

RESEARCH ARTICLE

Increased Frequency of Micronuclei in Binucleated Lymphocytes among Occupationally Pesticide-exposed Populations: A Meta-analysis

Hai-Yan Yang^{1&}, Ruo Feng^{2&}, Jing Liu¹, Hai-Yu Wang³, Ya-Dong Wang^{3*}

Abstract

Background: The cytokinesis-block micronucleus (CBMN) assay is a standard cytogenetic tool employed to evaluate chromosomal damage subsequent to pesticide exposure. **Objectives:** To evaluate the pooled levels of total micronuclei (MN) and binucleated cells with micronuclei (MNC) in 1000 binucleated lymphocytes among population occupationally exposed to pesticides and further determine the more sensitive biomarker of CBMN. **Materials and Methods:** A meta-analysis on the pooled levels of MN and MNC in binucleated lymphocytes among occupationally pesticide-exposed populations was conducted using STATA 10.0 software and Review Manager 5.0.24 in this study. **Results:** We found significant differences in frequencies of MN and MNC in 1000 binucleated lymphocytes between pesticide-exposed groups and controls, and the summary estimates of weighted mean difference were 6.82 [95% confidence interval (95% CI): 4.86-8.78] and 5.08 (95% CI: 2.93-7.23), respectively. However, when we conducted sensitivity analyses further, only the MN remained statistically different, but not the MNC, the summary estimates of weight mean difference were 2.86 (95% CI: 2.51-3.21) and 0.50 (95% CI: -0.16-1.17), respectively. We also observed pesticide-exposed subjects had significantly higher MN frequencies than controls among smokers and nonsmokers, male and female populations, and American, Asian and European countries in stratified analyses. **Conclusions:** The frequency of MN in peripheral blood lymphocytes might be a more sensitive indicator of early genetic effects than MNC using the CBMN assay for occupationally pesticide-exposed populations.

Keywords: Cytokinesis-block micronucleus - pesticides - peripheral blood lymphocytes - meta-analysis

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Introduction

Pesticides are a group of natural or synthetic chemical substances, being designated to fight against plagues that generally attack, harm or transmit illness to living organisms including humans. Since they are extensively used throughout the world, large amounts of pesticides are set free into the environment annually. It is reported that many of them have adverse biological effects on non-target organisms including humans. Therefore, concerns for their potential hazard to human health have been increasing. Human exposure to pesticides can occur via dermal contact, inhalation, ingestion, or across the placenta (Gilden et al., 2010). Individuals occupationally exposed to pesticides included farm workers, floriculturists, pesticides applicators and pesticides manufacturing workers, etc (Alavanja, 2009). Most pesticides are of acute and chronic toxicity to humans.

Chronic health effects have been related to environmental exposure to pesticides, including

neurological effects, reproductive or developmental dysfunctions and certain cancer. Epidemiological studies have shown that there was an etiologic link between occupational exposure to pesticides and several human neoplastic diseases. In particular, a significant increase was observed in the incidence of multiple myeloma (Rusiecki et al., 2009), Hodgkin's lymphoma (Orsi et al., 2009), non-Hodgkin's lymphoma (Fritschi et al., 2005; Merhi et al., 2007; Balasubramaniam et al., 2013; Yildirim et al., 2013), soft tissue sarcoma (Hardell et al., 1995), and lung (Lee et al., 2004), pancreas (Andreotti et al., 2009), liver (Weichenthal et al., 2010), colon and rectum (Lee et al., 2007), leukemia (Van Maele-Fabry et al., 2007; Rajabli et al., 2013; Kumar et al., 2014), prostate (Alavanja et al., 2003; Van Maele-Fabry et al., 2006) and bladder cancer (Koutros et al., 2009).

