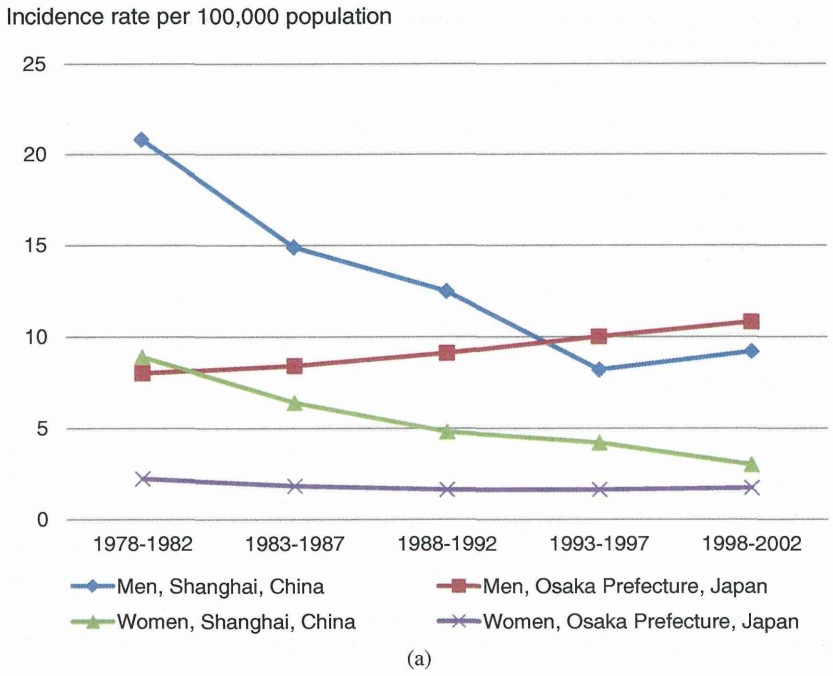
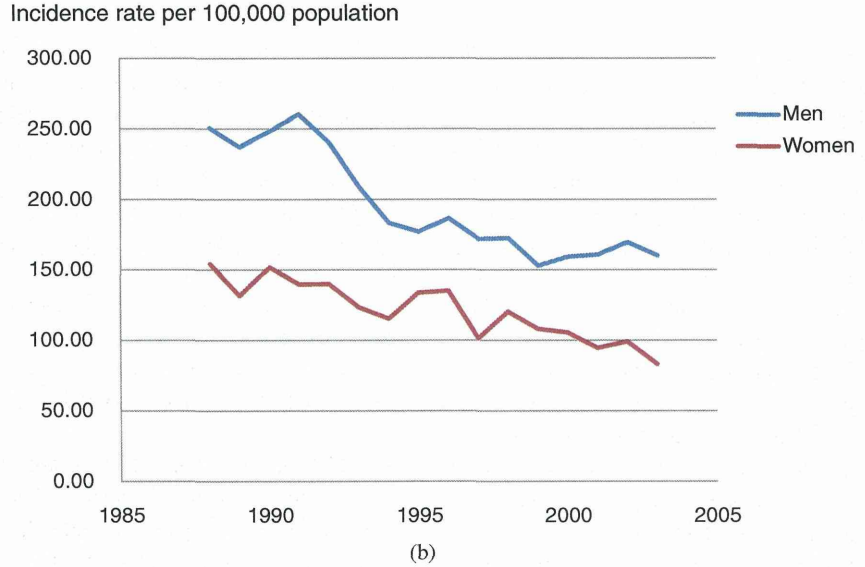


(a)



(b)

Figure 2. (a) Trend in age-adjusted incidence rates of esophageal cancer in Shanghai, China (representative low incidence area) and Osaka, Japan. Source: Cancer Incidence in Five Continents. (b) Trends in age-adjusted incidence rates of esophageal cancer in Cixian, a high-risk rural area in China. Source: He YT, et al. Trends in incidence of esophageal and gastric cardia cancer in high-risk areas in China. Eur J Cancer Prev. 2008;7:71–6. Reprinted with permission from the authors.

response relationship was apparent in all 4 cohort studies and in 5 case-control studies. The synergistic effect of alcohol consumption and cigarette smoking on esophageal cancer risk is well documented in Japanese studies: a greater than 10-fold increased risk was observed among Japanese with both habits.[20] The fraction of esophageal cancer associated with alcohol consumption and cigarette smoking was estimated at 84.8% among Japanese men and 51.6% among Japanese women.[21] After reviewing all epidemiologic evidence, the Research Group for the Development and Evaluation of Cancer Prevention Strategies concluded that there was convincing evidence that alcohol consumption and cigarette smoking strongly increase the risk of ESCC in the Japanese population.[18,19]

