A NEW EXPERIMENTAL MODEL OF RAT HEPATOCELLULAR CARCINOMA

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НОВАЯ ЭКСПЕРИМЕНТАЛЬНАЯ МОДЕЛЬ ГЕПАТОКАНЦЕРОГЕНЕЗА

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To develop rat hepatocellular carcinoma (HCC) within a short period of time, weaning rats were chosen due to comparatively high mitotic index of the liver and diethyl nitrosamine (DEN) and partial hepatectomy (PH) were used as initiating and promoting agents respectively. Within 30 weeks after initiation, premalignant liver lesions were observed in both groups with/without PH, whereas HCC was developed only in the rats where PH was performed. We have also analyzed the involvement of *c-myc* in the development of HCC and observed comparatively high c-myc expression in both premalignant and malignant liver lesions irrespectively to the PH.
***Key Words*: hepatocellular carcinoma, diethyl nitrosamine, c-myc, partial hepatectomy, weaning rats.**

Предложена новая модель экспериментального гепатоканцерогенеза (ГКГ), вызванного у детенышей крыс диэтилнитрозамином, с последующей частичной гепатэктомией (ЧГЭ). Через 30 дней после инициации ГКГ у животных, подвергнутых ЧГЭ, и таковых без ЧГЭ в ткани печени были выявлены предопухолевые очаги, хотя развитие опухолей было отмечено только у животных после ЧГЭ. При этом относительно высокая экспрессия гена *c-myc* наблюдалась как в опухолевых, так и предопухолевых очагах ткани печени независимо от ЧГЭ.
***Ключевые слова*: карцинома печени, диэтилнитрозамин, c-myc, частичная гепатэктомия, детеныши крысы.**

Hepatocarcinogenesis is a multistep process [1–3]. Models of chemically induced hepatocarcinogenesis allow us to evaluate the sequence of epigenetic and genetic changes involved in the initiation, promotion and progression stages [4]. DEN, a chemical carcinogen, is widely used to initiate hepatocarcinogenesis in rats while the further promotion of cancer phenotype might be caused by phenobarbital, carbon tetrachloride, dichlorodiphenyltrichloroethane, partial hepatectomy (PH), etc [5].

Among various proto–oncogenes activated in rat liver carcinomas initiated by DEN, c–myc, a nuclear proto–oncogene, is found to play an important role in tumor development [6–8]. Overexpression of c–myc within a range of 1.5–20 fold has been observed at precancerous stages to HCC [9, 10]. However, overexpression of c–myc has been seen in the altered hepatic foci (AHF) when only necrogenic doses of DEN were used for tumor initiation and phenobarbital — for tumor promotion [11]. On the other hand 3–12 fold amplification as well as rearrangement of the c–myc locus have been seen in the premalignant liver lesions to HCC, suggesting a role of c–myc alterations in progression of adenomas to malignancy [7, 9, 10, 12]. Thus, there is an ambiguity in c–myc alteration during the development of HCC.

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Abbreviations used: AHF — altered hepatic foci; DEN — diethyl nitrosamine; HCC — hepatocellular carcinoma; I.P. — intra peritoneum; PH — partial hepatectomy.

In regenerating rat liver, high expression of c–myc and increased rate of cellular proliferation are well–established facts [13]. The expression of c–myc has been seen to be high (10–15 folds) within 1–3 h after PH followed by gradual decrease in expression; however, within 48 h after PH, a second transient peak has also been observed [13].

In the popular models for the development of hepatocarcinogenesis, adult rats (90–220 g) are chosen as the subject [7, 9–12]. In the adult liver, the hepatocytes have a low mitotic index [14]. An initiating dose of DEN causes necrotic damage and forces the quiescent hepatocytes to enter the cell cycle. These initiated hepatocytes undergo clonal expansion and proliferation when only appropriately and differentially stimulated by carcinogens or hepatonecrogenic agents or PH [5]. In this method malignancy usually sets in after a lag period of 52 to 64 weeks after initiation [7, 9–12]. To reduce the time lag for the development of HCC in rat, attempts have been made to develop HCC in weaning rat where the mitotic index of hepatocytes is high compared to that in adult rats.

