Cancer Chemother Pharmacol (2010) 65:309–318
DOI 10.1007/s00280-009-1035-4

ORIGINAL ARTICLE

Effect of antisense oligomer in controlling c-raf.1 overexpression during diethylnitrosamine-induced hepatocarcinogenesis in rat

Tanushree Das · Falguni Patra · Biswajit Mukherjee

Received: 18 November 2008 / Accepted: 10 May 2009 / Published online: 5 June 2009

Abstract
Purpose In ras-mediated signal transduction pathway, c-raf.1 is believed to have predominant oncogenic potential and has been found to be highly expressed in certain human and animal malignancies including hepatocellular carcinoma. In the present study, anticancer efficacy of antisense c-raf.1 oligomer on the inhibition of c-raf.1 mRNA overexpression during hepatocarcinogenesis was determined.
Methods Initially antiproliferating effect of the antisense oligomers was studied in vitro by measuring the rate of tritiated thymidine incorporation into DNA in rat hepatocellular carcinoma cells in culture medium. Based on the findings, the antisense treatment was carried out in rat hepatocarcinogenesis model-initiated with diethylnitrosamine and promoted using 2-acetylaminoflourene. Different drug-metabolizing enzymes, lipid peroxidation, liver morphology and histopathological studies along with c-raf.1 gene expression by in situ hybridization were performed.
Results c-raf.1 antisense oligomers exhibited an inhibitory effect (~68%) on cancer cell proliferation in vitro. Gross and microscopic examination of liver showed fewer (29%) and smaller hyperplastic nodules and preneoplastic lesions (30%) in carcinogen and antisense oligomer-treated group as compared with carcinogen control group. Treatment of antisense c-raf.1 oligomers enhanced cytochrome P-450 content (81%) and reduced glutathione *S*-transferase activity (33%), UDP glucuronosyltransferase activity (74%) and MDA concentration (30%) in carcinogen and antisense oligomer-treated group as compared with carcinogen control animals. The oligomer treatment also resulted in less expression in terms of c-raf.1 expressed lesion count as compared to carcinogen control group.
Conclusion The study demonstrates that the antisense oligomer targeted against c-raf.1 mRNA inhibits the overexpression of c-raf.1 gene during hepatocellular carcinoma in rats.

Keywords Hepatocarcinogenesis · Hyperplastic nodule · c-raf.1 · Marker enzymes · In situ hybridization

Introduction

The process of hepatocarcinogenesis is complex, resulting from alterations in the normal patterns of cellular growth [1, 2]. The development of hepatocellular carcinoma (HCC) is believed to be caused by the accumulation of genetic alterations resulting in a distorted expression of thousands of genes. It is preceded by the early appearance of morphologically and genetically altered "resistant cells" termed as hepatic focal lesions or preneoplastic lesions [3, 4]. Experimental hepatocarcinogenesis in the rodent occurs in three distinctly defined stages—initiation, promotion, and progression [5]. Following an exposure to a suitable chemical carcinogen, initiated cells are formed from normal liver parenchyma cells, the hepatocytes. This chemical carcinogen, which acts as an initiator for its cancer inducing ability, results in genetic alteration following an interaction with various macromolecules, commonly DNA. There are several carcinogens, which are reported to induce tumors in various tissues or organs, but not in the liver unless they are associated with a suitable proliferative stimulus [1]. It is believed that subsequent tumor promotion with another carcinogenic compound termed as promoter results in a

T. Das · F. Patra · B. Mukherjee (✉)
Department of Pharmaceutical Technology, Jadavpur University, Kolkata 700032, India
e-mail: biswajit55@yahoo.com

selective clonal expansion of initiated cells to give rise to the formation of hepatic altered foci [6, 7]. This expansion is caused by a selective increase in cell proliferation with a selective decrease in apoptosis of preneoplastic hepatocytes [8, 9]. When initiated cells are persistently exposed to a promoter, it helps the cells to form lesions by a round of cell division thereby making the cells more susceptible to further genetic changes [10]. Cell proliferation for preneoplastic lesions is essential for the amplification of the initiated cell population during promotion whereby the initiated cell population gets altered cells and undergoes a series of irreversible changes to become more susceptible to further malignant transformation [11]. During preneoplastic development of the initiated cell population, several cycles of cell divisions not only increase the size of the preneoplastic lesion but also endow lesion with an intrinsic prerequisite for the further expression of the malignant phenotype [11]. The neoplastic transformation of hepatocytes results from accumulations of various genetic as well as epigenetic changes which act together to affect both positive mediators (e.g. cellular proto-oncogenes, signaling molecules) and negative mediators (e.g. tumor suppressor genes) of cellular proliferation thereby giving rise to cells with autonomous growth potential [12]. The hepatic altered foci that persist after the withdrawal of promoting agent is reported to have the highest potential to progress into hepatocellular carcinoma [13]. During the neoplastic transformation the first phenotypic cellular change that occurs is the excessive storage of glycogen in liver focal lesions [14–16]. Thus the sequence of cellular changes starts with glycogenetic clear and acidophilic (smooth endoplasmic reticulum-rich) hepatocytes and progresses through intermediate phenotypes in mixed cell populations to glycogen-poor, homogeneously basophilic (ribosome-rich) hepatoma cell.

The disorder in various biochemical pathways is also believed to be associated with hepatocarcinogenesis [17]. The alterations in some of the enzymes, e.g., cytochrome P-450 (cyt P-450) monooxygenases, glutathione *S*-transferase (GST), and UDP glucuronyl transferase (UDPGT) observed during hepatocarcinogenesis manifest the abnormalities in the regulatory state of primary hepatocyte cells [18]. During hepatocarcinogenesis the changes in the expression levels of these enzymes, which play important role in carcinogen metabolisms, make them useful biomarkers in characterizing hepatic altered foci as precursors for the development of hepatic neoplasia or hepatocellular carcinoma [19–21]. Again, studies on changes in lipid peroxidation end product, malonaldehyde (MDA), has shown the possible role of end product of lipid peroxidative degradation during hepatocarcinogenesis [22].

