instability and age, exposure distance from site and smoking status, while regression analysis provided supporting evidence.

THE NEXT GENERATION

Children in developing nations are at a higher risk of dual exposure to infectious diseases and chemical hazards. Their burden of exposure-based disease is much greater than that of adults due to compounding factors such as immature metabolic pathways, easily disrupted growth and development processes and longer lifespan, which provide more time for harboring chronic diseases triggered

Table 4. Summarized investigations on molecular studies with reference to MIC

Molecular studies	Parameters studied	References
Molecular studies with reference to MIC	Assessed the genotoxic potential of methyl isocyanate in cultured mammalian cells in three different normal cell lines MM55.K, B/CMBA.Ov, and NIH/3T3	[60]
	Chromosomal anomalies, expression of pericentrin protein, ISSR PCR-microsatellite instability in colonocytes	[61]
	Ovarian epithelial cells manifested DNA damage response, GADD45, p21, p16 (INK4A) and pRb proteins	[62]
	Chromosomal aberrations, telomere anomaly, aneuploidy and microsatellite repeat in cultured mouse spermatogonial cell line	[63]
	The effects of exposures among a cross-section of current residents suffering from COPD by ISSR PCR-microsatellite instability	[64]

by early exposure. Unfortunately, the Bhopal Gas tragedy appears to be no exception, rather a harbinger of conditions yet to develop [65–67].

Studies by Ghosh et al. [27] and Dikshit and Kanhere [18] indicate probable long term effects of MIC and hint at the inevitability of focusing on cohort-based investigations in order to understand the molecular mechanisms of chromosomal damage, their extent in the population and chances of enhanced susceptibility of DNA damage due to a genetic predisposition in the following generation, creating a high risk group which may be carrying a genetic load [68]. In the assessment of the effects of exposure to toxic chemicals such as MIC; the age, sex of the subject, physiological and nutritional statuses, as well as other confounding factors, have to be taken into account [69,70]. There is a compelling need for detailed investigations into the health implications of children's exposure to environmental toxins, with special reference to the high prevalence conditions, such as childhood cancer, asthma, endocrine and sexual disorders, and neurobehavioral toxicants [71]. Anthropometric studies in adolescents carried out by Ranjan et al. [72] identified selective growth retardation in boys either exposed to the toxic MIC gas as toddlers or born to the exposed patients but not in girls. Based on survey studies conducted by Irani and Mahashur [74] on children exposed to MIC and complaining of persistent respiratory, gastrointestinal and eye problems, Woolf and Sandel [73] state that there are still many unanswered questions concerning the long-term residual effects of MIC exposure on the health of children, which require specific and cohort-based cytogenetic studies and their appropriate interpretation.

TOOLS AVAILABLE

Cytogenetic analysis of peripheral blood lymphocytes (PBLs) has been accepted as a suitable assay for the biological monitoring of genetic damage induced by excessive exposure to clastogenic agents in the workplace [75–77]. Also, the evaluation of micronuclei in PBLs is a valuable cytogenetic biomarker in human populations occupationally exposed to genotoxic compounds [78,79]. A high frequency of chromosomal aberrations (CAs) and micronuclei (MN) in PBLs is a predictor of an increased risk of cancer [80–82]. Preliminary results of genomic studies suggest that certain genotypes of drug-metabolizing enzyme genes, such as GSTM1 null genotype, NAT1 slow-acetylator genotype, and GSTP1 slow-activity genotype, may confer an increased risk of isocyanate-induced asthma, and hence, can be used for risk assessment [83]. SKY has been applied to various tumor groups including hematological malignancies, sarcomas, carcinomas and brain tumors, with the intent to identify specific chromosomal abnormalities that may provide an insight into the genes involved in disease process, as well as identify recurrent cytogenetic markers for clinical diagnosis and prognostic assessment [84]. Researchers have shown a slight correlation between single nucleotide polymorphisms (SNPs) and environmentally responsive genes [85], and another between the airway inflammatory proteins, glutathione-S-transferase P1 and tumour necrosis factor, interacting with geocoded ambient levels of nitrogen oxides [86]. Variants of chromosome 17q21 were strongly associated with the risk of early-onset asthma in a replication study of SNPs on exposure to environmental tobacco smoke [87]. Therefore, molecular cytogenetic studies of allelic variability and resequencing data can play a pivotal role in the evaluation of a subjects' adaptation to environmental xenobiotics [88–90]. However, there is a need to use cutting-edge molecular technologies which can provide data to understand the genesis of environment-related disorders, and thus identify the causal links between exposure and a specific human disease [91]. Chromosomal abnormalities represent Nature's guide to the molecular basis of many unexplained disease conditions. More than fifty years have passed since the discovery of the number of human chromosomes in 1956. New techniques have developed since then, ranging from