Table 2. Major risk factors for esophageal cancer in Japan and China

Risk factors	Information on strength of association
Japan	
Cigarette smoking	Summary RR for ever smokers was 3.01 (95% CI: 2.30–3.94), based on 4 cohort and 11 case-control studies [Ref 19]
Alcohol drinking	Summary RR for ever drinkers was 3.30 (95% CI: 2.30–4.74), based on 9 cohort and 9 case-control studies [Ref 18]
Gastric atrophy	Positive associations observed in 3 clinical studies [Refs 42–44], but no prospective cohort studies confirmed these associations.
China	
Low-incidence areas[a]	
Cigarette smoking	RR was 2.06 (95% CI: 1.11–3.82) for those who smoked for ≥40 years in a cohort of Shanghai residents [Ref 32]
Alcohol drinking	RR was 2.02 (95% CI: 1.31–3.12) for regular drinkers in a cohort of Shanghai residents [Ref 32]
Drinking tea at high temperature	OR was 3.1 (95% CI: 2.2–4.3) in a case-control study in Jiangsu Province [Ref 33], but definitive evidence is lacking
High-incidence areas[b,c]	
Cigarette smoking and alcohol drinking (probable modest association)	RR was 1.32 (95% CI: 1.15–1.51) for current smokers and 1.12 (0.83–1.51) for currents smokers of ≥20 cigarettes per day [Ref 29] No significant association among those who consumed alcohol in the previous 12 months [Ref 29]
Family history	RR was 1.42 (95% CI: 1.29–1.56) for individuals with family history of esophageal cancer [Ref 67]
Nutritional deficiency	High intake of meat, eggs, and fresh fruit associated with decreased risk [Ref 29]
Food mutagens including nitrosamine and its precursors	Ecologic studies showed that concentration of nitrate nitrogen was higher in high-incidence areas than in low-incidence areas [Refs 40, 41]

RR: relative risk; OR: odds ratio.
[a]In general, low-incidence areas are distributed in urban cities, including Beijing, Guangdong, Qidong county, Shanghai, and Zhongshan (Table 1).
[b]High-incidence areas are defined as areas with an age-standardized rate >30 per 100 000 population, including rural areas such as Cixian and Changle (Table 1).
[c]The main findings in high-incidence areas are based on a prospective study of risk factors for esophageal and gastric cancers in the Linxian General Population Trial Cohort in China [Ref 29].

Table 3. Findings from genome-wide association studies of esophageal cancer in Japan and China

References	Sample size	Ethnic group	Loci associated with susceptibility to ESCC
Wang et al 2004[56]	1077 ESCC cases 1733 controls	Chinese	*PLCE1* (10q23 rs2274223; per allele OR = 1.43 [1.37–1.49]) and *C20orf54* (20p13; per allele OR = 0.86 [0.82–0.90])
Abnet et al 2010[57]	2115 ESCC cases 3302 controls	Chinese	*PLCE1* (10q23 rs2274223; per allele OR = 1.34 [1.22–1.48])
Wu et al 2011[58]	2031 ESCC cases 2044 controls	Chinese	Identified 7 susceptibility loci on chromosomes 5q11 (rs10052657; OR = 0.67 for minor variant allele), 21q22 (rs2014300; OR = 0.70 for minor variant allele), 6p21 (rs10484761), 10q23 (rs2274223), and 12q24 (rs2074356, rs11066280) (OR = 1.30–1.56 for minor variant alleles)
Cui et al 2009[24]	1070 ESCC cases 2836 controls	Japanese	*ALDH2* (4q21-23, rs671; per allele OR = 1.67 [1.58–1.76]) and *ADH1B* (12q24, rs1229984; per allele OR = 1.79 [1.69–1.88])
Tanaka et al 2010[25]	1071 ESCC cases 2762 controls	Japanese	*ALDH2* (4q23, rs671; per allele OR = 1.78 [1.60–1.98]) and *ADH1B* (12q24.11–13, rs1229984; per allele OR = 1.82 [1.63–2.03])

ESCC: esophageal squamous cell carcinoma; GCA: gastric cardia cancer; OR: odds ratio; ALDH2: acetaldehyde dehydrogenase; ADH1B: alcohol dehydrogenase.

Alcohol and its metabolic pathway have a central role in predisposing individuals to ESCC. Acetaldehyde, the primary metabolite of ethanol, forms adducts with DNA and is thus responsible for the carcinogenic effect of alcohol beverages.[22] Polymorphisms in the genes that encode alcohol dehydrogenase (ADH) and acetaldehyde dehydrogenase (ALDH)—2 important enzymes in the alcohol-metabolizing pathway—may contribute to variation in the amount of acetaldehyde produced. Differences in the activity of these enzymes, and the potential of acetaldehyde to cause mutations, may explain why ESCC risk varies among individuals with the same level of alcohol consumption. Yokoyama and colleagues from Japan clearly showed that drinkers who were *ALDH*1/2* heterozygotes had a significantly increased risk of developing ESCC.[23]

Since 2009, there have been 2 genome-wide association (GWA) studies reporting functional variants that were significantly associated with susceptibility to esophageal cancer in the Japanese population.[24,25] The first GWA study identified 4q21-23 and 12q24 as susceptibility loci, in which 2 functional variants in *ADH1B* and *ALDH2* showed significant associations with ESCC risk (Table 3).[24] *ADH1B* and *ALDH2* are crucial in the metabolism of alcohol. That study also found a strong gene–environment interaction: individuals who had genetic risk variants and were both smokers and drinkers had more than 100-fold the risk of developing ESCC. The second GWA study reported similar findings, ie, clear synergistic effects of *ADH1B* and *ALDH2* SNPs, alcohol consumption, and cigarette smoking on ESCC risk.[25]

China: In low-risk urban areas like Shanghai, 1.9% of 74 942 women were current alcohol drinkers at baseline in the Shanghai Women's Health Study.[26] In another sample of 3953 Shanghai adults, 26.6% of men and 1.8% of women were current drinkers.[27] Surveys of men in 2 rural areas showed prevalences of 61.4% and 68.2%, respectively.[28] In Linxian, a representative high-incidence rural area in China, 23% of the 9584 baseline population reported drinking alcohol in the Linxian General Population Trial Cohort.[29] Among women, 8% of esophageal cancer cases were current drinkers.[29] According to the 2010 Global Adult Tobacco Survey

