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# Application of human cell transformation assay on assessment of carcinogenic potential of river organic pollutants

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**Abstract**: The approach for assessing carcinogenic potential of complex mixtures of environment pollutants is still under development. In this study, human cell transformation assay was used to evaluate the carcinogenic potential of organic extracts of water pollutants collected from Jialu River (S1), one of the main tributaries of the Huai River, Henan province, China. The incidence of the digestive cancers in the basin rose dramatically within the past three decades. In addition, we collected water samples from the local wells in two liver cancer patients' homes. The distance of these two wells is 1 km (S2) and 20 km (S3) from the site of S1. Organic chemicals were extracted using hydrophilic-lipophilic balance solid phase cartridges and the fraction was dissolved in DMSO. Human hepatic immortal cells (HL-7702) were treated with each extract and cytotoxicity was measured. The cells were treated with each extract and the efficiency of cell transformation was examined periodically. The subcutaneous injection of treated cells in immune-deficient mice was performed to confirm the malignant cell transformation. The latency of malignant transformation for sample S1, S2, and S3 was 14, 14, and 16 weeks, respectively at the lowest concentrations of 1.0, 0.5, and 2.0 microlitre (μL) of extracts per milliliter medium (mL), enriched from 10, 50, and 200 mL original water, respectively. Moreover, we analyzed 16 polycyclic aromatic hydrocarbons (PAHs) in organic extracts using GC-MS analysis and found 13 components appearing in all water samples. Our study indicates that human cell transformation assay can be potentially used for assessing carcinogenetic potential of mixture of environmental pollutants.

## 1. Introduction

Current toxicity test methods are mostly conducted using laboratory animals. The prediction of human health effects from animal results that involves a number of assumptions and extrapolations remains controversial[1]. The development of alternative methods with rapid, less expensive, and more relevant to human exposure is extremely urgent in order to meet the requirement of testing huge numbers of emerging chemicals. In addition, there is an increasing demand for addressing the complexes issue and cumulative exposures.

Recent epidemiological studies have revealed an association between water pollution and high incidence of human digestive cancers in the Huaihe River basin, China, where the "cancer villages" were reported[2, 3]. The mortality rates of stomach, esophageal, liver, and colorectal cancer in the study areas were 2~6 times higher than average[2]. Organic chemical pollutants, including the PAHs were detected in the Huaihe River, China[4-6]. The surface water pollution originated from activities of industrial, agricultural, and domestic contamination undoubtedly reduces the ground water quality[7]. For a long time, ground water has been the main source of water supply for the residents living along the Huaihe river basin. However, the toxicity assays that assess the health risks of the complexes of environmental pollutants have not been established. Thus, it is urgent to develop assays that fulfill the need for quickly screening the carcinogenic potential of organic water pollutants.

Cell-based bioassays that predict health-relevant biological endpoints may therefore in keeping with the criteria for risk assessment. The *in vitro* cell

transformation assay (CTA) has been recommended as an alternative method for rodent carcinogenicity test[8] due to its accuracy and expeditiousness in prediction of chemical carcinogens[9].

In our prior work, we developed *in vitro* models of human cell transformation and demonstrated their efficiency in detecting both genotoxic and non-genotoxic carcinogens[10]. We established a series of human bronchial epithelial (HBE) cells by expressing oncogene *H-Ras* (HBER) or *c-Myc* (HBEM)[10], or defects in DNA repair genes including *excision repair cross-completion 1* (*ERCC1*), *excision repair cross-completion 2* (*ERCC2*), *ataxia-telangiectasia mutated* (*ATM*) or *mutS homolog 2* (*MSH2*)[11]. We demonstrated that HBER and HBEM cells were more sensitive to carcinogen-induced cell transformation with a shortened latency of induction. Thus, we speculate that human cell transformation models are feasible in assessing the carcinogenic potential of water pollution mixtures.

In this study, organic pollutants were extracted from the surface water of the Jialu River and well water in the basin. The carcinogenic potential was evaluated using *in vitro* human cell transformation assay. In addition, sixteen of USEPA priority controlled polycyclic aromatic hydrocarbons (PAHs) were also detected in all water samples, implying a relevance of carcinogenicity.