Human biomonitoring is a useful tool of great interest in cancer risk assessment once it allows estimating genetic risks deriving from environmental exposure to chemicals (Costa et al., 2006). The genotoxic effects of pesticides are

¹Department of Epidemiology, School of Public Health, Zhengzhou University, ²Department of Histology and Embryology, School of Basic Medicine, Zhengzhou University, ³Department of Toxicology, Henan Center for Disease Control and Prevention, Zhengzhou, China *Equal contributors *For correspondence: wangyd76@163.com

primary factors for carcinogenesis, and thus, cytogenetic biomonitoring will become useful in human population occupationally exposed to pesticides. Recently, many biomonitoring studies have investigated biomarkers of cytogenetic damage including chromosomal aberrations (CA), sister chromatids exchange (SCE) and cytokinesis-block micronucleus (CBMN) among pesticide-exposed population. Among them, CBMN in human peripheral blood lymphocytes is one of the most extensively studied biomarkers of cytogenetic damage (Bolognesi et al., 2011). However, a single study with relatively small sample may not be sufficient to present a robust evidence of the relationship between pesticides exposure and increased frequencies of CBMN in human peripheral blood lymphocytes. Moreover, various types of study populations and study designs may also have contributed to diversify the findings. Meta-analysis represents the ideal statistical tools for calculating pooled estimates of a biomarker using data from different studies, which seems to be one of the most informative ways to extract information from different studies on biomarkers when the evidence from single study is too sparse to provide definite conclusions (Taioli et al., 2002). Therefore, we collected the published data to evaluate the validation of CBMN in human peripheral blood lymphocytes as cytogenetic biomarkers of occupationally pesticide-exposed population comprehensively.

Materials and Methods

Literature source and analytical methods

We searched the databases of Medline/PubMed, EMBASE and web of science, using the combinations of the following key words: “micronucleus”, “pesticide”, “insecticide”, “fungicide”, “herbicide”, “farmer”, “floriculturist” and “lymphocyte”, the ending date of searched publications was December 31, 2013. A cited reference search of the retrieved papers was conducted, and further publications were also identified by retrieving the bibliographies of the retrieved papers.

Criteria of literature inclusion: (1) The papers should be published in English; (2) The genetic damage was assessed by CBMN assay in human peripheral blood lymphocytes; (3) The papers should include occupational exposure to pesticides and the frequency of MN or MNC in peripheral blood lymphocytes; (4) The paper must offer the exposed group and control group; (5) The paper must offer the size of the sample, arithmetic means and standard deviations (SD) or the information that can help infer the results; (5) When more than one article was identified for the same study population, we included the most recent population or publication including more information. Accordingly, reviews and repeated or overlapping literatures were excluded.

In total, 36 published studies were identified with the frequencies of CBMN in peripheral blood lymphocytes of occupationally pesticide-exposed population. We reviewed all papers in accordance with the criteria defined above and excluded ten overlapping articles, one review and two papers that did not offer full information. Therefore, twenty-three studies were determined to enter

our study. Among them, twenty-two studies focused on MN and twelve studies focused on MNC.

Data extraction

Two data managers tabulated the data first, and then inputted them to an electronic database, independently. The following information was extracted from the studies: authors, publishing year, arithmetic means and standard deviations, sample size of exposed group and control group, origin of country, study design, statistical test, duration and stratified factors.

We calculated the summary arithmetic means and standard deviations, if the study provided stratum information. Several characteristics of individual study were summarized in Table 1.

Quantitative data synthesis

To evaluate the association between CBMN frequency and occupational exposure to pesticides, we performed a meta-analysis of identified studies. Data were combined using either a fixed-effects model or a random-effects model (DerSimonian et al., 1986). The Cochran Q statistics test was carried out for the assessment of heterogeneity. A fixed-effects model is employed when the effects are assumed to be homogenous, while a random-effects model is employed when they are heterogeneous. We computed the weighted mean difference and 95% confidence interval (95% CI) for each study. Publication bias was concerned in this meta-analysis. The presence of publication bias signified that non-significant or negative finding remained unpublished. The funnel plot was drawn to evaluate publication bias and Egger's test was used to test the funnel plot symmetry (DerSimonian et al., 1986; Egger et al., 1997). Begg's rank correlation test was employed to check the publication bias as well (Begg et al., 1994).