The signaling molecule c-raf.1 is one of the three highly conserved members of the raf gene family, which code for serine threonine-specific protein kinases best known for its role in ras-mediated signal transduction pathway [23–25]. The Ras/Raf/MEK/ERK signaling cascade is frequently deregulated in tumorigenesis and is known to be involved in proliferation and transformation of cells [26]. Expression of c-raf-1 modifies cell growth, proliferation, and survival in a variety of cellular systems. c-raf.1 Protein kinase itself is believed to have oncogenic potential and found to be up regulated in tumors [27, 28]. It has been shown to be highly expressed in hepatocellular carcinoma as well [24, 29].

Since c-raf.1 is direct downstream effector of ras, an appropriate antisense therapy directed against c-raf.1 might be useful in the treatment of ras-dependent tumors. Antisense therapy directed against c-raf.1 protein kinase employs short, synthetic oligonucleotides which act by hybridizing with complementary mRNA sequence thereby inhibiting c-raf.1 protein expression through different mechanisms, including RNaseH-mediated degradation of target mRNA [30, 31]. One of the most attractive features of antisense oligomers is their great specificity to the intended molecular target [30]. The ability to use antisense oligonucleotides to target selectively the genetic processes involved in cancer provides not only a new class of chemotherapeutic agents to fight cancer, but also a better understanding of the critical molecular signaling events involved in development of cancer.

In this study, efforts have been made to test the effectiveness of phosphorothioate antisense oligonucleotides targeted against rat c-raf.1 mRNA to inhibit specifically c-raf gene expression. Attempt was also made to study the various enzymatic changes such as cyt P-450 content, GST, UDPGT activities as well as lipid peroxidation during hepatocarcinogenesis, which might provide an insight into the understanding of cancer biology during the development of hepatocellular carcinoma.

Materials and methods

Oligonucleotide

The 20-base sequence 5′-UCCCGCCUGUGACAUCGAU U-3′ (Neuprocell, Kolkata, India) used in this study was sulfur-substituted oligonucleotides having a phosphorothioate linkage and contained extra modification in form of digoxigenin-labeled uracil bases. As per supplier's datasheet, the sequence was synthesized on a 0.2 μmol scale.

[^{3}H]-thymidine incorporation assay to measure cellular proliferation

The antiproliferative effect of c-raf.1 phosphorothioate antisense oligos was studied by determining the incorporation

of [^{3}H]thymidine into cellular nucleic acids in cell culture medium. The rate of tritiated thymidine incorporation into DNA was used here to estimate the rate of cell proliferation in culture medium. The cell line used in this experiment was a rat hepatoma H4IIEC3 cells (B.D Biosciences, Mumbai, India) which is a clonal line of Reuber H-35 rat hepatoma [32, 33]. Cells were seeded in 6-well tissue culture plate in DMEM medium supplemented with 10% FCS, 100 U/ml penicillin–streptomycin (sigma) at the density of 2×10^5 cells per well and allowed to incubate at 37°C in a humidified 5% CO_2 incubator (INNOVA-CO-170, New Brunswick Scientific) overnight. The following day media were removed from wells and cells were rinsed with serum-free media and incubated for 24 h. Cells from three wells were treated with 20 base c-raf.1 antisense oligonucleotides (5′-UCCCGCCUGUGACAUCGAUU-3′) (Neuprocell, Kolkata, India) at concentration of 50 μg/ml in the presence of cationic lipid; DOTMA and both treated and untreated cells were incubated at 37°C for 16 h. 1 μCi of [^{3}H]thymidine (Indian Institute of Chemical Biology, Kolkata, India) was added to each well and again cells were incubated at 37°C in a humidified 5% CO_2 incubator for another 24 h. Following day, [^{3}H] thymidine-labeled DNA was extracted and the assessment of radioactive nucleotide incorporation was performed in a scintillation counter (TRI-CARB 2100TR, Analyzer Packard, Meriden, USA) from both treated and untreated cells.

Development of rat hepatocarcinogenic model

Experiments were conducted on hepatic tissues of male Sprague–Dawley rats purchased from the Indian Institute of Chemical Biology, Kolkata, West Bengal, India. The initial body weights of the animals were approximately 190–200 g in all the cases. They were maintained under constant conditions and fed standard rat diet ad libitum and drinking water. All animals were kept in polypropylene cages and housed in an animal room at a temperature (25 ± 1°C) and humidity (55%) environment with a normal day and night photoperiod. They have been accustomed to the facility for 2 weeks. All animal experiments were conducted following the guideline of the "Principles of laboratory animal care" (NIH publication no. 85-23, 1985) and only after receiving the approval of the institutional animal ethics committee.

Animals were divided into four groups (Fig. 1). Each group contained seven animals. Normal untreated control group animals (group I) had free access to food and water. Group II animals were carcinogen control. Carcinogenesis was initiated in liver by the carcinogen diethylnitrosamine (DENA) (Sigma Aldrich, Bangalore, India) at a dose of 200 mg/kg body weight once in 0.9% solution of NaCl (in emulsified form in PBS buffer, pH 7.4 using Tween 20 as emulsifier) intraperitoneally at the start of the experiment, i.e., day 0. The animals were then treated with 0.025% w/w of 2-acetylaminofluorene (2-AAF) in the diet to promote carcinogenesis. They were given a small amount of food mixed with 2-AAF every morning. After 6 h they were given the basal diet [34]. The carcinogenesis was promoted from the 2–18 weeks, i.e., for a period of 17 weeks. Group III animals received the same treatment along with the iv injection of c-raf.1 antisense oligo suspension (5 mg/kg body weight) thrice a week. The antisense oligomer treatment was started from 18th week and continued up to 30th week of the experimental period. Group IV animals had free access to food and water along with the treatment of c-raf.1 antisense oligomers as described in case of group III