## 2. Materials and methods

### 2a Chemicals and reagents

Cell culture medium and glutamine were obtained from GIBCO BRL-Life

Technologies (Grand Island, NY). Cytochalasin B was purchased from Sigma-Aldrich (St. Louis, MO). Sixteen PAHs (specified on US EPA Method 610 [12]) standards, including acenaphthenene (Acp), acenaphythylene (AcPy), anthrancenen (Ant), benz[a] anthrancene (BaA), benzo[a]pyrene (BaP), chrysene (Chr), dibenz[a,h]anthracene (Daa), fluorine (Flu), fluoranthene (Flua), naphthalene (Nap), phenanthrene (Phe), pyrenen (Pyr), benzo[b]fluoranthene (BbF), benzo[k]fluoranthene (BkF), benzo[g,h,i]perylene (Bgp), and indeno[1,2,3-c,d]pyrene (Ind) were purchased from Accu Standard Inc (Akron, Ohio).

**2b Sample collection and preparation**

Water samples from three locations were collected in March 2010, where was no rainfall at least one week before sampling and during the sampling periods. The organic pollutants were extracted according to the method developed by Ma et al[7]. Briefly, the water samples were collected in amber glass bottles and kept on ice while being transported to the laboratory. Then the water samples were passed through the glass fiber filters (GF/C, 1.2 μm, Whatman, Maidstone, UK) using a vacuum system. The pH of the water samples was adjusted to 3.0 with hydrochloric acid. The water samples were concentrated by Oasis HLB cartridges (Waters, USA) at a flow rate of 5-10 mL/min. The HLB cartridges were pre-conditioned with 10 mL of hexane, 20 mL of acetone, 10 mL of methanol, and 10 mL of ultrapure water consecutively. After extraction, the cartridges were dried under the flow of nitrogen gas. Subsequently, the cartridges were eluted by 20 mL of acetone and dried by nitrogen blowing. The

residues were dissolved in DMSO. Experimental blank was prepared by extracting ultrapure water using the same method as described above.

In particular, samples used for the analysis of PAHs were dissolved in 500 μL hexane instead of DMSO. Then the solvents were subjected to purification by C18 cartridges (Waters, USA) that were pre-conditioned with 25 mL of hexane and eluted with 5 mL hexane, 5 mL of a mixture of hexane and dichloromethane (1:1 in volume), and 5 mL of a mixture of hexane and dichloromethane (1:4 in volume). This purified extract was further concentrated to 100 μL and spiked with known amounts of the internal standards prior to instrumental analysis.

**2c Cell lines and cell treatment**

Human hepatic immortal cell line HL-7702 was obtained from Shanghai Cell Biology Institute, Chinese Academy of Sciences. Cells were cultivated in RPMI-1640 medium with 10% (v/v) heat-inactivated fetal calf serum at 37°C in an atmosphere containing 5% $CO_2$.

For carcinogenic potential analysis, the cells were treated at least 4 rounds with organic extracts. After 24-hour exposure, the medium was discarded and the cells were washed twice with PBS and refilled with 2 mL fresh complete medium for continuous cultivation. Cells were subcultured at 90% confluence, followed by the next round of treatment. It took one week for one round of treatment, and after the $4^{th}$ time of treatment cells were subjected to soft agar assay. Cells which formed colonies in soft agar and grew into tumors in nude mice were considered malignantly

transformed, and the organic extract treatment would be stopped. Treatment of the other groups would continue until the 20th week.

### 2d Soft agar assay

Anchorage-independent cell growth was detected using soft agar assay. Briefly, $1\times10^5$ cells/well were seeded in six-well culture plates and treated with water extracts when cells reached 60% confluence. After 24h, the medium was discarded, and fresh complete medium was added. Cells were subcultured at 90% confluence, and another round of treatment was followed. After the 4th round of treatment, cells were trypsinized every two weeks and subjected to soft agar assay.

Soft agar assay was performed as previously described[11]. Briefly, $6\times10^4$ of HL-7702 cells were suspended in DMEM plus 10% FBS in 0.4% agar above a layer of 0.6% agar base. The transformed HBERST cells expressing SV40 large T (LT), the telomerase catalytic subunit *(hTERT*) and an oncogenic allele *H-Ras*, were used as positive controls. The culture plates were examined 5 days later for signs of cell growth by using phase-contrast microscopy. Colonies with a diameter of greater than 100 μm were counted 4 weeks after treatment.

### 2e Tumorigenesis study

The BALB nu/nu mice were purchased from Experimental Animal Center of Guangzhou University of Traditional Chinese Medicine (Guangzhou, P.R. China). Two hundred microliter cell suspension containing $1\times10^7$ cells was injected

subcutaneously into the nude mice, with transformed HBERST cells and DMSO-treated HL-7702 cells as positive controls and negative controls, respectively. Tumors were observed as early as the 5$^{th}$ day after subcutaneous injection. After 5 weeks, the mice were sacrificed for examination. All the procedures herein were approved by the Animal Care and Use Committee of Sun Yat-sen University.