All of the statistical analyses were conducted with STATA 10.0 software package (Stata Corporation, College Station, Texas) and Review Manager (Version 5.0.24, The Cochrane Collaboration). All the tests were two-sided, and a P value of less than 0.05 for any test or model was considered to be statistically significant.

Results

Meta-analysis databases

We established a database according to the extracted information from each article. General information of included studies was listed in Table 1. It indicates first author, year of publication, exposure of pesticides, duration, outcome measure, origin of country, study design, covariate accounted for and statistical test. There were a total of 22 studies with 1278 exposed individuals and 1026 controls concerning MN frequency (Figure 1A) and 12 studies with 880 exposed individuals and 560 controls concerning MNC frequency (Figure 1B).

Test of heterogeneity

Table 2 shows the association between CBMN frequency and exposure to pesticides. The heterogeneity of studies on MN and MNC was analyzed for the 23 studies.

The results showed that there was not heterogeneity for meta-analysis on MN in female population. Thus, we computed the summary estimate of weighted mean difference for it with a fixed-effects model. The rest had heterogeneity with P value being less than 0.05. Therefore, we analyzed the summary estimates of weighted mean difference for them with a random-effects model.

Quantitative data synthesis

Table 2 indicates the summary estimates of weighted mean difference of the frequencies of MN and MNC, There were statistically significant differences in the frequencies of MN and MNC in peripheral blood lymphocytes between pesticide-exposed group and control, and the summary estimates of weighted mean difference were 6.82 (95%

Table 1. General Information of the Studies Included in this Meta-analysis

Author	Year	Exposure to pesticide	Outcome measure	Duration (Mean \pm SD)	Country	Study design	Covariates accounted for	Statistical test
Ali et al	2008	Carbamate, pyrethroid, and Organophosphate	MN and MNC	10.26 \pm 6.14 years	Pakistan	Retrospective	Age and duration	Mann-Whitney U tests
Bhalli et al	2006	Complex mixtures of pesticides	MN and MNC	13.48 \pm 3.84 years	Pakistan	Retrospective	Smoking and duration	Mann-Whitney U test
Bolognesi et al	1993	Complex mixture of compounds	MN	25.3 \pm 13.23 years	Italy	Retrospective	Age, sex, smoking and duration	Poisson regression analysis
Bolognesi et al	2004	Complex mixture of pesticides	MNC	26.35 \pm 14.46 years	Italy	Retrospective	Sex, age and smoking	Mann-Whitney U test
Bolognesi et al	2009	Glyphosate and other pesticides	MN and MNC	Unknown	Colombia	Prospective	Region, sex and age	One-way ANOVA
Coskun et al	2011	Mixing of pesticides	MN and MNC	Unknown	Turkey	Retrospective	Age and sex	Student's t-test
Costa et al	2006	Insecticides, Rodenticide, Acaricides and Herbicides	MN	15 \pm 13 years	Portugal	Retrospective	Sex and smoking	Analysis of variance
Costa et al	2011	Fungicides, Insecticides, Herbicides and Aphicides	MN	23.0 \pm 16.1 years	Portugal	Retrospective	Sex and smoking	Mann-Whitney tests
Davies et al	1998	Farmworkers	MN and MNC	7.33 years (range:1-24)	British	Retrospective	Duration, age, folate, meat, coffee and recent vaccination	ANOVA and Student's t-test
Figgs et al	2000	2, 4-dichlorophenoxyacetic acid	MN	Unknown	USA	Prospective	Unknown	t-test
Garaj-Vrhovac et al	2002	Atrazine, alachlor, cyanazine, 2, 4-dichlorophenoxyacetic acid and malathion	MN	22.25 years (range:4-30)	Croatia	Retrospective	Smoking	Chi-squared test
Joksic et al	1997	Herbicide and Fungicide	MN	12.1 \pm 6.02 years	Yugoslavia	Prospective	Unknown	Wilcoxon rank-sum test
Kehdy et al	2007	Several pesticides	MN and MNC	5.28 \pm 3.23 years	Brazil	Retrospective	Age, smoking, drinking and duration	Analysis of covariance
Lucero et al	2000	Complex mixture of pesticides	MN and MNC	9.82 \pm 8.08 years	Spain	Retrospective	Age, smoking and duration	One-way analysis of covariance
Marquez et al	2005	Insecticides, Fungicide and Herbicide	MN and MNC	8.0 \pm 4.8 years	Chile	Retrospective	Smoking and duration	Mann-Whitney U test
Pasquini et al	1996	Insecticides, Herbicides and Fungicides	MN	18.35 \pm 12.42 years	Italy	Retrospective	Age, smoking and duration	Variance analysis
Pastor et al	2003	Insecticides, Fungicides, Herbicides and Bactericides	MN and MNC	13.92 \pm 9.11 years	Greece, Spain, Poland and Hungary	Retrospective	Sex	Generalized linear model
Rohr et al	2010	Pesticides	MN	29.8 6 \pm 14.2 years	Brazil	Retrospective	Genotype	Mann-Whitney U test
Scarpato et al	1996	Greenhouse floriculturists	MN	Unknown	Italy	Retrospective	Smoking and sex	Multiple linear or Poisson Regression analysis
Titenko-Holland et al	1997	Malathion and MNC	MN	Exposure in the last 6 month	USA	Prospective	Sex	Chi-square test for trend
Tope et al	2006	Complex mixture of pesticides	MN	18.2 \pm 5.03 years	USA	Retrospective	Unknown	One-way ANOVA
Venegas et al	1998	Mixtures of pesticides	MN and MNC	About 7 years	Chile	Retrospective	Age and drinking	t-test
Zeljczic et al	2007	Carbofuran	MN	15.7 years (range:1-29)t	Croatia	Retrospective	Sex, age, smoking, drinking and X-ray exposure	Multivariate analysis

