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Research article

# Carcinogenicity and proteomic analysis of *N*-nitrosodiethylamine in rats

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## Abstract

*N*-nitrosodiethylamine (NDEA), a probable human carcinogen, is commonly used in rodent experimental hepatocarcinogenesis research. We evaluated both clastogenicity and carcinogenicity in rats using various concentrations of NDEA via a liver micronucleus assay and a medium-term carcinogenicity test. The administration of repeated NDEA injections ranging from 10 to 50 mg/kg bw together with partial hepatectomy resulted in significantly more liver micronuclei compared to control rats, while the mitotic index was indifferent. However, the highest dose (50 mg/kg bw) was lethal to the rats. Similarly, medium-term exposure at 100 mg/kg bw of NDEA increased the formation of glutathione *S*-transferase placental form (GST-P) positive foci, a preneoplastic lesion of liver cancer in rats. A proteomic analysis revealed overexpression of hepatic proteins involved in the PI3K/Akt/mTOR pathway after treatment with NDEA. This pathway may play a key role in the mechanisms that influence NDEA-initiated hepatocarcinogenesis in this model. In conclusion, the amount of NDEA used in the present study can be considered as clastogenic and carcinogenic to rat hepatocytes. This finding can further our knowledge concerning the establishment of experimental cancer chemoprevention models in rats.

**Keywords:** Clastogenicity, Carcinogenicity, *N*-nitrosodiethylamine, Proteomics

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# INTRODUCTION

In everyday life, we are exposed to a number of food-borne carcinogens. Nitrosamines are mainly present in smoked foods, fermented foods, and many alcoholic beverages as well as in tobacco smoke (Brunnemann et al., 1978; Sen et al., 1980). *N*-nitrosodiethylamine (NDEA), also known as diethylnitrosamine (Figure 1), is an extensively occurring nitrosamine. It is commonly used as a rodent carcinogen to induce either preneoplastic lesions or liver tumors in experimental carcinogenesis. NDEA, when metabolized to carbonium ion by CYP2E1, is capable of initiating DNA mutations and subsequent liver tumor formation in mice and rats via the formation of DNA adducts with either a nitrogenous base or the oxygen of guanine, resulting in $N^7$-ethylguanine or $O^6$-ethylguanine (Appel et al., 1984; Kang et al., 2007; Tolba et al., 2015). As a result, long-term consumption has been associated with hepatocellular carcinoma (HCC) in humans (Tolba et al., 2015). However, liver carcinogenesis is step-wise and can be reversed or attenuated by certain substances, including dietary antioxidants and anticarcinogens (Glauert et al., 2010; Thumvijit et al., 2014; Punvittayagul et al., 2016). In this study, we applied short-term micronucleus tests (Krishna et al., 2000; Charoensin et al., 2010) and a medium-term liver carcinogenesis bioassay (Thumvijit et al., 2014; Punvittayagul et al., 2016) to determine dose-response and molecular alterations of NDEA during the early stages of hepatocarcinogenesis. The concept is based on the two-step initiation and promotion of carcinogenesis. Evaluation of micronucleus frequency *in vivo* is the primary assay in a battery of genotoxicity tests (Krishna et al., 1992; Krishna et al., 2000). Such *in vivo* medium-term carcinogenesis assays have been extensively exploited to quantify the carcinogenic and/or tumor-promoting potential of test compounds (Ito et al., 1989; Puatanachokchai et al., 2006; Tolba et al., 2015). Data from the present work would allow us to set up a suitable model for further investigation of cancer chemopreventive properties of indigenous medicinal plants.

