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Full Length Research Paper

Organic pollution from the Songhua River induces NIH 3T3 cell transformation: Potential risks for human health

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Epidemiological investigation has shown that organic contamination of the Songhua River is a risk factor for tumor development among residents who live nearby. A mutagenesis is induced by organic contamination using short-term genotoxic bio-assays. To further investigate the risk of carcinogenic potential to human health, the NIH3T3 cell line was used to examine the induction of transformation by diethyl ether extracts of water samples taken from the Songhua River in the summer of 1994 and the winter of 1995. The results indicated that the malignant transformed foci were induced by diethyl ether extracts. Cellular transformation frequencies showed a dose response. Malignant cells possessed typical characteristics in cell growth and in the cellular anchorage dependent test while the control cells did not. Thus, this study demonstrates diethyl ether extracts of water samples could induce cell transformation of NIH3T3 cells. Evidence was provided the possible relationship between organic pollution and carcinogenic potential.

Key words: Diethyl ether extracts, NIH3T3 cells, cell transformation, malignant cells, the Songhua River.

INTRODUCTION

The Songhua River is the third largest river in the People's Republic of China, with a catchment area of about 556,800 km^2. The Songhua River crosses three provinces including Heilongjiang, Jilin and Inner Mongolia and consists of the NenJiang River, the Second Songhua River, and the Songhua River. The Songhua River is the major freshwater source for industry and agriculture, as well as the source of drinking water for 62.25 millions residents living along it (from a 2005 government report). It is known that the Songhua River has been heavily polluted by waste water from industry and domestic sewage. Xu et al. (1990) reported that 152 organic compounds were detected in the Songhua River by methods of gas chromatography (GC), Gas chromatography/mass spectrometry (GC/MS), high pressure liquid chromatography (HPLC) and total ion chromatography (TIC). Of these compounds, 19% were polycyclic aromatic hydrocarbon (PAHs), 14% were chloro-compounds, 13% were aromatic compounds and 54% were other compounds. An epidemiological investigation indicated that organic contamination of the Songhua River is a risk factor for tumor development among the residents living along it. Organic pollutants from the Songhua River were tested for mutagenicity using the *Salmonella typhimurium* assay and diethylether

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Abbreviations: MNNG; N-methyl-n'-nitro-n- nitrosoguanidine, **DMSO;** dimethyl sulphoxide, **EDTA;** ethylenediamine tetraacetic acid.

extracts of 1.7 or 3.5 L water equivalent / plate were positive with TA98 (-S9) and TA1538 (-S9) (Zhu et al., 1985). Yang's study also reported that the frequency of chromosome aberrations (CA) and sister chromatid exchanges (SCE) of blood lymphocytes in residents living along the Songhua River were higher than that of the control group (Yang et al., 1991). However, there is no further direct evidence to elucidate the high tumor development of residents who lived along with the Songhua River except for genotoxicity assays that only respond to genotoxic agents.

In vitro cell transformation tests using NIH3T3 cells can simulate the process of animal two-stage carcinogenesis by treating the cells under different growth conditions, and therefore potentially detecting not only initiating activity, but also promoting activity of chemicals. Cell transformation is the induction of certain phenotypic alterations in cultured cells that are characteristic of tumorigenic cells. These phenotypic alterations can be induced by exposing mammalian cells to carcinogens. Transformed cells that have acquired the characteristics of malignant cells have the ability to induce tumors in susceptible animals (Berwald and Sachs, 1963; Berwald and Sachs, 1965). *In vitro* transformed cells exhibit morphological changes related to neoplasia. The phenomenon of morphological cell transformation involves changes in behavior and growth control of cultured cells, such as alteration of cell morphology, disorganized pattern of colony growth, and acquisition of anchorage-independent growth (Weber et al., 1976; Combes et al., 1999). Transformed cells then become able to grow in semi-solid agar (anchorage-independent growth), produce autocrine growth factors and can evolve to tumorigenicity when injected into appropriate hosts. Although partly data in conclusion of cell transformation were mentioned in previous study (Liu et al., 2007), the detail information about cell transformation in NIH 3T3 cells should be stated because there is no further direct evidence to elucidate the high tumor development of residents who lived along with the Songhua River except for genotoxic assays that only respond to genotoxic agents. Thus, the objectives of this study were to determine the transformational effect of organic extracts taken from the Songhua River and the risk of carcinogenic potential to human health.