In the present study we have taken a new approach to develop HCC induced by DEN and PH in weaning rat and have analyzed the alterations of c–myc in different liver lesions developed in this experimental procedure.

MATERIALS AND METHODS

Treatment of animals. Sprague Dawley weaning rats 2 weeks old weighing 50 g were divided into 2 groups (I and II) (Table 1). The group I was injected with DEN through I.P. route at a dose of 100 mg/kg of body weight

Table 1. Development of liver lesions in rats

Group	Initiating agent	Promoting agent	No. of rats	Incidence of premalignancy at 8 weeks	Incidence of premalignancy at 32 weeks	Incidence of malignancy at 32 weeks
I	DEN	—	10	n. a.*	5/10 (50%)	0/10
II	DEN	PH	5	1/5 (20%)	3/5 (60%)	2/5 (40%)

* Indicates not applicable.

twice with two weeks interval. The rats were sacrificed at 32 weeks of age (i.e 30 weeks after initiation). The group II was also injected with DEN in the same way as in group I and underwent PH at the age of 8 weeks; then animals were followed up for another 24 weeks and sacrificed. All animals procedures were carried out according to the Rules of Ethic Committee.The PH was performed as described by Nagy et al and Ray et al [12, 15]. The portion of altered foci developed in the liver from each sample was stored in formalin for histology and rest of the altered foci was stored at –80 °C.

Histology. For histological analysis of the liver samples, haemotoxylin and eosin staining was applied.

DNA and RNA isolation. High molecular weight genomic DNA was isolated from rat liver by phenol: chloroform extraction [16].Total cellular RNA was extracted from rat liver using guanidine isothiocyanate method [16].

Probes. The myc 1.8 probe is a 1.1 kb BamHI restriction fragment encompassing parts of exon 1 and intron 1 of rat *c–myc* gene cloned in pGem vector [17]. The rat mlvi–4 probe is a 0.9 kb Hind III–EcoRI restriction fragment cloned in pUC18 vector [18]. The rat IgH probe is a 6 kb Hind III–BamHI restriction fragment cloned in pUC 18 vector [19]. The IgH probe was used as an internal control in Southern blot hybridization to standardize the amount of DNA loaded and to access the relative gene copy number of the *c–myc/mlvi*–4 gene. The GAPDH probe is a 0.5–kb PstI restriction fragment cloned in pUC 18 and was used as an internal control in RNA slot blot hybridization [20]. The probes were labeled with [α–^{32}P]dCTP by random priming method and used for analysis [21].

Southern blot analysis. 10 μg of genomic DNA from each sample was digested with EcoRI overnight at 37 °C, electrophoresed in 0.8% agarose gel over night at 30 V, and transferred to Genescreen nylon membrane (NEN, USA) by capillary transfer method. The pre–hybridization of the membranes was done in a solution containing 2 X SSC, 1% SDS, 10% dextran sulphate, 50% deionized formamide and 5X Denhardht's solution overnight at 42 °C. The purified labeled probe with specific activity 10^8–10^9 CPM/μg of DNA was added to the pre–hybridisation solution and hybridized overnight at 42 °C. After hybridization, the membranes were washed for 10 min at room temperature in 2X SSC, then — at 42 °C in 2X SSC, 1% SDS twice for 20 min and finally — in 0.2 X SSC, 1% SDS at 42 °C twice for 20 min. The membranes were then exposed to Kodak X–Omat film at –80 °C for 7–8 days with intensifying screen. The intensity of the hybridized bands on the autoradiographs was determined using densitometric scanner (Shimidazu CS–1900). The intensity of c–myc/mlvi–4 band in each sample was normalized with respect to the intensity of the IgH band of the corresponding sample. The copy number of c–myc/mlvi–4 loci in the liver lesions was calculated from the ratio of the normalized intensities of the liver lesions and normal liver. The c–myc/mlvi–4 locus was considered to be amplified when the ratio was ≥ 2.5 [10].