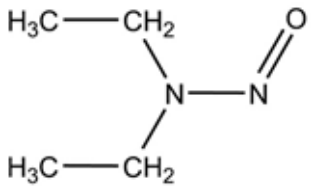


**Figure 1** Structure of *N*-nitrosodiethylamine.

# MATERIALS and METHODS

## Chemicals and reagents

*N*-nitrosodiethylamine (NDEA) was purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan. Collagenase type IV prepared from *Clostridium histolyticum* and 4'-6-Diamidino-2-phenylindole (DAPI) as a fluorescent dye were purchased from Invitrogen Corp. (CA, USA). The rabbit polyclonal anti-GST-P antibody was purchased from Medical and Biological Laboratories

Co., Ltd (Nagoya, Japan). The Vectastain Elite ABC kit was purchased from Vector Laboratories Inc. (CA, USA). Diaminobenzidine tetrahydrochloride (DAB) was purchased from Sigma-Aldrich (St. Louis, USA). The other reagents used were analytical grade.

## Animals

Six-week-old male outbred Wistar rats (190-200 g) were obtained from the National Laboratory Animal Center, Mahidol University, Salaya, Nakorn-Prathom, Thailand. Three rats per cage were acclimatized for at least seven days prior to the start of the study. Animals were kept in the Animal House, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand. They were maintained at an environmental condition of about 25˚C, with 12 hours of an alternate light and dark cycle. Commercial pellet diet and chlorinated tap water were available *ad libitum*. All rats were housed and treated with the permission of the Animal Ethics Committee of the Faculty of Medicine, Chiang Mai University (AEC number 17/2556).

## Experimental design for the dose-response study of NDEA clastogenicity

Rats were placed at random into six groups (Figure 2). Control rats (G1) were intraperitoneally administered normal saline solution (NSS). Treated groups (G2-G6) were administered NDEA ranging from 5-50 mg/kg twice on the first and fourth days. After the second NDEA injection, partially hepatectomy (PH) was applied to all rats according to published methods (Higgins et al., 1931). All rats were left for four consecutive days for compensatory proliferation of hepatocytes after liver incision. At the end of protocol, rats were sacrificed by two-step collagenase perfusion.

## Isolation of hepatocytes by two-step collagenase perfusion

Isolation of hepatocytes was done essentially according to Tates et al. (1980) with several modifications. In brief, anesthetized rats were first perfused with preperfusion medium, pH 7.4, 37 °C, at a flow rate of 15 ml/min to remove blood. Secondly, collagenase medium in the same conditions was continuously perfused to isolate hepatocytes. The isolated hepatocytes were washed with phosphate buffer saline, pH 7.4 followed by 10% buffered formalin, pH 7.4 three times. The hepatocytes were suspended in 10% buffered formalin, pH 7.4 and kept in a 4 °C refrigerator until the micronucleus analysis.

## Micronucleated hepatocyte analysis

Prior to the micronucleus analysis, the hepatocyte suspension was mixed with 20 µg/ml 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) stain solution for fluorescence microscopy. Stained hepatocytes were dropped onto a glass slide and covered with a coverslip. The number of micronucleated hepatocytes was counted and recorded based on analysis of 2,000 hepatocytes from each animal under a fluorescence microscope (x400, excitation/emission (nm): 358/461) equipped with an ultraviolet system. Round or distinct nuclei (fragmented chromosomes) stained with the same color as the nucleus and having with diameters of ¼ or less than that of the main nucleus were scored as micronuclei (Cliet et al., 1989). Mitotic index (MI), indicative of mitotic activity, was calculated from mitotic cells in 2,000 hepatocytes.