Table 2. Summary Results of Meta-analysis on Cytokinesis-block Micronuclei Induced by Pesticide Exposure

Biomarker	Exposure /control	Heterogeneity test		Analysis Model	Summary estimate of weighted mean difference (95%CI)	Hypothesis test		df	Egger's test		Begg's test	
		Q	P			Z	P		t	P	Z	P
MN	1278/1026	695.2	<0.00001	Random-effects	6.82 (4.86-8.78)	6.82	<0.00001	21	1.93	0.068	1.80	0.071
Stratification by smoking												
Smokers	63/74	100.43	<0.00001	Random-effects	10.09 (4.11-16.07)	3.31	0.001	5	1.51	0.205	1.13	0.260
Nonsmokers	208/222	62.95	<0.00001	Random-effects	6.65 (3.32-9.98)	3.91	0	5	1.74	0.156	0.75	0.452
Stratification by sex												
Male	199/178	15.97	0.007	Random-effects	2.73 (1.29-4.17)	3.71	0	5	0.99	0.380	0.75	0.452
Female	103/117	7.77	0.169	Fixed-effects	4.76 (2.94-6.57)	5.14	<0.00001	5	0.06	0.954	0.00	1.000
Stratification by origin of country												
America	488/229	183.26	<0.00001	Random-effects	5.89 (2.86-8.92)	3.8	0.0001	7	0.88	0.413	0.12	0.902
Asia	114/152	35.95	<0.00001	Random-effects	8.82 (4.80-12.85)	4.29	<0.0001	2	0.58	0.668	0.00	1.000
Europe	646/645	300.66	<0.00001	Random-effects	7.28 (4.03-10.54)	4.38	0	10	2.19	0.057	1.56	0.119
MNC	880/560	332.55	0	Random-effects	5.08 (2.93-7.23)	4.63	<0.00001	11	0.98	0.350	0.75	0.451