(GATS), which included a nationally representative sample of individuals aged 15 years or older, the prevalence of current smoking was 56.1% among males and 2.2% among females in rural areas.[30] In urban areas, prevalence was 49.2% among males and 2.6% among females.[30] Overall, alcohol consumption and cigarette smoking have been shown to be associated with increased ESCC risk in the Chinese population. For alcohol consumption, the results from a meta-analysis of the association between alcohol consumption and cancer risks were published in 2011.[31] For esophageal cancer, the meta-analysis included 34 case-control studies and 2 cohort studies. Most of the case-control studies found a positive association with alcohol consumption (summary odds ratio, 1.79; 95% CI, 1.47–2.17). However, the findings from the 2 cohort studies were inconsistent. In 1 cohort study, conducted in Linxian, no significant positive association was found between alcohol consumption and ESCC risk.[30] By contrast, the other cohort study, conducted in Shanghai, reported a 2.02-fold risk of ESCC among current drinkers.[32] In 2011, a case-control study of 1000 patients with ESCC and control subjects found that smoking and drinking were associated with a significantly increased risk of ESCC among men, but not among women, in Jiangsu Province, a high-risk area in China.[33] The proportion of esophageal cancer attributed to alcohol drinking was estimated at 15.2% in Chinese men and 1.3% in Chinese women.[34] For cigarette smoking, in a cohort study conducted in Linxian, the RR for ESCC was 1.34 (95% CI, 1.15–1.53) among ever smokers, and the risk increased with increasing years of cigarette smoking.[30] The proportion of esophageal cancer attributed to cigarette smoking was estimated at 17.9% in Chinese men and 1.9% in Chinese women.[35]

Diet and dietary habits

Japan: The association of diet and eating habits with ESCC risk in the Japanese population remains unclear. Among Japanese, high consumption of fruit and vegetables seems to protect against ESCC. The relationship of fruit and vegetable intake with ESCC was examined in the Japan Public Health Center-based Prospective Study, and the results showed that a 100-gram per day increase in consumption of total fruit and vegetables was associated with an 11% decrease in ESCC incidence.[36] Intake of pickled vegetables, however, was not associated with ESCC risk in that study. Another prospective study found that consumption of green-yellow vegetables and fruit reduced the risk of esophageal cancer, but the association was not statistically significant.[37]

China: Nutritional deficiency is believed to have an important role in ESCC development, especially in high-risk areas. Studies in Linxian found that general malnutrition, as well as deficiencies in selenium, zinc, folate, riboflavin, and vitamins A, C, E, and B_{12}, was associated with increased risk of ESCC.[38] Since the 1970s, in areas of North Central China with exceptionally high incidence rates, efforts have focused on identifying food mutagens and environmental carcinogens. Earlier studies found high concentrations of nitrates and nitrites, the precursors of nitrosamine, in drinking water samples, and nitrosamine in food samples.[39] Two ecologic studies, 1 conducted in Cixian[40] and the other in Shexian,[41] found that high concentrations of nitrate nitrogen in well water correlated with ESCC incidence. These findings highlighted the possible role of high levels of nitrate exposure in the pathogenesis of ESCC in high-risk areas.

Other etiologic factors

Japan: Apart from alcohol consumption and cigarette smoking, drinking tea at a high temperature was associated with 1.6-fold increased risk of esophageal cancer in a cohort study.[42] Three case-control studies consistently showed a strong, positive association between gastric atrophy and ESCC risk,[43–45] but there have been no studies on the association between *Helicobacter pylori* and ESCC in the Japanese population. In previous studies that used a candidate-gene approach, genetic polymorphisms in alcohol-metabolizing genes, DNA repair genes, and folate-metabolizing genes were linked to ESCC risk.[46,47]

China: Epidemiologic studies of Chinese populations have examined the association of esophageal cancer with *H. pylori* infection,[48] gastric atrophy,[49] human papillomavirus infection,[50] and drinking green tea at a high temperature[51]; however, the evidence is not sufficient to draw any definitive conclusions. A case-control study of Linxian residents showed no significant increased risk of ESCC among individuals infected with *H. pylori*.[48] Previous studies yielded mixed results on the association between green tea consumption, consumption of hot drinks, and risk of esophageal cancer. While a cohort study conducted in Linxian did not find any significant association,[29] a population-based case-control study in Jiangsu Province found that drinking tea at a high temperature significantly increased risk of esophageal cancer, after adjustment for confounding factors, including alcohol consumption and cigarette smoking.[51]

Numerous studies targeting certain genes have reported an association of genetic polymorphisms, including *CYP1A1*, *CYP2E1*, and *MTHFR*, with ESCC risk in the Chinese population.[52] In particular, the *ADH1*47 Arg*, *ALDH2*2*, and *MTHFR* 677 TT genotypes appear to act synergistically with alcohol consumption.[53–55]

Since 2010, 3 GWA studies of esophageal cancer in the Chinese population have been published, and 10q23 was consistently identified as a susceptibility locus for ESCC.[56–58] The main findings are summarized in Table 3. Variants at 10q23 in *PLCE1* were significantly associated with ESCC and gastric cardia cancer in GWA studies by Wang et al and Abnet et al.[56,57] *PLCE1* encodes a phospholipase and is involved in regulating cell growth, differentiation, apoptosis, and angiogenesis. In addition to *PLEC1*, *C20orf54* on chromosome 20p13 was significantly associated with