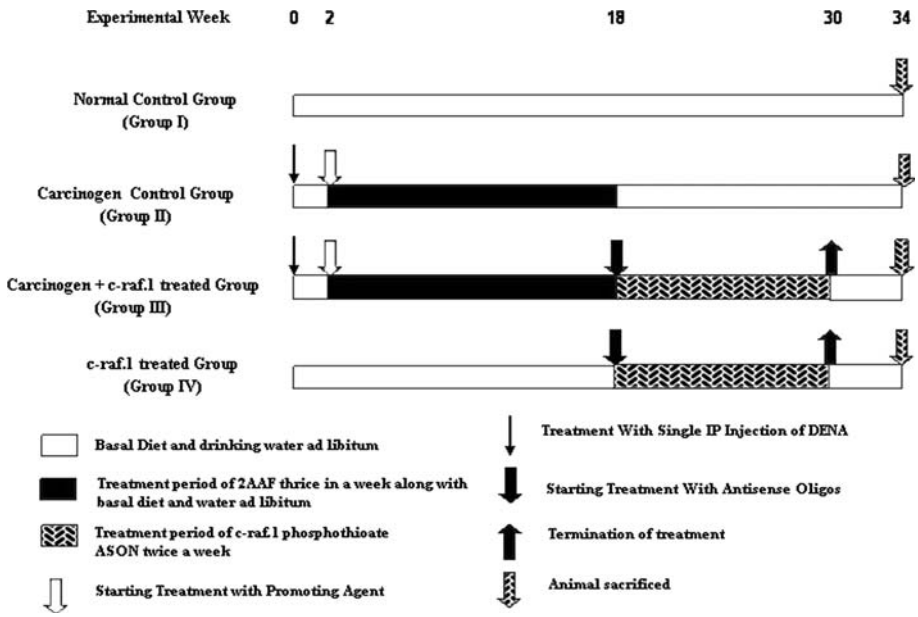

Fig. 1 Experimental regimen. *ASON* antisense oligonucleotide, *DENA* diethylnitrosamine

animals. Daily food and water consumptions were observed and body weights were taken every alternate day. All the treatments were stopped at the end of 30th week. At the 34th week from day 0, the animals were dissected and further experiments were conducted.

Tissue processing

Following the treatment schedule according to the experimental protocol, animals were killed. They were killed after anesthetizing with chloroform. The livers were removed, sliced (5–10 mm thick) and were snap-frozen in liquid nitrogen. The tissues were stored at −80°C until further used.

Liver morphology study

Liver tissues were subjected to macroscopic examination on the surfaces for visible lesions. The advanced hepatic altered foci (HAF), which were greater than 1 mm^2 in cross-section area were termed as hyperplastic nodules (HN). The macroscopic nodules were then identified and differentiated from the reddish brown non-nodular surrounding liver parenchyma with clear demarcation (Figs. 2, 3). Nodules were measured in two perpendicular directions to obtain the diameter of each nodule. According to their respective size and the total area of the occupied liver parenchyma, nodules were divided into three categories, namely <1 mm, >1 to <3 mm, and >3 mm [35].

Fig. 2 An external morphology of liver tumor of a carcinogen–control rat showing multiple hyperplastic nodules on the liver surface (*white arrowheads* indicate bile containing nodules and *white arrows* indicate nodules without bile)

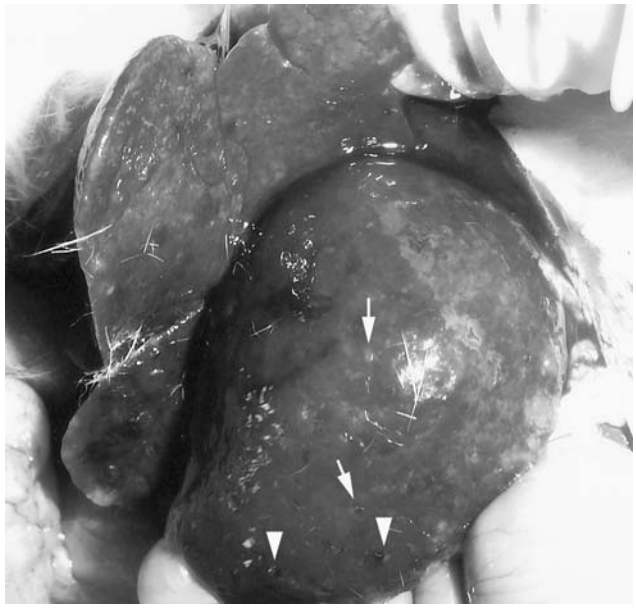

Fig. 3 An external morphology of liver tumor in an animal treated with carcinogen and c-raf.1 antisense oligomers (group III) showing fewer and smaller size hyperplastic nodules on the liver surface (*white arrowheads* indicate bile containing nodules and *white arrows* indicate nodules without bile)

Histopathological study

Various histochemical studies were performed on 5 µm thick serial sections of hepatic tissues using reagents such as Periodic acid-Schiff's reagent (PAS), hematoxylin–eosin (H&E), and toluidine blue to detect different hepatic altered foci [36–39]. Liver lesions were detected as hepatic altered foci and classified as clear endoplasmic reticulum rich acidophilic foci or glycogen stored foci (identified by PAS and H&E staining), mixed cell foci (identified by PAS and H&E staining) and ribosome rich basophilic foci (identified by toluidine blue staining) [40]. The analysis of number and size of focal lesions were carried out in two steps. In the first step, histological specimens were scanned and digitized using Zeiss light microscope driven by AxioVision software. In step two, lesions were measured and counted using image analyzer software SigmaScan Pro by marking individual lesions.

Enzyme assays

Preparation of hepatic cytosolic and microsomal fraction

Frozen liver tissue (stored at −80°C) of different experimental group of rats were blotted dry, and weighed quickly after thawing. The hepatic tissues were separately homogenized with ice-cold 0.1 N KCl (pH 7.4) in a precooled teflon-coated

glass homogenizer for few minutes to make 10% w/v tissue homogenate. The liver homogenate (LH) was centrifuged at 8,000×*g* for 15 min and an aliquot of this supernatant was used as cytosolic fraction to study glutathione *S*-transferase (GST) activity. The remaining portion was further processed to obtain the microsome fraction. The supernatant was once subjected to ultracentrifugation at 78,000×*g* for 90 min. The pellet thus obtained was resuspended in 0.1 N KCl and was ultracentrifuged at 10,5000×*g* for 1 h to obtain microsomes for assaying UDP-glucuronyl transferase (UDPGT) and cytochrome P-450 content.