MATERIALS AND METHODS

Reagents and chemicals

The chemicals were obtained from the following sources: N-methyl-n'-nitro-n- nitrosoguanidine (MNNG), and dimethyl sulfoxide (DMSO) from the Sigma Chemical Company (St. Louis, MO); Trypsin, ethylenediamine tetraacetic acid (EDTA), Giemsa, agar (low melting point), and Trypan Blue Stain from Tian Xiang Ren Chemical Company (Beijing, China). Methanol, acetone, diethyl ether, and anhydrous sodium sulfate were analytical grade and obtained from Harbin Chemical and Bio-Reagent Co. (Harbin, China).

Sampling collections and extraction

During the summer of 1994 and the winter of 1995, we collected 820 L and 990 L water samples directly taken from the intake of the most distant water treatment plant of Harbin, located 15 km above Harbin. The pH value in each sample was 7.30. For the extraction of pollutants, GDX-102 (40 - 60 bead mesh) resin (Tianjin Chemical Co., China) was washed by swirling and decanting 4 times with 10 resin-volumes of acetone for 12 h each time, followed by absolute methanol for another 48 h in the same volume and distilled water 3 times. The extract was stored in distilled water at 4℃ (Junk et al., 1974; Wang et al., 1983). Glass columns, 30 mm (inside diameter) × 350 mm, were filled with distilled water before the addition of sufficient washed GDX-102 resin (100 g dry weight) to give a bed height of 15 - 20 cm. Flow was regulated with a 3-way nylon stopcock. Distilled water (about 250 ml) was passed through the GDX-102 resin before using these columns.

Water samples (80 - 100 L) taken from the Songhua River were passed through each column. Water was dropped onto glass wool, protecting the resin from splatter, at 8-10ml/min. Finally, each column was washed with 3 resin-volumes of distilled water 3 times prior to diethyl ether extraction. Three volumes (resin column) of diethyl ether were added to the column. The resin in the column was swirled with glass rod during each exaction until the final ether eluted solution was colorless. Distilled lab grade water was used as the control. The effluent was dehydrated with anhydrous sodium sulfate and evaporated within a KD concentrator until nearly dry. The residue was dissolved in diethyl ether (< 1ml) and kept in a -30℃ refrigerator for cell transformation assays. The diethyl ether in the samples was evaporated and the residues were dissolved in DMSO prior to the assay. The cell transformation assay was performed when the samples were done in October 1995.

Cells, media, and culture conditions

NIH3T3 cell line, original from NIH Swiss mouse embryo, was bought from Cancer Research Institute of Beijing (China). NIH3T3 cells were cultured in RPMI 1640 medium (Gibco Co. Los Angeles, USA) supplemented with 10% FBS (fetal bovine serum, FBS, Gibco Co.) at 37℃ in a humidified incubator under 95% air and 5% CO_2. Cells were passaged before confluence by a mixture of ethylenediamine tetraacetic acid (EDTA) and trypsin, usually at 3 day intervals. In order to obtain constant transformation results, many frozen stock ampoules of the cells were not passaged more then 4 times after recovering cells from liquid nitrogen. One ampoule was thawed and used in each individual cell toxicity and transformation experiment.

Test procedures

Cytotoxicity assay

A preliminary cytotoxicity assay was carried out for determination of test concentrations. For the evaluation of growth inhibition and cytotoxic effect of initiating chemicals, NIH3T3 cells were seeded at a density of 5.0×10^3 cells per 25 ml culture bottle (20 cm^2 of bottle surface, four bottles per dose). The glass culture bottle was clear, did not have any blotches, and had a smooth surface to show that cells attached to the bottom of the glass bottle. Twenty-four hours later, the cultures were exposed to diethyl ether extracts of water samples and the medium containing the diethyl ether extracts was discarded after 24 h exposure. The cells in the bottles were washed three times with PBS (phosphate buffered saline, pH 6.8). Then the culture medium was replaced with fresh RPMI 1640 medium with 10% FBS. The same procedures were used for a positive (MNNG 1.5 μg/ml), negative (DMSO, 0.05 % v/v) and blank controls. After

Table 1. Cytotoxicity in NIH 3T3 cells treated with MNNG and diethyl ether extracts of water samples taken from the Songhua River in the summer of 1994 and the winter of 1995 (n = 4).