**2f Cytokinesis-blocked micronucleus (CBMN) assay**

HL-7702 cells were seeded on the six-well cell culture plates at the density of $2.5\times10^5$ cells/well. After 24 hours, the medium was removed and the cells were exposed to 4 doses of extracts and 1/3 $IC_{50}$ was used as the highest treatment concentration. Mitomycin C (MMC, 0.1 μg/mL) was used as the positive control and DMSO (0.1%, v/v) as the vehicle control. After treatment, cells were washed twice with PBS and cytochalasin B (3 μg/mL) was added. After 36 hours of incubation at 37°C, cells were then trypsinized and slides were coded according to the standard protocol proposed by Fenech [13] with minor modifications. The formation of CBMN was viewed and scored under microscope at 1000 × magnification. For each group, 1000 cytokinesis-blocked (CB) cells were examined for the presence of micronuclei, following the criteria described by Fenech[14]. All experiments were performed in triplicate.

**2g Detection of PAHs in water sample extract**

Sixteen PAHs were detected by using GC-MS (QP2010, SHIMADZU) in the

electron impact ionization mode (70 eV). Separation was carried out at the following conditions: helium as carrier gas at a constant flow rate of 1.0 mL/min and the temperature of injector and ion source at 280 and 200 ℃, respectively using a DB-5 (30 m × 0.25 mm, ID 0.25 μm) capillary column. A 1.0 μL sample was injected in a splitless mode. The initial temperature was 50°C and held for 3 min, increased to 300°C at a rate of 10°C and held for 5 min. The GC conditions were as follows: helium was the carrier gas at a constant flow rate of 1.0 mL/min and methane was the ionization gas at the flow rate of 40 mL/min; the injection was carried out in splitless mode with a splitless time of 2 min. The temperature program started at 50°C and held for 3 min, increased to 150°C with a rate of 10°C, then at 5 °C to 250°C and reached the final temperature of 300°C with 50°C (held for 5 min). Selective ion monitoring (SIM) was selected to analyze each PAHs component according to the corresponding *m/z*. The GC peaks were identified by accurate assignment of retention time for each standard. For quantification purposes, calibration curves based on a set of six concentration standards 5, 10, 25, 50, 100, and 250 μg/L were drawn. The instrumental detection limit was 1 μg/L.

### 2h Statistical analysis

Data are presented as the mean ± standard deviation (SD) for at least three independent experiments. Statistical analyses were performed using SPSS 13.0 (SPSS, Chicago, IL). The differences among different groups were analyzed using One-way analysis of variance. $P < 0.05$ was considered statistically significant. Specific post

hoc comparisons were used to examine differences among groups.

## 3. Results

### 3a Selection of sampling sites and preparation of organic extracts

Jialu River is one of the branches of Huaihe River. It has been contaminated by the direct discharge of industrial and domestic waste water, and farmland and livestock contaminants for over 30 years. There are about 1.38 million residents living along the river[5, 15]. The study region and sampling sites are selected as shown in Figure 1. In this region, shallow groundwater is the main source of drinking water consumed by the local residents. The sample S1 was the organic extract from the surface water of Jialu River. The other two samples S2 and S3 were from the shallow groundwater, which was 1 km (S2, about 10 m in depth) and 20 km (S3, about 10 m in depth) away from the Jialu River. The organic pollutants were extracted as described above and were finally dissolved in defined volume of DMSO. In particular, one microliter DMSO extract was equivalent to 10 mL of original river water, 100 mL of groundwater, and 100 mL of ultrapure water (control), respectively.

### 3b Hepatic cell transformation induced by organic extracts of water samples

To investigate the carcinogenic potential of the organic mixtures, *in vitro* cell transformation assay was performed using HL-7702 cells. Cell viability was determined by trypan blue exclusion assay. The highest concentration of each sample used in this experiment has no obvious cytotoxicity (<5%). Three doses for each