RNA slot blot analysis. 10 μg of the total cellular RNA from each sample was slot blotted to the Genescreen nylon membrane (NEN, USA) and fixed according to the instructions of manufacturer. The prehybridisation of the membranes was done in a solution containing 5X SSPE, 50% deionized formamide, 5X Denhardt's solution, 1% SDS, 10% dextran sulphate and 100 μg/ml of denatured sheared salmon sperm DNA for 4 h at 42 °C. The hybridization was carried out in a solution of similar composition, with the omission of salmon sperm DNA, containing purified labeled probe with specific activity 10^8–10^9 CPM/μg of DNA at 42 °C overnight. After hybridization, the membranes were washed with 2X SSPE twice for 15 min at room temperature, in 2X SSPE, 2% SDS twice for 45 min at 65 °C, and in 0.1X SSPE twice for 15 min at room temperature. The membranes were then exposed to Kodak X–Omat film at –80 °C for 7–8 days with intensifying screen. The level of expression of the *c–myc* gene was quantified by densitometric scanning as described earlier and the readings were normalized to the GAPDH gene expression.

Statistical analysis. The results in this experiment are expressed as mean ± standard deviation.

RESULTS AND DISCUSSION

The histological alterations that occur during the development of rat liver carcinogenesis are well–documented [22]. In histological analysis it was revealed that 50% (5/10) of the group I rats developed premalignant changes in the liver at 32 weeks (see Table 1; Fig. 1, *b*, *c*). Focal atypia with dilated central vein and abnormal mitotic figures with multinucleated cells were the predominant features here. In group II rats (see Table 1; Fig. 1), 1/5 (20%) rats showed early premalignant changes in liver like fatty changes and early cirrhotic changes (Fig. 1, *d*) while others (4/5) showed focal atypia at 8 weeks i.e. at the time of PH. After PH at 32 weeks, 60% (3/5) of the group II rats showed premalignant changes in liver where fatty changes and abnormal mitotic figures with multinucleated cells could be seen (Fig. 1, *e*). However, the rest of the rats (40%, 2/5) had malignant changes in liver (including the rat that developed premalignant changes at 8 weeks) with abnormal mitotic figures and gross changes in liver architecture (Fig. 1, *f*).

The manifestation of premalignancy at 8 weeks could be due to the fact that neonatal liver has higher mitotic index compared to the adults [14]. DNA damage caused by initiating agents like DEN and promotion by PH accelerates cell–cycle entry. As a result, the neoplastic process is developing in more short period (30 weeks after initiation) as compared to the other methods of HCC development in rats where about 52–64 weeks after initiation were necessary for the development of HCC [7, 9–12]. The absence of HCC in group

