Brief Communication

Overexpression of histone variant H2A.1 and cellular transformation are related in N-nitrosodiethylamine-induced sequential hepatocarcinogenesis

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Abstract

Histones through a complex repertoire of non-allelic variants and their post-translational modifications regulate gene expression. Though alterations in histone-modifying enzymes and post-translational modifications of histones have been studied in cancer, expression of histone variants has not been clearly associated with dedifferentiation and malignant transformation of hepatocyte *in vivo*. In the present work, the pattern of variants of histones was investigated during N-nitrosodiethylamine (NDEA)-induced hepatocarcinogenesis. Our studies show for the first time *in vivo* overexpression of a major histone H2A variant H2A.1 and a decrease in H2A.2 at protein and mRNA levels by sodium dodecyl sulfate-Acetic acid-Urea-Triton (SDS-AUT) two-dimensional gel electrophoresis followed by matrix-assisted-laser desorption/ionization time-of-flight (TOF)/TOF mass spectrometry and reverse transcriptase-polymerase chain reaction analysis during sequential development of hepatocellular carcinoma (HCC). H2A.1 and H2A.2 are highly homologous, replication-dependent, non-allelic variants of histone H2A differing at only three amino acid positions. Our results of increase in proliferating cell nuclear antigen expression indicate that with increase in replicating population of transformed cells in HCC, H2A.1 expression increases, suggesting association of H2A.1 overexpression with hyper-proliferation of hepatocytes during cellular dedifferentiation and progressive transformation of normal liver to preneoplastic and neoplastic stages of HCC.

Keywords: histone variants, NDEA, HCC

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Introduction

Histones wrap DNA into nucleosome core particles and compact it into chromatin. The five types of histones (H1, H2A, H2B, H3 and H4) have multiple non-allelic subtypes called variants. Histone variants are synthesized in different relative amounts during the cell cycle and it has been proposed that they could be necessary for distinct chromatin-mediated functions. Histone variants, in combination with site-specific post-translational modifications, play crucial roles in chromosome segregation, transcriptional regulation, DNA repair and other processes.[1]

The chromatin field has seen a renewed interest in histone variants, homo- or heteromorphous, due to the structural and functional specificity they impart to chromatin. However, deciphering the alterations in major core histone variants that are associated with larger fraction of genome in various pathological conditions remains one of the most exciting challenges in chromatin research.

Epigenetic events like dysregulation of histone-modifying enzymes in various malignancies[2] and the role of histone deacetylase inhibitors as anticancer agents[3] indicate relevance of histones in the process of carcinogenesis. Genome-wide alterations in site-specific post-translational modifications of histones[4] and histone variants (e.g. H2A.Z, mH2A and CENPA)[5–7] have also been reported in various malignancies and are proposed to carry diagnostic and prognostic significance. Alteration in histone-modifying enzymes e.g. overexpression of histone methyltransferases Smyd3[8] and Poly-[ADP-ribose] polymerase 1[9] and reduced expression of another histone methyltransferase Riz1[10] have been reported in hepatocellular carcinoma (HCC). However, global changes in histone variants have not yet been studied during initiation and progression of HCC.

In this study, we determined whether qualitative and/or quantitative global changes in histone variant profile occur during cellular transformation of normal liver to preneoplastic

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and neoplastic stages of HCC. The study of liver histone profiles in N-nitrosodiethylamine (NDEA)-induced foci, dysplastic and neoplastic nodules and frank stages of HCC in experimental rats revealed overexpression of major histone H2A variant H2A.1 during the process of carcinogenesis. Another major H2A variant H2A.2 showed decreased expression in the process while minor H2A variants remained unaltered. Thus, both histone variants may be involved in the *in vivo* 'construction' of distinct nucleosomes with specialized functions in different pathological stages of carcinogenesis.