Table 4. Summary of similarities and differences between Japan and China in epidemiology of esophageal cancer

	Japan	China
Similarities		
Histologic type	ESCC: predominant histologic type	
Incidence and mortality: men vs women	Higher rates in men than in women	
Risk factors	Two established risk factors: cigarette smoking and alcohol drinking	
Differences		
Health burden	Relatively low vs other major cancers	High, especially in rural areas
Pattern of incidence/mortality according to geographic area	Not noted	Wide variations between rural and urban areas
Risk factor profiles according to geographic area	Not noted	Probably different
Strength of associations concerning major risk factors: cigarette smoking and alcohol drinking	Strong	Relatively weak, especially in high-incidence rural areas
Association with gastric cardia adenocarcinoma	Not noted	Reported in recent GWAS Studies
Loci associated with susceptibility to ESCC in GWAS	*PLCE1* and *C20orf54*	*ADH1B* and *ALDH2*
Prevention strategy	Smoking cessation and avoidance of excessive drinking, especially in individuals with certain susceptibility risk variants, such as *ALDH 2*1* genotypes.	Diet, alcohol consumption, and cigarette smoking are essential components. In rural areas, must improve nutritional status, drinking water quality, food preservation, and cooking practices.

ESCC: esophageal squamous cell carcinoma; GWAS: genome-wide association study; PLCE1: phospholipase C epsilon 1; C20orf54: chromosome 20 open reading frame 54; ADH1B: alcohol dehydrogenase; ALDH2: acetaldehyde dehydrogenase.

susceptibility to ESCC in the GWA study by Wang et al.[56] The biologic function of *C20orf54* is not clear, but it may be involved in modulating riboflavin absorption. Furthermore, 3 susceptibility loci for ESCC—on chromosomes 5q11, 6p21, and 21q22—were recently identified in the GWA study by Wu et al in 2011.[58]

Epidemiologic studies in China suggest that gastric cardia adenocarcinoma (GCA) and ESCC have a similar geographic distribution in incidence and common risk factors.[29,59–61] In particular, GCA was more prevalent in ESCC high-risk areas such as Linxian and Cixian.[10,29] Case-control and cohort studies in high-risk areas reported that family history of esophageal cancer, low socioeconomic status, and low intake of vegetables and fruit were significant risk factors for GCA and ESCC.[29,61] Interestingly, in the GWA studies by Wang et al and Abnet et al, variants in *PLCE1* were also significantly associated with GCA risk.[56,57] These findings strongly suggest that the pathogenic processes of ESCC and GCA are similar.

DISCUSSION

Similarities and differences in esophageal cancer between Japan and China

International comparisons of cancer epidemiology are challenging. We closely examined patterns of incidence, mortality rates, and risk profiles to identify similarities and differences between Japan and China in esophageal cancer epidemiology. The identified similarities were as follows (Table 4). First, ESCC is the predominant histologic type, and the incidence of EAC is extremely low in both countries. Second, numerous epidemiologic studies in both countries have confirmed that alcohol consumption and cigarette smoking are important risk factors for ESCC. Third, although a number of putative risk factors have been investigated, such as gastric atrophy and drinking hot beverages, the associations have been inconsistent and inconclusive.

Despite these similarities, there are obvious differences between Japan and China in many aspects of ESCC (Table 4). First, the health burden of esophageal cancer is greater in China than in Japan. Overall incidence and mortality rates are higher in China. Indeed, China alone accounts for about half of new cases worldwide and has many areas with incidence rates exceeding 100 per 100 000 population. In Japan, however, esophageal cancer appears to be less of a burden than other digestive malignancies, such as cancers of the liver, stomach, and colorectum. Among Japanese women, in particular, mortality from esophageal cancer is among the lowest of all cancers—even lower than that from leukemia. Second, variation in mortality and incidence patterns is greater in China than in Japan, eg, the decline in age-adjusted mortality rates was more apparent in rural areas in China than in Japan for the available period (1987–2000). Third, risk factor profiles may differ between high-incidence and low-incidence areas in China, although this is not the case in Japan. Overall, the association between smoking, drinking, and ESCC risk might be weaker in China than in Japan, where compelling evidence confirms the central roles of alcohol consumption and cigarette smoking. In China other potent, but unidentified, risk factors may exist and account for a considerable proportion of ESCC (especially in high-incidence areas), in light of the very low prevalences of smoking and drinking among Chinese women. Fourth, studies in high-risk areas of China have shown that gastric cardia cancer and ESCC have many similarities, including geographic distribution, environmental risk factors, and genetic susceptibility alleles. By contrast, no such findings have been reported in Japan. Fifth, GWA studies in China showed that variants in several chromosome regions confer increased risk, suggesting the involvement of multiple genes in the carcinogenic process. However, GWA studies in Japan found that ESCC risk was associated only with genetic variants in alcohol-metabolizing genes.