Assays for cytochrome P-450, UDPGT and GST

The total amount of microsomal cytochrome P-450 was measured as described by Omura and Sato [41]. Glutathione-*S*-transferase activity was measured according to Habig et al. [42] using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. The GST-catalyzed formation of CDNB-GSH produces a dinitrophenyl thioether, which can be detected by spectrophotometer at 340 nm. One unit of GST activity is defined as the amount of enzyme producing 1 mmol of CDNB-GSH conjugate/min under the conditions of the assay using an extinction coefficient of 9.6/mM/cm. UDP glucuronosyltransferase activity towards *p*-nitrophenol was determined according to the standard procedure of Anderson et al. [43]. The specific activity of UDPGT is expressed as activity/mg microsomal protein/min.

Protein was determined from cytosolic fraction as well as microsomal fraction by the method of Lowry et al. [44] using bovine serum albumin (BSA) as standard at 660 nm.

Lipid peroxidation

Lipid peroxidation in liver was estimated colorimetrically using thiobarbituric acid-reactive substrate (TBARS) by the method of Nichans and Samuelson [45]. Since the assay estimates the amount of TBA reactive substance, e.g., MDA, it is also known as TBARS (thiobarbituric acid reactive substance) test. It gives an index of the extent of progress of lipid peroxidation. Both cytosolic and microsome fraction were assayed for the presence of lipid peroxides.

In situ hybridization study to locate c-raf.1 gene expression

In situ hybridization (ISH) was conducted on hepatic cryosections (6 µm) of the experimental samples [46]. All steps prior to and during hybridization were conducted under RNase-free conditions. The sections were fixed in 4% paraformaldehyde in phosphate buffer saline, pH 7.4 (PBS) for 1 h followed by dehydrating through the ascending concentration of ethanol [70–100% (1 min × 2 times each)] and xylene. The sections were dried and then treated with proteinase K, 0.5 µg/ml in Tris–$CaCl_2$ buffer, pH 8.0 at room temperature for 5 min and washed briefly with triethanolamine buffer, pH. 8.0. The sections were then acetylated for 20 min with 0.25% (v/v) acetic anhydride in 0.1 M triethanolamine (TEA), pH 8.0 and the slides were washed twice in 4× SSC (10 min each). Prehybridization was conducted using hybridization buffer except dextran sulfate and Denhardt's solution for 1 h at 52°C. Each 10 ml of hybridization buffer contained 5 ml deionized formamide, 2.4 ml diethyl pyrocarbonate-treated water, 2 ml 20× SSC, pH 7.0, 1 g dextran sulfate, 200 µl 50× Denhardt's solution, denatured yeast t-RNA (10 mg/ml) 200 µl and denatured salmon sperm DNA (10 mg/ml) 200 µl along with the respective probes denatured by heating at 80°C for 3 min followed by instant cooling on ice. This was followed by hybridization with DIG-labeled antisense c-raf.1 mRNA sequence at 50°C overnight in a humid chamber. After hybridization, sections were washed in 2× SSC at room temperature (RT) for 30 min, then again with 2× SSC at 60°C, for 30 min. This was followed by washing with 1× SSC for 30 min and 0.1× SSC for 1 h, at 60°C with a frequent moderate shaking. After the stringency wash, the slides were incubated with Tris–NaCl buffer, pH 7.5 containing 0.5% blocking agent (Boehringer-Mannheim, Mannheim, Germany) at RT on a shaking platform with a low speed. Sections were then incubated with anti-DIG-polyclonal antibody conjugated with alkaline phosphatase (Boehringer-Mannheim, Mannheim, Germany) (1:5,000) in Tris–NaC1 buffer, pH 7.5 for 2 h at RT on the shaking platform with a low speed. After incubation, sections were rinsed in washing buffer (Tris–NaC1 buffer, pH 7.5, containing 0.3% tween 20) for 10 min at room temperature. This was followed by two 15-min washes with Tris–NaCl buffer, pH 7.5. The sections were equilibrated with detection buffer (100 mM Tris–HC1, 100 mM NaC1 and 50 mM $MgC1_2{\cdot}6H_2O$), pH 9.5 for 5 min and staining was done using nitroblue tetrazolium/bromochloroindolylphosphate (NBT/BCIP) in detection buffer. Counter staining was also done occasionally.

Statistical analysis

Data were analyzed statistically for differences between the normal control and other treated groups using Dunnett's *t* test and between cancer control group and carcinogen-c-raf.1 treated group using Student's *t* test, following ANOVA.

Results

Antiproliferative effect of c-raf.1 antisense oligo

An increased incorporation of [^{3}H]thymidine has been observed in the untreated rat HCC cells, whereas the cells

treated with c-raf.1 antisense oligo showed a decreased uptake of tritiated thymidine as indicated by the measured radioactivity (Fig. 4). Again, the significant ($P < 0.001$) percentage of inhibited cell proliferation (68.25 ± 4.26) showed that treatment of cells with c-raf.1 antisense oligo resulted in a considerable inhibition of cancer cell proliferation in culture. This experiment boosted us to study the effect of the c-raf.1 antisense oligomers in in vivo model.

Effect of c-raf.1 antisense oligos on numbers and size distribution of hyperplastic nodules (HN) and preneoplastic lesions

Macroscopically, liver from animals of cancer control groups showed a multiple solid grayish/whitish nodules. Out of the four experimental groups, only group II and group III animals were shown to have developed hyperplastic nodules. Again, the maximum numbers of nodules were observed in group II (carcinogen control) animals (Table 1). Compared to animals from group II, group III animals (carcinogen and antisense treated group) showed only less number of nodules with smaller sizes (Figs. 2, 3). Hyperplastic nodules from group II animals were mostly greater than 3 mm in size, while group III animals had nodules mostly less than 1 mm in size and few between 1 and 3 mm in size. Histological examination of liver tissues from animals of group III treated with c-raf.1 antisense oligos demonstrated a significant reduction in occurrence of hepatic altered foci (HAF) as compared with group II or carcinogen control group. The number of preneoplastic lesions was found to be less in the oligomers and carcinogen-treated group than the carcinogen-treated group alone. In this count, early preneoplstic (glycogen stored) lesions [15] were found to be more than the late preneoplastic basophilic lesions (Fig. 5) [15] which were found to be more in group II animals. Gross and microscopic examination of the liver revealed no histological changes in experimental group IV and I. This suggests that c-raf.1 antisense oligomers administration does not alter normal hepatocellular architecture.