Materials and methods

NDEA-induced sequential hepatocarcinogenesis

All the experiments were performed on male Sprague-Dawley rats (spp. *Rattus norvegicus*) after approval of the Institute Animal Ethics Committee, Advanced Centre for Treatment Research and Education in Cancer and the Committee for the Purpose of Control and Supervision on Animals, India standards. The protocol for sequential stages liver carcinogenesis is as described.[11] Rats ($n = 40$), 6–8 weeks old, were randomized and divided into five groups ($n = 8$). The first group was given normal drinking water for four months and acted as negative control. Groups 2–5 were given NDEA (Sigma, Steinheim, Germany; Cat#N0756) at a dose of 1 ppm (w/v) through drinking water for 1–4 months (Figure 1). One week after completion of treatment, all the animals were euthanized and blood samples were collected for liver function tests (Supplementary method 1). Liver tissues were excised, washed with ice-cold saline and either fixed in formalin for hematoxylin and eosin (H&E) staining or snap-frozen in liquid nitrogen.

Sodium dodecyl sulfate-Acetic acid-Urea-Triton two-dimensional polyacrylamide gel electrophoresis of histones

Nuclei were isolated from liver tissues by sucrose density gradient centrifugation[12] and histones were extracted from nuclei by acid extraction method.[13] Histones were reconstituted in 0.1% (v/v) 2-ME and estimated by Bradford method.[14] Histones (20–25 μg) were separated on sodium dodecyl sulfate-Acetic acid-Urea-Triton (SDS-AUT) two-dimensional (2D) gel as described.[15] In brief, histones separated on first-dimensional 18% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were stained by Coomassie staining. The core histone region was cut out from the SDS-gel and equilibrated in equilibration buffer (1% w/v protamine sulfate, 5% v/v 2-ME, 0.75 mol/L potassium acetate pH 4.8, 20% v/v glycerol, 1% cysteamine-HCl and 6 mol/L urea). The gel pieces containing core histones were applied horizontally to the top of 15% AUT-PAGE and sealed using sealing buffer (1% w/v agarose, 0.75 mol/L potassium acetate pH 4, 20% v/v glycerol and 0.001% pyronin Y). The gel was electrophoresed at a constant voltage of 200 V and was subjected to 'SDS-silver' staining method.[16] Gels were documented as image files (.TIF) and proteins spots that appeared on gel were quantitated by ImageJ software (v1.42q, National Institutes of Health). Individual spot intensities were normalized to total spot intensity. Alterations in normalized band intensities were analyzed by one-way analysis of variance with Bonferroni's multiple-comparison test using GraphPad Prism software (version 5.0 for Windows).

Mass spectrometry

Histone spots of interest were subjected to matrix-assisted-laser desorption/ionization-mass spectrometry (MALDI-MS) (Bruker Daltonics, Bremen, Germany; Ultraflex II).[17,18] In brief, gel pieces were washed, destained, reduced, alkylated and were subjected to in-gel digestion using 20 μg/mL trypsin protease (Sigma, Cat#T5266). Peptides recovered using 25 mmol/L ammonium bicarbonate in 50% (v/v) acetonitrile were mixed with peptide matrix (HCCA) and applied onto sample target. Mass spectra were acquired on reflector ion positive mode. Database searching for protein masses was carried out using the MASCOT