Prevention strategies

Although screening for precursor lesions and detection of early-stage ESCC in selected populations is performed in both

Japan and China, prevention remains the best way to decrease the burden of esophageal cancer. Epidemiologic evidence indicates that ESCC is preventable through risk factor modification. Given the above-mentioned differences in various aspects of ESCC, the components of a prevention strategy would be different in Japan and China. In Japan, the decisive roles of alcohol consumption and cigarette smoking in ESCC have been clearly demonstrated; thus, efforts should focus on smoking cessation and avoidance of excessive drinking, particularly among individuals who harbor certain susceptibility risk variants, such as *ALDH 2*1* genotypes. Ideally, the strategy would evolve to personalized prevention based on different genetic backgrounds and varied sensitivity to environmental carcinogens. In China, improved diet and reduced alcohol consumption and cigarette smoking should constitute the essential components of a prevention strategy. Educating the general public regarding risk factor modification is urgently needed in rural areas. Although diet-related mechanisms are not fully understood, improving nutritional status and eating habits would reduce risk. Because nitrosamine, heterocyclic amines, and polycyclic aromatic hydrocarbons are known food mutagens,[62] improving drinking water quality, food preservation, and cooking practices are also important strategies in high-incidence areas.

Future research directions

Given the complex pathogenesis of esophageal cancer, we would like to highlight 3 important research areas for future studies. First, recent GWA studies of esophageal cancer in Japanese and Chinese populations have yielded novel insights into the pathogenesis of ESCC. While GWA studies in the Japanese population found that the major susceptibility variants are located in alcohol-metabolizing genes, GWA studies in the Chinese population did not replicate this finding. Instead, susceptibility to esophageal cancer may be determined by many variants in different genes that have mostly small effects. Differences in study methodology and the frequency or effect size of the alleles at a given locus may explain differences in findings from GWA studies in these countries. Extremely high incidence rates in certain areas of China suggest that high-risk variants remain to be discovered. With the increasing availability of next-generation sequencing technologies, it would be interesting to attempt to identify such high-risk variants. Furthermore, the functional significance of variants identified in extant GWA studies and their interaction with environmental exposures should be clarified in future studies.

Second, there is a great need for a multidisciplinary approach to address the complex role of diet and eating habits in esophageal cancer development. As compared with Japan and low-risk areas of China, a variety of different factors may contribute to development of esophageal cancer in high-risk areas of China. If smoking and drinking do indeed have minor roles, then a high prevalence of potent, but unidentified, factors might be contributing to the lingering high incidence in those areas. It is highly likely that nitrosamine and its precursors are very strong risk factors.[39] Although evidence from ecologic studies suggests a correlation between nitrosamine precursors and ESCC incidence,[40,41] very few studies have measured nitrosamine and its precursors and evaluated their associations with esophageal cancer risk. The main challenge for such studies is correctly determining exposure to nitrosamine from various sources, including exogenous exposure and endogenous formation. Endogenous formation of nitrosamine depends on a variety of factors, including nitrate and nitrite sources, oral bacteria activity, vitamin C level, and secondary amine.[63,64] Moreover, the interaction between these factors remains largely unknown. To unravel the complex mechanisms underlying the nitrosamine–esophageal cancer association, we need to target the whole process by using improved epidemiologic methods, specific biomarkers, and biological pathway analyses. For example, DNA adductome analyses, combined with epidemiologic data on environmental exposure and lifestyle, would provide valuable information on exposure to exogenous or endogenous carcinogens such as nitrosamine.[65]

Third, while there is convincing evidence that *H. pylori* is strongly associated with increased risk of noncardia gastric cancer, studies of its association with ESCC have been limited and have yielded inconsistent results. One mechanism to explain the association between *H. pylori* infection and the increased risk of gastric cancer is that hypochlorhydria in individuals with long-term *H. pylori* infection allows overgrowth of other bacteria, which increasingly convert ingested nitrites to N-nitrosamines.[66] Determining whether this mechanism can also be applied to ESCC warrants further study. Moreover, prospective studies are needed to address the role of *H. pylori* infection and gastric atrophy in ESCC development.

In summary, while evidence from the latest GWA studies has advanced our understanding of esophageal cancer pathogenesis, the best strategy for preventing esophageal cancer in Japan and China remains risk factor modification, namely smoking cessation and avoidance of excessive drinking. It is hoped that the role of diet and eating habits will be clarified in a future well-designed multidisciplinary epidemiologic study.

ONLINE ONLY MATERIALS

Abstract in Japanese.

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Genotoxicity of multi-walled carbon nanotubes in both *in vitro* and *in vivo* assay systems

Tatsuya Kato[1,2], Yukari Totsuka[1], Kousuke Ishino[1], Yoko Matsumoto[1,3], Yukie Tada[4], Dai Nakae[5,6], Sumio Goto[3], Shuichi Masuda[2], Sayaka Ogo[7], Masanobu Kawanishi[7], Takashi Yagi[7], Tomonari Matsuda[8], Masatoshi Watanabe[9], & Keiji Wakabayashi[2]

[1]Division of Cancer Development System, National Cancer Center Research Institute, Chuo-ku, Tokyo, Japan, [2]Graduate School of Nutritional and Environmental Sciences, University of Shizuoka, Yada, Shizuoka, Japan, [3]Laboratory of Environmental Risk Evaluation, School of Life and Environmental Science, Azabu University, Sagamihara, Kanagawa, Japan, [4]Department of Environmental Health and Toxicology, Tokyo Metropolitan Institute of Public Health, Shinjuku-ku, Tokyo, Japan, [5]Department of Pharmaceutical Sciences, Tokyo Metropolitan Institute of Public Health, Shinjuku-ku, Tokyo, Japan, [6]Tokyo University of Agriculture, Setagaya-ku, Tokyo, Japan, [7]Graduate School of Science, Osaka Prefecture University, Sakai, Osaka, Japan, [8]Research Center for Environmental Quality Management, Kyoto University, Otsu, Shiga, Japan and [9]Division of Materials Science and Engineering, Graduate School of Engineering, Yokohama National University, Hodogaya-ku, Yokohama, Japan