Season	Doses (L water/ml)	Number of seeded cell	No. of colonies Mean ± SD	Absolute CFE (%)	Relative CFE (%)
Winter	0.198	5000	1050 ± 300	21.0**	41.9
	0.099	5000	1600 ± 452	32.0**	63.9
	0.050	5000	2230 ± 402	44.6	89.1
Summer	0.205	5000	800 ± 202	16.0**	31.9
	0.102	5000	1025 ± 201	20.5**	40.9
	0.051	5000	1395 ± 355	27.9**	55.7
	0.026	5000	1710 ± 669	34.2*	68.2
MNNG	1.5 µg/ml	5000	1940 ± 595	38.8	77.5
DMSO	0.05 % (v/v)	5000	2505 ± 574	50.1	100.0
Blank	0	5000	2410 ± 375	48.2	96.2

CFE: colony-forming efficiency; * P < 0.05, ** P < 0.01, compared to the negative control group (DMSO) using χ^2 test.

$$\text{Absolute CFE (\%)} = \frac{\text{Number of colonies formed}}{\text{Number of cells seeded}} \times 100\%$$

$$\text{Relative CFE (\%)} = \frac{\text{Number of colonies on treatment}}{\text{Number of colonies on negative control}} \times 100\%$$

one week, the cells were fixed with methanol and stained with Giemsa. Cell colonies, cells which originated from one cell and contained more than 20 cells per colony were counted under the microscope.

The colony-forming efficiency (CFE) was calculated as follows:

Transformation assay

The assay procedure for transformation experiments was described in detail in previous reports (Huang, 1985; Dunkel et al., 1991). Exponentially growing NIH3T3 cells were plated at a density of 3.0 × 10^3 cells per 25 ml glass bottle in RPMI 1640 medium with 10% FBS. Fifteen bottles were used for each dose. After 24 h incubation, different levels of the diethyl ether extracts were added as the initiation treatment for 24 h at 37℃ in
95% air and 5% CO_2. Then, the medium containing the diethyl ether extracts was discarded. The cells were washed three times with PBS. Then the culture medium was replaced with 3 ml RPMI 1640 fresh medium with 10% FBS at three day intervals until 21 days after sample treatment (Huang, 1985). Both a negative and the positive controls were used in this experiment. Cells in most bottles were fixed with methanol at 21 days after treatment, stained with Giemsa and scored for transformed foci. Other bottles which were not fixed by methanol were used to determine the cell growth curve and semi-solid agar culture. A rubber scraper ring (10 mm diameter) was used to separate transformed foci from surface of bottles with trypsin / EDTA mixture. Verification of the transformed morphology was made with an invert microscope at ×10, ×20, and × 40 magnifications.

(a) Criteria for transformation: Scoring of transformed foci was characterized by the following morphological criteria, which discriminate transformed foci by four morphological characteristics: (1) foci of more than 2 mm in diameter, (2) deep basophilic staining, (3) piling up of cells forming a dense multi-layer, and (4) random orientation of cells at the edge of foci, overlapping nuclei, and basophilia. The criteria were considered suspect when either of criteria (1) without (4) or (3) without (1) and (4) was met (Huang, 1985; Dunkel et al., 1991).

The transformation frequency (TF) per bottle was calculated as follows:

$$\text{TF/bottle (\%)} = \frac{\text{Average transformed foci per bottle}}{\text{Average survival foci per bottle}} \times 100$$

(b) Transformed cell growth (Huang, 1985): For each extract and control, 1 × 10^5 cells from transformed foci, including suspect foci, from the cellular transformation test were seeded in 6-well plates. Beginning the next day, three wells for each extract or control were selected at random and the number of living cells was determined by Trypan Blue Stain. The mean for each set of 3 wells was determined and the curves of cell growth were plotted.