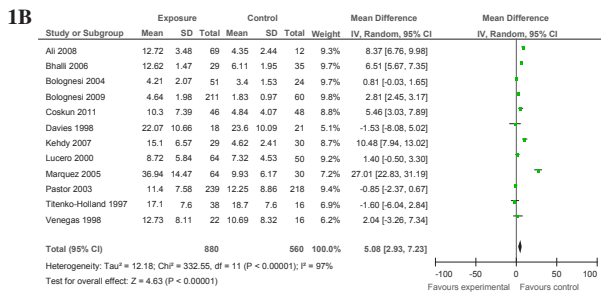
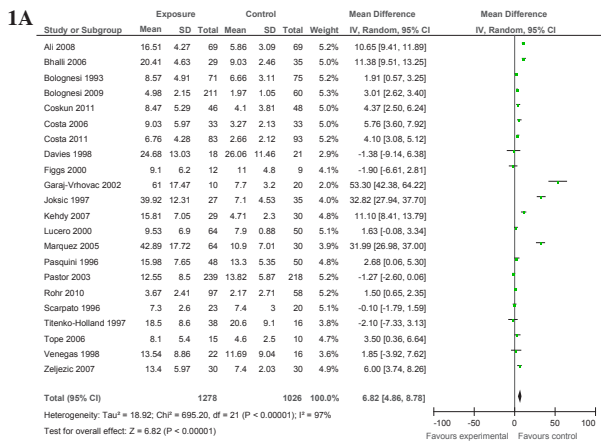


Figure 1. Meta-analysis of Total Micronuclei (MN) (1A) and Binucleated Cells with Micronuclei (MNC) (1B) in 1000 Binucleated Lymphocytes Among Total Population. Each estimate of weighted mean difference on MN and MNC was designated by a solid square, and the 95% confidence interval (95%CI) of each subgroup was shown by transverse line. The blank rhombus at the bottom was the pooled estimate of weighted mean difference by random-effects model

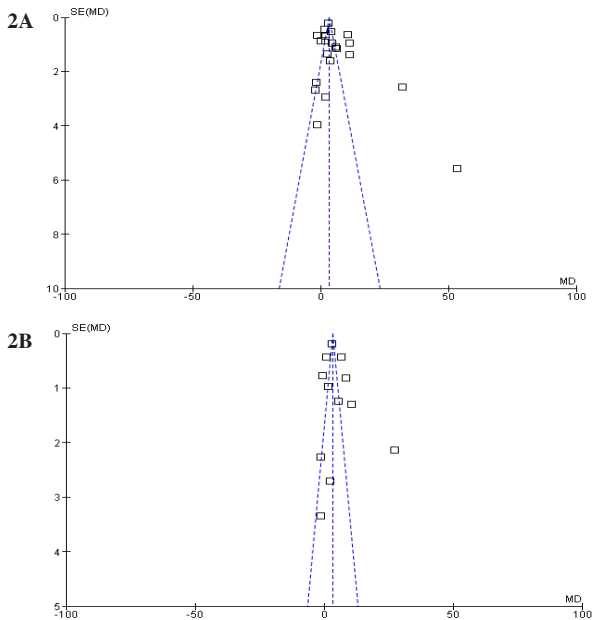


Figure 2. Funnel Plot Analyses to Detect Publication Bias for MN (2A) and MNC (2B)

CI: 4.86-8.78) (Figure 1A) and 5.08 (95% CI: 2.93-7.23) (Figure 1B), respectively. We performed meta-analysis on MN frequency stratified by cigarette smoking, sex and origin of country further, our findings showed that there were statistically significant differences in the frequency of MN in peripheral blood lymphocytes between pesticide-