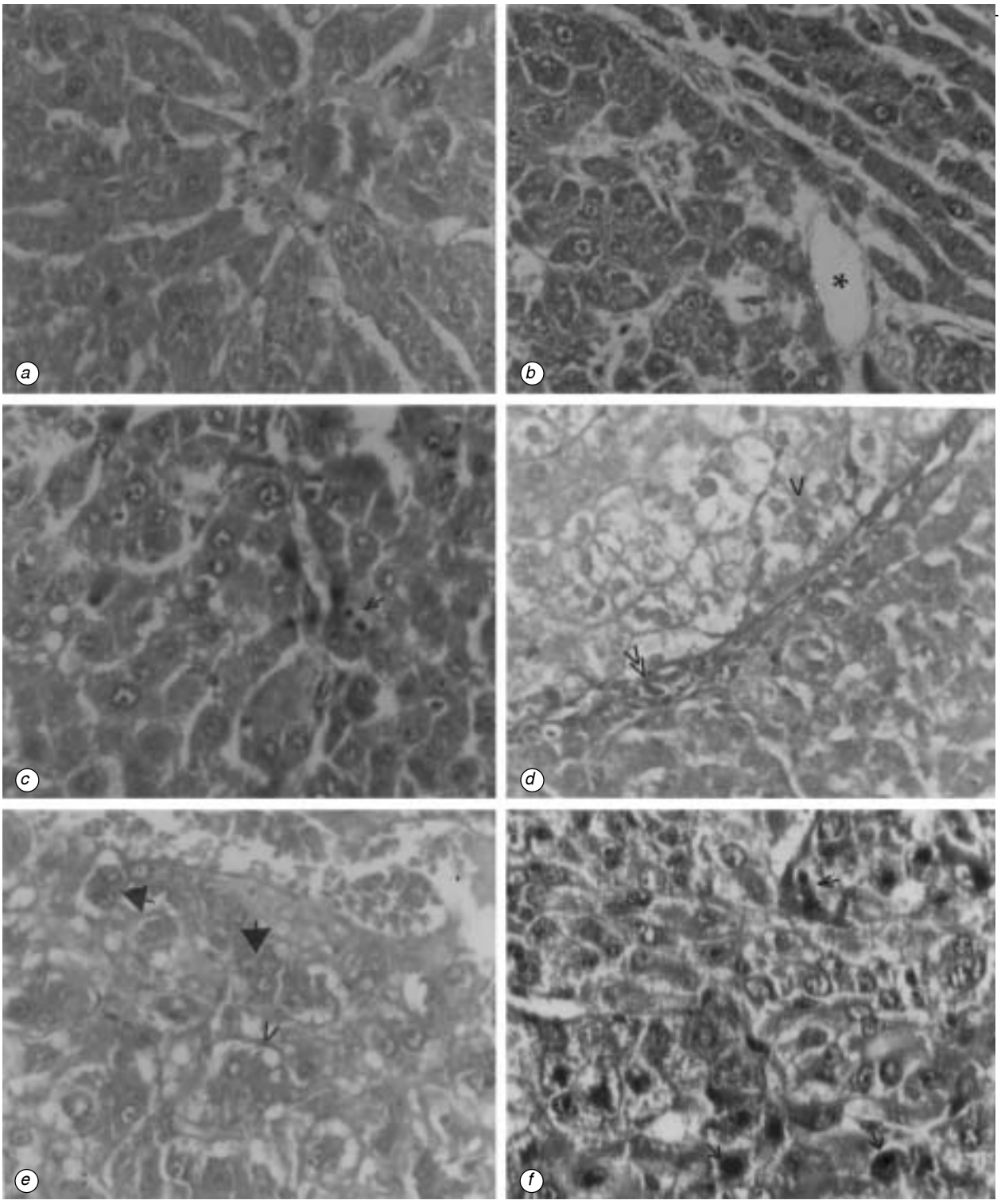

Fig. 1. Photomicrographs of the sections of the normal rat liver (*a*), premalignant liver lesions of group I rats at 32 weeks (*b* and *c*), early premalignant liver lesion of group II rats at 8 weeks (*d*), premalignant liver lesion of group II rats at 32 weeks (*e*), malignant liver lesion of group II rats at 32 weeks (*f*) (magnification x 1000) where * indicates dilated central vein; → indicates abnormal mitotic figures and pleomorphic nuclei; > indicates fatty changes; >> indicates early cirrhotic changes; ▸ indicates multinucleated cells

l rats has indicated that the initiation by DEN and pro–motion by PH in weaning rats seem to be a better me–thod for HCC development.

