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Rat ferritin-H: cDNA cloning, differential expression and localization during hepatocarcinogenesis

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Introduction

Hepatocellular carcinoma (HCC) is one of the most frequent malignancies of humans, mainly in areas of Asia and Africa (1). Development of cancer results from a stepwise process involving different preneoplastic lesions that reflect multiple genetic events like activation of proto-oncogenes, inactivation of tumour-suppressor genes and over- or re-expression of growth factors (2,3). It is generally acknowledged that the majority of HCC cases is associated with interactive effects of hepatitis virus and chemical carcinogens. However, the latter have been widely used for analysing multistep carcinogenesis and for generating animal models (4,5). Although the pathogenesis of experimental liver (pre)neoplasia has been studied since 1970 (6), the molecular mechanisms underlying hepatic carcinogenesis, and the factors responsible for initiation and progression of HCC remain still largely unknown. Furthermore, the requirement for specific and sensitive tumour marker of human HCC has not been satisfied yet.

Since altered gene expression is a common feature of neoplastic cells, the steady state level of particular transcripts may also provide information on the differentiation status of the hepatocytes, both during carcinogenesis and in fully developed tumours (7). We previously reported that by utilizing the subtraction-enhanced display technique, 36 up- and down-regulated gene products including c-myc, p21 and glutathione-S-transferase Yb1 (GST) from rat HCC were identified (8). Among these, one clone was found to be 88 and 96% homologous with respect to human and mouse ferritin-H cDNA (9,10).

In eukaryotic cells, ferritin serves as the major intracellular iron storage protein. It consists of 24 subunits of the H and L type. H and L subunits associate in various ratios depending on the type of tissue and the physiological state of cells (11,12). A number of previous studies have suggested that a correlation may exist between ferritin and cancer. For instance, serum ferritin level, particularly the H subunit, is frequently elevated in patients with cancer (13,14). However, no consistent relationship between the over-expression of ferritin-H and carcinogenesis has been documented.

In this study, we show that ferritin-H is a highly conserved protein, that rat ferritin-H mRNA is differentially expressed at the early stage of rat HCC development, and that over-expression of mRNA is exclusively localized to preneoplastic foci and to the parenchymal nodules with histopathological evidence of malignancy.

Materials and methods

Animal model

HCC was induced in male Wistar rats, weighing 200–250 g, by supplying diethylnitrosamine (DENA) at a concentration of 1:10,000 via drinking water (8). The rats were kept in a temperature and humidity controlled environment, and fed rat chow and water ad libitum. At the time points of 0, 1 day, 3 days, 1 week and 3 weeks after treatment with DENA, two rats were killed, while four rats at the time points of 6, 9, 12 and 16 weeks were killed. In addition, four rats were pair-fed during the entire experimental procedure by giving water without DENA as control. For a 70% partial hepatectomy, the medial and left hepatic lobe were excised according to the method of Higgins and Anderson (15). All procedures involving animals were performed according to the guidelines for Animal Use and Care of the University of Amsterdam.

Histology

Liver tissues were examined by standard histopathologic techniques employing haematoxylin-eosin (HE) and reticulin staining on paraffin-embedded liver sections.

cDNA cloning and sequencing

A cDNA fragment of ferritin-H (0.45 kb) isolated by the subtraction-enhanced display technique (8) was used to screen a rat liver cDNA library constructed in the pCDNA3 vector (INVITROGEN, San Diego, CA). Eight independent clones were isolated and sequenced from both 3' and 5' ends using a PCR cycle sequencing kit (Perkin-Elmer, Nieuwerkerk a/d IJssel, The Netherlands) with T7 and SP6 primer. The sequence was compared with those reported in GenBank database via the Blast search.

Preparation of RNA

Total RNA was extracted from frozen liver tissue of the rats treated with DENA at the above-indicated time points, of control rats and of 70% hepatectomized rats by using Trizol according to the vendor’s protocol (GIBCO BRL, Breda, The Netherlands). The amount of RNA was determined.
by measuring the absorbance at 260 nm, and RNA quality was confirmed by electrophoresis on a agarose gel stained with ethidium bromide.