(c) Cellular anchorage dependent test (Ruhaut, 1979): Briefly, 6-well plates were prepared with a 1.5 ml underlay of 0.6% soft agar in RPMI 1640 containing 10% FBS. 1 × 10^4 cells in 1.5 ml of 0.3% soft agar (low melt) were placed on top in RPMI 1640 containing 10% FBS. The plates were incubated at 37℃ in 95% air and 5% CO_2. Cell colonies were observed after 3 - 4 weeks. The number of colonies was counted and the result was reported as mean ± standard deviation.

Data analysis and statistical evaluations

All analyses were conducted using the SPSS for Windows statistical package, version 11.0 (SPSS Inc., Chicago, IL, USA). For each experimental condition (that is, the same cell number in the cytotoxicity assay and anchorage dependence test or per bottle in the transformation assay), foci data were expressed as mean ± SD. The statistical analysis of experimental values in the cell transformation test was done using the χ^2 test with a one-sided Fisher's Exact Test (Armitage, 1971). A *P*-value of ≤ 0.05 was considered statistically significant.

A positive result was recorded if there was a significant increase in the number of foci (P < 0.05), with one of the following: (1) a dose-response relationship, (2) 2 or 3 transformed foci in two successive concentrations, or (3) 3 or more transformed foci in a concentration (Huang, 1985; Dunkel et al., 1991).

Table 2. Activity of transformation in NIH3T3 cells induced by MNNG and diethyl ether extracts of water samples taken from Songhua River in the summer of 1994 and the winter of 1995.

Season	No. of bottles fixed	Doses	Total no. of cell foci			Cell foci/bottle			TF/bottle
		(L water/ml)	TCF	SCF	SVF	TCF	SCF	SVF	(%)
Winter	8	0.198	16	8	179	2.0 ± 1.3**	1.0 ± 0.8	22.4 ± 11.3**	8.9**
	9	0.099	15	4	452	1.7 ± 1.0**	0.4 ± 0.5	50.2 ± 21.8	3.3**
	8	0.050	1	3	507	0.1 ± 0.4	0.4 ± 0.5	63.4 ± 26.8	0.2
Summer	11	0.205	14	6	431	1.3 ± 1.1**	0.6 ± 0.7	39.2 ± 14.7**	3.3**
	10	0.102	16	22	800	1.6 ± 1.2**	2.2 ± 1.6**	80.0 ± 24.1	2.0**
	8	0.051	7	10	523	0.9 ± 0.8	1.3 ± 1.3*	65.4 ± 15.1	1.3**
	9	0.026	0	5	635	0	0.6 ± 0.7	70.6 ± 26.7	0
MNNG	9	1.50	22	5	305	2.4 ± 1.7**	0.6 ± 0.9	33.9 ± 10.8**	7.2**
DMSO	9	0.05% (v/v)	0	3	600	0	0.3 ± 0.5	66.8 ± 22.5	0
Blank	8	0	0	0	665	0	0	83.1 ± 20.4	0

*P < 0.05, **P < 0.01, compared to the negative control group (DMSO) using χ^2 test. MNNG: N-methyl-n'-nitro-n-nitrosoguanidine; TCF: transformed cell foci; SCF: suspect cell foci; SVF: survival cell foci; Total TF: frequencies of (transformed + suspect) cell foci.

RESULTS

Cytotoxicity

The results of cytotoxicity are as shown in Table 1. Diethyl ether extracts of water samples taken from the Songhua River were toxic to NIH 3T3 cells and the frequency of cellular colonies was reduced as the concentration increased. Both the absolute CEF and relative CEF showed a dose-response relationship. The maximal doses for each sample were selected according to the cytotoxicity assay.