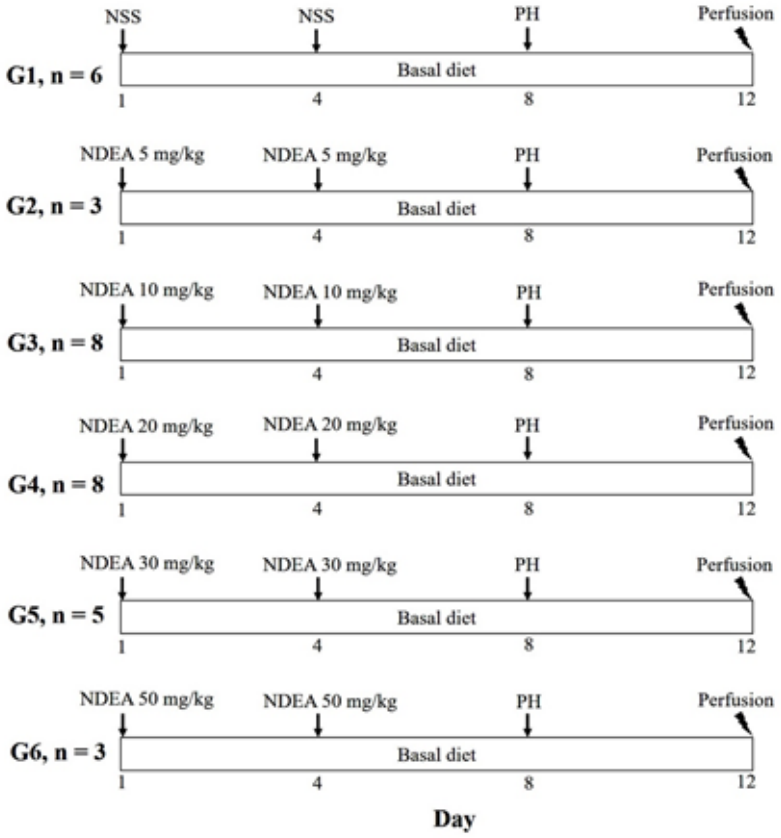


**Figure 2** Treatment protocol for testing clastogenic effects of *N*-nitrosodiethylamine in Wistar rat livers. Rats were divided into six groups. NSS: normal saline solution (G1), NDEA: *N*-nitrosodiethylamine (G2-G6). PH: partial hepatectomy, G: group, and n: numbers of rats.

## Experimental design for proteomic analysis of NDEA carcinogenicity

Six-week-old male Wistar rats were divided into two groups, 6-8 rats per group. Group 1, a negative control group, was injected with NSS. Another group was twice intraperitoneally injected with 100 mg/kg body weight of NDEA at weeks 1 and 2 of the experiment. Body weight, water and food intake were recorded weekly. All rats were anesthetized with isoflurane at nine weeks after the last injection. After rats were sacrificed, the final body weights and liver weights were recorded. Rat livers were collected and used for determination of GST-P positive foci and proteomic analysis.

## Immunohistochemistry for GST-P positive foci in rat livers

Liver sections 4 μm in thickness on prepared slides were immunohistochemically analyzed for GST-P positive foci by the avidin–biotin complex method according to Thumvijit et al. (2014). After deparaffinization, liver sections were treated with hydrogen peroxide to inactivate endogenous peroxidase. Skim milk was applied to block non-specific staining, and slides were sequentially incubated with rabbit polyclonal anti-rat GST-P antibody. Reactivity with the primary antibody was determined with anti-rabbit IgG biotinylated

antibody conjugated with the avidin-biotin-peroxidase complex. Finally, the sites of peroxidase binding were visualized with the substrate diaminobenzidine tetrahydrochloride. The tissue sections were lightly counterstained with hematoxylin to facilitate orientation under microscopic examination.

### Proteomic analysis of NDEA-initiated rat livers

The liver protein was extracted with 0.5% SDS, and total protein was determined by the Lowry method. Proteins were separated on SDS-PAGE mini slab gels (8×9×0.1 cm) and a separating gel containing 12.5% acrylamide. One-dimensional gel electrophoresis was performed in SDS electrophoresis buffer (25mM Tris-HCl, pH 8.3, 192 mM glycine, 0.1% SDS) followed by silver staining of the gels (Blum et al., 1987). The protein bands were excised, the gel plugs were dehydrated, and in-gel digestion of proteins was performed using trypsin solution containing 10 ng/µl trypsin in 50% ACN/10 mM ammonium bicarbonate. The peptides were extracted by adding of 30 µl of 50% ACN in 0.1% formic acid and were then dried by vacuum centrifugation. The peptide suspension was injected to LC-MS/MS (ESI-QUAD-TOF mass spectrometry). The MS/MS data were analyzed by the Mascot software and identified using the NCBI database. The molecular and biological functions of the identified proteins were assigned according to the Uniprot database (http://www.uniprot.org).