Abstract

The genotoxic effects of multi-walled carbon nanotubes (MWCNTs) were examined by using *in vitro* and *in vivo* assays. MWCNTs significantly induced micronuclei in A549 cells and enhanced the frequency of sister chromatid exchange (SCE) in CHO AA8 cells. When ICR mice were intratracheally instilled with a single dose (0.05 or 0.2 mg/animal) of MWCNTs, DNA damage of the lungs, analysed by comet assay, increased in a dose-dependent manner. Moreover, DNA oxidative damage, indicated by 8-oxo-7,8-dihydro-2′-deoxyguanosine and heptanone etheno-deoxyribonucleosides, occurred in the lungs of MWCNT-exposed mice. The *gpt* mutation frequencies significantly increased in the lungs of MWCNT-treated *gpt* delta transgenic mice. Transversions were predominant, and G:C to C:G was clearly increased by MWCNTs. Moreover, many regions immunohistochemically stained for inducible NO synthase and nitrotyrosine were observed in the lungs of MWCNT-exposed mice. Overall, MWCNTs were shown to be genotoxic both in *in vitro* and *in vivo* tests; the mechanisms probably involve oxidative stress and inflammatory responses.

Keywords: Micronuclei, sister chromatid exchange, DNA damage, *gpt* mutation, oxidative stress

Introduction

Multi-walled carbon nanotubes (MWCNTs) are among the most extensively researched and developed nanomaterials, finding use in electrochemical devices and for many biomedical applications. Accordingly, the market for MWCNTs is predicted to grow on a global scale and would be released into the human environment and subsequent inhalation would occur, especially in workplaces. Since MWCNTs are not only nanosized particles, but also rod-shaped fibres with a superbly high aspect ratio, their carcinogenic potential have attracted attention over the years. In fact, mesothelioma could be induced by a single intraperitoneal administration of MWCNTs in both cancer susceptible $p53^{+/-}$ mice and F344 rats (Takagi et al. 2008; Sakamoto et al. 2009). Although intraperitoneal application is not relevant to human exposure, the findings point to possible major hazard.

Due to high cost and the need for special equipment for inhalation studies to create mock conditions for the situation of human exposure, only a few reports on MWCNT inhalation are available so far (Porter et al. 2010; Morimoto et al. 2011). On the other hand, intratracheal instillation is less expensive and more easily performed, and there have been several reports of MWCNT exposure by this route using rats (Takaya et al. 2010; Reddy et al. 2012; Morimoto et al. 2011). MWCNTs induced strong inflammatory reactions, including formation of granulomas and fibrosis in the lungs. Translocation into lung-associated lymph nodes was also observed in mice and rats with both inhalation and intratracheal exposure (Porter et al. 2010, 2002; Ellinger-Ziegelbauer & Pauluhn 2009; Pauluhn 2010a). In addition to MWCNT-induced pulmonary toxicity, genotoxicity, such as micronucleus induction, chromosome aberration and DNA damage, using *in vitro* and *in vivo* assay systems have been reported and are a little controversial (Wirnitzer et al. 2009; Asakura et al. 2010; Ghosh et al. 2011; Patlolla et al. 2010a,b,c; Migliore et al. 2010). For example, Wirnitzer et al. reported

Correspondence: K. Wakabayashi, PhD, Graduate School of Nutritional and Environmental Sciences, University of Shizuoka, 52-1, Yada, Shizuoka 422-8526, Japan. Tel: +81 54 264 5784. Fax: +81 54 264 5904. E-mail: kwakabayashi@u-shizuoka-ken.ac.jp

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that MWCNTs demonstrated neither cytotoxic, clastogenic activities against mammalian cells nor bacteriotoxic, mutagenic activities for bacterial strains. However, most reports revealed that MWCNTs, indeed, are genotoxic and clastogenic for cultured mammalian cells, plants and mice. Among these, chromosomal damage by carbon nanotubes (both single-wall and multi-wall) has been well documented (Asakura et al. 2010; Muller et al. 2008; Sargent et al. 2009, 2011 unpublished data). However, *in vivo* genotoxicity including mutagenicity of MWCNTs has not been fully elucidated yet.

The present study, therefore, aimed to examine the genotoxicity/clastogenicity of this nanomaterial, MWCNTs, in both *in vitro* micronucleus and sister chromatid exchange (SCE) tests. Genotoxic effects were also examined by *in vivo* comet assay, DNA adduct formation and mutation assay using wild type and transgenic mice. In the present study, MWCNTs were, thereby, demonstrated to be genotoxic both in *in vitro* and *in vivo* tests, and possible mechanisms were also suggested. Finally, health concerns raised by the use of MWCNTs are also discussed.

Materials and methods

Materials

High-purity MWCNTs (MITSUI MWCNT-7, identical to those used in the Fischer 344 rats study of Sakamoto et al. 2009) were provided by Mitsui & Co., Ltd. (Ibaraki, Japan). MWCNTs were suspended in saline containing 0.05% Tween 80 (Nacalai Tesque, Kyoto, Japan) and sonicated well on ice. Width distribution of MWCNTs used in the present study indicated a Gaussian distribution with a peak at 90 nm, and more than 80% of particles belonged in a range of 70–110 nm. The length of MWCNTs distributed with a peak at 2 μm, and more than 70% of particles belonged in a range of 1–4 μm (Sakamoto et al. 2009). Detailed information, such as elemental contents of MWCNTs, can be found in a previous report (Sakamoto et al. 2009).