sample were chosen at 0.11, 0.33 and 1.00 μL/mL medium for S1, or at 0.06, 0.17 and 0.50 μL/mL medium for S2, or at 0.22, 0.67 and 2.00 μL/mL medium for S3. Treatment of 0.1% (v/v) of DMSO was served as a negative control. HL-7702 cells were treated with organic extract weekly at the concentration described above. After four weeks treatment, we examined the anchorage-independent cell growth by soft agar assay. 14 weeks after treatment, we observed transformed phenotypes in HL-7702 cells at doses of 1.0 μL/mL (S1) and 0.5 μL/mL (S2) (Fig. 2), respectively. 16 weeks after treatment, we found that sample S3 at a dose of 2.0μL/mL showed a phenotype of malignant transformation (Fig. 2). In contrast, we did not observe cell transformation phenotype in DMSO-treated control cells even for 20 weeks. The transformed cells displayed significant morphological changes including cell enlargement, turning round and overlapping growth, while the non-transformed cells remained long fusiform shaped and growing in a monolayer manner (Fig. 3). The malignant cell transformation was confirmed by subcutaneously injection of treated cells in immunodeficient mice. Collectively, although all kinds of water samples could induce malignant cell transformation, the concentrations began to show malignant cell transformation varied greatly and might be relevant to the carcinogenic potential of the mixtures.

### 3c Genetic toxicity of the organic extracts examined by CBMN assay

To assess the genetic toxicity of organic extracts, we performed the CBMN assay on HL-7702 cells treated with different concentration of organic extracts. The CBMN

method is a simple, sensitive, repeatable, and high accuracy assay to detect genetic damage. Four doses at 0.11, 0.33, 1.00, and 3.00 μL/mL medium for S1, or at 0.06, 0.17, 0.50 and 1.50 μL/mL medium for S2, or at 0.07, 0.22, 0.67 and 2.00 μL/mL medium for S3, respectively were used for administration. DMSO (0.1%, v/v) and MMC (0.1 μg/mL) was served as the negative and positive control, respectively. 24 h after treatment, significant elevation of micronuclei formation (MNi‰) in cytokinesis-blocked cells ($P$ <0.05) was observed in sample S1 at concentration of 0.33, 0.17 and 0.67 μL /mL medium, and in sample S2 and S3 (at 3.3, 16.7 and 66.7 mL of original water per millimeter medium), respectively (Fig. 4). The image of representative micronuclei was shown in figure 5. These findings indicate that all water samples displayed DNA damage potential, and the order of genotoxicity of three different sites from high to low was S1>S2>S3, which is well correlated with the activity of cell transformation.

### 3d Detection of PAHs in water samples

To examine whether the organic extracts of Jialu River and ground water were contaminated by polycyclic aromatic hydrocarbons (PAHs), we detected 16 PAHs listed as the priority pollutants for monitoring the quality of water and wastes (U.S. EPA) by GC-MS analysis. As shown in Table 1, 13 out of 16 PAHs appeared in all water samples, among which BaP, BaA, BkF, BbF, and Chr belonged to group B2 according to the USEPA IRIS database. The total amount of PAHs in water sample S1, S2 and S3 was 20.34 ng/L, 8.87 ng/L and 7.75 ng/L, respectively, which were lower

than the safety level of the Standard for Drinking Water Quality (GB5749-2006)[16] executed in China. The BaP concentration of sample S2 and S3 water extract was 0.246 ng/L and 0.136 ng/L, respectively, which is lower than the recommended level (10 ng/L) according to the Standard GB5749-2006. Although at a low level of exposure, we are unable to exclude the possibility that the adverse health effects might develop in residents exposed to combined PAHs.

## 4. Discussion

The approach of combination of multiple toxicological endpoints with a system-based impact assessment allows us to get an insight into time- and dose-dependent molecular perturbations of specific biological pathways[17]. Human cell-based *in vitro* systems can be applied for testing the biological effects of single toxicant or environmental mixtures and for evaluation of the quality of environment in time course studies. In this study, organic extracts from the surface water and shallow groundwater of the Jialu River basin were subjected to *in vitro* bioassays. The results from human cell transformation assay showed that water extracts of the Jialu River and the nearby underground water could transform hepatic HL-7702 cells after 14 or 16 weeks of treatment. Genotoxicity was also observed in a dose-dependent manner. Chemical analysis revealed 13 priority PAHs emerging in all water samples, some of which are human carcinogens. The findings implicated a causative role of the organic pollutants in the development of digestive cancers. This study indicated that *in vitro* cellular assay can effectively predict the potential of carcinogenicity of environmental

mixtures.