### Statistical analysis

The experimental data were reported as mean ± S.D. of each variable for each group. Differences between treated groups and the control group were determined by one-way ANOVA; $p < 0.05$ was considered as significant.

## RESULTS

### Clastogenicity of NDEA in rat livers

As the endpoint marker of liver cancer during the initiation stage, we visualized micronuclei using the fluorescent dye DAPI. Figure 3 shows a representative DAPI imaging of normal nuclei (A) and micronuclei (B, white arrows), which were separated from cellular nuclei and suspended in the cytosolic compartment. The concentration of NDEA ranged from 10 to 50 mg/kg bw, which significantly induced hepatic micronucleus formation when compared with the control group ($P < 0.05$) (Figure 4A), whereas there was no significant difference in mitotic indices between control and NDEA-treated rats (Figure 4B). In addition, two of three rats injected with 50 mg/kg of NDEA were dead two days after partial hepatectomy.

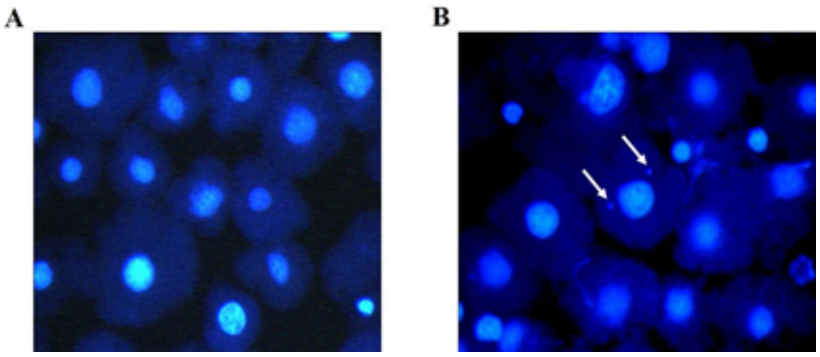


**Figure 3** Representative DAPI visualizations of normal hepatic nuclei (A) and hepatic micronuclei (B, white arrows) from rats administered 30 mg/kg of NDEA. Cells were stained with DAPI for fluorescence imaging (x400 magnification, excitation/emission (nm): 358/461).