Transformation test

Based upon the cytotoxicity results, all concentrations of samples were used in the transformation test. As shown in Table 2, the diethyl ether extracts from water samples taken from the Songhua River showed a positive response in the transformation test. The TCF per bottle and suspect cell foci (SCF) per bottle showed a dose-response relationship. A significant low frequency of formation of the survival cell foci (SVF) per bottle was observed in 0.198 L water / ml of winter sample, 0.205 L water /ml of summer sample when compared to the negative control group (P < 0.01 and P < 0.05). Dose-dependent relationships were also observed in the frequency of cellular transformation (TF) per bottle as the concentrations of samples increased. There was no evidence of transformation in the negative control group. As noted in Table 2, the suspect transformed foci were noted in all samples, including the negative control groups except for the blank control. STF would further determine whether there have malignant cells in the foci, especially, in the negative control. In contrast to the normal cellular pattern of NIH3T3 cells (Figure 1A), Figure 1B shows at lower power (×40) evidence of cell transformation (invasive and piling up properties) and Figure 1C, at higher power (×100), shows evidence of cellular transformation (fibroblastic, criss-crossing, densely stained cells, random orientation at edges of foci and basophilia).

Malignant cellular transformed foci measurement

To further characterize the transformed cells from transformed foci, cell growth curves and colony formation assays in semi-solid agar were performed. The cell growth curves showed that the MNNG and the diethyl ether extract of the winter sample did not differ in growth compared to the blank control group within 3 days. After 4 days, the transformed cells grew rapidly while the growth of cells in the blank control group was slow (Figures 2A and 2B). The cells from transformed foci of the winter sample grew quickly and the shape of cell growth curves was nearly the same after 5 days. The cell growth curve in the summer

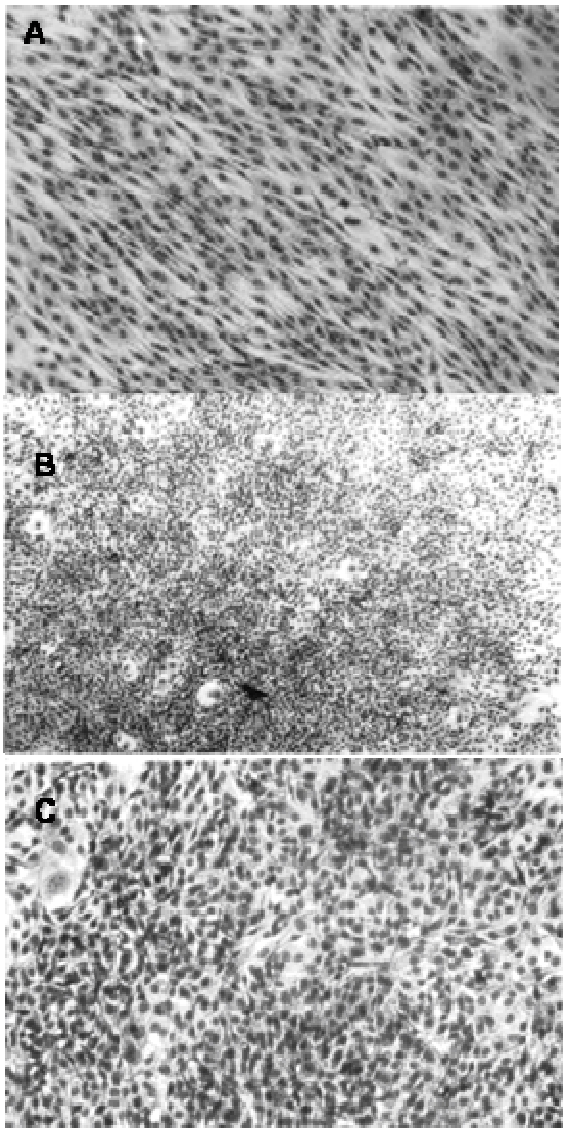

Figure 1. Morphological difference in the cell foci. (A) Non-transformed NIH 3T3 cells showing contact inhibition and growth in monolayer (×100). (B) Typical transformed cell foci showing invasive and piling up properties (×40). (C) Typical transformed foci showing fibroblastic, criss-crossing, densely stained cells, random orientation at edges of foci, and basophilia (×100).

sample was only different from the blank control group after 6 days. In the Colony formation assay, the plates were incubated for 3 - 4 weeks and the number and size of colonies were analyzed. The transformed cells formed colonies while no colonies were observed in the control groups including blank control and DMSO groups (Table 3 and Figure 3). These results confirm that transformed cells from all diethyl ether extracts lost anchorage dependence and grew in an anchorage independent manner, whereas the normal cells did not.