Standards of DNA adducts and their stable isotopes

8-Oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) was purchased from Sigma-Aldrich Japan (Tokyo, Japan). [U-$^{15}N_5$]-8-oxodG was supplied by Dr Shibutani at SUNY Stony Brook, NY, USA. 4-Oxo-2(*E*)-nonenal-derived DNA adducts, heptanone etheno-2'-deoxycytidine (HεdC), heptanone etheno-2'-deoxyguanosine (HεdG) and heptanone etheno-2'-deoxyadenosine (HεdA) were synthesised according to previously published methods (Rindgen et al. 1999, 2000; Pollack et al. 2003).

Micronucleus test

A micronucleus test using human lung carcinoma A549 cells (RIKEN Cell Bank, Wako, Japan) was performed, as described previously (Totsuka et al. 2009). Briefly, A549 cells were seeded in plastic cell culture dishes (ϕ60 mm) 1 day before treatment. Particles were suspended in physiological saline containing 0.05% (v/v) Tween-80 with sonication (for 5–10 min at room temperature). One volume of the suspension was mixed with nine volumes of the culture medium with serum (altogether 3.3 mL/dish), and then cells were treated at indicated concentrations for 6 h. After treatment, cells were further cultured for 42 h. Then, cells were trypsinized and counted and centrifuged. Cells were resuspended in 0.075 M KCl, and incubated for 5 min. Cells were then fixed four times in methanol:glacial acetic acid (3:1) and washed with methanol containing 1% acetic acid. Finally, cells were resuspended in methanol containing 1% acetic acid. The cell solution was dropped onto slides and the nucleus was stained by mounting with 40 μg/mL acridine orange (Nacalai Tesque) solution and immediately observed by fluorescence microscopy using blue excitation. The number of cells with micronuclei was recorded based on observation of 1000 interphase cells.

SCE test

Chinese hamster ovary (CHO) AA8 cells were cultured in RPMI 1640 (Sigma-Aldrich, Japan) supplemented with 10% foetal bovine serum (JRH Biosciences, Lenexa, KS) in a 5% CO_2 atmosphere at 37°C. The cells were treated with MWCNTs for 1 h and cultured in medium containing 10% serum and 10 μg/mL 5-bromodeoxyuridine (Sigma-Aldrich, Japan) for 26 h. Colcemid (Nacalai Tesque) was added for the last 2 h at a final concentration of 60 ng/mL. Cells were trypsinized and centrifuged, resuspended in 0.075 M KCl, and incubated for 30 min. The cells were fixed four times in methanol:glacial acetic acid (3:1). The cell solution was dropped onto slides in a Metaphase Spreader HANABI (AD Science Technology, Funabashi, Japan). The slides were soaked in 50 μg/mL Hoechst #33258 (Sigma-Aldrich, St. Louis, MO, USA). The slides were covered with 0.01 M sodium phosphate buffer (pH 7.6) and cover glasses and irradiated with black light at 365 nm for 3 h. Subsequently, slides were stained with 6% Giemsa (Merck KGaA, Darmstadt, Germany) in 0.06 M sodium phosphate buffer (pH 6.4) for 15 min. SCE was scored under a microscope. The experiments were repeated until acquiring at least 50 cells that were suitable for scoring SCEs in each dose.

Animals

Male ICR mice (6 weeks old) and guanine phosphoribosyltransferase (*gpt*) delta mice (9 weeks old) were purchased from Japan SLC (Shizuoka, Japan). The *gpt* delta mice carry ~80 copies of *lambda* EG10 DNA on each chromosome 17 on a C57BL/6J background (Nohmi & Masumura 2005). The animals were provided with food (CE-2 pellet diet; CLEA Japan, Inc., Tokyo, Japan) and tap water *ad libitum* and maintained under controlled conditions: a 12 h light/dark cycle, 22 ± 2°C room temperature and 55 ± 10% relative humidity. After quarantine for 1 week, the experiments were conducted according to the "Guidelines for Animal Experiments in the National Cancer Center" of the Committee for Ethics of Animal Experimentation of the National Cancer Center.

In vivo comet assay

Each group of five male ICR mice was intratracheally instilled with nanoparticles using a polyethylene tube under anaesthesia with 4% halothane (Takeda Chemical,

Table I. Sister chromatid exchange (SCE) in CHO AA8 cells following a 1 h treatment with MWCNTs.

Treatment [μg/mL]	SCEs/cell*
0†	3.87 ± 1.82
0.1	6.65 ± 1.30‡
1.0	11.3 ± 2.58‡
2.0	10.1 ± 1.52‡

*Mean ± SD of at least 50 cells; †Solvent control (treatment with 0.05% (v/v) Tween 80); ‡$p < 0.01$ (*versus* solvent control) by Student's *t*-test.