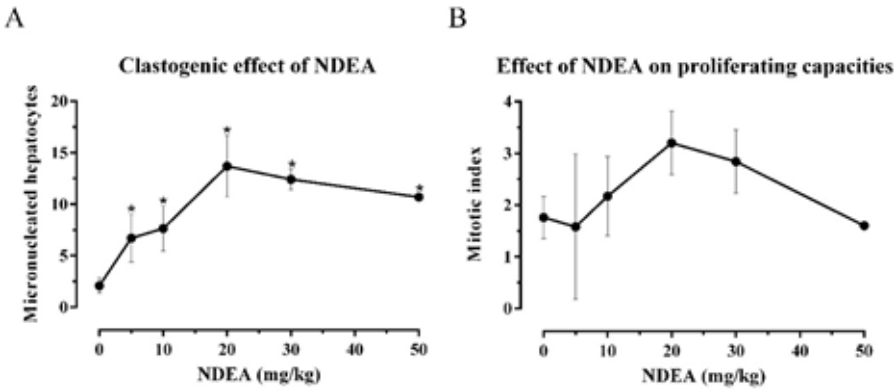


**Figure 4** Micronucleated hepatocytes and mitotic index. Curves showing average number of micronucleated hepatocytes from control and NDEA-treated rats (A) and proliferating capacities of liver cells (B). Averages were numbers of micronucleated hepatocytes per 1,000 hepatocytes with respective standard deviations. Significant differences were observed between control and NDEA-treated rats ($P < 0.05$), n = 5 (control), n = 3 (5 mg/kg NDEA), n = 6 (10 mg/kg NDEA), n = 6 (20 mg/kg NDEA), n = 4 (30 mg/kg NDEA) and n = 1 (50 mg/kg NDEA, two rats died from NDEA hepatotoxicity).

## Carcinogenicity of NDEA in rats

After NDEA treatment, the body weights of rats tended to decrease, but this difference did not reach statistical significance compared to negative control rats. Moreover, there was no significant difference in relative liver weight between NDEA and negative control groups (Table 1). GST-P positive foci were only found after NDEA treatment. The number and area of GST-P positive foci were significantly increased in NDEA-treated rats compared to negative control rats. The results suggest that treatment with NDEA could induce the formation of preneoplastic lesions in rat livers.

**Table 1** General appearance and number of GST-P positive foci in rats.

| Parameters | NSS | NDEA |
|---|---|---|
| Initial body weight (g) | 206 ± 15 | 206 ± 12 |
| Final body weight (g) | 448 ± 21 | 409 ± 31 |
| Relative liver weight (%) | 2.70 ± 0.24 | 2.74 ± 0.34 |
| Number of GST-P$^+$ foci/cm$^2$ | 0.00 ± 0.00 | 2.96 ± 1.42$^a$ |
| Area of GST-P$^+$ foci/liver area (mm$^2$/cm$^2$) | 0.00 ± 0.00 | 0.12 ± 0.06$^a$ |

The results are shown as mean ± SD. GST-P$^+$: Glutathione *S*-transferase placental form positive foci. $^a$significantly different from negative control (NSS) group ($P < 0.05$).

## Proteomic profile of proteins in the liver of NDEA-initiated rats

A total of 152 differentially expressed proteins were identified. Of these, 44 up-regulated and 108 down-regulated proteins were detected in NDEA-initiated rats compared to negative control rats. These proteins could be categorized into 14 groups according to their molecular and biological functions. The up-regulated proteins involved signal transduction and cell growth; this group included phosphoinositide 3-kinase regulatory subunit 6 (Pik3r6) and eukaryotic translation initiation factor 4E (eIF4E), which were found in NDEA-treated rats (Table 2). In contrast, the expressed proteins involved in DNA repair such as X-ray repair cross-complementing protein 5 (XRCC5) and UV excision repair protein RAD23 homolog A isoform X1 were down-regulated in NDEA-treated rats (Table 3). Protein network association and other correlated proteins were analyzed via the STRING database version 10.5 (http://string-db.org/). Seven major proteins were expressed after treatment with NDEA, including Pik3r6, eIF4E, XRCC5, sister chromatid cohesion protein homolog A (Pds5a), CLIP-associating protein 2 (Clasp2), Nipped-B homolog (Nipbl) and missmatch repair endonuclease PMS2 (Pms2). Notably, the interaction of proteins involved in the cell signaling pathway and cell growth, especially Pik3r6 and eIF4E were observed (Figure 5). The signal transduction pathway in NDEA-induced rats was related to phosphatidylinositol 3-kinase (PI3K/Akt) and the mammalian target of rampamycin (mTOR) pathways.