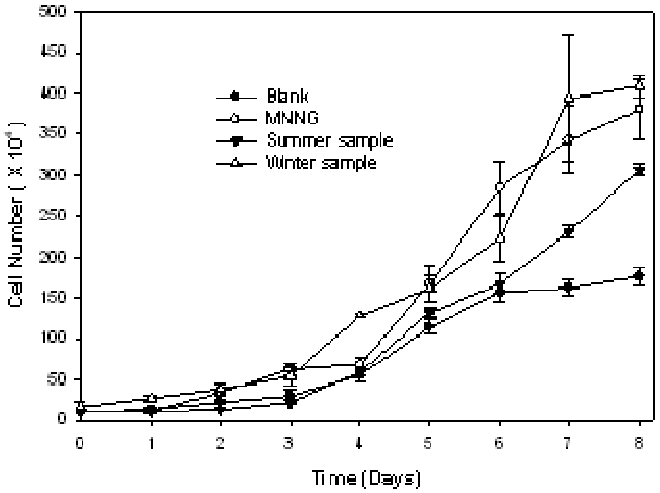

Figure 2. The cell growth curves of NIH 3T3 cells from the experimental and control groups. To determine the rate of cell growth from the experimental and control groups, the cell growth curves were plotted. The cell growth curve of the diethyl ether extract of the winter sample and the positive control did not differ in growth compared to the blank control group within 3 days. After 4 days, the transformed cells had grown rapidly while the growth of cells in the blank control group was slow. The cells from transformed foci of the winter sample grew quickly and the shape of cell growth curves was nearly the same as the positive control group after 5 days. The cell growth curve in the summer sample was only different from the blank control group after 6 days.

DISCUSSION

Surface water pollution is a very serious public health and aquatic ecosystem problem, especially in rivers that are used as a source of drinking water Ohe, et al. 2004, Liu, et al, 2007, 2009 Ohe, et al. (2004) reported that pollutants from surface waters in Europe, Asia, South America, etc. were determined to be mutagenic / genotoxic using a variety of bioassays which demonstrated that these environmental mixtures contain many toxicants that may have the risk of carcinogenic potential. Umbuzeiro et al. (2001) reported that compiled data obtained during the last 20 years from more than a thousand samples and found that TA98 was more sensitive than TA100 and 79% of the mutagenicity was detected by this strain, regardless of the presence of S9-mix. However, *in vitro* mammalian cells also can further predict carcinogenic potential. Quantitative cell culture systems provide procedures for detecting potential carcinogens in a relatively short period of time compared with the years required to complete *in vivo* tests (Dunkel et al., 1991). Mammalian cells in culture are particularly appropriate in quantitative cell culture systems (Saffiotti, 1983). Quantitative dose-response relationships have been demonstrated with established mammalian cell systems in fibroblasts derived from mouse embryo cells. With most of these systems, the dose-response relationship for

Table 3. Colony formation of transformed cells picked up from suspect foci of bottle in semi-solid agar.

Groups	Total no. of foci picked up	No. of colonies/1,000 cells
		Mean ± SD
Transformed foci from experimental groups	15	53 ± 39**
MNNG	18	133 ± 48**
DMSO	5	0
Blank	5	0