**Northern blot analysis**

Total RNA of the liver (10–20 µg) from DENA-treated rats, the control and sections were dipped in Ilford Nuclear Research Emulsion K-5 (Ilford Photo, 70% Na₂S₂O₅·H₂O, 30% 3% sodium acetate solution) for 1 hour, the Northern blots were prehybridized for 2 h at 65°C in 6× SSC, in distilled water and stained with 0.1% nuclear-fast-red. Denhardt’s solution, 0.5% SDS, 100 µg/ml of herring sperm DNA. Rat GST Yb1 which was previously shown to be overexpressed in tumour (8), as well as ferritin-H cDNA were isolated from low melting gel agarose and labelled according to the hexamer-random primed method following the manufacturer’s protocol (Promega, Leiden, The Netherlands). Membranes were hybridized under the same conditions as stated for prehybridization and afterwards were washed four times for 15 min with 1× SSC/0.1% SDS and once with 0.2× SSC/0.1% SDS at 65°C. The membranes were exposed to X-ray film (Kodak X-Omat AR) for 2 h to 3 days, then exposed to X-ray film (Amersham, Arlington Heights, IL) for 1 week, and finally scanned with a Phosphorimager radioanalytic scanning system (Molecular Dynamics, USA).

**Results**

**Sequencing analysis of ferritin-H cDNA.**

Figure 1 shows the complete cDNA sequence of 830 base pairs (bps), which is in agreement with the size estimated from Northern blots, is 96 and 88% similar to cDNA isolated from mouse (819 bps), and human (790 bps), respectively. The first ATG codon is at nucleotide 130 and in-frame termination codon is 96 and 88% similar to mouse and the human analogue, respectively.

**Differential expression of ferritin-H during hepatic carcinogenesis.**

To detect the temporal alterations of ferritin-H mRNA levels during hepatic carcinogenesis, equal amounts of total RNA, isolated from liver of the rats before and 1 day, 3 days, 1 week, 3, 6, 9, 12 and 16 weeks after treatment with DENA...
were blotted, and hybridized with the ferritin-H probe and with the GST probe. As shown in Figure 2, up-regulation of ferritin-H mRNA was evident as early as 6 weeks after treatment with DENA, paralleling with the expression pattern of GST (Figure 2A). The mRNA levels of ferritin-H from rat livers after 9 weeks were >10-fold higher than those seen at early stages or in control rat liver (Figure 2B).

Constant mRNA level of ferritin-H during liver regeneration

In order to rule out the possibility that over-expression of ferritin-H may be due to regeneration nodules which may co-exist during hepatocarcinogenesis, mRNA from the rat liver obtained at various time points after a 70% partial heptectomy as well as a sham-operation was compared with that from a rat liver with HCC. As shown in Figure 3 the ferritin-H mRNA level, remained unchanged during rat liver regeneration.

Localization of ferritin-H mRNA in rat livers treated with DENA

To study the tissue distribution of over-expressed ferritin-H mRNA, an anti-sense RNA probe was radio-labelled and hybridized on serial liver sections examined by HE staining. The microscopic appearance of liver parenchyma appeared to be normal until 6 weeks after initiation with DENA. The liver lobule at week 6 showed a small group of basophilic cells indicated by arrowheads (Figure 4A), where GST reaction was positive (data not shown). This feature was described as the early preneoplastic focus (18). Figure 4B showed increased ferritin-H mRNA expression in these cells representing early preneoplastic foci in contrast to the low expression in normal liver cells (Figure 4B). After 9 weeks of DENA-administration, islands of tumour cells embraced by fibrotic stroma were seen in some parts of the liver (Figure 3C) and the adjacent section with reticulin staining showed that the normal hepatocellular network was destroyed (data not shown). On the serial section, a high level of ferritin-H mRNA was present in the tumour cell islands (Figure 4D) as compared to the surrounding non-neoplastic tissue. After 16 weeks, all rat livers showed characteristic features of parenchymal malignancy, e.g. central necrosis, haemorrhage in tumour nodules and vaso-invasion, as indicated by arrows in Figure 4E. Correspondingly, over-expression of ferritin-H was exclusively localized to the well-defined tumour nodules and to the invading tumour cells in the blood vessels as indicated with arrowheads (Figure 4F).

Discussion

The extensive use of hepatocarcinogenesis models in rodents has provided an important and useful framework for studying the multistep process by which malignant tumours develop (1,19). We have recently reported that several genes, like c-myc, α-prothymosin, p21, GST Yb1 and ferritin-H were isolated, and shown to be over-expressed in the DENA-induced HCC model in Wistar rats by employing the subtraction-enriched display technique (8). In this study, we first cloned and sequenced the rat full-length ferritin-H cDNA with 830 bps which is 96 and 88% similar to mRNA of mouse and human, while the coding region predicts a 182 amino acids which is 100 and 97%
Fig. 4. Gene expression and localization of ferritin-H mRNA in the serial liver sections from rats exposed to DENA for 6 (A,B), 9 (C,D) and 16 weeks (E,F) with magnification of ×200, ×160 and ×40, respectively. Histological appearance of rat liver sections were examined with haematoxylin-eosin staining (A, C and E). Over-expressed ferritin-H mRNA was shown in the early preneoplastic foci (B), tumour nodules (D) and these tumour cells invading blood vessels indicated with arrowheads. An asterisk shows the necrotic centre of the tumour nodule.

similar to mouse and human ferritin-H, respectively (9,10). This indicates that ferritin-H is highly conserved during evolution.