**Table 2** Some upregulated hepatic proteins in NDEA-induced rats.

| Protein name | Accession number | ID score | Fold change | Function |
|---|---|---|---|---|
| Phosphoinositide 3-kinase regulatory subunit 6 | gi124517728 | 6.05 | 22.26 | Signal transduction |
| RAS protein activator like-3 | gi197381464 | 15.21 | 17.53 | Signal transduction |
| Latent-transforming growth factor beta-binding protein 4 precursor | gi281371412 | 2.49 | 19.72 | Proliferation/differentiation |
| Interferon-related developmental regulator 1 | gi53733404 | 3.24 | 18.05 | Proliferation/differentiation |
| Eukaryotic translation initiation factor 4E | gi13540382 | 8.99 | 20.36 | Cell cycle/cell division |
| MHC class II transactivator isoform 1 | gi399154119 | 5.41 | 20.94 | Inflammatory/immune response |
| MHC class II antigen | gi18958188 | 8.59 | 19.81 | Inflammatory/immune response |
| COX assembly mitochondrial protein homolog isoform 2 | gi207080032 | 4.56 | 15.75 | Inflammatory/immune respons e |
| Polyhomeotic-like protein 1 | gi157823775 | 7.85 | 22.62 | Ubiquitin conjugation pathway |
| Ubiquitin carboxyl-terminal hydrolase 26 | gi157818981 | 9.45 | 18.48 | Ubiquitin conjugation pathway |

## DISCUSSION

Numerous *in vitro* and *in vivo* protocols are available to evaluate the carcinogenic potential of environmental chemicals. The liver micronucleus assay is an acceptable method of choice for testing mutagenicity and cancer-inducing ability of chemical compounds. When performed appropriately, the assay can detect both clastogenicity associated with chromosome breakage and aneugenicity as indicated by dysfunction of the mitotic apparatus caused by chromosome lagging (Krishna et al., 1992; Krishna et al., 2000). Clastogenicity of various chemicals has been considered as the classical hallmark of initiation of chemical carcinogenesis (Cliet et al., 1989; Krishna et al., 2000). In this study, we investigated the in vivo clastogenic and carcinogenic potential of NDEA. Fluorescence visualization via DAPI clearly identified the morphology of fragmented chromosomes forming round-shaped micronuclei (Figure 3). Our results are comparable to those reported by Charoensin and colleagues (2010). NDEA administration ranging from 10-30 mg/kg bw was sufficient to induce initiation stage of liver chromosomal damage as seen by significant increased number of micronucleated hepatocytes (Figure 4).

Although the LD50 of intraperitoneal NDEA injection is approximately 216 mg/kg bw (Heath et al., 1962), two-thirds of the partially hepatectomized rats did not survive after double injection of 50 mg/kg bw of NDEA. This may have been due to poor detoxifying ability of the remaining liver. This dose is therefore not suitable for rat liver micronucleus induction models.

**Table 3** Some downregulated hepatic proteins in NDEA-induced rats.

| Protein name | Accession number | ID score | Fold change | Function |
|---|---|---|---|---|
| Eukaryotic translation initiation factor 2 alpha kinase 3, isoform CRA_b | gi149036361 | 3.94 | 22.78 | Apoptotic process |
| Death associated transcription factor 1, isoform CRA_b | gi149034011 | 2.30 | 16.23 | Apoptotic process |
| Ran GTPase-activating protein 1 | gi349501086 | 9.92 | 24.30 | Cell adhesion |
| SH3-domain GRB2-like endophilin B2, isoform CRA_b | gi149039098 | 10.77 | 24.17 | Cell adhesion |
| Integrin alpha-4 | gi157819949 | 11.67 | 20.12 | Cell adhesion |
| X-ray repair cross-complementing protein 5 | gi41056215 | 7.12 | 17.18 | DNA damage/DNA repair |
| UV excision repair protein RAD23 homolog A isoform X1 | gi564394955 | 9.55 | 23.69 | DNA damage/DNA repair |
| Mismatch repair endonuclease PMS2 | gi157787060 | 6.01 | 18.55 | DNA damage/DNA repair |
| Protein kinase, DNA activated, catalytic polypeptide | gi149019691 | 7.23 | 14.97 | DNA damage/DNA repair |
| Acetyl-Coenzyme A dehydrogenase, long-chain, isoform CRA_b | gi149016010 | 2.30 | 22.04 | Lipid metabolism |
| Lysosomal acid lipase/cholesteryl ester hydrolase | gi20138456 | 3.24 | 18.53 | Lipid metabolism |
| Sterol regulatory element-binding protein 2 | gi76096332 | 4.57 | 17.97 | Lipid metabolism |
| Homeobox protein PKNOX2 isoform 1 | gi404312683 | 6.85 | 22.44 | Transcription/translation |
| Cyclic AMP-dependent transcription factor ATF-6 alpha | gi157821879 | 6.19 | 16.43 | Transcription/translation |
| 4F2 cell-surface antigen heavy chain isoform 1 | gi402743472 | 13.60 | 23.01 | Transport |