**P < 0.01, compared to the negative control group (DMSO).

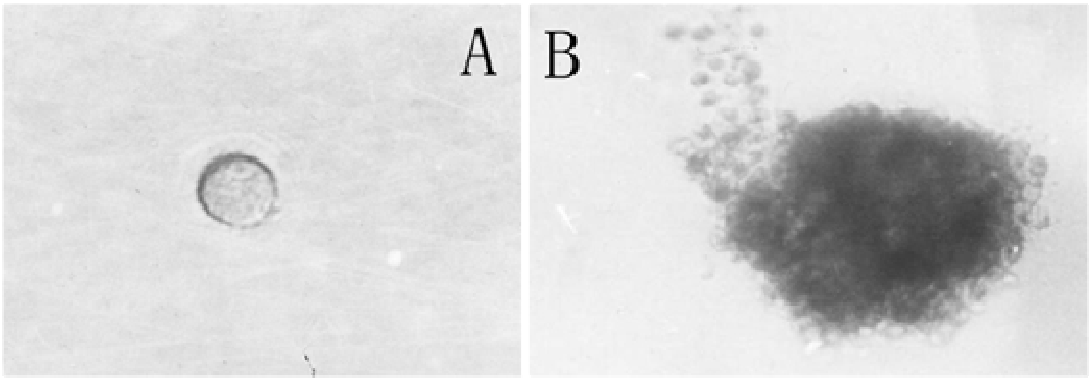

Figure 3. Growth of NIH3T3 cells transformed in semi-solid agar. To determine the cells' ability for anchorage independent growth, about 1×10^4 cells were suspended in 1.5ml of 0.3% top agar containing PRMI 1640 plus 10% FBS and overlaid onto 1.5ml of bottom agar (0.6% agar in RPMI 1640 containing 10 % FBS) in a 6-well plate. After incubation for 3 - 4 weeks at 37℃, we found that the cells that had transformed had acquired the ability to grow in soft agar and colony formation (B, ×100). In contrast, the non-transformed cells did not produce any anchorage independent colonies (A, ×400).

for transformation by an organic chemical carcinogen is consistent, indicating a direct cause- and -effect relationship between carcinogen and transformation. The use of mammalian cell culture (*in vitro*) transformation systems is an important technique for short-term testing for potential carcinogens, since the mechanisms involved in such systems may be similar to the processes of neoplastic transformation *in vivo* (IARC, 1980, Barrett, 1985). Hu et al. (2004) reported that arsenic at the low doses of 0.1 and 0.5 μmol/L for 110 days could induce the morphological transformation of NIH3T3 cells. The transformed cells had the characteristics of malignant cells, not only growing in semi-solid agar, but also still surviving over 50 passages.

The Songhua River is located at the junction of the temperate and cold-temperate zones. The region has a long, cold winter, a torrid, rainy summer; and a dry, windy spring. Early August was chosen for the summer sample because it had more rain and early January was chosen for the winter season because the level of water in the Songhua River was at its lowest. The Songhua River is a major freshwater source for industry and agriculture in the area. However, the increasing population, industry and agricultural activities around the Songhua River lead to the introduction of contaminants and the possible pollution of the river. Zhu et al. (1985); Zhang et al. (1988) and Zhu et al. reported that compiling mutagenicity tests of diethyl ether extracts of fish and water samples taken from the Songhua River and epidemiological investigations on cancer mortalities for residents of Harbin and Zhaoyuan county along with the Songhua River revealed the following results: (1) Mutagenicity tests showed that diethyl ether extracts from fish and water samples are mutagenic. Treatment of 1.7 L water equivalent / plate and 6.0 g fish equivalent / plate in the *Ames* test indicated a positive response in a dose-dependent manner; (2) a case-control study of gastric and liver cancers showed that the cancer mortalities were significantly higher for residents who drank water or ate fish from the Songhua River. The cancer mortalities were significantly higher for residents of Harbin, who drank water of the Songhua River, than for those residents drinking ground water. The cancer mortalities were significantly higher for residents of Zhaoyuan county near the Songhua River, as they ate more fish; (3) The certain relationship between cancer mortalities and organic contamination of the Songhua River was noted; (4) Further laboratory evidence was needed to explain this relationship. Yang's study (Yang et al., 1991) also investigated the frequencies of CA and SCE of blood lymphocytes from 59 residents who lived near the Songhua River over 5 years and 52 residents as

a control group. The results of CA in residents living near the Songhua River were higher than that in the control residents but the difference was not significant. However, the frequency of SCE in these same residents was significantly higher than in the control group. This indicated that water pollution including mercury and methyl-mercury from the Songhua River is a main factor for increasing frequencies of CA and SCE in the blood lymphocytes of residents who lived along the river.