Subsequently we focused on studying temporal and spatial alteration of ferritin-H gene expression during hepatic carcinogenesis, of which little is known. Both gross (data not shown) and microscopic liver morphology showed that 9 weeks of DENA treatment was critical for the generation of parenchymal neoplasms. After 16 weeks, all microscopically characteristic features of HCC could be observed. On Northern blot, an increase in ferritin-H mRNA started dramatically after 6 weeks of DENA treatment, paralleling the alteration in GST expression, a well established early tumour marker (20). However, a definitive histological diagnosis of malignancy at this time point could not be made. Hence, the over-expression of ferritin-H, like GST, appeared as a sensitive indicator for the early stage of rat hepatocarcinogenesis.

It has been suggested that cytoplasmic ferritin expression is associated with cellular proliferation as shown in an immunohistochemical study of ferritin in colorectal carcinomas (21). Therefore, we considered the possibility that benign liver regeneration nodules could contribute to the over-expression of ferritin-H during hepatocarcinogenesis. For this reason,
Ferritin-H over-expressed in hepatocarcinogenesis

Ferritin-H mRNA expression was studied during liver regeneration at various time points after a 70% partial hepatectomy by Northern blot analysis. Contrary to our expectation, no change in ferritin-H mRNA level was found in this study. This suggests that the up-regulation of ferritin-H during hepatocarcinogenesis is tumour-associated rather than regeneration-related.

The origin of the elevated serum ferritin-H in many malignancies seems quite equivocal, although it has been generally acknowledged that ferritin is a multiple tumour marker (22–24). In the present study, we demonstrated that over-expression of ferritin-H mRNA was an early event in hepatocarcinogenesis and was exclusively localized to these preneoplastic foci, to well-defined tumour nodules and to the vaso-invasive cancer cells.

Ferritin is the major intracellular iron storage protein in tissues. It is composed of 24 subunits of the H and L type. It has been reported that preneoplastic foci and neoplastic lesions are refractory to iron accumulation, which was considered to be an early marker of chemical-induced carcinogenesis in the presence of hepatic siderosis produced by iron overload. Also in neoplastic lesions in human haemochromatotic livers, resistance to iron accumulation has been observed (6,25). This seems to be in contrast to our finding of exclusive localization of overexpressed ferritin-H in preneoplastic foci and in vaso-invasion tumour cells. Therefore, we propose that over-expression of ferritin-H during hepatocarcinogenesis is more related to carcinogenesis than to iron storage. This is in agreement with the studies in which ferritin-L expression was associated with iron overload but not with tumour development (26–28).

The exact mechanism underlying the over-expression of ferritin-H during carcinogenesis remains to be characterized. Tsuji and coworkers have shown a correlation between oncogene expression and ferritin-H expression (26). Somewhat surprisingly, ferritin-H expression was preferentially repressed by E1A, while H-ras did not affect ferritin-H composition. This suggests that there must be other elements modulating the over-expression of ferritin during carcinogenesis. One of such factors that has been proposed by Modjtabehdi et al. is c-myc. Ferritin-H was found to be overexpressed in the tumourigenic clones of the SW 613-S human colon carcinoma cell line which also have a high level of c-myc amplification. Furthermore, the steady-state level of the corresponding mRNA is increased in most of the tumourigenic cell lines obtained after transfer of c-myc gene copies into nontumourigenic SW 613-S clones (27). We previously reported that c-myc, together with ferritin-H, was also isolated in our subtraction procedure and was shown to be overexpressed in chemically induced rat HCC (8). This is consistent with reports in which c-myc amplification was found to be an early and critical event in the hepatocarcinogenesis model induced by a choline-devoid diet (29), and even in altered hepatic foci induced by single dose of DENA and promoted by 0.05% phenobarbital (30). Further research is necessary to detect directly gene expression of ferritin in rat liver epithelial cell transformed by c-myc (31) or other early oncogenes like c-jun and c-fos. In summary, we conclude that overexpressed ferritin-H mRNA is an early molecular marker for hepatocarcinogenesis.

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References


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