To find out the role of *c–myc* in the development of HCC the expression of *c–myc* was analyzed in the dif–ferent liver lesions (Table 2; Fig. 2). The expression of *c–myc* was increased by about 1.8 fold in the prema–lignant lesion (1/5) of 8 weeks old rat (group II) whilst its expression in the rest of the samples (4/5) in this group did not change significantly as compared to the normal liver (see Table 2). Interestingly, the rat that had early premalignant liver lesions at 8 weeks (see Ta–ble 1 and 2) also developed HCC at 32 weeks without change in the level of *c–myc* expression. From other hand, other HCC sample (see Table 1 and 2) that pos–sessed focal atypia at 8 weeks with normal *c–myc* le–

Table 2. Expression of c–myc in premalignant and malignant liver lesions compared to normal liver

Sample	*c–myc* expression	
	Premalignant	Malignant
Group I [DEN]	2.88 ± 0.26	
Group II [DEN + PH]		
a) before PH	1.84	
b) after PH	1.4 ± 0.54	1.7 ± 0.14

Values are expressed as mean ± standard deviation.

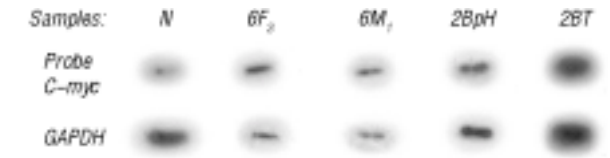

Fig. 2. Expression of c–myc in rat liver lesions from normal rat liver (*N*), premalignant rat liver lesions of group I ($6F_3$ and $6M_1$), premalignant rat liver lesion of group II at 8 weeks (*2 BpH*), from malignant rat liver lesion of group II at 32 weeks (*2 BT*)

vel also had 1.7 fold–higher *c–myc* expression. However, at 32 weeks in the premalignant liver lesions of animals from both groups I and II, *c–myc* expression was increased about 1.4–3 folds (see Table 2). Thus, the upregulation of *c–myc* expression in the early premalignant liver lesions of 8 weeks old rats may be due to the necrosis of the liver induced by DEN. At 32 weeks, the upregulation of *c–myc* was sustained at comparable level in both the premalignant liver lesions and HCC. Thus, it indicates that the upregulation of *c–myc* after DEN treatment may provide the continuous proliferative signal to the AHF to remain in the cell cycle. For the development of HCC, PH–dependent promotion of liver carcinoma development may provide proliferative stimulus in addition to *c–myc*.

To find out the mechanism of the *c–myc* gene upregulation, we have analyzed the genetic alterations in the *c–myc* locus by Southern blot analysis and found no amplification or rearrangement in this locus in the liver lesions (Table 3). It has also been reported that the rearrangement/amplification in the flanking regions of *c–myc* gene could increase its expression [23]. For this reason we have analyzed the alterations in the mlvi–4 locus located 30 kb 3′ of the *c–myc* gene in liver samples and didn't register any rearrangements or amplification (see Table 3). It seems that the persistent upregulation of *c–myc* in the liver lesions induced by DEN could be due to the activation of some transactivating factors that regulates *c–myc* gene expression [6, 24].

Table 3. *c–myc* and *mlvi–4* gene copy number in premalignant and malignant liver lesions compared to normal liver

Group	*c–myc* gene copy number		*mlvi–4* gene copy number	
	Premalignant	Malignant	Premalignant	Malignant
I [DEN]	0.9 ± 0.37	—	0.94 ± 0.26	—
II [DEN + PH]				
a) before PH	0.9	—	0.7	—
b) after PH	0.93 ± 0.4	1 ± 0.54	1.44 ± 0.23	1.6 ± 0.21

Values are expressed as mean + standard deviation

Thus, we showed that DEN–induced liver carcinogenesis in weaning rats followed by PH seems to be a better method for the development of HCC within a short period of time (i.e. 30 weeks after initiation). The comparatively high *c–myc* expression was observed in both premalignant and malignant liver lesions irrespectively to PH, but for the development of HCC the comparatively high *c–myc* expression along with extra proliferative stimulus provided by PH was required.

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