During the past three decades, the Huai River has been seriously polluted. The term of "cancer villages" has been conferred to the areas with high incidence of digestive tract tumor[2, 3]. The previous studies indicated that the organic pollutants such as PAHs were the major contaminants attributable to the adverse health outcome[6]. In this study, we revealed the presence of 13 PAHs in the complexes, albeit at the levels lower than the permissible limit adopted by the Standard GB5749-2006[16]. Drinking water pollution predisposes people to complex contaminant mixtures, and the combined adverse effects of pollution mixtures should be taken into consideration of risk assessment. It has been reported that although the concentration of pollutants in aquatic environment is very low, the complex interactions, such as synergistic or antagonistic effects, may occur[18, 19]. Proper models that evaluate the joint effects of environmental mixtures at low levels would be helpful for addressing this issue using integrated biological and informatics approach[18, 19]. Herein, *in vitro* cell transformation assay has shown its potential in risk assessment of environmental mixture exposure.

*In vitro* cell transformation assay has been proposed as an alternative method of 2-year rodent cancer bioassay for screening of potential chemical carcinogens. A recent report showed that this method could effectively detect 90% and 95% of the chemicals or compounds listed in group 1 and group 2 of carcinogens (IARC), respectively[9]. Three rodent cell transformation models, SHE, BALB/c 3T3 and C3H10T1/2, were widely used, which have good accordance with rodent bioassay

data. However, these animal cell-based CTAs have limitations such as difficulties in species extrapolation, while the human cell-based CTAs are recommended for prediction of human carcinogenic potential[9].

Recently, the U.S. National Research Council (NRC) launched a new risk assessment approach based on the ‘‘toxicity pathways’’ obtained through well-designed *in vitro* human cell assays[20]. The mechanism underlying specific toxic effect could be studied using human cells or cell lines, and key pathways could be obtained and used as cell state biomarker[1]. Our previous studies have indicated that human CTAs are feasible and effective in testing the carcinogenic potential of chemicals[10]. Using these chemical carcinogen-transformed human cell models, we identified critical regulatory pathways and epigenetic mechanisms involved in malignant cell transformation[21, 22]. In this study, we demonstrate that human CTAs assay is able to evaluate the potent carcinogenicity of environmental mixtures. Therefore, the *in vitro* cell transformation could be developed as an alternative method for assessment of carcinogenicity.

## 5. Conclusion

In this study, we used *in vitro* cell transformation assay to study the carcinogenic potential of the organic extracts from polluted water samples. The effects of genotoxicity of 13 PAHs components are associated with the phenotype of human cell transformation. The human cell-based transformation assay exhibits its advantages in high-speed, high-sensitivity, and accurate prediction of carcinogenic potential of

single or mixture pollutants. It can be applied for dynamically monitoring the water quality, in particular at a situation that unknown carcinogens appearing in water environment.

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**Table 1 Concentration of PAHs in water samples (ng/L)**

| Compound | S1 | S2 | S3 |
|---|---|---|---|
| naphthalene (Nap) | 4.152 | 0.709 | 0.534 |
| acenaphythylene (AcPy) | 0.392 | 0.098 | 0.106 |
| acenaphythylene (Acp) | 3.105 | 1.215 | 1.160 |
| fluorine (Flu) | 2.470 | 1.219 | 1.200 |
| phenanthrene (Phe) | 3.870 | 2.331 | 3.632 |
| anthrancenen (Ant) | 0.476 | 0.052 | 0.163 |
| fluoranthene (Fla) | 0.822 | 0.574 | 0.730 |
| pyrenen (Pyr) | 0.857 | 0.666 | 0.662 |
| benz[a] anthrancene (BaA) | 0.255 | 0.122 | 0.095 |
| chrysene (Chr) | 0.417 | 0.251 | 0.245 |
| benzo[b]fluoranthene (Bbf) | 0.145 | 0.092 | 0.140 |
| benzo[k]fluoranthene (Bkf) | 0.130 | 0.075 | 0.068 |
| benzo[a]pyrene (BaP) | 0.274 | 0.202 | 0.111 |
| $\Sigma$ (polycyclic aromatic hydro-carbons , PAHs) | 17.363 | 7.603 | 8.848 |

Concentration levels of the PAHs in the Jialu River surface water (S1), the adjacent groundwater S2 and S3.