Medium-term (within 8 weeks) carcinogenicity tests in rats have been established for analysis of chemical compounds. Such tests are able to detect both genotoxic and non-genotoxic carcinogens in rat livers (Ito et al., 2003; Tsuda et al., 2003). NDEA is a potent hepatocarcinogen that can act a tumor initiator and promoter in multistage of hepatocarcinogenesis models (Aparicio-Bautista et al., 2013).

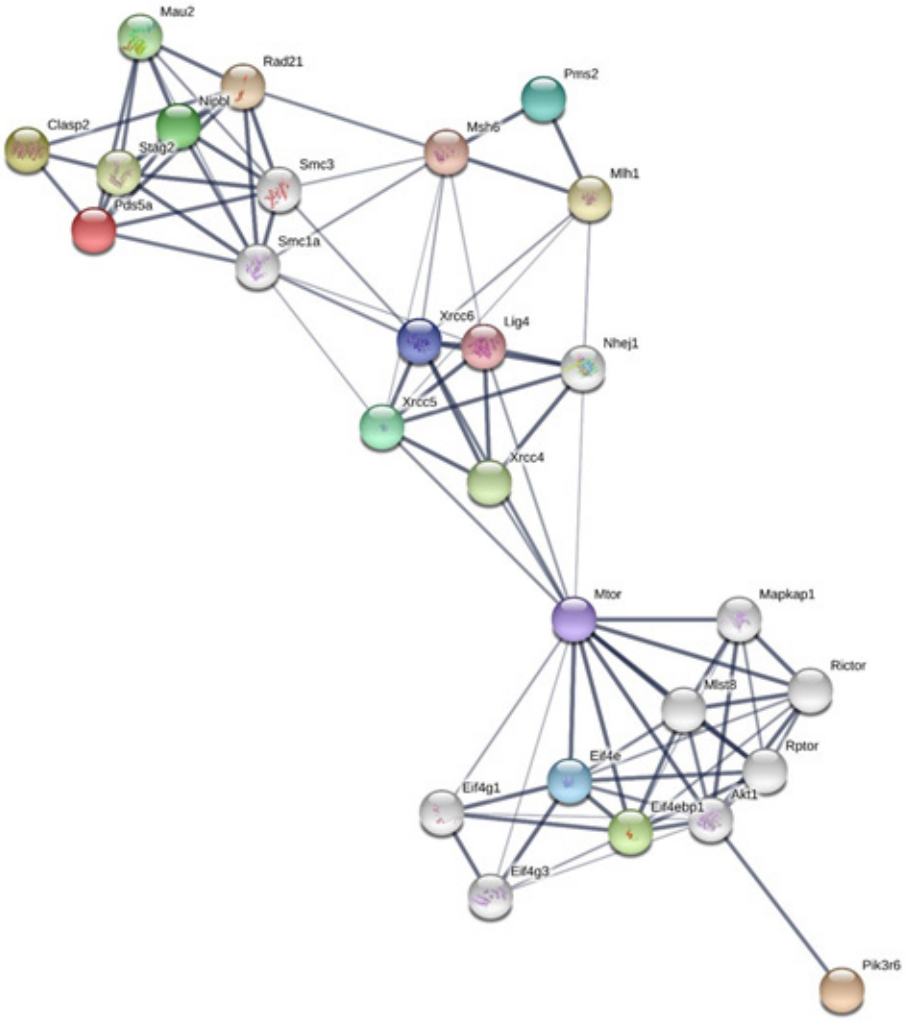


**Figure 5** The protein network complex using the STRING database. Protein-protein interactions are indicated by the bold black lines.