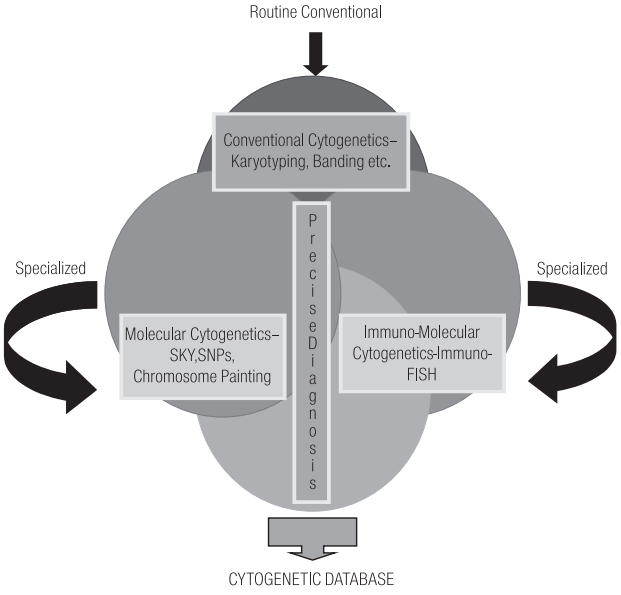

Fig. 1. Schematic representation of various tools available for cytogenetic studies to create a cytogenetic database

the conventional banding techniques to the currently used molecular array comparative genomic hybridization. With a combination of these conventional and molecular techniques, cytogenetics has become an indispensable tool used in the diagnosis of various genetic disorders, simultaneously paving the way for possible treatment and management [92]. This can be achieved by an amalgamation of conventional and advanced molecular tools that capitalize on the power of available cutting edge techniques to build up a cytogenetic database (Figure 1) of the surviving population in order to aid diagnosis of genetic disorders and their possible treatment and management. To summarize, it is necessary to undertake studies to examine the molecular toxicological effects of gaseous toxins, including those mimicking MIC, using whole genome scanning approaches.

FUTURE PERSPECTIVES

Chromosomal aberrations induced by environmental exposure represent a proportion of genetic disorders often being transmitted as a germline mutations. A higher incidence of chromosomal abnormalities at the prenatal or postnatal stages in human beings is relatively common in a population with any kind of genotoxic exposure. The MIC catastrophe has not been the last such industrial disaster. There have been other accidents of various magnitude like leakage of chlorine gas from the water filtration plant of BHEL-Bhopal, India [93] and radiation leakage from Fukushima Nuclear plant in Japan [94], with slightly different toxins involved but very similar after-effects.

The effect(s) of such environmental toxins in children are mediated, partly, by their genetic make-up and partly by the existence of genetic variants and polymorphisms of certain genes which confer relative age-related vulnerability or resistance to molecular perturbations and cellular dysfunctions which may manifest downstream as phenotypic variation(s) and clinical toxicity. Also there may be environmental influences on epigenetic factors that contribute to variations in the functional expression of genes. A study of transgenerational epigenetic signatures, especially in the offspring of Bhopal gas tragedy victims is an area seeking scientific attention. Although conventional cytogenetics using banded chromosomal analysis remains a simple and popular technique to get an overview of the human genome, a combination of routine banded karyotype analysis with Immuno-FISH and other various molecular techniques can achieve a more precise diagnosis of various syndromes in children [95]. Microarray-based formats using large insert genomic clones, cDNAs or oligonucleotides can replace metaphase chromosomes as DNA targets, providing higher resolution and the ability to directly map the copy number changes to the genome sequence [92].

Both conventional and molecular technique-based investigations, must now be focused on understanding the molecular mechanisms of chromosomal damage; delineating a high risk population and its susceptibility to DNA damage and genetically-predisposed cancers, with the

identification of individuals hypersensitive to selected genotoxins. This research can prove to be beneficial in designing clinical management strategies to overcome or minimize the disastrous effects of similar exposure in the future.

CONCLUSION

Intense research on *in utero* exposure and progeny of the exposed population is being augmented. Molecular cytogenetic studies using techniques like FISH, Immuno-FISH, SKY and SNP analysis, are warranted to build up a cytogenetic database of the surviving cohort to substantiate the sparse data available concerning threshold values of various cytogenetic markers so as to identify and define susceptibility indicators, if any.

ACKNOWLEDGEMENT

Authors are grateful to Bhopal Memorial Hospital & Research Centre for IRB sanctioned Research Project on Cytogenetic Workstation (IRB/11/Research/2010). The authors have made a careful attempt to cite all available reports on cytogenetic studies pertaining to MIC exposure, however, exclusions, if any, are regretted.

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