Figure legends:

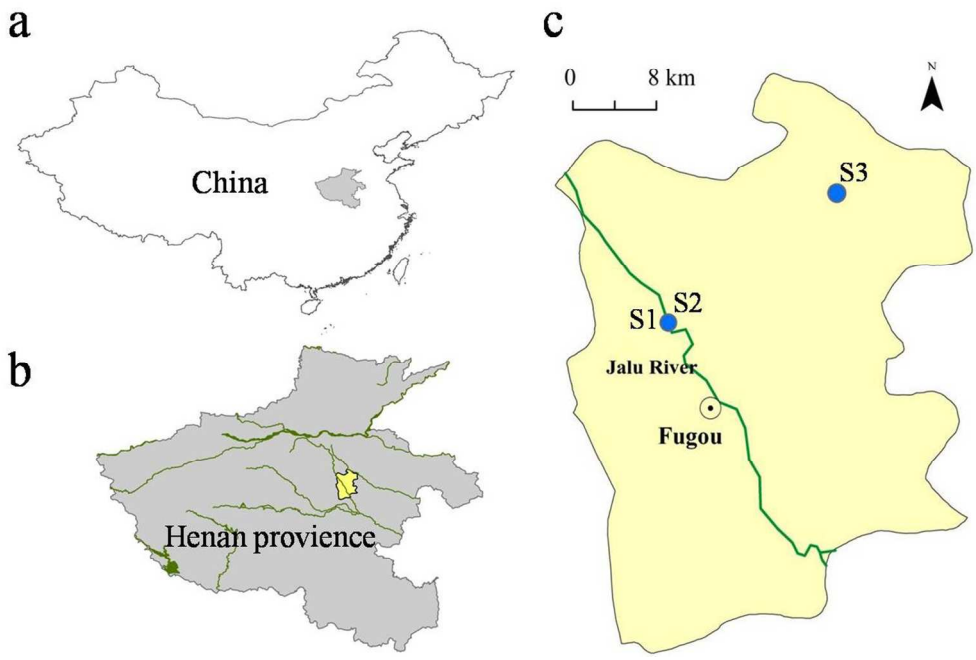
Fig.1 The study area is one of the towns of Henan province (b), locates in the middle of China (a). S1 was the surface water collection site of the Jialu River. S2 and S3 were adjacent groundwater sites, which were 1 km and 20 km away from S1. In this district, groundwater served as the source of drinking water (c).

Fig.2 Numbers of cell colonies in anchorage-independent growth assay. HL-7702 cells were treated with the extracts of water samples from (a) S1, (b) S2, and (c) S3 at indicated concentrations expressed as μL extracts/mL medium. The data were expressed as mean ± SD from three independent experiments. *$P$ <0.05 statistically significant difference compared to the negative control (NC). The indicated highest dose of each group induced 100% tumor formation in nude mice (#).

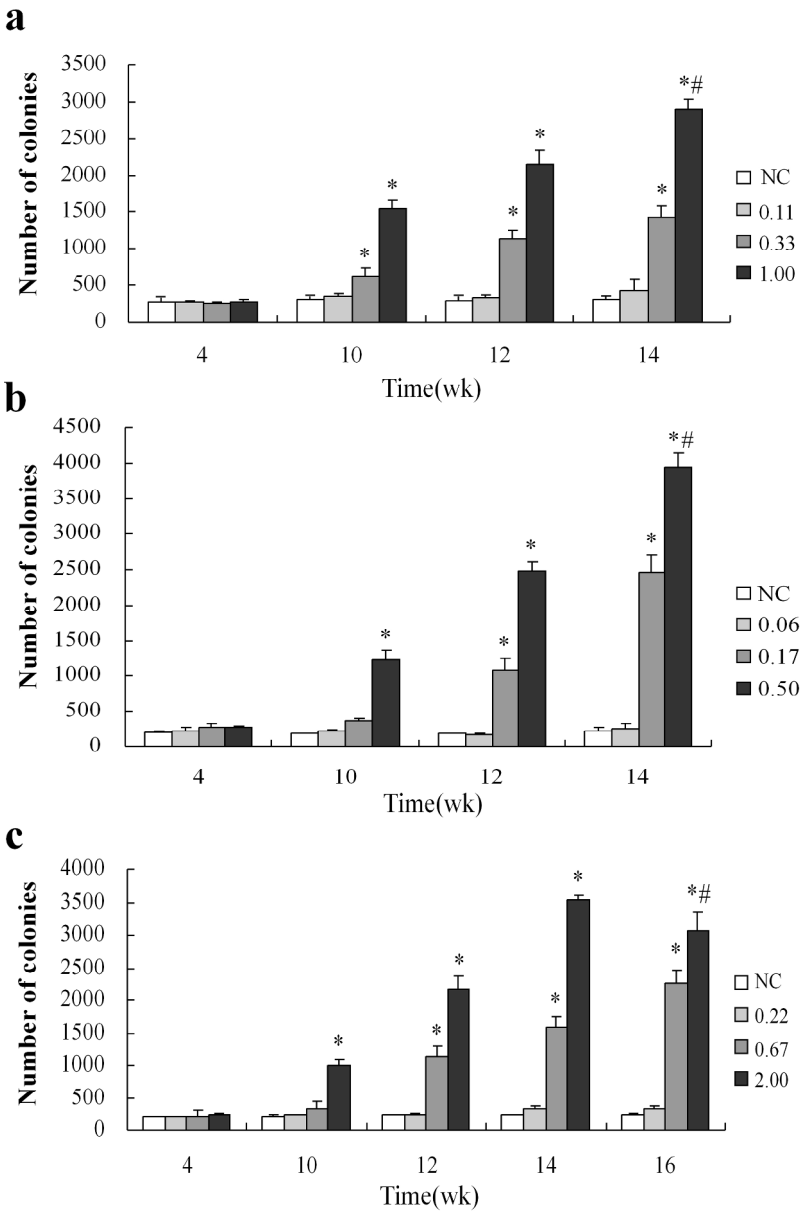
Fig.3 The malignant cell transformation induced by river water extracts. The cells were treated with 0.1% DMSO (a, e) or the lowest dose (b, f). A representative image shows colony formation in soft agar plate 14 weeks post-treatment. The cells treated with organic extracts at concentration of 0.33 (c, g) and 1.00 (d, h) μL/mL medium formed obvious colonies.