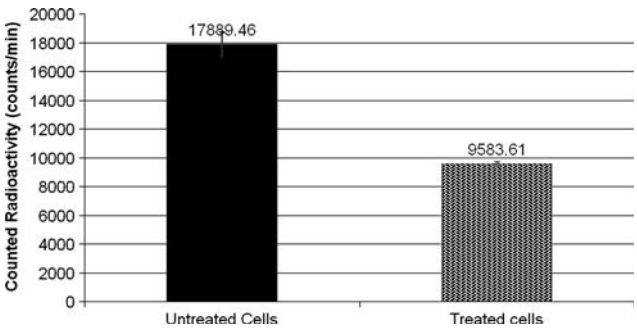

Fig. 4 Counted radioactivity of both untreated and treated rat HCC cells as measured in counts per minute. Treated cells denote cell treated with c-raf.1 antisense oligomers (50 μg/ml for 16 h). Each value represents mean ± SD ($n = 4$); *$P < 0.001$ as compared to the untreated group

Changes in cytochrome P-450 content, enzyme expression patterns and altered lipid peroxidation

During chemical hepatocarcinogenesis in rats, cyt P-450 showed a reduction in its content, whereas the enzymes such as GST and UDPGT showed an increased activity. When the cyt P-450 content of antisense-treated group was compared with carcinogen control group, it was observed that group III animals had the higher values of cyt P-450 content (Table 2). Again the group IV animals and group I had higher values of cyt P-450 content than that of the group III animals.

Hepatic GST activity was increased significantly ($P < 0.001$) by 1.93-fold in carcinogen control animals. The GST activity was found to be significantly ($P < 0.001$) lower in c-raf.1 antisense oligomer-treated carcinogen administered group (group III) as compared with group II animals.

UDP glucuronosyltransferase activity was markedly increased in carcinogen control group (1.98 ± 0.08 μmol/mg protein) as compared with the normal control animals. This was followed by group III (0.73 ± 0.11 μmol/mg protein) animals. UDPGT activity was significantly ($P < 0.001$) reduced in group III animals upon c-raf.1 antisense oligomer treatment. No predominant difference in activity of UDPGT was observed between group I (0.56 ± 0.014 μmol/mg protein) and group IV (0.42 ± 0.031μmol/mg protein) animals.

A significant elevation ($P < 0.001$) in MDA concentration was observed in group II animals as compared with the normal control animals. Lipid peroxidation increased by 1.74-fold following DENA/2-AAF treatment as compared with normal control rats. However, there was about 30% significant ($P < 0.001$) reduction of lipid peroxidation upon c-raf.1 antisense oligomer treatment (Table 2).

c-raf.1 expression and c-raf.1 expressed hepatic lesions assessed by in situ hybridization

The in situ hybridization study revealed that increased c-raf.1 expressions occurred in neoplastic nodules as well as in hepatocellular carcinoma since the gene was found to express predominantly in the hepatocytes of carcinogen control animals (Fig. 6). Group III animals, which were treated with phosphorothioate c-raf.1 antisense oligos (5′-UCCCGCCUGUGACAUCGAUU-3′) (Neuprocell, Kolkata, India) showed significantly ($P < 0.01$) less expression in terms of c-raf.1 expressed lesion count as compared with carcinogen control group (Table 1). There was no c-raf.1

Table 1 Effect of c-raf.1 antisense oligos on numbers and size distribution of hyperplastic nodules (HN) from experimental animals

Group	Number of nodule bearing rats/total no. of rats	Nodule incidence (%)	Number of nodules/cm^2 of hepatic tissue	Size distribution of nodules (% of total number)			Number of HAF/cm^2 of hepatic tissue
				<1 mm	>1 to <3 mm	>3 mm	
I	0/7	0	–	–	–	–	
II	7/7	100.00	16	19.57 ± 0.85	38.15 ± 0.87	42.28 ± 0.84	HAF = 46.7 ± 2.62 GSF = 11.7% MCF = 35.3% BSF = 53.0% REL = 37.59 ± 2.21
III	5/7	71	09	50.00 ± 2.94	24.26 ± 1.81	5.71 ± 1.13	HAF = 32.9 ± 1.31 GSF = 43.1% MCF = 29.5% BSF = 27.4% REL = 10.58 ± 1.72
IV	0/7	0	–	–	–	–	–

Values represent mean ± SD (n = 7)

HAF Hepatic altered foci/preneoplastic lesions, *GSF* glycogen storage foci, *MCF* mixed cell foci, *BSF* basophilic foci, *REL* c-raf.1 expressed lesions, *Gr I* Normal control group, *Gr. II* Carcinogen control group, *Gr. III* Carcinogen and antisense c-raf.1oligomer-treated group, *Gr. IV* c-raf.1 antisense oligomer-treated group

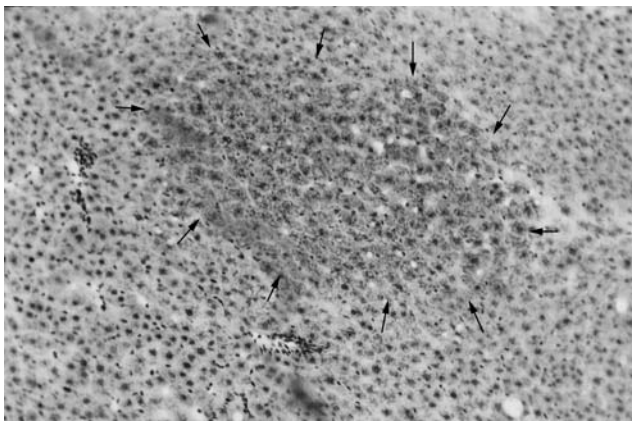

Fig. 5 A distinct hepatic basophilic lesion (shown by *arrows*) in carcinogen control animals using toluidine blue ×20

gene overexpression in group I and group IV animals. There was also no hybridization noticed in case of the tissues exposed to sense oligos kept as control.