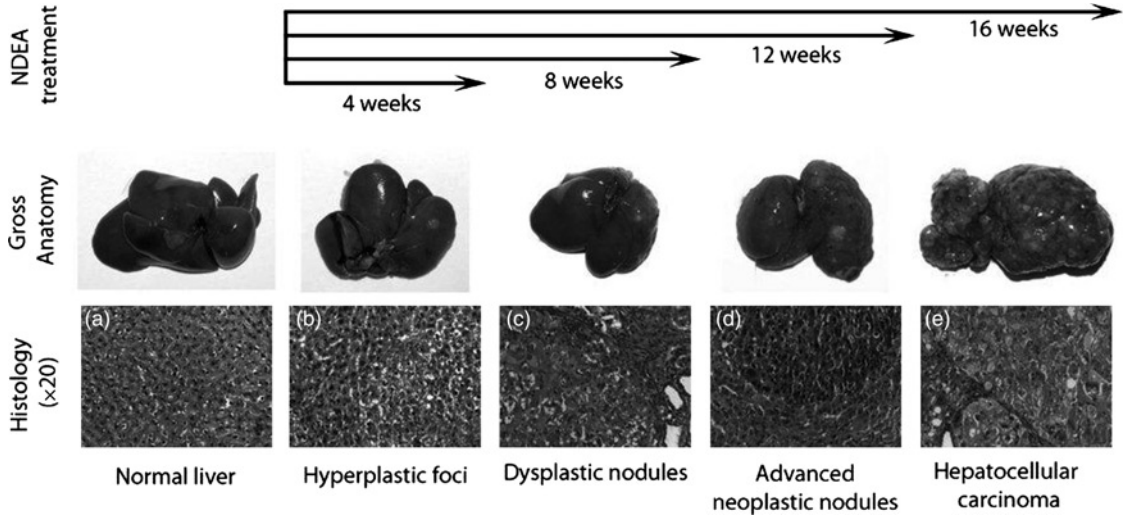

Figure 1 NDEA treatment regimen, gross anatomy and H&E-stained sections of normal liver and different stages of hepatocellular carcinoma. NDEA treatment from one to four months typically showed increased gradation of liver cell hyperplasia to dysplasia and development of HCC. NDEA, N-nitrosodiethylamine; H&E, hematoxylin and eosin; HCC, hepatocellular carcinoma

search engine (version 2.2.03) by comparing peptide masses with those in the NCBInr protein database (database version: NCBInr_20080812.fasta) in rattus spp. The searches were carried out with trypsin digestion, one missed cleavage, fixed carbamidomethylation of cysteine residues and optional oxidation of methionine with 100 ppm mass tolerance for monoisotopic peptide masses.

Semi-quantitative reverse transcriptase-polymerase chain reaction analysis

Total RNA was prepared from liver tissues using TRIzol (Invitrogen, Carlsbad, CA, USA; Cat#15596–026) and cDNA synthesis was carried out with 5 μg total RNA using random hexamer primers (Fermentas, Glen Burnie, MD, USA; Cat#K1632) as per the manufacturer's instructions. cDNA were subjected to polymerase chain reaction (PCR) for analysis of genes coding for H2A.1, H2A.2, H2A.X, H2A.Z, proliferating cell nuclear antigen (PCNA) and 18S rRNA. Primers were designed using primer3plus online software.[19] Table 1 summarizes the information about primers and PCR conditions. PCR was carried out using an Invitrogen kit (Cat#11615–010) according to the manufacturer's instructions. Amplification products were subjected to 1.5% TAE agarose gel electrophoresis and stained with ethidium bromide. Band intensities of PCR products of histone variants and PCNA were normalized to those of 18S rRNA and analyzed by GraphPad Prism software as described earlier.

Results

NDEA treatment for one to four months resulted in increase in gradation of liver disease (Supplementary Figure 1). Histological analysis of H&E-stained slides showed normal architecture of the liver in the control group of animals (group 1, Figure 1a). NDEA treatment for one to two months (groups 2 and 3, Figures 1b and c) resulted in preneoplastic stages, i.e. hyperplastic foci and dysplastic nodules. Three to four months of treatment (groups 4 and 5, Figures 1d and e) resulted in development of advanced neoplastic nodules and frank HCC.

To study alterations in histones during sequential hepatocarcinogenesis, SDS-AUT 2D PAGE was used to increase the reliability of subsequent mass spectrometry results. Core histones from normal liver tissues and different stages of hepatocarcinogenesis were subjected to SDS-AUT 2D gel electrophoresis and stained by mass spectrometry compatible 'SDS-silver' staining method (Figure 2a). Core histones were separated into multiple spots depending on alteration from unmodified canonical histones. According to gel analysis, a characteristically different H2A band pattern was detected with sequential transformation of normal liver to malignant tumor (Figure 2b). One spot in the H2A region which showed gradual and significant increase (Figure 2c) during progressive transformation of normal liver to HCC yielded good mass spectrum and was identified as H2A.1 by peptide mass fingerprinting (Table 2, Supplementary Figure 2 and Supplementary Table 1). Another spot immediately below H2A.1 showed a slight decrease during HCC progression (Figure 2c). This spot also yielded good mass spectrum and was identified as H2A.2 by MALDI-MS (Table 2, Supplementary Figure 2 and Supplementary Table 1). Protein sequence alignment of H2A.1 and H2A.2 indicated high homology with differences at 16th, 51st and 99th amino acid positions (Figure 2d). Spots in the vicinity that did not show any significant changes during development of HCC were identified as H2A.X and H2A.Z (mass spectrometry data not shown).

Semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) was used to detect gene expressions of histone H2A variants, identified by MALDI-MS using 18s rRNA as standard. In rat, H2A.1 is coded by two genes, *Hist1h2ah* and *hist1h2ao*, which share 89% homology. H2A.2 is also coded by two genes, *LOC690131* and *Hist2h2aa3*, which are 100% homologous to each other. We designed separate detection grade PCR primer pairs for two H2A.1 coding genes and a single primer pair for both H2A.2 coding genes. RT-PCR analysis suggested a gradual significant increase in H2A.1 coding gene *hist1h2ah* transcript and a decrease in H2A.2 coding gene transcript from one to four months of NDEA treatment (Figures 3a and b). Another H2A.1 coding gene, *Hist1h2ao*, did not show expression in any sample. *Hist1h2ao* PCR primers were validated by PCR with genomic DNA used as a template (data not shown). Minor H2A variant

Table 1 Primer sequences for histone variants, PCNA and 18S rRNA coding genes with expected product sizes and PCR conditions

Protein/RNA	Coding gene/s (official symbol/GeneID)	Primer sequence (F: forward, R: reverse)	Expected product size/number of cycles
H2A.1	Hist1h2ah/502125	F: CTGTGCTGGAGTACCTGACG R: TGTGGTGGCTCTCAGTCTTC	216 bp/30
	Hist1h2ao/364723	F: TGCTGTTTGTCATGTCTGGA R: TTATTTCCCCTTGGCCTTGT	405 bp/30
H2A.2	Hist2h2aa3/365877 and LOC690131/690131	F: GAAGACGGAGAGCCACCATA R: GGAAGAGTAGGGCACACGAC	220 bp/30
H2A.X	H2afx/500987	F: GTGAGTTGATTGCCGGACTT R: CCCGGCTCCTCTAGTCTTCT	222 bp/35
H2A.Z	H2afz/58940	F: CTATCCGCGGAGATGAAGAG R: CTGGAATCACCAACACTGGA	190 bp/30
PCNA	PCNA/25737	F: TCACAAAAGCCACTCCACTG R: CATCTCAGAAGCGATCGTCA	193 bp/30
18S rRNA	M11188	F: CGCGGTTCTATTTTGTTGGT R: AGTCGGCATCGTTTATGGTC	219 bp/18