Fig.4 Frequencies of MNi in HL-7702 cells treated with extracts of water samples S1(a), S2 (b) and S3 (c). Treatment of MMC at 0.1 mg/mL was served as a positive control. The data were expressed as mean ± SD from three independent experiments. * $P < 0.05$, # $P < 0.01$ statistically significant difference compared to the negative control (NC).

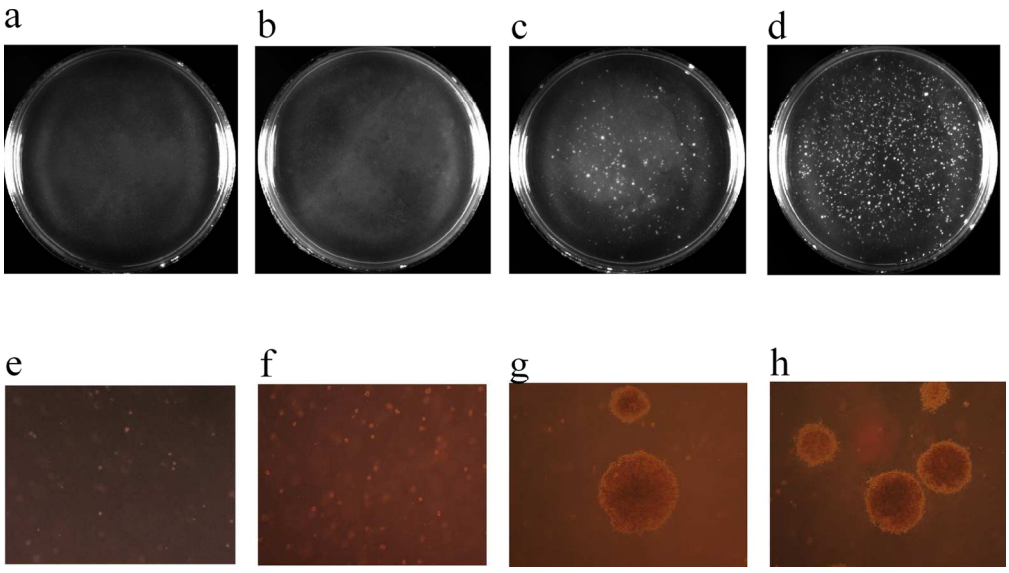
Fig.5 Micronuclei formation in cells treated with MMC or organic extracts (b), while no micronuclei were observed in normal cells (a). The black arrow indicated micronuclei (1000 × magnification).