Discussion

In this study, various biochemical changes and antisense-mediated inhibitory effect exerted by c-raf.1 antisense oligos during chemical hepatocarcinogenesis in rats have been demonstrated. The treatment of cells with c-raf.1 antisense oligo in culture media exhibited an inhibitory effect on cancer cell proliferation. Labeled thymidine was incorporated into dividing cells, i.e., during log phase or exponential phase of cell growth and the level of this incorporation which was measured using a liquid scintillation counter, was proportional to the amount of cell proliferation. The higher the proliferation rate, the more radioactivity is incorporated into DNA [47, 48]. The findings from the radioactive cell culture study suggest that the experimental c-raf.1 antisense oligo has a potential inhibitory effect on cell proliferation.

Again in in vivo study, the numbers and sizes of hyperplastic nodules and preneoplastic lesions in antisense oligo-treated carcinogen administered animals (group III) were greatly decreased as compared to those in carcinogen control animals (group II). The lineage of glycogen-stored/clear cell/acidophilic focal lesions to basophilic lesions through the mixed cell focal lesions is believed to lead ultimately into HCC [49]. In our study, group III animals had more percentage of GSF and less percentage of BSF and MCF as compared with group II animals. It was also noticed that animals treated with carcinogen and c-raf.1 antisense oligo (group III) had improved physical condition assessed in terms of body weight, food, and water intake after they started receiving c-raf.1 antisense oligo suspension (data not shown).

MDA is one of the major end products of peroxidative degradation of the polyunsaturated fatty acid constituents of biological membranes and its mutagenic and carcinogenic properties have been shown in in vitro systems and in experimental animals [50, 51]. The present study shows a decrease in lipid peroxidation in the antisense-treated carcinogen administered animals (group III) as

Table 2 Changes in cytochrome P-450 content, enzyme expressions and lipid peroxidation in different experimental animals

Group	cyt P-450 content (nmol/mg protein)	GST activity (μMol CDNB/min/mg protein)	UDPGT activity (μMol/mg protein)	MDA concentration (μM)
I	0.516 ± 0.089	0.73 ± 0.026	0.56 ± 0.096	7.9432 ± 1.13
II	0.161 ± 0.096[a]*	1.41 ± 0.52[a]*	1.22 ± 0.19[a]*	14.628 ± 3.42[a]*
III	0.291 ± 0.090[a]*,[b]**	0.94 ± 0.016[a]*,[b]**	0.32 ± 0.11[a]*,[b]**	10.131 ± 2.71[a]*,[b]**
IV	0.490 ± 0.023[a]*	0.59 ± 0.038[a]*	0.42 ± 0.031[a]*	7.698 ± 0.75[a]*

Values represent mean ± SD ($n = 7$)

Gr. I Normal control group, *Gr. II* carcinogen control group, *Gr. III* carcinogen and antisense c-raf.1 oligomer-treated group, *Gr. IV* c-raf.1 antisense oligomer-treated group, [a]* <0.05, [b]** <0.001

[a] Statistical level of significance (using Dunnett's *t* test) when group II, III, and IV were compared with group I

[b] Statistical level of significance (using Student's *t* test) of the data obtained of group III against group II

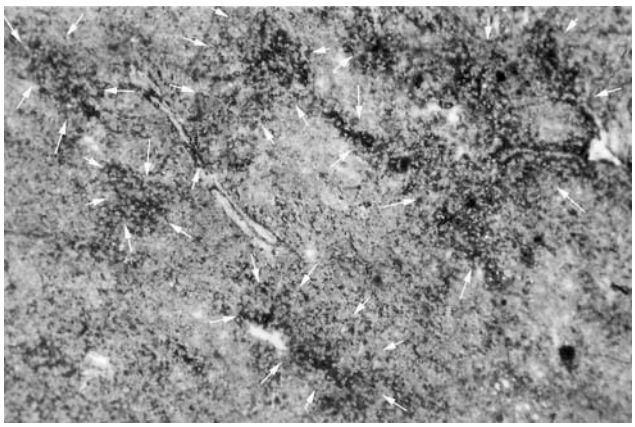

Fig. 6 c-raf.1 expressed scattered lesions in a carcinogen control liver (×10), shown by *white arrows*

compared to that in carcinogen control animals (group II). This decrease in lipid peroxidation in antisense-treated group III may be due to the antisense-mediated inhibition of c-raf.1 gene expression, which is believed to exert proliferative effects on the tumor cells by several biochemical modulations including lipid peroxidation [52].

cyt P-450, a supergene family of heme-containing oxidase enzymes is found in the smooth endoplasmic reticulum of centrilobular hepatocytes [53, 54]. The decrease in cyt P-450 content during hepatocarcinogenesis is reported to alter the regulation of homeostasis, which affects both the expression of the monooxygenases and the growth-controlling components of the preneoplastic cell population [18]. In our study, antisense-treated group showed a relatively lower level of cyt P-450 content as compared with normal control and group IV animals. No predominant differences in P-450 content were found in normal control and group IV animals. The fact that animals from carcinogen control group demonstrated the lowest level of cyt P-450 among these four groups indicates that the expression of this detoxifying isoenzyme was suppressed during hepatocarcinogenesis and the appropriate antisense treatment significantly improved the isoenzyme level in group III animals.

UDP glucuronyltransferase and GST, the two important phase II enzymes, play predominant role in carcinogen metabolism and protect the liver against carcinogen-induced hepatotoxicity [55, 56]. They have been found to increase in preneoplastic and neoplastic lesions [57, 58]. In this study it has been shown that the hepatic UDPGT activities toward *p*-nitro phenol decreased in group III animals, whereas an elevated level of the enzyme was observed in group II animals (carcinogen control). A similar trend was noticed in case of GST activity. A reduction in the levels of GST and UDPGT activities in antisense c-raf.1 oligo-treated carcinogen administered animals (group III) as compared to carcinogen control group clearly indicates that the role of inhibitory effect exerted by the experimental phosphorothioate antisense oligos of c-raf.1 in the development of hepatocellular carcinoma may directly or indirectly be produced through modulation of the enzymes.