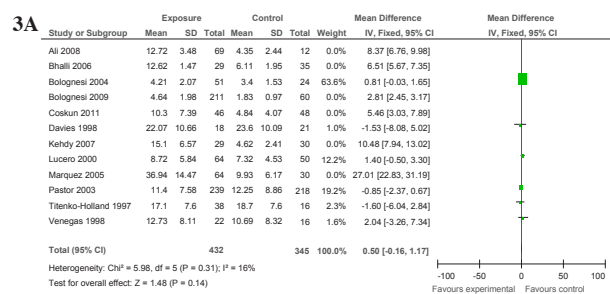
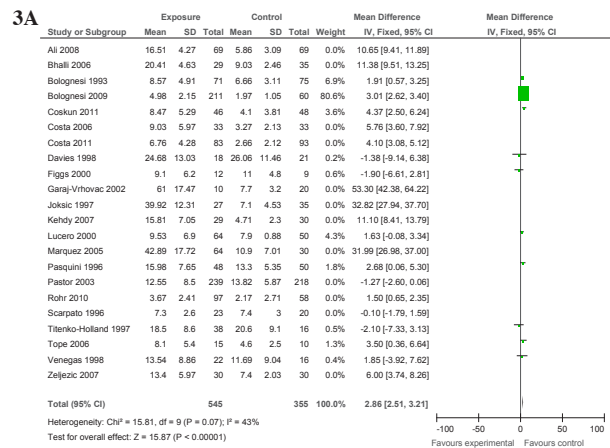


Figure 3. Results of Sensitivity Analyses

exposed group and control group among smokers and nonsmokers, male and female population, and American, Asian and European country in stratified analyses, and the summary estimates of weighted mean difference were 10.09 (95% CI: 4.11-16.07) and 6.65 (95% CI: 2.73-9.98), 2.73 (95% CI: 1.29-4.17) and 4.76 (95% CI: 2.94-6.57), and 5.89 (95% CI: 2.86-8.92), 8.82 (95% CI: 4.80-12.85) and 7.28 (95% CI: 4.03-10.54), respectively (Table 2).

Bias diagnosis

Publication bias was assessed for MN (22 studies) and MNC (12 studies). The results for MN (Figure 2A) and MNC (Figure 2B) were a symmetric funnel plot. Our results from Egger's test and Begg's test showed that there was no publication bias for MN and MNC among total population as well (Table 2). Publication bias might not have significant influences on the summary estimates of MN in stratified analyses as well (Table 2).

Sensitivity analyses

We conducted the sensitivity analyses and found that the study including Bolognesi et al., 1993, Bolognesi et al., 2009, Coskun et al., 2011, Davies et al., 1998, Figgs et al., 2000, Lucero et al., 2000, Pasquini et al., 1996, Titenko-Holland et al., 1997, Tope et al., 2006 and Venegas et al., 1998 was homogenous for MN, the *Q* value for test of heterogeneity was 15.81 (*df*=9, *p*=0.07) and the summary estimate of weighted mean difference was 2.86 (95% CI: 2.51-3.21) (Figure 3A). The study was homogenous for MNC, while including Bolognesi et al., 2004, Davies et al., 1998, Lucero et al., 2000, Pastor et al., 2003, Titenko-Holland et al., 1997 and Venegas et al., 1998, the *Q* value for test of heterogeneity was 5.98 (*df*=5, *p*=0.31) and the summary estimate of weighted mean difference was 0.50 (95% CI: -0.16-1.17) (Figure 3 B).

Discussion

Micronuclei originate mainly from chromosome breaks or whole chromosomes that fail to engage with the mitotic spindle and therefore lag behind when cells divide. It is the only biomarker that permits the assessment of both clastogenic and aneugenic effects in a vast range of cells, since they are determined in interphase. CBMN assay in human peripheral blood lymphocytes was firstly established by Fenech in 1985, which had several advantages such as speed and ease of analysis, no requirement for metaphase cells and reliable identification of cells that have completed only one nuclear division, comparing with chromosomal aberrations and the conventional micronucleus (Fenech, 1997). Frequencies of CBMN in peripheral blood lymphocytes were the most frequent cytogenetic biomarkers of occupational population exposed to pesticides. Recently, El-zein has reported that CBMN assay might be a good predictor of lung cancer risk (El-Zein et al., 2006).