PCNA, proliferating cell nuclear antigen; PCR, polymerase chain reaction

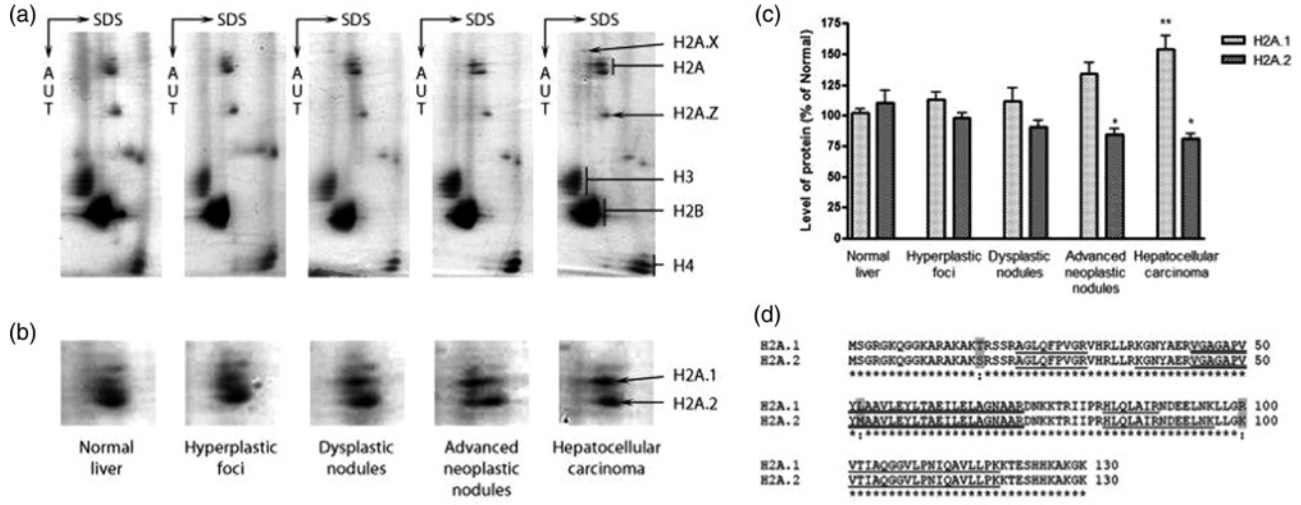

Figure 2 SDS-AUT 2D PAGE of histones, spot-densitometry analysis and sequence alignment of H2A.1 and H2A.2. (a) Core histones isolated from normal liver and different stages of HCC separated on SDS-AUT 2D gel electrophoresis, stained by 'SDS-silver' staining method. (b) Enlarged view of H2A regions of 2D gels. The gel pictures are representative of an experiment repeated thrice with similar results. (c) Spot densitometry indicated increase in H2A.1 and decrease in H2A.2 proportion during sequential hepatocarcinogenesis. Each histogram represents mean densitometry ± SEM ($^{*}P < 0.05$, $^{**}P < 0.01$). (d) H2A.1 and H2A.2 show high degree of homology. Underlined sequence was covered by MALDI-TOF mass spectrometry. Double underlined sequence was confirmed by MALDI-TOF/TOF MS (data not shown). HCC, hepatocellular carcinoma; MALDI-TOF, matrix-assisted-laser desorption/ionization time-of-flight; MS, mass spectrometry

Table 2 Mass spectrometry analysis of H2A.1 and H2A.2 bands

Protein (GeneID)	Number of peaks	Matched peaks	Sequence coverage (%)	Score	*P* value
H2A.1 (502125)	9	4	50	70	0.0069
H2A.2 (365877)	27	6	52	66	0.018

H2A.Z coding gene *H2afz* transcript and H2A.X coding gene *h2afx* transcript did not show any change. RT-PCR analysis of PCNA expression confirmed increase in proliferating cell population during precancerous to cancerous stages of HCC (Figure 3a).

Discussion

Histone variants constitute a small, but essential fraction of a cell's histone complement and play distinct functional and presumably structural roles in chromatin dynamics. Major histone variant mRNAs harbor stem loop structures at their 3′ ends and are believed to be replication-dependent in their expression whereas minor variant mRNAs undergo polyadenylation and express throughout the cell cycle.

Major histone variants are often coded by multiple genes which are located in clusters along with other histone coding genes. Though proliferating cells generally show higher proportion of replication-dependent variants, regulation of histone variant expression seems to be driven by additional mechanisms as tissue and developmental stage-specific differences in variant expression are known.[20] The mammalian histone H2A family consists of major variants e.g. H2A.1, H2A.2 and minor variants e.g. H2A.X, H2A.Z, etc. Interestingly, H2A.1 and H2A.2 coding genes are located on two different clusters. While H2A.1 coding genes are located on chromosome 6p21–22 in humans and