Although the overexpression of c-raf.1 starts early in preneoplastic lesions [59], it is predominantly expressed in the basophilic tumors [60]. The c-raf.1 overexpression was not observed in normal untreated control group. In fact the difference between the basal levels of c-raf.1 expression in normal hepatocytes and in preneoplastic lesions was reported to be minimal [61]. The administration of phosphorothioate antisense oligonucleotide targeted against c-raf.1 markedly inhibited c-raf.1 expression as evident by ISH study in group III animals. The expression of c-raf.1 was significantly higher in carcinogen control than in antisense-treated carcinogen administered group and this suggests that c-raf.1 phosphorothioate antisense oligos exerted its inhibitory effect on the expression of the gene during the development of hepatocellular carcinoma.

Acknowledgments This work has been carried out with the financial grants from UGC; grants no. 7-5/2004 (SR) and DST; grant no SR/SO/HS-30/2004.

References

1. Farber E (1991) Hepatocyte proliferation in stepwise development of experimental liver cell cancer. Dig Dis Sci 36:973–978
2. Pitot HC (1986) Fundamentals of oncology, 3rd edn. Mercel Dekker, New York, p 532
3. Pitot HC, Sirica AE (1980) The stages of initiation and promotion in hepatocarcinogenesis. Biochem Biophys Acta 605:191–215
4. Schulte-Hermann R (1985) Tumor promotion in the liver. Arch Toxicol 57:147–185
5. Pitot HC, Dragan YP (1991) Facts and theories concerning the mechanisms of carcinogenesis. FASEB J 5:2280–2286
6. Klaunig JE, Kamendulis LM (1999) Mechanism of cancer chemoprevention in hepatic carcinogenesis: modulation of focal lesion growth in mice. Toxicol Sci 52:101–106
7. Bannasch P (1984) Sequential cellular changes during chemical carcinogenesis. J Cancer Res Clin Oncol 108:11–22
8. Schulte-Hermann R (1987) Initiation and promotion in hepatocarcinogenesis. Arch Toxicol 60:179–181
9. Schulte-Hermann R, Bursch W, Grasl-Kraupp B et al (1995) Role of active cell death (apoptosis) in multi-stage carcinogenesis. Toxicol Lett 82:143–148
10. Farber E, Sarma DS (1987) Hepatocarcinogenesis: a dynamic cellular perspective. Lab Invest 56(1):4–22
11. Rabes HM (1983) Development and growth of early preneoplastic lesions induced in the liver by chemical carcinogen. J Cancer Res Clin Oncol 106:85–92
12. Coleman WB (2003) Mechanisms of human hepatocarcinogenesis. Curr Mol Med 3(6):573–588
13. Kolaja KL, Stevenson DE, Walborg EF Jr et al (1996) Reversibility of promoter induced hepatic focal lesion growth in mice. Carcinogenesis 17(7):1403–1409
14. Bannasch P (1968) The cytoplasm of hepatocytes during carcinogenesis. Recent Results Cancer Res 19:1–100
15. Bannasch P, Mayer D, Hacker HJ (1980) Hepatocellular glycogenosis and hepatocarcinogenesis. Biochim Biophys Acta 605:217–245
16. Bannasch P, Hacker HJ, Klimek F et al (1984) Hepatocellular glycogenosis and related pattern of enzymatic changes during hepatocarcinogenesis. Adv Enzym Reg 22:97–121
17. Roomi MW, Ho RK, Sarma DSR et al (1985) A common biochemical pattern in preneoplastic hepatocyte nodules generated in four different models in the rat. Cancer Res 45:564–571
18. Buchmann A, Schwarz M, Schmitt R et al (1987) Development of cytochrome P-450-altered preneoplastic and neoplastic lesions during nitrosamine-induced hepatocarcinogenesis in the rat. Cancer Res 47:2911–2918
19. Lindahl R, Feinstein R (1876) Purification and characterization of aldehyde dehydrogenase from 2-acetylaminofluorene induced rat hepatomas. Biochim Biophys Acta 452:345–355
20. Levin W, Lu AYH, Thomas PE et al (1978) Identification of epoxide hydrolase in rat liver hyperplastic nodules. Proc Natl Acad Sci USA 75:3240–3243
21. Satoh K, Kitahara A, Soma Y et al (1985) Purification, induction and distribution of placental glutathione transferase: a new marker enzyme for preneoplastic cells in rat chemical hepatocarcinogenesis. Proc Natl Acad Sci USA 85:3964–3968
22. Sánchez-Pérez Y, Carrasco-Legleu C, García-Cuellar C et al (2005) Oxidative stress in carcinogenesis. Correlation between lipid peroxidation and induction of preneoplastic lesions in rat hepatocarcinogenesis. Cancer Lett 217(1):25–32
23. Daum G, Eisenmann-Tappe I, Fries HW et al (1994) The ins and outs of Raf kinases. Trends Biochem Sci 19:474–480
24. Rapp UR (1991) Role of Raf-1 serine/threonine protein kinase in growth factor signal transduction. Oncogene 6:495–500
25. Sebolt-Leopold J (2000) Development of anticancer drugs targeting the MAP kinase pathway. Oncogene 19:6594–6599
26. Avruch J, Khokhlatchev A, Kyriakis JM et al (2001) Ras activation of the Raf kinase: tyrosine kinase recruitment of the MAP kinase cascade. Recent Prog Horm Res 56:127–155
27. Störm SM, Rapp UR (1993) Oncogene activation: c-raf-1 gene mutations in experimental and naturally occurring tumors. Toxicol Lett 67:201–210
28. Stanton VP Jr, Cooper GM (1987) Activation of human raf transforming genes by deletion of normal amino-terminal coding sequences. Mol Cell Biol 7:1171–1179
29. Störm SM, Brennscheidt U, Sithanandam G et al (1990) Raf oncogenes in carcinogenesis. Crit Rev Oncog 2:1–8
30. Crooke ST (1993) Therapeutic application of oligonucleotides. Annu Rev Pharmacol Toxicol 32:329–376
31. Crooke ST (1999) Molecular mechanisms of action of antisense drugs. Biochim Biophys Acta 1489:31–44
32. Pitot HC, Peraino C, Morse PA Jr, Potter VR (1964) Hepatomas in tissue culture compared with adapting liver in vivo. Natl Cancer Inst Monogr 13:229–245
33. Reuber MD (1961) A transplantable bile-secreting hepatocellular carcinoma in the rat. J Natl Cancer Inst 26:891–897
34. Mukherjee B, Basu M, Chatterjee M (2001) Effect of selenomethionine on *n*-methylnitrosoguanidine-induced colonic aberrant crypt foci in rats. Eur J Cancer Prev 10:347–355
35. Moreno FS, Rizzi MB, Dagli ML et al (1991) Inhibitory effects of β-carotene on preneoplastic lesions induced in Wistar rats by the resistant hepatocyte model. Carcinogenesis 12(10):1817–1822
36. Bannasch P (1976) Cytology and cytogenesis of neoplastic (hyperplastic) hepatic nodules. Cancer Res 367:2555–2562
37. Bannasch P, Jahn UR, Hacker HJ et al (1997) Focal hepatic glycogenosis: a putative preneoplastic lesion associated with neoplasia and cirrhosis in explanted human livers. Int J Oncol 10:261–268
38. Yang D, Alt E, Rogler CE (1993) Coordinate expression of N-myc2 and insulin-like growth factor II in precancerous altered hepatic foci in woodchuck hepatitis virus carriers. Cancer Res 53:2020–2027
39. Mukherjee B, Ghosh S, Das T et al (2005) Characterization of insulin-like-growth factor II (IGF II) mRNA positive hepatic altered foci and IGF II expression in hepatocellular carcinoma during diethylnitrosamine-induced hepatocarcinogenesis in rats. J Carcinogenesis 4:12
40. Omura T, Sato N (1964) The carbon monoxide-binding pigment of liver microsome. J Biol Chem 239:2370–2378
41. Habig WH, Pabst MI, Jacob WB (1975) Glutathione *S*-transferase: the first enzymatic step in mercapturic acid formation. J Biol Chem 249:7130–7139
42. Anderson T, Pesonens M, Johansson C (1985) Differentiatial induction of cytochrome P-450 dependent monoxygenase, epoxide hydrolase, glutathione transferase and UDP-glucuronosyl transferase activities in the liver of the rainbow trout by β-naptoflavone or clophen A 50. Biol Chem Pharmacol 34:3309–3314
43. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin-phenol reagent. J Biol Chem 193:265–275
44. Nichans WG, Samuelson B (1968) Formation of MDA from phospholipid arachidonate during microsomal lipid peroxidation. Eur J Biochem 6:126–130
45. Braissant O, Wahli W (1998) A simplified in situ hybridization protocol using non-radioactively labeled probes to detect abundant and rare mRNAs on tissue sections. Biochemica 1:10–16