Several studies have shown that certain pesticide induced an increase frequency of MN and MNC in assays performed in vitro. Zeljezic et al found that the total number of micronuclei was significantly increased in cytokinesis-blocked lymphocytes treated with 0.4 μ g/ml and 4 μ g/ml 2, 4-dichlorophenoxyacetic acid, compared to the negative controls (Zeljezic et al., 2004). A significant increase in micronucleated cells was found in isolated lymphocytes at high dose levels (75-100 μ g/ml) of malathion in comparison with negative controls (Titenko-Holland et al., 1997). The total number of micronuclei observed in binuclear peripheral blood lymphocytes of the *p*, *p'*-DTT-exposed samples (ranging from 32 to 47) was significantly greater than that detected in the unexposed control sample, where the total number of micronuclei was 7 (Garaj-Vrhovac et al., 2008). Surralles et al reported that significantly increased frequencies of micronuclei and micronucleated binucleated cells in cultured human lymphocytes were induced by a 48-hour treatment with alachlor, compared with negative controls (Surralles et al., 1995).

Our meta-analysis showed that the frequency of MN in peripheral blood lymphocytes was significantly higher in the pesticide-exposed group than that in control group evidenced by a random-effects model, where the summary estimate of weighted mean difference was 6.82 (95% CI: 4.86-8.78). A significantly increased frequency of MNC in peripheral blood lymphocytes was also observed in the pesticide-exposed group compared with control group, the summary estimate of weighted mean difference was 5.08 (95% CI: 2.93-7.23), but the summary estimate of weighted mean difference was 0.50 (95% CI: -0.16-1.71) in sensitivity analysis. Our findings indicated that exposure to pesticides could induce significantly increased levels of chromosome damage in peripheral blood lymphocytes measured by MN, which might be a more sensitive indicator of early genetic effects than MNC by using CBMN assay for occupationally pesticide-exposed population.

There are some limitations inherent in this present

meta-analysis. Firstly, only published literatures were included in this study. Therefore, publication bias may have occurred. To address this issue, both Egger's test and Begg's test were conducted simultaneously. Our results showed that the likelihood of key publication bias was negligible in this present study. Secondly, several factors such as sex, age, duration, smoking status, category of pesticide and levels of environmental exposure to pesticides might affect the frequency of MN in peripheral blood lymphocytes. Bolognesi et al observed an age-related increase of MN in human lymphocytes (Bolognesi et al., 1997); Bonassi et al reported that sex had an influence on MN evidenced from a large sample and review of the literature (Bonassi et al., 1995). Smoking status and sex were stratified in this meta-analysis for MN frequency further, and we also observed a significantly higher frequency of MN in peripheral blood lymphocytes among smokers and nonsmokers, male and female population exposed to pesticides in comparison with their corresponding control group, respectively. However, other confounders were not stratified in this meta-analysis, since only a few investigators reported such results and stratified range was not uniform for some factors. Thirdly, since both studies on MN and MNC were heterogeneous, we performed sensitivity analysis further, and found that the studies were homogenous for MN and MNC, when some articles were excluded.

Considering that the origin of studied population might have effects on the MN frequency among subjects exposed to pesticides, country of studied population was stratified in this meta-analysis further. We observed that there were significantly increased frequencies of MN in exposed group among American, Asian and European country, compared with their corresponding control group.

It is acknowledged that there is the main limitation of the classification of pesticides. Pesticides encompass many distinct chemicals and chemical classes including herbicides, insecticides, acaricides, rodenticides and fungicides. Some of them are used to kill weeds, while others kill insects and other pests via a variety of mechanisms of action. Perhaps, different pesticides had different cytogenetic effects on organisms. Stratification on category of pesticides should be performed in this meta-analysis. However, almost all of studies included in this study reported complex pesticides exposure rather than single pesticide exposure. Therefore, we could not perform sub-analysis on pesticides classification.

In summary, results from this current meta-analysis showed a significant increase in MN frequency in peripheral blood lymphocytes among pesticide-exposed population. MN might be a more sensitive biomarker than MNC while being used to evaluate the genetic damage induced by occupational exposure to pesticides. However, our meta-analysis was performed on population-based study, meta-analysis based on individual data might provide more precise and reliable results. When sufficient individual data are available, it may be likely to deal with the issue of confounders including sex, age, duration, smoking status, category of pesticides and levels of exposure to pesticides.

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