Osaka, Japan). Single doses of 0.05 or 0.2 mg/animal were employed. The control mice ($n = 5$) were instilled intratracheally with 0.1 mL of the solvent alone. The mice were sacrificed 3 h after particle administration; the lungs were removed and then used immediately for comet analysis. The alkaline comet assay was performed according to previously described procedures (Totsuka et al. 2009). Fifty cells were examined per mouse. The tail moment of DNA was automatically measured using a Comet Analyzer from Youworks Co. (Ibaraki, Japan). The distance between the centre of nucleus and centre of tail was defined as tail distance, and the fluorescence intensity of damaged area was divided by that of the whole area of cell to achieve the damage ratio. The tail moment was calculated by multiplication of tail distance and damage ratio. Furthermore, percentage of DNA in the tail, another index of DNA damage, was also calculated.

DNA adduct analysis

For DNA adduct analyses, each group of five male ICR mice was intratracheally instilled with MWCNTs at a single dose of 0.2 mg/animal, and sacrificed 3, 24, 72 or 168 h after nanoparticle administration. Control samples were obtained from the lungs of mice given vehicle. Mouse lung DNA was extracted and purified using a Gentra® Puregene™ tissue kit (QIAGEN, Valencia, CA, USA). The protocol was performed according to the manufacturer's instructions except that desferroxamine (final concentration: 0.1 mM) was added to all solutions to avoid the formation of oxidative adducts during the purification step.

DNA samples in 40 μg aliquots were digested into their constituent 2'-deoxyribonucleoside-3'-monophosphate units by the addition of 15 μL of 17 mM citrate plus 8 mM $CaCl_2$ buffer that contained micrococcal nuclease (22.5 U) and spleen phosphodiesterase (0.075 U) plus internal standards. The solutions were mixed and incubated for 3 h at 37°C, then alkaline phosphatase (1 U), 10 μL of 0.5 M Tris-HCl (pH 8.5), 5 μL of 20 mM $ZnSO_4$ and 67 μL of distilled water were added and the reactions were incubated for a further 3 h at 37°C. The digested sample was extracted twice with methanol. The methanol fractions were evaporated to dryness, resuspended in 50 μL of distilled water and subjected to liquid chromatography tandem mass spectrometry (LC/MS/MS). LC/MS/MS analyses were performed using a Waters 2795 LC system (Waters, Manchester, UK) interfaced with a Quattro Ultima triple stage quadrupole MS (Waters). The LC column was eluted over a gradient that began at a ratio of 5% methanol to 95% water, changed to 30% methanol over a period of 30 min, changed to 85% methanol from 30 to 45 min, and was then maintained at 85% methanol from 45 to 55 min. Sample injection volumes of 20 μL each were separated on a Shimpack FC-ODS column (150 × 4.6 mm, 3 μm, Shimadzu, Kyoto, Japan) and eluted at a flow rate of 0.4 mL/min. Mass spectral analyses were carried out in positive ion mode with nitrogen as the nebulising gas. The ion source temperature was 130°C; the desolvation gas temperature was 380°C. Nitrogen gas was also used as the desolvation gas (700 L/h) and cone gas (35 L/h) and argon was used to provide a collision cell pressure of 1.5×10^{-3} mbar. Positive ions were acquired in multiple reaction monitoring mode. The multiple reaction monitoring transitions were monitored; each cone voltage and collision energy used was as follows: 8-oxodG [284->168, 35 V, 14 eV], HεdG [404->288, 35 V, 10 eV], HεdA [388->272, 35 V, 10 eV], HεdC [364->248, 35 V, 10 eV].

gpt and Spi⁻ mutation assays

For mutation analysis, each group of six to seven male *gpt* delta mice was intratracheally instilled with particles at a single dose or multiple doses of 0.2 mg/animal as follows. Group 1 served as the vehicle control (0.1 mL of saline containing 0.05% Tween 80), Groups 2–4 were the study groups and received single or multiple doses of MWCNTs (Group 2: single dose of 0.2 mg/animal; Group 3: 0.2 mg/animal for each of two instillations 2 weeks apart; Group 4: 0.2 mg/animal, once a week for 4 weeks). The mice were sacrificed at 22 weeks of age; this was 8–12 weeks after particle administration. Lungs were removed, and stored at −80°C until high-molecular-weight genomic DNA was extracted using a RecoverEase DNA Isolation Kit (Stratagene, La Jolla, CA, USA), according to the manufacturer's instructions. *Lambda* EG10 phages were rescued using Transpack Packaging Extract (Stratagene). The *gpt* and Spi⁻ mutagenesis assay was performed, according to previously described methods (Nohmi et al. 2000).

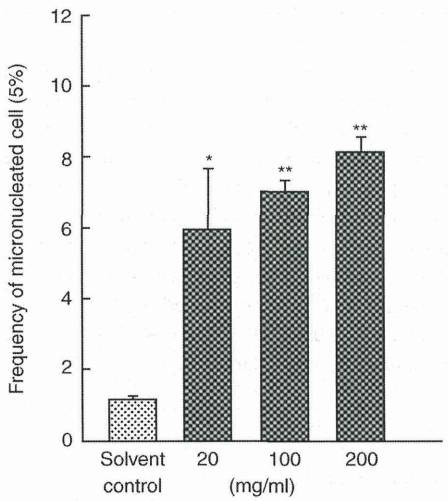

Figure 1. Frequency of micronucleated A549 cells. The frequency was calculated after counting the number of cells with micronuclei based on observation of 1000 interphase cells. Mean values ± SD of three independent experiments are shown. Solvent control represents treatment with 0.05% (v/v) Tween 80; *$p < 0.05$ and **$p < 0.01$, by Student's *t*-test.