46. Jasper HG, Brasel A (1980) Rat liver DNA synthesis during the "Catch-up" growth of varied nutritional rehabilitation. J Nutr 110:2336–2340
47. Jeffrey WH, Paul JH (1988) Underestimation of DNA synthesis by [3H] thymidine incorporation in marine bacteria. Appl Environ Microbiol 54(12):3165–3168
48. Su Q, Benner A, Hofmann WJ et al (1997) Human hepatic preneoplasia: phenotypes and proliferation kinetics of foci and nodules of altered hepatocytes and their relationship to liver cell dysplasia. Virchows Arch 431(6):391–406
49. Esterbauer H, Schaur RJ, Zollner H (1991) Chemistry and biochemistry of 4-hydroxynonenal, malondialdehyde and related aldehydes. Free Radic Biol Med 11:81–128
50. Vaca CE, Wilhelm J, Harms-Ringdahl M (1988) Interaction of lipid peroxidation products with DNA. A review. Mutat Res 195:137–149
51. Ji C, Rouzer CA, Marnett LJ et al (1998) Induction of cell cycle arrest by the endogenous product of lipid peroxidation, malondialdehyde. Carcinogenesis 19(7):1275–1283
52. Barnard DL, Hettich E, Chuang XF et al (1995) Identification of the sites of interaction between c-raf.1 and Ras-GTP. Oncogene 10:1283–1290
53. Watkins PB (1992) Drug metabolism by cytochromes P450 in the liver and small bowel. Gastroenterol Clin North Am 21:511–526
54. Dahm LJ, Jones DP (1996) Mechanisms of chemically induced liver disease. In: Zakin D, Boyer TD (eds) Hepatology: a textbook of liver disease, 3rd edn. Saunders, Philadelphia, pp 875–890
55. Lee WM (1995) Drug-induced hepatotoxicity. N Engl J Med 333:1118–1127
56. Bock KW, Lilienblum W, Pfeil H et al (1982) Increased uridine diphosphate-glucuronyltransferase activity in preneoplastic liver nodules and Morris hepatomas. Cancer Res 42:3747–3752
57. Tatematsu M, Mera Y, Ito N et al (1985) Relative merits of immunohistochemical demonstrations of placenta, A, B and C forms of glutathione *S*-transferase and histochemical demonstration of f-glutamyl transferase as markers of altered foci during liver carcinogenesis in rats. Carcinogenesis 6:1621–1626
58. Bannasch P (1996) Pathogenesis of hepatocellular cacinoma: sequential cellular, molecular, and metabolic changes. In: Boyer JL, Ockner RK (eds) Progress in liver diseases, vol 14. Saunders, Philadelphia, pp 161–197
59. Hwang Y, Choi J, Kim S et al (2004) Over-expression of c-raf-1 protooncogene in liver cirrhosis and hepatocellular carcinoma. Hepatology Res. 29:113–121
60. Beer DG, Neveu MJ, Paul DL et al (1988) Expression of the c-mf protooncogene, 7-glutamyltranspeptidase, and gap junction protein in rat liver neoplasms. Cancer Res 48:1610–1617