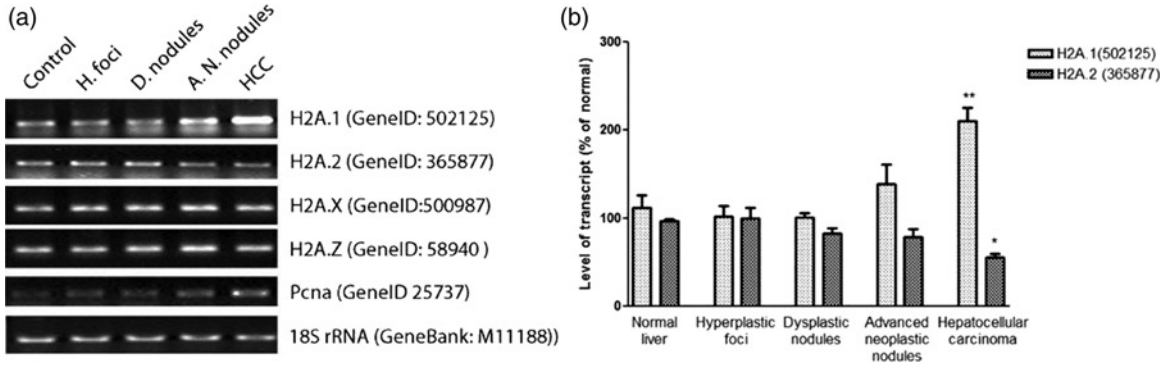

Figure 3 Semi-quantitative RT-PCR analysis of histone variants and PCNA. (a) PCR amplification products separated in 1.5% agarose gel. The gel picture is a representative of an experiment repeated thrice with similar results. (b) Spot densitometry indicates increase in H2A.1 coding gene *hist1h2ah* during HCC progression. H2A.2 expression decreases while minor variant expression remains unaltered in the process. Increase in cell proliferation during carcinogenesis confirmed by PCNA expression. Each histogram represents mean densitometry ± SEM ($^{*}P < 0.05$, $^{**}P < 0.01$). RT-PCR, reverse transcriptase-polymerase chain reaction; PCNA, proliferating cell nuclear antigen; HCC, hepatocellular carcinoma

17p11 in rats, H2A.2 coding genes are part of chromosome 1q21 in humans and 2q34 in rats. In this *in vivo* study, variations in the steady-state amounts of the H2A variants have been observed under different stages of tumor development. For instance, in normal and preneoplastic stages the more predominant form is H2A.2 whereas in HCC, H2A.1 is more abundant.

The biosynthesis of histones is known to be coordinated with changes in the proliferating population of cells. Our studies suggest gradual and significant decrease in tetraploid and increase in diploid and dividing (S-phase) population of cells during the process of hepatocarcinogenesis (Supplementary Figure 3). Increase in proliferating population of cells during hepatocarcinogenesis coincided with increase in H2A.1 and decrease in H2A.2 expression. This replacement of highly homomorphous variants in nucleosome core particles may lead to functional heterogeneity.

H2A.1 and H2A.2 proteins share a very high degree of homology with each other. In rats, H2A.1 and H2A.2 differ at three amino acid positions viz. 16 (serine→threonine), 51 (leucine→methionine) and 99 (arginine→lysine). The leucine to methionine alteration at the 51st amino acid position occurs in the H2A–H2B interaction domain and is highly conserved across species. The *in vivo* replacement of H2A.2 with H2A.1 may therefore alter the dynamics of H2A/H2B interaction in nucleosomes during tumor development. Earlier studies have shown gradual global hypomethylation of DNA[21] and chromatin decondensation by a micrococcal nuclease sensitivity assay[11] during NDEA-induced sequential hepatocarcinogenesis in rats.

Interestingly, the H2A.1/H2A.2 ratio has been shown to decrease during development and differentiation of rat brain cortical neurons[22] and during *in vitro* differentiation and aging of fibroblasts.[23] Further, H2A.1 has also been shown to predominantly express in fetal tissues and lung carcinoma.[24] These findings suggest that the H2A.1/H2A.2 ratio is an important player in cell proliferation and dedifferentiation.

The *in vivo* replacement of H2A.2 by H2A.1 modulates the highly dynamic nature of the nucleosomes and may contribute to alterations in the transcriptional activities of a wide range of genes associated with malignant transformation of hepatocytes by controlling the access of regulatory factors to altered chromatin configuration. Future studies in this field will provide insights into the mechanisms through which specific histone variants exert their functions on the development of neoplastic phenotype.

Author contributions: SPK carried out NDEA treatment, SDS-AUT 2D-PAGE, MALDI-TOF/TOF mass spectrometry, RT-PCR analysis and drafted the manuscript. AS participated in carrying out SDS-AUT 2D-PAGE and in drafting the manuscript. KKD carried out histopathological analysis. SG designed and planned the experiments and participated in drafting the manuscript. All authors read and approved the final manuscript.

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