Glutathione *S*-transferase placental form (GST-P) positive foci, a sign of preneoplastic lesions of HCC in rats, is widely used as the endpoint marker (Ito et al., 1998; Ichihara et al., 1999). Normally, GST-P is strongly up-regulated in placental tissues, absent in adult tissues, and is increased in preneoplastic and neoplastic cells (Higashi et al., 2004). A previous study showed that only single GST-P positive hepatocytes in rats were found after treatment with 30 mg/kg body weight of NDEA (Wang et al., 2009), and this may not develop into malignant tumors. From our preliminary observation, the number of GST-P positive foci induced by the twice injection of 100 mg/kg body weight of NDEA was comparable to the administration of NDEA in the standard protocol for medium-term rat carcinogenicity test (Ito et al., 2003). Therefore, NDEA at the repeated dose of 100 mg/kg body weight was selected to use in our study. We found that NDEA enhanced the formation of preneoplastic lesions by increasing both the number and size of GST-P positive foci in rat livers.

Interestingly, our proteomic analysis revealed the overexpression of phosphoinositide 3-kinase regulatory subunit 6 (Pik3r6) and eukaryotic

translation initiation factor 4E (eIF4E), which are involved in PI3K/Akt/mTOR signaling in NDEA-initiated rats. The PI3K/Akt/mTOR pathway is a well-known oncogenic kinase that plays an important role in cell growth, survival and cancer development (Buphathi et al., 2015; Golob-Schwarzl et al., 2017). PI3K is usually found in various types of human cancer (Matsuda et al., 2013). Activation of PI3K induces the phosphorylation and activation of Akt, which further phosphorylates multiple downstream targets to control cell proliferation, including the mTOR complex (Fortin et al., 2016). The mTOR phosphorylates the ribosomal protein S6 kinases and eukaryotic translation initiation factor 4E-binding protein 1 leading to the release of the eIF4E (Huo et al., 2011), which plays a vital role in the control of the initiation step of protein translation and is strongly expressed in HCC (Golob-Schwarzl et al., 2017). Moreover, overexpression of mTOR and pAKT have also been found in non-virus-related HCC (Golob-Schwarzl et al., 2017). The PI3K/Akt/mTOR pathway is most often involved in NDEA-induced hepatocarcinogenesis (Zhou et al., 2011; Wang et al., 2016), and may be another factor influencing preneoplastic formation in NDEA-induced hepatocarcinogenesis in rats of the present study.

In conclusion, we can reconfirm that NDEA is potentially clastogenic and carcinogenic to rat hepatocytes. The PI3K/Akt/mTOR pathway may play an essential role in preneoplastic formation in NDEA-initiated hepatocarcinogenesis in rats. Importantly, this study has established an experimental animal model for further cancer chemoprevention studies.

## ACKNOWLEDGEMENTS

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## AUTHOR CONTRIBUTIONS

Insuan, O., Charoensin, S., and Wongpoomchai, R. conceived and designed the experiments; Insuan, O., and Charoensin, S. performed the experiments; Insuan, O., Charoensin, S., Wongpoomchai, R., and Roytrakul, S. analyzed the data; Wongpoomchai, R., Roytrakul, S., Thumvijit, T., and Bunpo, P. contributed reagents/materials/analysis tools; Insuan, O., Charoensin, S., and Wongpoomchai, R. wrote the paper.

## CONFLICT OF INTEREST

There is no conflict of interest.

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