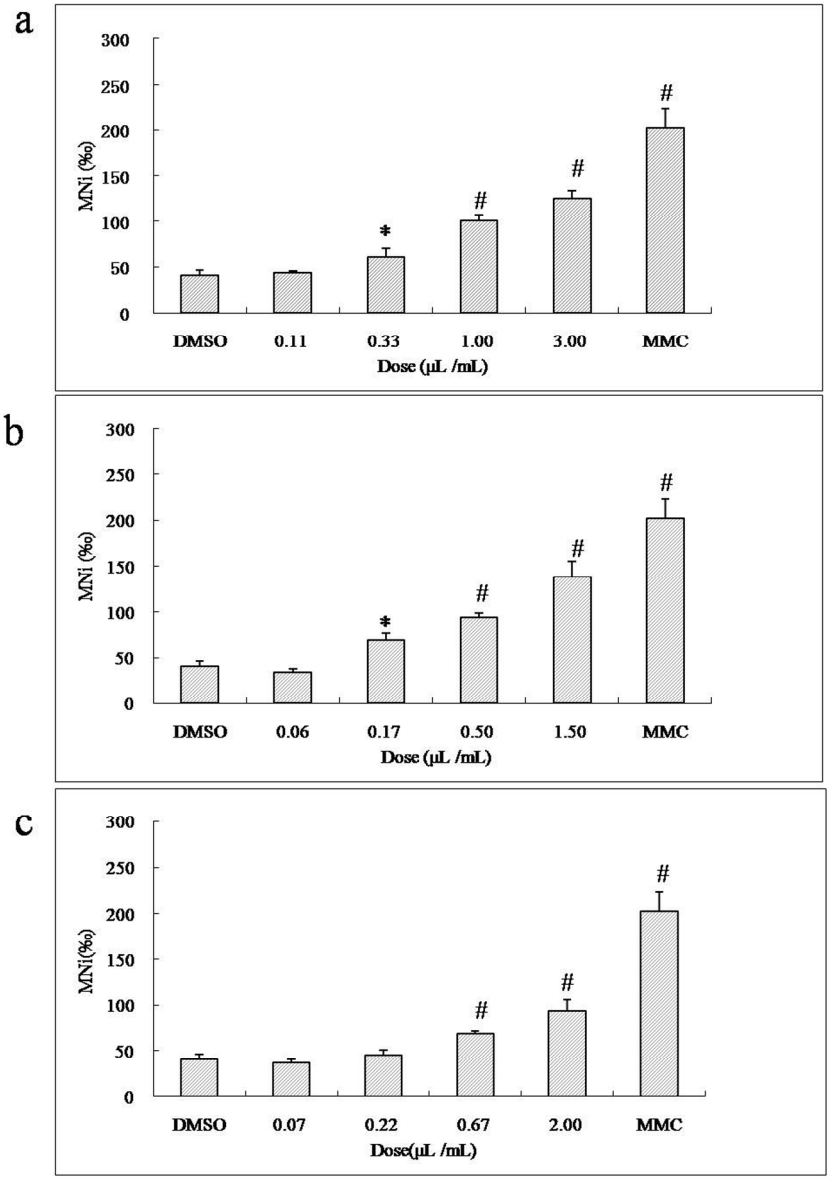

155x106mm (300 x 300 DPI)



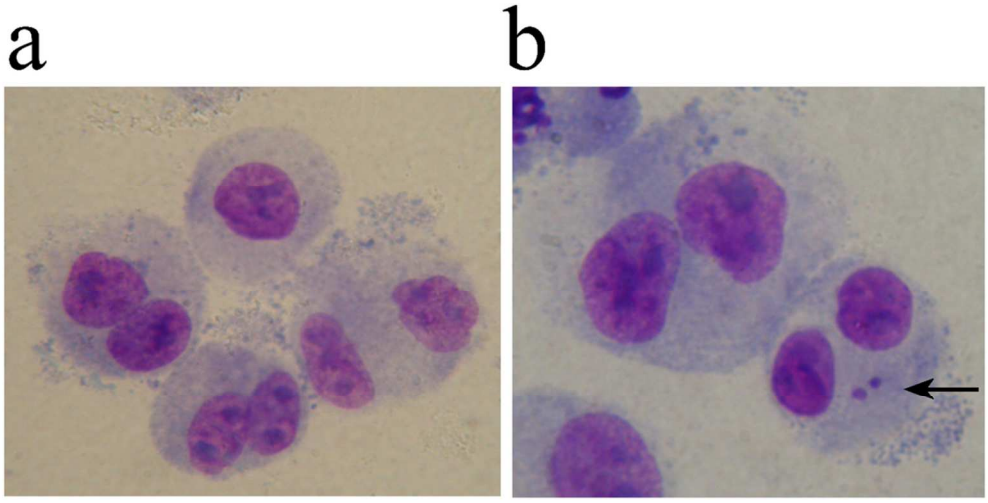

286x417mm (300 x 300 DPI)




148x82mm (300 x 300 DPI)




234x330mm (300 x 300 DPI)




97x50mm (300 x 300 DPI)