In this study, the TF per bottle for the blank control group is zero and 3 SCF for the DMSO control group; however, there were three foci in the DMSO control group which were suspect in that they possessed anchorage dependence in the semi-solid agar culture test. This result in the negative control is very astonishing to us, because a high transformation frequency was obtained in the cell line by searching related articles. Rubin (2005) reported that spontaneous neoplastic transformation develops within days in the NIH3T3 cell line through differential inhibition of their proliferation under contact inhibition. Contact inhibition and reduction in serum concentration select for the same cellular phenotype that increases saturation density and generates transformed foci. This allows the selection of cells that are better able to proliferate and reduces spontaneous transformation under restricted conditions such as low seeding cell density, low passage number, the same serum concentration in the media as well as short experimental period. Rubin et al. (1989), in another study, showed that NIH3T3 cells could produce transformed foci spontaneously if kept in the confluent state for more than 10 days. Cells maintained in continuous exponential multiplication in the sub confluent state by transfer every 2 - 3 days in medium with 10% calf serum failed to develop the capacity to produce foci in 2% calf serum, but those transferred in the same way in 2% calf serum or in 10% fetal bovine serum, which is a less potent growth stimulant, did develop that capacity to an increasing degree over time. The formation of foci depends on the type and concentration of bovine serum used in the medium and on the passage history of the cells. The number of transformed cells increased sharply with the time that a culture remained in the confluent state. In our study, concentrations of diethyl ether extracts of water samples taken from the Songhua River in the summer of 1994 and the winter of 1995 could induce typical transformed foci in NIH3T3 cells. TF per bottle showed a dose-response relationship. The TF also showed a dose-dependent manner as our previously study (Liu et al., 2007). Transformed NIH3T3 cells lost contact inhibition, and the speed rate of their growth increased. Normal cells reach contact inhibition and growth rate slows after 4 days. The cell growth curve in the DMSO control group were also determined in the previously study (Liu et al., 2007). The shape of cell growth curve in the DMSO control group is nearly the same as the blank control group. Although the cell growth curve from diethyl ether extract of water sample in the summer was a bit different in comparison with the blank control group, the number of transformed cells in cellular foci was less than those of the diethyl ether extract of water sample in the winter sample and the positive control group. The latter was proved by the anchorage dependent test. Thus, the cell growth speeds are different from each other. NIH3T3 cells are anchorage dependent, this is, and they must have a solid surface to adhere to in order to grow. Transformed NIH3T3 cells lose anchorage dependence and grow into cluster of cells in semi-solid agar.

No single method has been universally accepted for the statistical evaluation of chemical responses in cell transformation assays. The distribution of transformed foci is generally symmetric across culture vessels for all treatment conditions. However, some researchers have noted abnormal distributions, and relatively large numbers of foci occur at random in both control and carcinogen-treated cultures (Rundell, 1984). The current literature describes two main ways to evaluate the frequency of transformed foci. Frequencies can be evaluated using non-parametric χ^2 or one-tailed Fisher's Exact tests (Armitage, 1971). Alternatively, parametric methods can be employed following suitable transformation of the data. After examining several mathematical transformations, this study employed parametric analyses following logarithmic (that is, log10) transformation Matthews, 1986; Rundell and Guntakatta, 1983). ANOVA (analysis of variance) was carried out on the log10-transformed frequency values, and differences between responses were investigated using a modified student's t-test.

We have measured the cell transformation of diethyl ester extracts of water samples from the Songhua River, a major source of drinking water in the North Eastern region of China. This study is unique in that it used a cell transformation assay to demonstrate that the water samples possess the risk of potential carcinogenesis. Cell morphology changed dramatically as reflected by transformed foci. The cells from transformed foci have the characteristics of malignant cells. These results indicate that organic extracts from the Songhua River samples have induced cell transformation of NIH3T3 cells and provide further an evidence of a relationship between organic pollution and carcinogenic potential. Based on these results, we recommend that future analyses of organic pollutants be both qualitative and quantitative and a further epidemiological study of the population is needed.

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